Diss. ETH No. 13553

Analytical and Phytochemical Investigations on Hypericin and Related Compounds of *Hypericum perforatum*

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Natural Sciences

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Zürich 2000

Acknowledgments

The presented thesis was carried out at the Swiss Federal Institute of Technology (ETH) Zurich, Institute of Pharmaceutical Sciences, Section Pharmacognosy and Phytochemistry, under the supervision of **Prof. Dr. Otto Sticher**. I would like to express my gratitude to Professor Sticher for giving me the opportunity to work in his research group and providing excellent working facilities.

I would like to heartily thank my co-examiner **Prof. Dr. Beat Meier** (Zeller AG, Herbal Remedies) for his guidance during this study. I thank him for many constructive and helpful discussions, his advises, encouragement and interest.

I would like to thank **Dr. Jörg Heilmann** (ETH Zurich) for accepting to act as co-examiner and for his friendly support.

On behalf of the structure elucidation, I would like to especially thank **Dr. Oliver Zerbe** (ETH Zurich) for his practical NMR support and helpful discussions. I greatly acknowledge the advises on structure elucidation that I obtained from **Prof. Dr. Ihsan Çalis** (Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University). I thank him for his interest and the help with the acetylation of the skyrin derivatives. I am grateful to **Mr. Oswald Greter** (ETH Zurich) and **Dr. Walter Amrein** (ETH Zurich) for recording mass spectra and **Dr. Engelbert Zass** (ETH Zurich) for performing literature search.

Great thanks are due to **Dr. Urs Simmen** (Department of Pharmacy, University of Basel) for performing the receptor binding studies.

I also wish to thank all my **colleagues** and the **staff** of the Institute of Pharmaceutical Sciences for the pleasant working atmosphere and the enjoyable time we had together.

Last but not least, I would like to thank my **parents** and my **friends** for their encouragement, support and patience during this work.

Contents

General Abbreviations

$A^{1\%}$	coefficient of absorbance for 1% (w/v) solutions
ACN	acetonitrile
CD	circular dichroism
chloroform-d_1	deuterated chloroform
CRH	corticotropin releasing hormone
δ	chemical shift
d	doublet
2D	two-dimensional
DAC	Deutscher Arzneimittel Codex
DAD	diode array detector
DCCC	droplet countercurrent chromatography
dd	double doublet
DEPT	distortionless enhancement by polarization transfer
D_2O	deuterated water
DQF-COSY	double quantum filter correlation spectroscopy
ε	molar coefficient of absorbance
EI-MS	electron impact - mass spectrometry
ESI-MS	electrospray ionization - mass spectrometry
EtOAc	ethyl acetate
EtOH	ethanol
FAB-MS	fast atom bombardment - mass spectrometry
HCOOH	formic acid
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum correlation
H_2O	water
HPLC	high-performance liquid chromatography
HSCCC	high-speed countercurrent chromatography
Hz	hertz
IC_{50}	50% inhibition concentration
IR	infrared spectroscopy
J	coupling constant
m	multiplet
MeOH	methanol
methanol-d_4	deuterated methanol
MLCCC	multi-layer countercurrent chromatography
m/z	mass-to-charge ratio
MS	mass spectrometry
NMR	nuclear magnetic resonance

Ph. Helv.	Pharmacopoea Helvetica (Swiss Pharmacopoeia)
Ph. Eur.	European Pharmacopoeia
ppm	parts per million
pyridine-d_5	deuterated pyridine
r	correlation coefficient
Rf	retention factor (TLC analysis)
ROESY	rotating-frame overhauser spectroscopy
RP	reversed phase
rpm	rotation per minute
RT	(a) room temperature (b) retention time (HPLC)
s	singlet
s	standard deviation
s_{rel}	relative standard deviation
$t_{1/2}$	half-time
tetrahydrofuran-d_8	deuterated tetrahydrofuran
TFA	trifluoroacetic acid
TLC	thin layer chromatography
UV	ultraviolet
VIS	visible
VLC	vacuum liquid chromatography

Summary

This study is dedicated to questions concerning the quality control of products of *Hypericum perforatum*. It is divided into six chapters. Each of the chapters two to six starts with a literature overview on the subject examined, followed by the description of experiments, results, discussion, conclusions and references mentioned in the relevant chapter.

Chapter 1 presents factors influencing the quality of extracts of Hyperici herba and discusses tools allowing its evaluation. At present, most of the commercial extracts are standardized on a certain content of hypericin, as hypericin is a very characteristic constituent and as the active principle(s) and the mode of action of *Hypericum perforatum* have still not been clarified yet. This requires that hypericin is available as reference substance of constant high quality.

On the basis of this background, an isolation process for hypericin and also pseudohypericin has been developed, which is described in **chapter 2**. The isolation procedure starts with a liquid-liquid partition of a dry extract of Hyperici herba in a separatory funnel followed by High-Speed Countercurrent Chromatography. The identity and purity of the two substances were ratified by TLC, HPLC, UV/VIS spectroscopy, EIMS and NMR measurements.

Chapter 3 deals with the physicochemical properties of hypericin and pseudohypericin, which must be known to be able to assess influences of the reference substance on the quantification result. The evaluation of the absorbance data of hypericin and pseudohypericin revealed the ***molar/specific coefficients of absorbance*** in methanol-pyridine (99:1, v/v) at the maximum of the longest wavelength to be 51936/1030 and 43486/836, respectively. The absorbance data of hypericin were also determined in methanol. They were not significantly differing from those in the presence of pyridine. The decrease of the coefficients by water addition was found to be the same for hypericin and pseudohypericin. It was concluded that hypericin and pseudohypericin reveal the same ***homoassociation*** behavior. The ***solubility*** of hypericin turned out to be enhanced by the addition of pyridine. In pure methanol only 37.17 µg/ml could be dissolved. In comparison, 320.91 µg hypericin were soluble in one ml methanol-pyridine (99:1, v/v). Tests on the ***stability*** of hypericin and pseudohypericin in extracts and standard solutions were done under different temperature and light conditions monitored by VIS spectroscopy and HPLC-VIS / DAD measurements. All solutions were stable at -20 °C in darkness over the investigated time period of 140

days. Higher temperatures, light and the presence of pyridine turned out to accelerate the degradation of pseudohypericin, while exposure to light was most aggressive. Hypericin showed higher stability, light being the only factor investigated that decreased the concentration of hypericin. The instability in the presence of light was more pronounced in the extract solution both for hypericin and pseudohypericin. Under all the other storage conditions, the stability of pseudohypericin in the extract solutions was improved. Cyclopseudohypericin was assumed to be one of the transformation products of pseudohypericin.

Besides the reference substance, various other factors, not completely known yet, are affecting the evaluation of the content of hypericin in drug samples and dry extracts of Hyperici herba. Therefore, **chapter 4** examined, which effect quantification techniques, extracting solvents and extracting methods in general had on the results. In **section 4.1**, some HPLC methods were compared with each other, with VIS spectroscopy and TLC-densitometry using a commercial dry extract. The modified HPLC method of Kerb turned out to be most suited for the quantification of naphthodianthrones, as it showed the shortest run time, best selectivity and highest reproducibility. Applying this method, pyridine did not influence run time and quantification, allowing preparing standard solutions with pyridine and doing extraction without. VIS spectroscopic results were higher than HPLC results caused by differences in selectivity. The ratio VIS spectroscopic to HPLC results was not the same for all the HPLC methods investigated. A drug sample was subjected to various extraction methods in **section 4.2** revealing the method of the Ph. Helv. 8, which uses tetrahydrofuran-water (8:2, v/v) for extraction, to be the most efficient. The application of the method of the DAC (1986) showed preextraction with dichloromethane to diminish differences between the results of the two quantification techniques. This could also be seen in **section 4.3**. There, it was investigated in which degree the drug sample itself affected the results, finding the ratio of blossoms to leaves to play a crucial role. To clarify if the factors affecting the quantification of naphthodianthrones were different for drug material and commercial extracts, **section 4.4** looked closer at the extraction procedure of a commercial dry extract. Ultrasonic extraction turned out to be the fastest, most efficient and precise method to extract naphthodianthrones from the dry extract Ze117. It was shown that the ratio of VIS spectroscopic to HPLC results was higher for the extracting solvent methanol than for acetone. Chlorophyll and other compounds, not definitely defined yet, were made responsible for the discrepancy. Polarity and selectivity of the extracting

solvent influence the extracted amount of these substances. Therefore, the extracting solvent has an impact on the final result.

In **chapter 5**, factors influencing the extractability of hypericin, pseudohypericin, hyperforin and adhyperforin were studied. Aqueous ethanol 70% provided best yields of *hypericin* and *pseudohypericin* among the extracting solvents investigated. An increase in temperature led to higher yields as was shown with ethanol 100%. In a tea preparation 12% of the total amount of hypericin and 29% of total pseudohypericin were found. An HPLC method was developed for the quantification of *hyperforin* and *adhyperforin*, which allowed baseline separation of the two compounds within a reasonable run time. Retention times were 14.5 min and 17.3 min for hyperforin and adhyperforin, respectively. Highest yields of the two phloroglucin derivatives were achieved with the extracting solvents aqueous ethanol 70% to ethanol 100%. Exclusion of light and oxygen during the extraction procedure improved stability of the two compounds. Tea preparations did not contain any hyperforin or adhyperforin.

As the antidepressant effect of *Hypericum perforatum* is assumed to result from a synergism of several active constituents, further investigations on this subject have been done in **chapter 6**. On the search for new active compounds, four new bisanthraquinone glycosides, S-(+)-skyrin-6-*O*-β-glucopyranoside (**1**), R-(-)-skyrin-6-*O*-β-glucopyranoside (**2**), S-(+)-skyrin-6-*O*-β-xylopyranoside (**3**) and S-(+)-skyrin-6-*O*-α-arabinofuranoside (**4**), have been isolated from an ethanol-water (1:1, m/m, Ze117) dry extract of Hyperici herba. The isolation procedure started with a liquid-liquid-partitioning (hexane-toluene-water-ethyl acetate-formic acid (75:225:135:120:15, v/v)). Subsequent RP-18 VLC (acetonitrile-water-trifluoroacetic acid (5:4.9:0.1, v/v/v)) and preparative TLC on silica gel (ethyl acetate-acetonitrile-water (10:1:1, v/v/v)) led to the isolation of the glycosides. The structures were elucidated by spectroscopic methods, mainly NMR and mass spectrometry. Acetylation of **1** and **2** was helpful to determine the site of glycosidation. Circular dichroism was used to determine their axial stereochemistry revealing **1** and **2** to be atropisomers. The stereochemistry of **3** and **4** was in accordance with **1**. **1** and **2** showed moderate potency to inhibit the binding to CRH-1 (corticotropin releasing hormone) receptors.

Zusammenfassung

Die vorliegende Arbeit behandelt Fragen der Qualitätskontrolle von Johanniskrautextrakten. Sie ist in sechs Kapitel unterteilt. Die Kapitel zwei bis sechs beinhalten eine Literaturübersicht über das entsprechende Thema, gefolgt von der Beschreibung der Experimente, den Resultaten, der Diskussion, den Schlussfolgerungen und der in den einzelnen Kapiteln zitierten Literatur.

Kapitel 1 stellt Faktoren vor, welche die Qualität von Johanniskrautextrakten beeinflussen und diskutiert Methoden zur Qualtätsbeurteilung. Da das aktive Prinzip und der Wirkungsmechanismus der antidepressiven Wirkung von Johanniskraut nicht bekannt sind und Hypericin einer der charakteristischsten Inhaltsstoffe darstellt, werden zur Zeit die meisten kommerziellen Extrakte auf einen bestimmten Gehalt an Hypericin standardisiert. Eine zuverlässige Standardisierung und Qualitätskontrolle setzen voraus, dass Hypericin als Referenzsubstanz von gleichbleibend hoher Qualität erhältlich ist.

Dies war der Anlass zur Entwicklung einer Isolierungsmethode für Hypericin und Pseudohypericin, welche in **Kapitel 2** beschrieben wird. Zur Anreicherung der beiden Substanzen wurde zunächst eine Flüssig-Flüssig-Verteilung eines Trockenextraktes von Johanniskraut in einem Scheidetrichter durchgeführt. Die definitive Trennung von Hypericin und Pseudohypericin erfolgte unter Anwendung der Hochgeschwindigkeits-gegenstromchromatographie (HSCCC). Der Nachweis der Identität und Reinheit der beiden Substanzen wurde mittels DC, HPLC, UV/VIS Spektroskopie, EIMS und NMR durchgeführt.

Kapitel 3 behandelt die physikochemischen Eigenschaften von Hypericin und Pseudohypericin. Diese Merkmale müssen bekannt sein, um den Einfluss der Referenzsubstanzen auf die Ergebnisse der Quantifizierung beurteilen zu können. Die Bestimmung der Absorptionsdaten von Hypericin und Pseudohypericin ergaben für die *molaren* bzw. *spezifischen Absorptionskoeffizienten* in Methanol-Pyridin (99:1, v/v) im Maximum der längstwelligen Absorptionsbande die Werte 51936 bzw. 1030 für Hypericin und 43486 bzw. 836 für Pseudohypericin. Die Absorptionsdaten von Hypericin wurden auch in Methanol bestimmt. Sie waren nicht signifikant verschieden von denen in Methanol-Pyridin (99:1, v/v). Wasserzugabe führte bei Hypericin und Pseudohypericin in gleichem Masse zur Abnahme des molaren Absorptionskoeffizienten. Daraus wurde gefolgert, dass Hypericin und Pseudohypericin dasselbe *Homoassoziationsverhalten* aufweisen. Die *Löslichkeit* von Hypericin wurde durch die Zugabe von Pyridin zu Methanol erhöht. In reinem Methanol liessen sich nur 37.17 µg/ml lösen.

In einem ml Methanol-Pyridin (99:1, v/v) waren dagegen 320.91 µg Hypericin löslich. *Stabilitätsuntersuchungen* von Hypericin und Pseudohypericin in Extrakt- und Standardlösungen wurden bei verschiedenen Temperatur- und Lichtverhältnissen durchgeführt. Transformations- und Degradationsprozesse wurden mittels VIS Spektroskopie und HPLC verfolgt. Bei -20 °C erwiesen sich alle Lösungen während der untersuchten Zeitperiode von 140 Tagen als stabil. Lagerung bei höheren Temperaturen, Licht und die Anwesenheit von Pyridin beschleunigten die Zersetzung von Pseudohypericin, wobei sich die Lichtexposition als am aggressivsten herausstellte. Hypericin erwies sich als stabiler. Nur die Bestrahlung mit Licht führte zu einer Abnahme der Hypericinkonzentration. Die Instabilität unter Lichteinfluss war ausgeprägter in Extraktlösungen; dies galt auch für Pseudohypericin. Unter all den anderen Lagerungsbedingungen war die Stabilität von Pseudohypericin in den Extraktlösungen dagegen erhöht. Die Resultate wiesen daraufhin, dass Cyclopseudohypericin ein Transformationsprodukt von Pseudohypericin ist.

Neben der Referenzsubstanz beeinflussen noch verschiedene andere zum Teil nicht bekannte Faktoren die Ermittlung des Hypericingehaltes in Drogenmustern und in Trockenextrakten von Johanniskraut. In **Kapitel 4** wurde untersucht, welche Auswirkungen die Quantifizierungsmethode, das Extraktionsmittel und die Extraktionsmethode auf das Resultat haben. In **Abschnitt 4.1** wurden anhand eines Johanniskrauttrockenextraktes verschiedene HPLC-Methoden mit einer VIS spektroskopischen und einer dünnschichtchromatographisch-densitometrischen Methode verglichen. Die modifizierte HPLC-Methode von Kerb stellte sich für die Quantifizierung von Naphthodianthronen als die geeignetste Methode heraus. Sie wies die kürzeste Laufzeit, die beste Selektivität und die höchste Reproduzierbarkeit auf. Bei Einsatz dieser Methode beeinflusste Pyridin weder die Retentionszeit noch die Quantifizierung. Dies ermöglicht die Herstellung von Standardlösungen mit Pyridin und die Durchführung der Extraktion ohne Pyridin. Die mittels HPLC erhaltenen Resultate waren tiefer als die VIS spektroskopisch ermittelten Werte. Ausschlaggebend für die Diskrepanz ist die unterschiedliche Selektivität der Methoden. Das Verhältnis der Resultate VIS Spektroskopie / HPLC war nicht für alle HPLC-Methoden dasselbe. In **Abschnitt 4.2** wurde ein Drogenmuster mehreren Extraktionsmethoden unterworfen. Die Extraktionsmethode der Ph. Helv. 8 mit Tetrahydrofuran-Wasser (8:2, v/v) erwies sich als die effizienteste. Die Anwendung der Methode des DAC (1986) zeigte, dass die Vorextraktion mit Dichlormethan die Differenzen der VIS spektroskopischen und der HPLC-Resultate verminderte. Dies sah man auch in **Abschnitt 4.3**. Dort

wurde untersucht, in welchem Ausmass das Drogenmuster selbst das Resultat beeinflusst. Es stellte sich heraus, dass das Verhältnis Blüten zu Blättern eine wichtige Rolle spielt. Um zu überprüfen, ob die Quantifizierung von Naphthodianthronen in Trockenextrakten und Drogenmaterial von denselben Faktoren abhängt, wurde in **Abschnitt 4.4** die Extraktion eines kommerziellen Trockenextraktes genauer angeschaut. Die Ultraschallextraktion erwies sich als die schnellste, effizienteste und präziseste Methode zur Extraktion von Naphthodianthronen aus dem Trockenextrakt Ze117. Es wurde gezeigt, dass das Verhältnis der Resultate der VIS Spektroskopie / HPLC für das Extraktionsmittel Methanol höher war als für Aceton. Chlorophyll und weitere bisher nicht näher definierte Substanzen wurden für die Differenzen verantwortlich gemacht. Polarität und Selektivität des Extraktionsmittels beeinflussen die extrahierte Menge dieser Substanzen und dadurch das Endresultat.

In **Kapitel 5** wurden die Faktoren, welche die Extrahierbarkeit von Hypericin, Pseudohypericin, Hyperforin und Adhyperforin beeinflussen, untersucht. Wässriger Ethanol 70% ergab unter den untersuchten Lösungsmitteln die besten Ausbeuten für Hypericin und Pseudohypericin. Eine Erhöhung der Temperatur führte zu höheren Ausbeuten, wie am Beispiel Ethanol 100% gezeigt wurde. Eine Teezubereitung enthielt 12% der gesamten Hypericinmenge und 29% des gesamten Pseudohypericins. Weiter wurde eine HPLC-Methode zur Quantifizierung von Hyperforin und Adhyperforin entwickelt. Sie erlaubte Basislinientrennung der beiden Substanzen innerhalb einer vernünftigen Laufzeit. Die Retentionszeiten waren 14.5 min für Hyperforin und 17.3 min für Adhyperforin. Die höchsten Ausbeuten der zwei Phloroglucinderivate wurden mit den Extraktionsmitteln Ethanol 70% bis 100% erzielt. Ausschluss von Licht und Sauerstoff während des Extraktionsprozesses erhöhte die Stabilität der beiden Substanzen. Teezubereitungen enthielten weder Hyperforin noch Adhyperforin.

Es wird angenommen, dass die antidepressive Wirkung von Johanniskraut durch einen Synergismus verschiedener aktiver Substanzen zustande kommt. Zu diesem Thema wurden in **Kapitel 6** weitere Untersuchungen durchgeführt. Auf der Suche nach noch nicht bekannten aktiven Inhaltsstoffen wurden vier neue Bisanthrachinonglykoside, S-(+)-Skyrin-6-*O*-β-glucopyranosid (**1**), R-(-)-Skyrin-6-*O*-β-glucopyranosid (**2**), S-(+)-skyrin-6-*O*-β-xylopyranosid (**3**) and S-(+)-skyrin-6-*O*-α-arabinofuranosid (**4**), aus einem Ethanol-Wasser (1:1, m/m, Ze117) Trockenextrakt des Johanniskrauts isoliert. Am Anfang des Isolierungsprozesses stand eine Flüssig-Flüssig-Verteilung (Hexan-Toluol-Wasser-Ethylacetat-Ameisensäure (75:225:135:120:15, v/v)). Es folgte eine RP-18 VLC

(Acetonitril-Wasser-Trifluoressigsäure (5:4.9:0.1, v/v/v)). Die abschliessende präparative Dünnschichtchromatographie auf Silicagel (Ethylacetat-Acetonitril-Wasser (10:1:1, v/v/v)) führte zur Isolierung der Glykoside. Die Strukturen wurden mittels spektroskopischer Methoden, hauptsächlich NMR Spektroskopie und MS aufgeklärt. Die Acetylierung der Verbindungen **1** und **2** war hilfreich, um die Position der Glykosidierung zu bestimmen. Der Circulardichroismus wurde eingesetzt, um die axiale Stereochemie zu bestimmen. Es konnte gezeigt werden, dass **1** und **2** Atropisomere sind. Die Stereochemie von **3** und **4** stimmte mit **1** überein. **1** und **2** zeigten eine moderate Hemmung der Rezeptorbindung an CRH-1 (Corticotropin-Releasing-Hormon) Rezeptoren.

Objectives

Hypericum perforatum has gained importance in the medical treatment of mild to moderate depression in the last few years. Its efficiency has been shown in various clinical studies. As the active principle(s) and the mode(s) of action of *Hypericum perforatum* have not been fully understood yet, criterions for high quality extracts are still under discussion. One of the tools to provide extracts with constant composition and efficiency is the standardization on a certain content of hypericin. However, the availability of hypericin of constant quality represents a problem. Physicochemical properties, as the molar coefficient of absorbance for example, are not consistent. The present study aimed at developing an efficient and repeatable isolation procedure for hypericin and pseudohypericin from *Hypericum perforatum*. Investigations on the absorbance data, homoassociation behavior, solubility and stability of the isolates should provide further insight into their physicochemical properties. Various methods for the quantification of hypericin exist. However, as not all the methods come up with the same result, further research on this subject seemed reasonable. Effects of the extraction and quantification method, the drug sample and commercial dry extracts, the extracting solvent and irradiation with light on the final result were to be examined. Hyperforin has been discussed as one of the possible active principles of *Hypericum perforatum* recently. Still, only few data about the extractability of hyperforin from the drug have been published. Therefore, it was a further scope of this study to find a solvent suited for the extraction of hyperforin and also to get further information about the stability of the phloroglucin derivatives. Finally, fractionation of extracts of *Hypericum perforatum*, isolation of new compounds and their testing in receptor binding studies were planned with the aim of finding further biologically active constituents that possibly contribute to the antidepressant effect.

1 Efficacy and Quality of Extracts from *Hypericum perforatum*

Hypericum perforatum is widely used for the treatment of mild to moderate depressive states. Its superiority to placebo has been shown in several extensive clinical studies. The main advantage over tricyclic antidepressants is the low prevalence of short-term side effects improving compliance (Linde and Mulrow, 1999). A recent study (Schrader, in press) came up with the same result for the selective serotonin re-uptake inhibitor fluoxetine. In addition, Schrader showed the extract Ze117 of *Hypericum perforatum* to be as efficacious as fluoxetine. Still, long term (over six weeks) evaluations of effectiveness and also of side effects have to be done in future studies (Linde and Mulrow, 1999).
Besides investigations on the effectiveness, intense research has been done on quality control to provide manufactured extracts with constant efficiency and safety. The comparability of the preparations is another matter of concern. Various approaches are existing to guarantee high and constant quality of extracts from Hyperici herba, as standardization on a certain amount of hypericin, application of fingerprint chromatography and determination of the drug-to-extract ratio (Figure 1). As hyperforin has recently been controversially discussed to be one of the active substances in *Hypericum perforatum*, its use for standardization is to consider. However, the questions concerning the stability of hyperforin have to be answered first (see section 5).

Standardization on a Certain Amount of Hypericin

As the active principle(s) and the mode of action of *Hypericum perforatum* have not been clarified yet, the pharmaceutical quality of the extracts was characterized on the basis of the content of hypericin, which is the most characteristic component of *Hypericum perforatum*. Its high coefficient of absorbance (51712, see section 3) at the specific wavelength of 588 nm makes it predestined to be a reference compound. However, its use is not problem-free. Various factors are influencing the evaluated content of hypericin, which have to be considered, if the amount of hypericin is made a criterion for the quality of an extract (Figure 1).

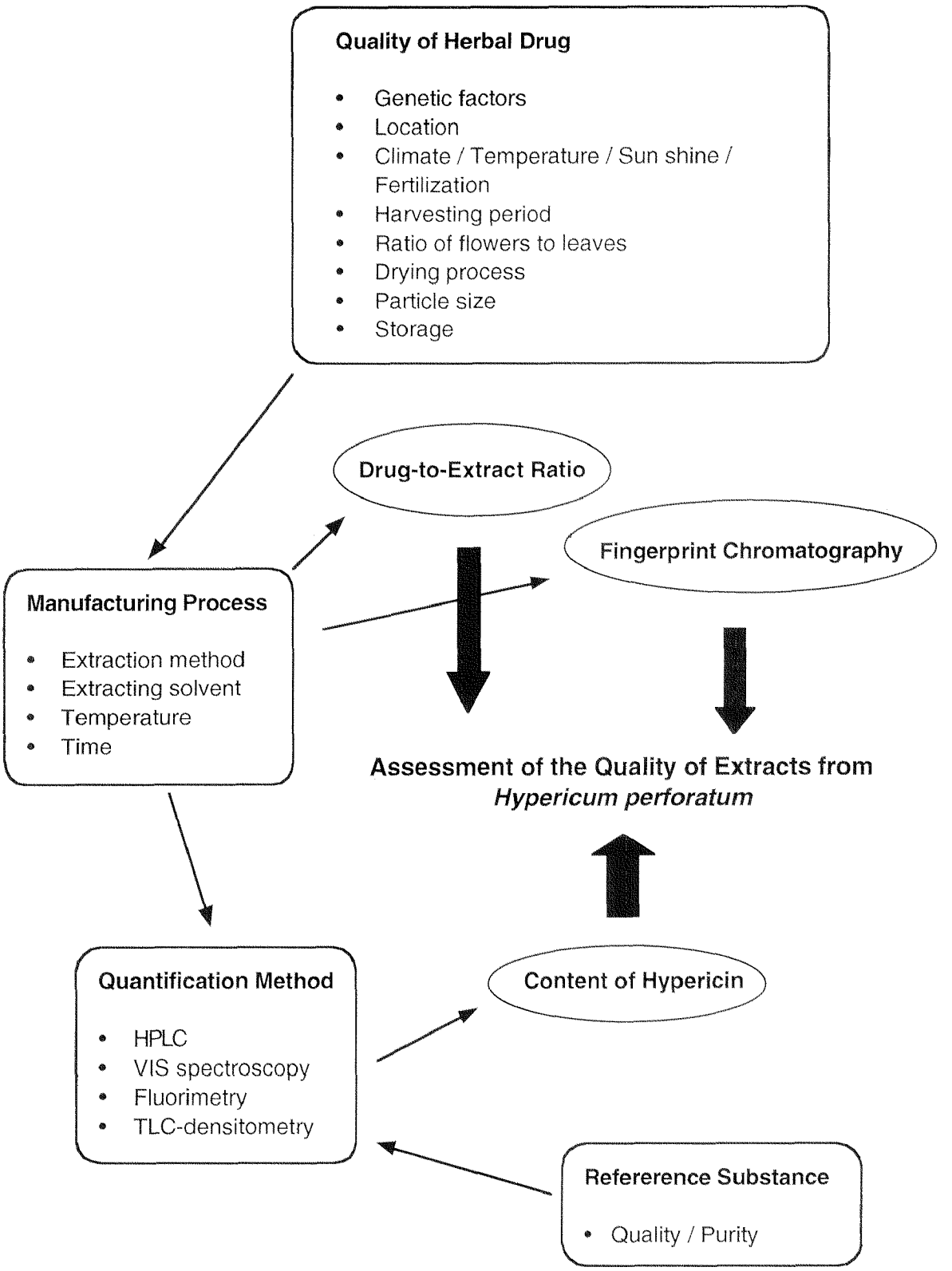


Figure 1 Tools for the assessment of the quality of extracts from *Hypericum perforatum* and some factors influencing the results

The **quality of the herbal drug** is one of the features affecting the content of hypericin. It already starts with the seedcorn: Büter *et al.* (1998) found genetic factors to play an important role. It goes on with environmental effects, Jensen *et al.* (1995) reporting location to have its influence. They also showed in a controlled-environment study that hypericin levels increased linearly with increasing temperatures. One possibility to diminish the variability of the plant material is cultivation.
Southwell and Campbell (1991) reported hypericin to be localized in glands in all parts of the topgrowth, while levels were higher in upper than in lower foliage. The highest concentrations were found in blossoms and capsules. Investigations of Braunewell (1991) showed the hypericin content to decrease from buds to blossoms to capsules. Therefore, the final content of hypericin in the drug sample also depends on the ratio of the flowering parts to leaves, for which the stage of development at the harvesting period is responsible. This goes together with the finding that highest amounts of hypericin in the herb are found at full blooming (Kartnig *et al.*, 1997). Brantner *et al.* (1994) investigated the drying process, finding drying of the plant material in the microwave to give highest amounts of hypericin. Storage conditions and particle size are further points of concern. The monograph in the European Pharmacopoeia (Ph. Eur. III, suppl. 2000) is a mean to guarantee high quality of the herbal drug.
The **manufacturing process** is decisive for the final content of hypericin in the extract. The extracting solvent, temperature, time and the method are influencing results (see section 4 and 5; Gaedcke, 1997).
Various **quantification methods** have been developed for the evaluation of the hypericin content making use of HPLC with UV/VIS or fluorimetric detection, VIS spectroscopy, fluorimetry and TLC-densitometry (see section 4). As their specificity is not the same, they do not all come up to the same content of hypericin (see section 4).
In connection with quantification, the availability and consistent quality of the **reference substance** is important (see section 3). Its physicochemical properties have to be known in order to assess influences of the reference substance on the result.

Büter *et al.* (1998) found no significant correlation between the content of hypericin and any other of the tested compounds. Therefore, standardization based exclusively on the content of hypericin does not guarantee a constant composition of the extracts. This is true at least if different sources of raw plant material are used. Furthermore, a direct

correlation of the content of hypericin and the therapeutic effect does not exist, not allowing a direct prediction of the effectiveness.

However, high amounts of hypericin in an extract indicate that the processing of the drug has been mild, preventing also other constituents from degradation. Too high drying temperatures, for example, would decrease the content of hypericin, as it is sensitive to heat. As the solubility of hypericin in common solvents is low, high yields of hypericin also correlate with high yields of better soluble compounds as flavonoid glycosides for example (Meier and Linnenbrink, 1996).

Fingerprint Chromatography

It is assumed that the antidepressant effect results from a synergism of several active constituents as hypericin, pseudohypericin, amentoflavone, procyanidins, hyperforin and others (Simmen *et al.*, 1999; Butterweck, 1997; Baureithel *et al.*, 1997). Extracts can differ in their content of potentially active ingredients depending among others on the plant material and the extraction method. The concentration of hyperforin, for example, is high in extracts prepared with aqueous ethanol 70% to ethanol 100% and low in extracts obtained with ethanol 50% (see section 5). Variations in the composition of the extracts can modify the effects of the preparations. Fingerprint chromatographs allow the evaluation of the whole range of constituents. They are tools for the comparison of extracts and products and the validation of extraction procedures. TLC-densitometry (Schütt, 1996) and HPLC methods (Table 4.1 C in section 4; e.g. Hölzl and Ostrowski, 1987) are in use. The HPLC methods consider hypericin, pseudohypericin, hyperforin and the whole range of flavonoids. Although the flavonoids of *Hypericum perforatum* are ubiquitous, their pattern is very characteristic for the species (Meier, 1999).
HPLC-fingerprints of common commercial extracts, standardized on the basis of hypericin, were in good agreement, also for minor peaks (Meier and Linnenbrink, 1996). This indicates that, besides all reservations, the content of hypericin can be used to get comparable products.

Drug-to-Extract Ratio

The drug-to-extract ratio is a further tool to describe the quality of an extract. It is influenced by the extractive matter of the plant material,

the manufacturing process and the extracting solvent. The common commercial extracts of Hyperici herba reveal a drug-to-extract ratio of 4-7:1 (Schulz, 1991). It allows to check the dosage recommendations of the commission E and ESCOP, which refer to the drug (Steinhoff, 1998).
The amount of extractive matter is influenced by the quality of the plant material. Extracts with high yields of hypericin reveal a low drug-to-extract ratio. Such extracts derive from plant material with high portions of blossoms and buds, which are rich in hypericin, yield high amounts of extractive matter and therefore lead to low drug-to-extract ratios. High portions of stems lead to extracts containing less hypericin and extractive matter, resulting in higher drug-to-extract ratios. Denke *et al.* (1999) found that N-fertilizing increased the yield of plant material, but lowered the concentration of secondary metabolites. The relative composition of the secondary metabolites was changed as well.
As the same drug-to-extract ratio does not guarantee similar compositions of the extracts, it is questionable if the drug-to-extract ratio alone is suited for the comparison of different commercial extracts.

Consequently, the meaningfulness of each approach on its own is limited. However, taken all together they provide a powerful tool to ensure the quality of extracts and products of Hyperici herba. They allow the comparison of extracts and the validation of extraction procedures. If preparations, which are similar on the basis of analytical investigations, also reveal the same antidepressant effect, is under discussion, as the active principles are not known. This brings up the question if clinical trials have to be done for each preparation or if analytical similarity with a tested product is proof enough for its efficiency.
To improve the comparability of products, declarations of the products of Hyperici herba should include the quality of the genuine drug, the amount of extract, the content of hypericin, the drug-to-extract-ratio, the extracting solvent and the manufacturing process.

References

Baureithel, K. H., Berger Büter, K., Engesser, A., Burkard, W. and Schaffner, W.: Inhibition of Benzodiazepine Binding in Vitro by Amentoflavone, a Constituent of Various Species of *Hypericum*. *Pharm. Acta Helv.* **72**: 153-157 (1997)

Brantner, A., Kartnig, Th. and Quehenberger, F.: Vergleichende phytochemische Untersuchungen an *Hypericum perforatum* L. und *Hypericum maculatum* Crantz. *Sci. Pharm.* **62**: 261-276 (1994)

Braunewell, H.: Ökologische, ontogenetische und morphogenetische Einflüsse auf Ertrag und Inhaltsstoffgehalt von *Hypericum spp.* (Johanniskraut). Thesis. Agrarwissenschaften. Justus-Liebig Universität Giessen, 1991

Büter, B., Orlacchio, C., Soldati, A. and Berger, K.: Significance of Generic and Environmental Aspects in the Field Cultivation of *Hypericum perforatum*. *Planta Med.* **64**: 431-437 (1998)

Butterweck, V.: Beitrag zur Pharmakologie und Wirkstoff-Findung von *Hypericum perforatum* L. Thesis. Mathematisch-Naturwissenschaftliche Fakultät. Westfälische Wilhelms-Universität Münster, 1997

Denke, A., Schempp, H., Mann, E., Schneider, W. and Elstner, E. F.: Biochemical Activities of Extracts from *Hypericum perforatum* L. *Arzneim.-Forsch./Drug Res.* **49**: 120-125 (1999)

European Pharmacopoeia (Ph. Eur. III, suppl. 2000), Hypericum Monograph, Strasbourg, Cedex, Council of Europe

Gaedcke, F.: Johanniskraut und dessen Zubereitungen. Qualitätsbeurteilung über ein selektives, reproduzierbares HPLC-Verfahren. *Dtsch. Apoth. Ztg.* **137**: 3753-3757 (1997)

Hölzl, J. and Ostrowski, E.: Johanniskraut (*Hypericum perforatum* L.). HPLC-Analyse der wichtigen Inhaltsstoffe und deren Variabilität in einer Population. *Dtsch. Apoth. Ztg.* **127**: 1227-1230 (1987)

Jensen, K. I. N., Gaul, S. O., Specht, E. G. and Doohan, D. J.: Hypericin Content of Nova Scotia Biotypes of *Hypericum perforatum* L. *Can. J. Plant Sci.* **75**: 923-926 (1995)

Kartnig, Th., Heydel, B. and Lässer, L.: Johanniskraut aus Schweizer Arzneipflanzenkultur. *Agrarforschung* **4**: 299-302 (1997)

Linde, K. and Mulrow, C. D.: St John's Wort for Depression (Cochrane Review). In: *The Cochrane Library* **3**: Oxford, 1999, Update Software. http://ovid.sams.ch/ovidweb/ovidweb.cgi

Meier, B. and Linnenbrink, N.: Status und Vergleichbarkeit pflanzlicher Arzneimittel. Wie schafft man Transparenz? *Dtsch. Apoth. Ztg.* **136**: 4205-4219 (1996)

Meier, B.: The Science Behind *Hypericum*. *Advances In Therapy* **16**: 135-147 (1999)

Schrader, E.: Equivalence of St John's Wort Extract Ze117 and Fluoxetine: A Randomised, Controlled Study in Mild-Moderate Depression. *International Clinical Psychopharmacology*, in press

Schulz, V.: Johanniskraut als Antidepressivum. In: *Rationale Phytotherapie: Ratgeber für die ärztliche Praxis.* Springer, Berlin, Heidelberg, New York, 1996, pp 55-71

Schütt, H.: Morphologische, phytochemische und botanische Untersuchungen zur Selektion hypericin-, pseudohypericin- und flavonoidreicher *Hypericum perforatum* L. Stämme. Thesis. *Dissertationes Botanicae* **263**. Cramer, J. Berlin, Stuttgart, 1996

Simmen, U., Burkard, W., Berger, K., Schaffner, W. and Lundstrom, K.: Extracts and Constituents of *Hypericum perforatum* inhibit the Binding of Various Ligands to Recombinant Receptors expressed with the Semliki Forest Virus System. *J. of Receptor & Signal Transduction Research* **19**: 59-74 (1999)

Southwell, I. A. and Campbell, M. H.: Hypericin Content Variation in *Hypericum perforatum* in Australia. *Phytochemistry* **30**: 475-478 (1991)

Steinhoff, B.: Standardisierung der Qualität von Johanniskrautdroge und -zubereitungen. *Z. Arz. Gew.pfl.* **3**: 63-66 (1998)

2 Isolation of the Reference Compounds Hypericin and Pseudohypericin

2.1 Introduction

As hypericin still plays an important role in standardization and quality control of *Hypericum perforatum*, the limited accessibility of pure hypericin and pseudohypericin as reference standards represents a problem.
Various methods to gain hypericin have been developed, including isolation from natural sources, synthesis and semi-synthesis (Meyer, 1993). Most methods are quite tedious, time-consuming and lacking from high yield. Some of them do not separate hypericin and pseudohypericin well. Moreover, the hypericins described in literature seem to differ in their physicochemical properties (see section 3). Difficulties in the isolation of hypericin from Hyperici herba arise from its bad solubility in most organic solvents and its low content in the herb.

The main steps of some selected isolation methods are described in the following:

- Hostettmann (1980) reported the isolation of pure **hypericin** and **pseudohypericin**, submitting a crude extract of *Hypericum perforatum* to Droplet Countercurrent Chromatography (DCCC) with chloroform - methanol - water (65:35:20).

- Knox and Dodge (1985) isolated **hypericin** from the glandular trichomes located on the calyx of *Hypericum hirsutum*. They were extracted with 90% aqueous acetone. Chlorophyll was removed by partitioning against light petroleum ether. Paper chromatography with acetone - light petroleum ether (3:7) and thin layer chromatography with chloroform - methanol (3:1) or diethyl ether - methanol (9:1) were used for purification.

- Meruleo *et al.* (1988) extracted **hypericin** and **pseudohypericin** from *Hypericum triquetrifolium*, removing chlorophyll with dichloromethane and doing extraction with acetone in a Soxhlet extractor. Column chromatography on silica gel with the solvents dichloromethane - acetone - methanol (75:15:10) and dichloromethane - acetone - methanol (68.8:18.7:12.5) led to the

isolation of hypericin and pseudohypericin. Hudson *et al.* (1991) applied a similar method to isolate hypericin.

- Maisenbacher (1991) extracted blossoms of *Hypericum perforatum* with acetone in a Soxhlet extractor. Acidification (pH 2.5) with acidic acid - water (25:75) led to precipitation. The precipitate was chromatographed on a polyamide column. The chromatography was started with methanol, hypericin and pseudohypericin remaining adsorbed on the column. The naphthodianthrones were eluted with methanol - acetone - water (4:4:2) containing 1% potassium hydroxide into a vessel that was filled with methanol - acidic acid. Precipitation with acidic acid - water (25:75) and HPLC separation of the precipitate led to the isolation of **hypericin** and **pseudohypericin.**

- The isolation procedure of Falk and Schmitzberger (1992) included extraction of blossoms of Hyperici herba with 2-butanone using an ultra turax device. The obtained extract was dissolved in methanol - water (4:1). Insoluble residues were taken off. Removal of chlorophyll with dichloromethane was followed by a separation on Sephadex®-LH-20 with dichloromethane - methanol - water (4:5:1). Final purification was done by Droplet Countercurrent Chromatography (DCCC) with dichloromethane - methanol - water (10:8:6), **hypericinate** and **pseudohypericinate** being the isolated products.

- Butterweck (1997) fractionated a commercial extract on Sephadex®-LH-20 with ethanol - water (3:1). The final isolation of **hypericin** and **pseudohypericin** was done by Multi-Layer Countercurrent Chromatography (MLCCC), using chloroform - methanol - water (65:35:20) as solvent system.

- Sattler (1997) started with the removal of chlorophyll from plant material of *Hypericum perforatum* with dichloromethane followed by extraction with acetone using a Soxhlet extractor. The extract was chromatographed on a polyamide column. The hypericin fraction was eluted with methanol - ammonia (99:1), after elution of the other constituents with methanol. Finial purification of **hypericin** was done with RP-18 HPLC with a mobile phase composed of methanol - acetonitrile - phosphoric acid (89.5:10:0.5).

- Vandenogaerde *et al.* (1998b) percolated Hyperici herba with dichloromethane, ethyl acetate and acetone to isolate **pseudohypericin**. The acetone extract was chromatographed on a silica gel column using ethyl acetate, ethyl acetate - water (100:2.5) and finally ethyl acetate - water - acetone (100:2.5:30) as eluent. The last step was a column chromatography on Sephadex®-LH-20 with methanol - acetone - dichloromethane (30:15:55) that led to pure pseudohypericin.

The methods described did not allow the application of high loading doses, making repeated running of the procedures necessary to get yields of several milligrams. Butterweck (1997), for example, had to carry out the MLCCC separation 10 times to get 100 mg hypericin and 200 mg pseudohypericin. The preceding fractionation on Sephadex®-LH-20, using a commercial extract of Hyperici herba as starting material, had to be done even more often.

Against this background, it was the aim of this study to develop a simple and efficient isolation method for hypericin and pseudohypericin. The method should improve the accessibility and provide a consistent quality of these reference standards.

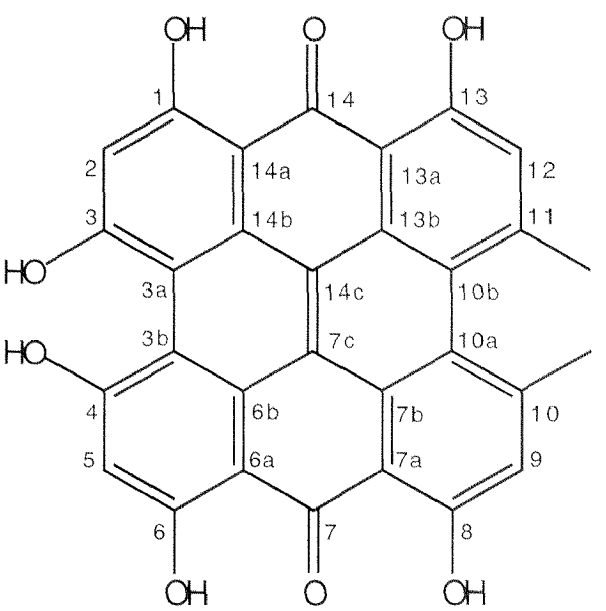


Figure 2.1 Structural formula of hypericin

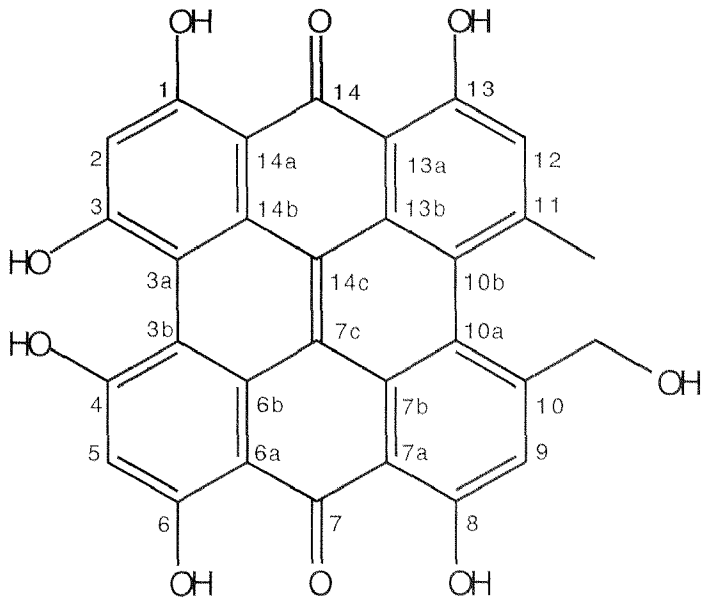


Figure 2.2 Structural formula of pseudohypericin

2.2 Experimental

Materials

The ethanol - water (1:1, m/m) dry extract (Ze117) from Hyperici herba was obtained from Zeller Company (CH-Romanshorn). Ethanol absolute, hexane, ethyl acetate, methanol, tetrahydrofuran and acetonitrile were of HPLC quality (Romil Chemicals, GB-Shepshed). Toluene (p.a.) was purchased from Scharlau (EGT Chemie, CH-Tägerig), formic acid (p.a.) from Merck (CH-Dietikon) and pyridine (p.a.) from Fluka (CH-Buchs). *Ortho*-phosphoric acid 85% (Ph. Helv. VI / Ph. Eur.), sulfuric acid 95% puriss (Ph. Eur.) and vanillin were purchased from Hänseler (CH-Herisau). Sodium dihydrogen phosphate (z.A.) was from Merck (CH-Dietikon). De-ionized water was obtained using an NANO-pure cartridge system (Skan, CH-Basel). Regenerated cellulose syringe filters (0.2 µm, 13 mm) were from Schleicher&Schuell (D-Dassel). Silica gel 60 F_{254} pre-coated aluminum sheets (layer thickness 0.2 mm) were obtained from Merck (CH-Dietikon). The NMR solvent dimethylsulfoxide-d_6 was purchased from Dr. Glaser AG (CH-Basel).

Instrumentation and Procedures

High-Speed Countercurrent Chromatography

The separation of hypericin and pseudohypericin was carried out with the countercurrent chromatography instrument KROMATON II from S.E.A.B. Company (F-Villejuif).
The cooling unit (Model SK 3390) was from RITTAL-Werk (D-Herborn). The manometric module 807 and the high-pressure pump (Model 305) were from Gilson (F-Villiers-le-Bel). The fraction collector LKB 7000 UltroRac was obtained from LKB Instrument G.m.b.H. (D-Gräfelfing).
The analytical column (total volume 75 ml) of the Kromaton II was used for preliminary tests and the preparative column (total volume 1 l) for the scale-up experiment. The solvent system of the final separation was toluene - acetonitrile - water - ethanol absolute (3:4:3:2, v/v) the upper phase being the stationary phase. The rotation speed was 460 rpm, the flow rate 7 ml/min and the column temperature 20 °C. The flow direction was from center to periphery of the column (C→P). The displaced amount of stationary phase was 940 ml, the fraction size 7 ml and the size of the sample loop 10 ml.

TLC

Analyses were carried out on silica gel $60F_{254}$ (Merck, CH-Dietikon) with the solvent system ethyl acetate - formic acid - water (30:2:3, v/v) over a distance of approximately 12 cm. For detection of potential impurities, the chromatograms were sprayed with a vanillin reagent, consisting of two solutions (5% ethanolic sulfuric acid and 1% vanillin in ethanol) applied one after another. UV_{254} and UV_{366} detection were also performed.

HPLC

Instrumentation. HPLC analyses were performed using a Hewlett Packard instrument (Model 79994A analytical workstation, model 1090 liquid chromatograph, model 1040 diode-array detector). For chromatographic separations a Knauer (D-Berlin) prepacked cartridge column (250 x 4 mm I.D., 5 µm) filled with Spherisorb S ODS2 and a guard column (10 x 4 mm I.D.) of the same material was used.

Chromatographic conditions. The partly modified HPLC method developed by Hölzl and Ostrowski (1987) was used to analyze the fractions of the isolation process and the purity of the isolated compounds. The mobile phase consisted of solvent A (methanol), B (acetonitrile - water - *ortho*-phosphoric acid 85% (19:80:1, v/v/v)) and C (acetonitrile - methanol - *ortho*-phosphoric acid 85% (59:40:1, v/v/v)). The elution profile was: 0-8 min 100% B, 8-30 min 100% to 50% B in C, 30-45 min 50% B in C to 100% C, 45-70 min 100% C, 70-75 min 100% A (wash out), 75-85 min 100% B (re-conditioning). All gradient steps were linear. The flow rate was 0.6 ml/min, the column temperature 25 °C and the injection volume 25 µl. The detection wavelength of the DAD was set at 254 nm. For the evaluation of the purity of the isolated compounds, the detection wavelength was set at 254, 290, 366, 450, 555 and 590 nm.
The modified HPLC method of Kerb *et al.* (1996) was applied to evaluate the yield of the isolation procedure. Solvents used were solvent A (methanol) and solvent B (methanol - tetrahydrofuran - buffer (9:6:5, v/v)). The buffer consisted of 13.8 g sodium dihydrogen phosphate in 1 litre water adjusted to pH 4.0 with *ortho*-phosphoric acid 85%. The elution profile was: 0-12 min 100% B, 12-17 min 100% A and 17-27 min 100% B. The flow rate was 0.8 ml/min for solvent B and 1 ml/min for solvent A. The column temperature was set at 25 °C and the injection volume at 25 µl.

NMR spectroscopy

NMR spectra of pseudohypericin were recorded on a Bruker AMX-300 spectrometer (operating at 300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C) (Spectrospin, CH-Fällanden). NMR spectra of hypericin were obtained from a Bruker AMX-600 spectrometer (operating at 600.13 MHz for ^{1}H and 150.92 MHz for ^{13}C). All spectra were measured in dimethylsulfoxide-d_6 and referenced to residual hydrogen (2.5 ppm) or carbon (39.7 ppm) resonances.

MS spectrometry

EIMS spectra were measured on a Hitachi-Perkin Elmer mass spectrometer at 70 eV.

Ultraviolet spectroscopy

See section 3.2.3.1

Isolation procedure

The isolation procedure is summarized in Figure 2.3 as a flow chart.
An ethanol - water (1:1, m/m) dry extract from Zeller Company (Ze117) was fractionated by liquid-liquid-partition. 20.0 g of the extract was shaken with the solvent system hexane - toluene - water - ethyl acetate - formic acid (75:225:135:120:15, v/v).
The lower level was shaken once more with a fresh upper level. The upper levels were combined, extracted with a fresh lower level, washed three times with 200 ml water and evaporated below 35 °C with reduced pressure. The residue was dissolved in 20 ml methanol and stored at 8 °C for 2 hours. The formed precipitate was removed by filtration through a filter paper. 20.0 g dry extract (Ze117) yielded 25.2 mg precipitate and 320.0 mg filtrate A. The procedure was repeated several times (further processing of filtrate A see section 6).
80.0 mg of the precipitate were dissolved in 170 mg pyridine, 4 ml upper and 4 ml lower level of the solvent system toluene - acetonitrile - water - ethanol absolute (3:4:3:2, v/v) and sonicated for 15 min. The sample was filtered through a regenerated cellulose syringe filter and subjected to High-Speed Countercurrent Chromatography (HSCCC) using the solvent system toluene - acetonitrile - water - ethanol absolute (3:4:3:2, v/v). The upper level was the stationary phase. The fraction size was 7 ml. The fractions containing hypericin (fractions 23-26) and pseudohypericin (fractions 42-123) were selected by TLC analysis (silica gel, ethyl acetate - formic acid - water (30:2:3, v/v)) and combined, respectively.

The solutions were evaporated under reduced pressure below 35 °C. This led to the isolation of 18.8 mg hypericin and 14.5 mg pseudohypericin. The procedure was repeated four times. The compounds were completely dried in a vacuum drying oven at 25-28 °C and then subjected to TLC, HPLC, UV/VIS spectroscopy, NMR and MS analysis. A further purification was not necessary.

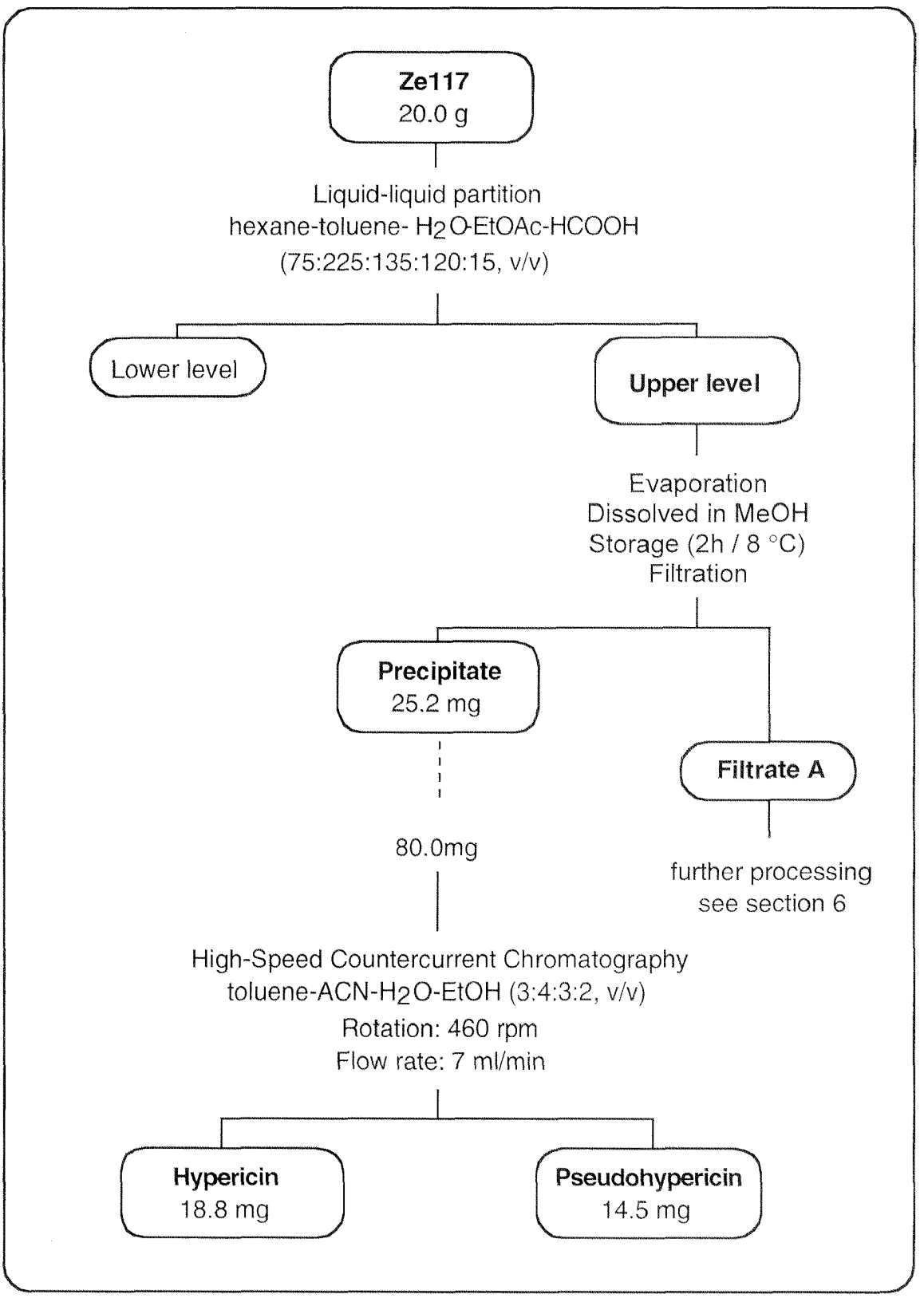


Figure 2.3 Isolation procedure of hypericin and pseudohypericin

2.3 Results and Discussion

2.3.1 Isolation Procedure

Elaboration of the Isolation Procedure

The isolation of hypericin is complicated by its bad solubility in most organic solvents (see section 3). The low content of hypericin and its derivatives in the dried herb of *Hypericum perforatum* (0.1%) is another limiting factor. Consequently, the first step was to find an enrichment process. It should be a simple process easily to be reproduced. A liquid-liquid partition with hexane - toluene - water - ethyl acetate - formic acid (75:225:135:120:15, v/v) was found to be suitable yielding a fraction enriched with hypericin and pseudohypericin. The second step was to separate hypericin and pseudohypericin. Various methods were examined, as open column on Sephadex® LH-20, preparative TLC (silica gel) and HPLC (RP-18). The yields were not satisfactory, mainly caused by the bad solubility limiting the loading dose. On silica gel, hypericin was partly irreversible adsorbed. In HPLC (RP-18) hypericin showed tailing already in analytical concentrations, preparative concentrations leading to extreme peak broadening. Besides bad solubility, formation of tautomers (Dax *et al.*, 1999) and homoassociates (Falk and Meyer, 1994), revealing different chromatographic properties could be an explanation. Extracts of Hyperici herba led to irreversible colouring of the chromatographic materials, proposing to look for a method that works without solid support. Finally High-Speed Countercurrent Chromatography (HSCCC) proved to be well adapted for separation of hypericin and pseudohypericin. It is a form of liquid-liquid chromatography that does not use a solid support and therefore avoids problems as adsorptive loss and damaged columns. It provides the mildest possible conditions for a chromatographic separation (McAlpine and Hochlowski, 1988). To achieve a separation of hypericin and pseudohypericin, a two-phase system had to be found in which the two compounds revealed different partition coefficients. For solvent selection, the dry extract Ze117 was partitioned between the two phases of the solvent systems, which were subjected to TLC analysis (silica gel, ethyl acetate - formic acid - water (30:2:3, v/v/v)) afterwards. The systems giving a slight separation of hypericin and pseudohypericin were examined by HSCCC, using the analytical coil. After having tested various solvent-systems mentioned in literature (Abbott and Kleiman, 1991) the system toluene - acetonitrile - water -

ethanol absolute (3:4:3:2, v/v) was found to be the most suitable. Variation of the ratio of the solvents proved the chosen one to give best results as well.

Composition of Various Fractions of the Isolation Procedure

The lower level, the precipitate and filtrate A (Figure 2.3) were analyzed by HPLC with the method of Hölzl and Ostrowski (1987).
The lower level consisted of the more polar compounds as chlorogenic acid, rutin, hyperoside and isoquercetin. The compounds of the precipitate were mainly hypericin and pseudohypericin accompanied by small amounts of quercetin, biapigenin, S-(+)-skyrin-6-*O*-β-glucopyranoside and R-(-)-skyrin-6-*O*-β-glucopyranoside. The filtrate A contained quercetin, biapigenin, S-(+)-skyrin-6-*O*-β-glucopyranoside and R-(-)-skyrin-6-*O*-β-glucopyranoside, accompanied by very small amounts of S-(+)-skyrin-6-*O*-β-xylopyranoside and S-(+)-skyrin-6-*O*-α-arabinofuranoside (see section 6).

Yield

The liquid-liquid-partition of 20.0 g Ze117 yielded 25.2 mg precipitate (0.13% of 20.0 g Ze117; Table 2.1). Its content of hypericin and pseudohypericin was analyzed by HPLC using the modified method developed by Kerb *et al.* (1996). The precipitate consisted of 41.0% hypericin and 32.2% pseudohypericin, corresponding to 0.0517% and 0.0406% of 20.0 g Ze117, respectively. The liquid-liquid-partition was carried out several times leading to similar results.

HSCCC separation of 80 mg of the precipitate led to the isolation of 18.8 mg hypericin and 14.5 mg pseudohypericin, corresponding to 23.5% and 18.1% of 80 mg precipitate, respectively. The procedure was done four times, results being in accordance.

The contents of hypericin and pseudohypericin in Ze117 determined by HPLC (see section 4, Tables 4.1.6 and 4.1.7, Kerb method) were 0.0711% and 0.143%, respectively. Referring to these results, the overall yield of the method including liquid-liquid-partition and HSCCC separation is 42.2% for hypericin and 16.1% for pseudohypericin.

In this way, 76 mg hypericin and 59 mg pseudohypericin were isolated.

Table 2.1 Yield of 20.0 g Ze117 subjected to liquid-liquid-partition and HSCCC

Method	Fraction / Compound	Yield [mg] of 20.0 g Ze117	Yield [%] of 20.0 g Ze117	Overall yield [1] [%]
Liquid-liquid-partition	Precipitate	25.2	0.13	
	Filtrate A	320.0	1.8	
HSCCC	Hypericin	5.93[2]	0.030	42.2
	Pseudohypericin	4.57[2]	0.023	16.1

[1] Percentage of hypericin and pseudohypericin isolated by liquid-liquid-partition and HSCCC, referring to the hypericin and pseudohypericin content of the extract determined by HPLC (see section 4, Tables 4.1.6 and 4.1.7, Kerb method)

[2] Yields of 80 mg precipitate were converted into yields of 25.2 mg precipitate, resulting from carrying out one liquid-liquid-partition with 20.0 g Ze117.

The loading dose in HSCCC was limiting the yield of the procedure, as low solubility and aggregation did not allow the application of more than 80 mg precipitate per run. Higher amounts led to bad separation of hypericin and pseudohypericin and the remaining of aggregates in the column system.

2.3.2 Characterization of the Isolated Compounds

TLC

TLC investigations of the isolated hypericin and pseudohypericin did not reveal any impurities.

HPLC

The method of Hölzl and Ostrowski (1987) was used to evaluate the purity of isolated hypericin and pseudohypericin. Solutions of the isolated compounds (10 µg/ml) showed no signals of impurities at the detection wavelengths 254, 366, 450, 555 and 590 nm. At 290 nm, however, there was an additional signal at 48 min, revealing an UV spectrum with absorption maxima at about 220 and 310 nm. Referring to the area integration values the HPLC purity at 290 nm was 93% for hypericin and pseudohypericin, whereas the other wavelengths showed a purity of 100%.

NMR

The measured NMR resonances of isolated hypericin and pseudohypericin were in agreement with the reported data. The assignments of published data, however, were not consistent for every signal. Tables 2.2 to 2.5 show measured and published NMR data of hypericin and pseudohypericin recorded in dimethylsulfoxide-d_6. The assignments of the isolated compounds were confirmed by HMQC and HMBC experiments. Further NMR data of hypericin and its salts recorded in tetrahydrofuran-d_8 and dimethylsulfoxide-d_6 were published by Kapinus *et al.*, 1999.
The compounds isolated by Falk and Schmitzberger (1992) revealed a resonance at 18.4 ppm in the ^{1}H NMR spectra. They assumed them to be the rapidly interconverting 3- and 4-phenolate ions, which were better soluble in common solvents than the non-ionized forms. As in the crude plant extract the same signals could be detected, they concluded that the extract contained hypericin and pseudohypericin in their ionized form. This finding was supported by the good extractability of the pigments from plant material, indicating that its genuine form is soluble (Falk and Schmitzberger, 1992). However, the isolates from own investigations did not reveal a resonance at 18.4 ppm and they were only sparingly soluble in common solvents (see section 3.3.3.2). It is possible that the ionized isolates were converted to non-ionized forms by the use of acid during the isolation process. Dax *et al.* (1999) stated the 3-hypericinate ion to be the predominating species in dimethylsulfoxide. As D_2O is usually present in deuterated solvents, the OH groups in position 3 and 4 were assumed to be involved in an isotope exchange with D_2O, resulting in the occasional absence of their resonances in ^{1}H NMR spectra (Dax *et al.*, 1999; Kapinus *et al.*, 1999).

MS

The EIMS of hypericin showed the molecular peak at m/z 504.2.
The EIMS of pseudohypericin gave a $[M - 2H]^-$ peak at 518.2

UV/VIS Spectroscopy

The position of the absorption maxima of hypericin and pseudohypericin were in accordance with literature data (Wynn *et al.*, 1995; Table 3.1). For further discussions see section 3.3.1.

Table 2.2 ^{1}H NMR spectral data (DMSO-d_6, δ ppm) of hypericin (1) and hypericinate (2)

H	Measured	Published data							
		Kapinus et al. (1999)	Smirnov et al. (1999)	Butterweck (1997)	Sattler (1997)	Falk, Meyer (1994)	Freeman et al. (1994)	Falk, Schoppel (1992)	Stock (1992)
	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
OH-3 or OH-4	-	-	-	-	-	-	-	-	-
OH-1,6	14.70 *s*	14.66	14.09	-	-	-	14.04 *s*	14.63 *s* broad	-
OH-8,13	14.06 *s*	14.00	14.74	-	-	-	14.67 *s*	14.02 *s* broad	-
CH-9,12	7.44 *s*	7.42	7.46	7.44 *s*	7.37 *s*	7.55 *s*	6.50 *s*	7.38 *s*	7.42 *s*
CH-2,5	6.58 *s*	6.57	6.59	6.58 *s*	6.50 *s*	6.68 *s*	7.35 *s*	6.53 *s*	6.57 *s*
CH_3-10,11	2.74 *s*	2.73	2.75	2.74 *s*	2.69 *s*	2.84 *s*	2.65 *s*	2.68 *s*	2.73 *s*

H	Published data							
	Maisenbacher (1991)	Berghöfer (1987)	Freytag (1984)	Banks et al. (1976)	Cameron, Raverty (1976)	Brockmann, Spitzner (1975)	Freeman et al. (1994)	Falk, Schmitzberger (1992)
	(1)	(1)	(1)	(1)	(1)	(1)	(2)	(2)
OH-3 or OH-4	-	-	-	-	-	-	18.34 *s*	18.39 *s*
OH-1,6	-	-	-	-	-	-	14.06 *s*	14.70 *s*
OH-8,13	-	-	-	-	-	-	14.67 *s*	14.06 *s*
CH-9,12	7.35 *s*	7.44 *s*	7.38	7.35 *s*	7.35 *s*	7.29 *s*	6.45 *s*	7.39 *s*
CH-2,5	6.48 *s*	6.58 *s*	6.53	6.52 *s*	6.52 *s*	6.44 *s*	7.34 *s*	6.51 *s*
CH_3-10,11	2.67 *s*	2.74 *s*	2.69	2.66 *s*	2.66 *s*	2.65 *s*	2.66 *s*	2.70 *s*

Table 2.3 ^{1}H NMR spectral data (DMSO-d_6, δ ppm, J Hz) of pseudohypericin (1) and pseudohypericinate (2)

H	Measured	Published data					
		Butterweck (1997)	Falk, Schmitzberger (1992)	Stock (1992)	Berghöfer (1987)	Cameron, Raverty (1976)	Brockmann, Spitzner (1975)
	(1)	(1)	(2)	(1)	(1)	(1)	(1)
OH-3	-	-	18.46[a] *s*	-	14.10[b] *d* (*J*= 6.5)	-	-
OH-4	-	-		-	14.73[b] *d* (*J*= 6.5)	-	-
OH-1,6	14.67 *s* (2H)	-	14.75 *s*	-	18.44	-	-
OH-8,13	14.15 *s* (2H)	-	14.12 *s*	-	18.44	-	-
CH-9	7.68 *s* (1H)	7.70 *s*	7.71 *s*	7.72 *s*	7.69 *s*	7.62 *s*	7.73 *s*
CH-12	7.41 *s* (1H)	7.44 *s*	7.46 *s*	7.46 *s*	7.44 *s*	7.31 *s*	7.44 *s*
CH-2,5	6.53 *s* (2H)	6.57 *s*	6.59 *s*	6.60 *s*, 6.56 *s*	6.57 *s*, 6.56 *s*	6.44 *s*, 6.43 *s*	6.56 *s*
CH_2OH-10	4.92 *s* broad (1H)		5.34 *s* broad	5.31 *t* (*J* = 5)	5.32 *t* (*J* = 5)		
CH_2OH-10 (B-part of the AB-system)	5.10 *d* (1H) (*J* = 13)	5.12 *d* (*J* = 13)	5.15 *d* (*J* = 12.8)	5.08 *dd* (*J* = 13)	5.08 *dd* (*J* = 13.5)	5.05 *d* (*J* = 13)	
CH_2OH-10 (A-part of the AB-system)	4.66 *d* (1H) (*J* = 12.9)	4.69 *d* (*J* = 13)	4.67 *d* (*J* = 12.8)	4.71 *dd* (*J* = 13)	4.68 *dd* (*J* = 13.5)	4.57 *d* (*J* = 13)	
CH_3-11	2.66 *s* (3H)	2.68 *s*	2.68 s	2.69 *s*	2.60 *s*	2.59 *s*	2.71 *s*

[a] Signal of OH-3 or OH-4

[b] Assignments bearing the same superscript may be interchanged

Table 2.4 ^{13}C NMR spectral data (DMSO-d_6, δ ppm) of hypericin (1) and hypericinate (2)

C	Measured	Published data				
		Kapinus et al. (1999)	Butterweck (1997)	Sattler (1997)	Falk, Schmitz-berger (1992)	Maisen-bacher (1991)
	(1)	(1)	(1)	(1)	(2)	(1)
1	168.04	168.0	168.07	174.41	166.0	174.5
2	105.49	105.5	105.54	105.47	105.4	105.4
3	174.12	173.6	174.31	161.24	174.7	161.2
3a	126.94	127.0	102.02	120.69	126.6[a]	119.3[a]
3b	126.94	127.0	102.02	120.69	126.6[a]	119.3[a]
4	174.12	173.6	174.31	161.24	174.7	161.2
5	105.49	105.5	105.54	105.47	105.4	105.4
6	168.04	168.0	168.07	174.41	166.0	174.0
6a	102.04	108.7	119.26	101.93	108.3	101.9
6b	119.12	126.1	126.77	126.85	126.0[a]	126.9[b]
7	183.56	184.9	183.56	183.35	183.3	183.3
7a	108.36	102.2	108.38	108.34	101.9	108.3
7b	126.05	119.0	121.42	121.25	119.2[b]	121.3[a]
7c	121.38	121.3	126.07	125.98	121.2	126.0[b]
8	161.35	161.5	161.34	168.04	161.2	168.0
9	118.85	118.4	118.85	118.69	118.6	118.6
10	143.81	144.0	143.81	143.53	143.5	143.5
10a	120.81	120.9	120.82	120.69	120.7[b]	120.7[a]
10b	120.81	120.9	120.82	120.69	120.7[b]	120.7[a]
11	143.81	144.0	143.81	143.53	143.5	143.5
12	118.85	118.4	118.85	118.69	118.6	118.6
13	161.35	161.5	161.34	168.04	161.2	168.0
13a	108.36	102.2	108.38	108.34	101.9	108.3
13b	126.05	119.0	121.42	121.25	119.2[b]	121.3[a]
14	183.56	184.9	183.56	183.35	183.3	183.3
14a	102.04	108.7	119.26	101.93	108.3	101.9
14b	119.12	126.1	126.97	126.85	126.0[a]	126.9[b]
14c	121.38	121.3	126.07	125.98	121.2	126.0[b]
CH_3-10,11	23.58	21.1	23.58	23.56	23.6	23.5

[a-b] Assignments bearing the same superscript may be interchanged

Table 2.5 ^{13}C NMR spectral data (DMSO-d_6, δ ppm) of pseudohypericin (1) and pseudohypericinate (2)

C	Measured	Published data	
		Butterweck (1997)	Falk, Schmitz-Berger (1992)
	(1)	(1)	(2)
1	168.04[a]	168.18[a]	168.2
2	105.38[b]	105.54[b]	95.1
3	173.79[c]	174.42[c]	174.9
3a	118.64	102.13[d]	130.6
3b	118.64	102.03[d]	130.6
4	173.56[c]	174.18[c]	174.9
5	105.34[b]	105.51[b]	95.1
6	167.93[a]	168.07[a]	168.2
6a	102.04[d]	119.10[e]	101.0
6b	126.82	126.95[f]	128.7
7	183.52[e]	183.56[g]	184.3
7a	109.14	109.21	99.9
7b	125.36[f]	121.32[h]	114.5
7c	121.14	125.45[i]	118.4
8	161.78	161.79	161.8
9	117.66	117.72	93.8
10	148.06	148.12	144.8
10a	119.07	119.10	115.0
10b	120.21	120.24	115.0
11	144.20	144.23	144.8
12	118.77	118.79	93.8
13	161.47	161.48	161.8
13a	108.31	108.32	99.9
13b	126.02[f]	121.36[h]	114.5
14	183.59[e]	183.59[g]	184.3
14a	102.15[d]	119.35[e]	101.0
14b	126.82	126.99[f]	128.7
14c	121.14	126.12[i]	118.4
CH_2OH-10	62.62	62.6	66.3
CH_3-11	23.03	23.2	27.8

[a-i] Assignments bearing the same superscript may be interchanged

2.4 Conclusions

The method described consists of two isolation steps, which can easily be reproduced: a liquid-liquid partition in a separatory funnel followed by High-Speed Countercurrent Chromatography. The avoidance of open column chromatography, used by most methods described in literature, simplifies the procedure. Overall yields are satisfactory, the method being more efficient for hypericin (42.2%) than for pseudohypericin (16.1%). The purity of the compounds was high, proven by TLC, HPLC, NMR, MS and UV/VIS spectroscopy. The molar coefficient of absorbance of the commercially available sample from Roth Company and literature data were lower than the value of the isolated hypericin. HPLC analysis, however, revealed an additional peak at 290 nm, which could not be identified. MS data and measured NMR resonances were in accordance with reported data.

Consequently, the developed procedure can be considered as an additional isolation method to gain hypericin and pseudohypericin. The purity of the isolates was sufficient allowing them to be used as reference standards. As the loading dose is limited, scaling-up is made difficult and therefore the method is not suited to improve accessibility of the two compounds. Still, the procedure described provides higher yields, is simpler and less time consuming than published methods.

2.5 References

Abbott, T. P. and Kleiman, R.: Solvent Selection Guide for Counter-Current Chromatography. *J. Chromatogr.* **538**: 109-118 (1991)

Amer, A. M., Falk, H. and Tran, H. T. N.: The Dissociation and Tautomerization Equilibria of Hypericin: Alkyl Protected Hydroxyl Derivatives. *Monatshefte für Chemie* **129**: 1237-1244 (1998)

Banks, H. J., Cameron, D. W. and Raverty, W. D.: Chemistry of the Coccoidea. Part II: Condensed Polycyclic Pigments from Two Australian Pseudococcids (Hemiptera). *Aust. J. Chem.* **29**: 1509-1521 (1976)

Berghöfer, R.: Analytik und Isolierung phenolischer Inhaltsstoffe von *Hypericum perforatum* L. aus Anbau und Wildvorkommen. Thesis. *Dissertationes Botanicae* **106**. Cramer, J. Berlin, Stuttgart, 1987

Brockmann, H. and Spitzner, D.: Die Konstitution des Pseudohypericins. *Tetrahedron Lett.* **1**: 37-40 (1975)

Butterweck, V.: Beitrag zur Pharmakologie und Wirkstoff-Findung von *Hypericum perforatum* L. Thesis. Mathematisch-Naturwissenschaftliche Fakultät. Westfälische Wilhelms-Universität Münster, 1997

Cameron, D. W. and Raverty, W. D.: Pseudohypericin and Other Phenanthroperylene Quinones. *Aust. J. Chem.* **29**: 1523-1533 (1976)

Dax, T. G., Falk H. and Kapinus E. I.: A Structural Proof for the Hypericin 1,6-Dioxo Tautomer. *Monatshefte für Chemie* **130**: 827-831 (1999)

Falk, H. and Meyer, J.: On the Homo- and Heteroassociation of Hypericin. *Monatshefte für Chemie* **125**: 753-762 (1994)

Falk, H. and Schmitzberger, W.: On the Nature of "Soluble" Hypericin in Hypericum Species. *Monatshefte für Chemie* **123**: 731-739 (1992)

Falk, H. and Schoppel, G.: On the Synthesis of Hypericin by Oxidative Trimethylemodin Anthrone and Emodin Anthrone Dimerization: Isohypericin. *Monatshefte für Chemie* **123**: 931-938 (1992)

Freeman, D., Frolow, F., Kapinus, E., Lavie, D., Lavie, G., Meruelo, D. and Mazur, Y.: Acidic Properties of Hypericin and its Octahydroxy Analogue in the Ground and Excited States. *J. Chem. Soc., Chem. Commun.*: 891-892 (1994)

Freytag, W. E.: Bestimmung von Hypericin und Nachweis von Pseudohypericin in *Hypericum perforatum* L. durch HPLC. *Dtsch. Apoth. Ztg.* **124**: 2383-2386 (1984)

Hölzl, J. and Ostrowski, E.: Johanniskraut (*Hypericum perforatum* L.). HPLC-Analyse der wichtigen Inhaltsstoffe und deren Variabilität in einer Population. *Dtsch. Apoth. Ztg.* **127**: 1227-1230 (1987)

Hostettmann, K.: Droplet Counter-Current Chromatography and its Application to the Preparative Scale Separation of Natural Products. *Planta medica* **39**: 13 (1980)

Hudson, J. B., Lopez-Bazzocchi, I. and Towers, G. H. N.: Antiviral Activities of Hypericin. *Antiviral Research* **15**: 101-112 (1991)

Kapinus, E. I., Falk, H. and Tran, H. T. N.: Spectroscopic Investigation of the Molecular Structure of Hypericin and its Salts. *Monatshefte für Chemie* **130**: 623-635 (1999)

Kerb, R., Brockmöller, J., Staffeldt, B., Ploch, M. and Roots, I.: Single-Dose and Steady-State Pharmacokinetics of Hypericin and Pseudohypericin. *Antimicrobial Agents and Chemotherapy* **40**: 2087-2093 (1996)

Knox, J. P. and Dodge, A. D.: Isolation and Activity of the Photodynamic Pigment Hypericin. *Plant, Cell and Environment* **8**: 19-25 (1985)

Maisenbacher, P.: Untersuchungen zur Analytik von Johanniskrautöl. Thesis. Fakultät für Chemie und Pharmazie. Eberhard-Karls-Universtität Tübingen, 1991

Maisenbacher, P. and Kovar, K.-A.: Analysis and Stability of Hyperici Oleum. *Planta Medica* **58**: 351-354 (1992)

McAlpine, J. B. and Hochlowski, J.: Countercurrent Chromatography. *Natural Products Isolation*. Wagman, G. H. and Cooper, R. Elsevier, New York, 1988

Meruelo, D., Lavie, G. and Lavie, D.: Therapeutic Agents with Dramatic Antiretroviral Activity and Little Toxicity at Effective Doses: Aromatic Polycyclic Diones Hypericin and Pseudohypericin. *Proc. Natl. Acad. Sci. USA* **85**: 5230-5234 (1988)

Meyer, J.: Assoziationsverhalten und spektroskopische Eigenschaften von Hypericin. Thesis. Technisch Naturwissenschaftliche Fakultät. Johannes-Kepler-Univesität Linz, 1993

Sattler, S.: Naphthodianthrone aus *Hypericum perforatum* L.: Isolierung, Pharmakokinetik, Löslichkeitsverbesserung und Absorptionsstudien am Caco-2 Zellkulturmodell. Thesis. Pharmazie und Lebensmittelchemie. Philipps-Universität Marburg/Lahn, 1997

Smirnov, A., Fulton, D. B., Andreotti, A. and Petrich, J. W.: Exploring Ground-State Heterogeneity of Hypericin and Hypocrellin A and B: Dynamic and 2D ROESY NMR Study. *J. Am. Chem. Soc.* **121**: 7979-7988 (1999)

Stock, S.: ^{14}C-Markierung und Pharmakokinetik von Naphthodianthronen. Thesis. Pharmazie und Lebensmittelchemie. Philipps-Universität Marburg/Lahn, 1992

Vandenbogaerde, A. L., Delaey, E. M., Vantieghem, A. M., Himpens, B. E., Merlevede, W. J. and de Witte, P. A.: Cytotoxicity and Antiproliferative Effect of Hypericin and Derivatives after Photosensitization. *Photochemistry and Photobiology* **67**: 119-125 (1998a)

Vandenbogaerde, A. L., Kamuhabwa, A., Delaey, E., Himpens, B. E., Merlevede, W. J. and de Witte, P. A.: Photocytotoxic Effect of Pseudohypericin versus Hypericin. *Journal of Photochemistry and Photobiology B: Biology* **45**: 87-94 (1998b)

Wynn, J. L. and Cotton, T.M.: Spectroscopic Properties of Hypericin in Solution and at Surfaces. *J. Phys. Chem.* **99**: 4317-4323 (1995)

3 Investigations on the Physicochemical Properties of Hypericin and Pseudohypericin used as Reference Compounds for Medicinal Plant Analysis

3.1 Introduction

The standardization of the commercial extracts of Hyperici herba is mostly done based on the content of naphthodianthrones. Using hypericin as reference substance, its consistent quality is a precondition for reproducible standardization. Therefore, its physicochemical properties are a matter of concern. *Absorbance data*, *solubility* and *stability* problems are subjects of this study.

Absorbance data

Absorbance data of hypericin and pseudohypericin are of interest, as most quantification methods apply absorption measurement for detection. Calibration graphs of hypericin are used for the calculation of both hypericin and pseudohypericin. Therefore, unknown differences in the absorption properties of the two compounds would lead to wrong results. Literature data of the molar coefficients of absorbance of hypericin are not consistent (Table 3.1A) and only few data are published for pseudohypericin (Table 3.1B). Various factors have been discussed to influence the absorption spectra of hypericin, as homoassociation, pH of the solvent and its tautomeric and conformational state. This study aimed to gain further insight into the problem.

Homoassociates of hypericin molecules reveal another molar coefficient of absorbance than monomolecularly dissolved hypericin, as has been shown by Falk and Meyer (1994). Hypericin dissolves monomolecularly in common polar solvents up to concentrations of 10^{-3} mol/l. Under certain conditions, however, it forms homoassociates, for example in the presence of water. Increasing percentages of water in dimethylsulfoxide - water mixtures lead to a decrease of the intensity of the long wavelength absorption band. Its extinction coefficient in water was reported to be about one fourth of that in pure dimethylsulfoxide (Falk and Meyer, 1994). Burel and Jardon (1996) and Lavie *et al.* (1990) did

similar experiments with ethanol - water mixtures. They described as well water addition to decrease the intensity of the absorption spectra. Lavie *et al.* (1990) determined the extinction coefficient of hypericin in water to be only one eighth of that in ethanol. Wynn and Cotton (1995) mentioned homoassociation to occur likewise in nonpolar organic solvents such as toluene, chloroform and hexane. Homoassociation can be broken down by the alkali metal cations potassium and sodium, according to Burel *et al.* (1997). Xia *et al.* (1998) observed that the addition of anthraquinone to hypericin solutions lowered the intensity of the absorption spectrum of hypericin, but did not change the shape of the spectrum. They thought aggregation to be responsible for the finding.

The form of the homoassociates depends on the tautomeric state of hypericin. The homoassociates of the 7,14-dioxo tautomer exhibit a stacking pattern due to the hydrophobic effect of the aromatic core. Hydrogen bonding is responsible for the formation of the homoassociates of the 1,6-dioxo tautomer (Figure 3.1).

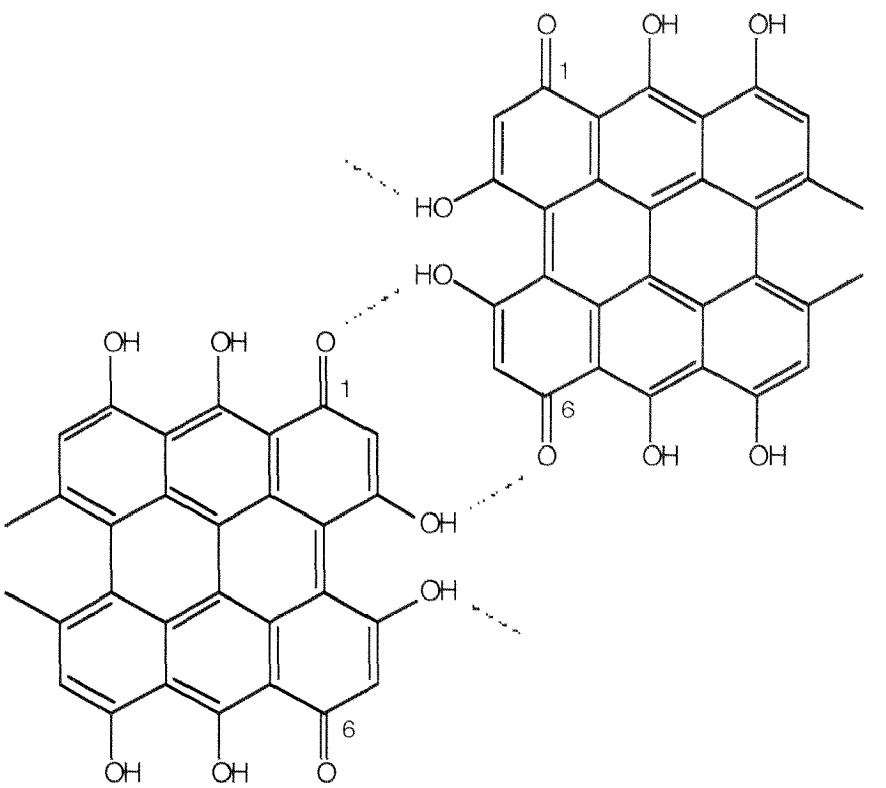


Figure 3.1 Intermolecular hydrogen bonding of the 1,6-dioxo tautomer

In contrast to the homoassociates of the 7,14-dioxo tautomer, the homoassociates of the 1,6-dioxo tautomer show high extinction

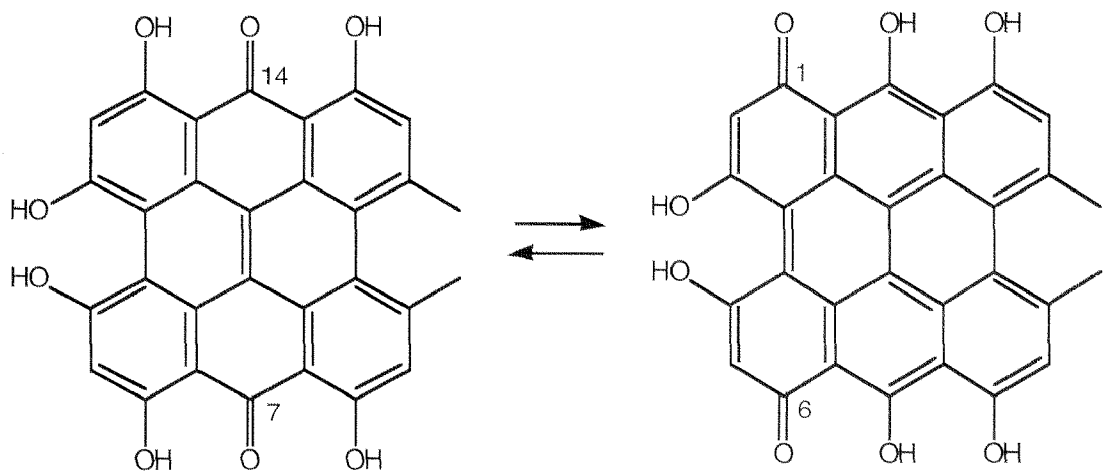
coefficients and narrow absorption bands (Falk and Meyer, 1994; Kapinus *et al.*, 1999).
The aggregation behavior of pseudohypericin has not been described yet, although being of some importance with regard to its quantification as hypericin. The quantification of hypericin and pseudohypericin by VIS spectroscopy and HPLC / DAD in extracts of Hyperici herba does not give the same results. As the two methods do not use the same solvent systems, it was speculated that different association behavior of hypericin and pseudohypericin could be one of the factors responsible. If the homoassociation of pseudohypericin in water were more pronounced than that of hypericin, the molar coefficient of absorbance of pseudohypericin in watery solvents would be lower. Therefore, HPLC / DAD results would be lower than spectroscopic results, as VIS spectroscopy (German Drug Codex) does not make use of water, but most HPLC methods do (Kerb *et al.*, 1996; Krämer and Wiartalla, 1992; Ostrowski, 1988).

Ionization was shown to influence absorbance data of hypericin (Falk and Schmitzberger, 1992). They found that concentrated solutions of the monoanion of hypericin ($1x10^{-2}$ mol/l) revealed a characteristic UV-VIS spectrum. In diluted solutions ($1x10^{-5}$ mol/l), however, the spectrum approached that of non-ionized hypericin (Table 3.1A).

The *conformational state* of hypericin is a further factor affecting the absorption spectra. The propeller and butterfly conformers were reported to reveal small spectroscopic shift differences (Etzlstorfer *et al.*, 1996).

Finally, *tautomerism* of hypericin has to be considered as well, when absorbance data are looked at. The absorption spectra of the tautomer with the carbonyl groups in position 1 and 6 reveal fairly intensive bands between 400 and 500 nm and long wavelength absorption bands, which are hypsochromically shifted compared to the tautomer with the carbonyl groups in positions 7 and 14 (Etzlstorfer *et al.*, 1993; Kapinus *et al.*, 1999). The following observations have been done by Kapinus *et al.* (1999). Both tautomeric forms (Figure 3.2) can occur in solid state. Transformation from one tautomer into another only occurs in solution. The 7,14-dioxo tautomer was shown the most stable hypericin isomer. It can be reverted to the 1,6-tautomer by acidification. Dissolution of the 1,6-dioxo tautomer in polar solvents as dimethylsulfoxide leads to its conversion to the 7,14-dioxo form. Saturated solutions of the 1,6-dioxo tautomer in tetrahydrofuran (or other low to moderate polar organic

media) reveal some stability. Dilution, rise in temperature, addition of pyridine and dimethylsulfoxide revert the 1,6-dioxo to the 7,14-dioxo tautomer in tetrahydrofuran as well. The 1,6-dioxo tautomers form homoassociates, which are stabilized by efficient intermolecular hydrogen bonding (Figure 3.1). Dilution, rise in temperature, addition of pyridine and dimethylsulfoxide disturb the hydrogen bonds by solvating the hypericin molecules, and therefore enhance the dissociation of the homoassociates. After dissociation, isomerization can take place. The stabilizing effect of the intermolecular hydrogen bonds on the 1,6-dioxo tautomeric form is most prominent in saturated solutions and in the solid state.



7,14-dioxo tautomer 1,6-dioxo tautomer

Figure 3.2 Tautomeric forms of hypericin

Solubility

A problem in the preparation of standard solutions of hypericin is its bad solubility in most common solvents. In contrast, salts of hypericin are generally soluble in polar solvents (Lavie *et al.*, 1995). Addition of 1% pyridine to methanol improves the solubility of hypericin as well (Freytag, 1984), may explainable by the formation of hypericin-pyridine-complexes or ionization. In this context, *pKa* values of hypericin are of interest. The subject has been discussed controversially in literature (Altmann and Falk, 1997). Various methods have been applied to derive *pKa* values of hypericin such as ^{1}H NMR (Falk and Schmitzberger, 1992), UV/VIS spectroscopy (Falk and Meyer, 1995; Freeman *et al.*, 1994),

mass spectroscopy (Ahrer *et al.*, 1998), electrophoresis (Altmann and Falk, 1997) and UV/VIS spectroscopy after derivatization of hypericin (Altmann and Falk, 1997; Amer *et al.*, 1998). From experiments, it was concluded that three main ionization steps, which are coupled in part to homoassociation equilibria, characterize the protonation and deprotonation behavior of hypericin. The protonation of C=O groups was assigned to the *pKa* value of -6 and the deprotonation of one of the hydroxyl groups in position 3 and 4 to the *pKa* value of 2 (Figure 3.3). The *pKa* value of 9 was found to characterize the second deprotonation step, which generates a diphenolate ion by ionization of one of the hydroxyl groups in position 1, 6, 8 and 13 (Amer *et al.*, 1998). These results indicate that hypericin is present as an anion under most circumstances, due to its rather low *pKa* value of 2. However, the finding that salts of hypericin are better soluble in common organic solvents, would be easier to explain by a higher *pKa* value, as found for example by Eloy *et al.*, 1996. They depicted two deprotonation equilibria (*pKa* = 7 and 11). The protonation of the carbonyl groups was proposed to have a *pKa* of 1.

As influences on the solubility of hypericin are not definitely clarified, reasons for the differing solubility properties of the various hypericins described in literature are discussed controversially. Sattler (1997) determined the solubility of hypericin to be 40 µg/ml in water and 500 µg/ml in methanol; the lowest solubility (10 µg/ml) showed the investigated buffer systems (pH=7.4). Butterweck (1997) found similar results for the solubility of hypericin in water (60 µg/ml). She discussed the enhanced solubility of hypericin and pseudohypericin in extract solutions, explaining the finding by the presence of solubilizers as procyanidin B2 and C1, forming better soluble naphthodianthron-procyanidin-complexes. Falk and Schmitzberger (1992) presented another explanation. It was deduced from NMR data that hypericin was present in the plant as phenolate ion, potassium being the counter-ion. As the non-ionized hypericin from Roth Company was only sparingly soluble in common solvents, salt formation was made responsible for the easy extractability from the plant. The hypericin isolated from blossoms of *Hypericum perforatum* allowed them the preparation of solutions containing at least 5 mg hypericin / ml methanol. Stock (1992) mentioned differing solubility properties of synthesized hypericin from Roth Company and hypericin isolated from Hyperici herba. She found the isolated hypericin to be better soluble than the synthetic one and assumed different amounts of water of crystallization to be responsible. The synthetic hypericin used by Liebes *et al.* (1991) allowed the preparation of solutions of 5 mg hypericin in 1 ml ethanol absolute. It

can be speculated that the hypericins with different solubility properties reveal different tautomeric forms. Strong hydrogen bonds are present in the 1,6-tautomer (Figure 3.1); therefore, this is possibly the form with lower solubility.

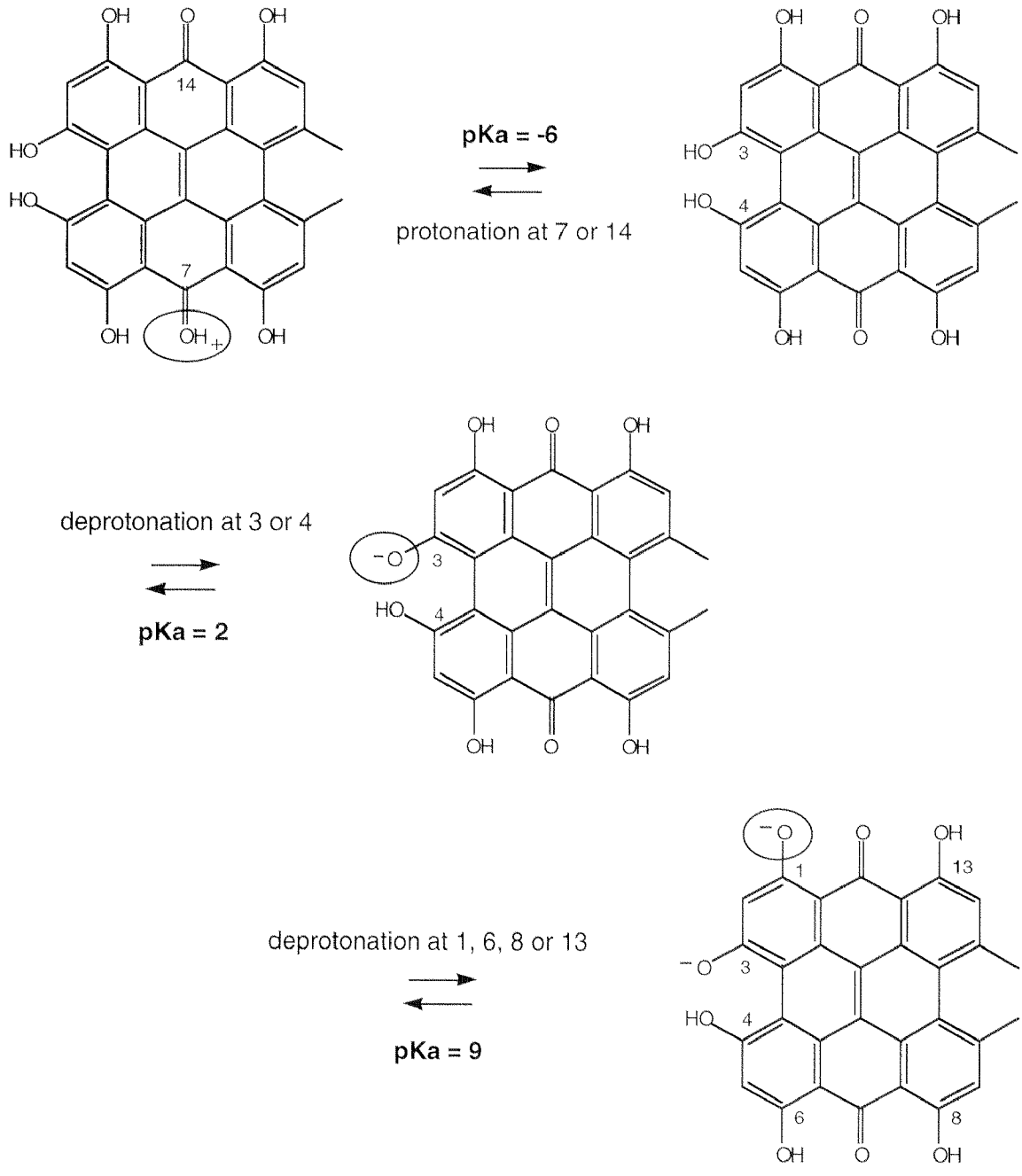


Figure 3.3 Protonation and deprotonation behavior of hypericin

Stability

The stability of reference solutions is a further point of concern. The stability of hypericin and pseudohypericin has been investigated in extract solutions of Hyperici herba and in monosubstance solutions.

Extract solutions: Studies on the stability of hypericin and pseudohypericin in solutions of Hyperici herba extracts have been done up to 48 hours. Butterweck (1997) used a commercial dry extract and showed the two compounds to be stable in methanol - dimethylsulfoxide (9:1) solutions, exposed to light, over night. Brolis *et al.* (1998) came up with the same result, storing methanol and methanol - water (9:1) solutions of a dried methanol extract of Hyperici herba at room temperature in the dark over 48 hours. Experiments at increased temperature were done by Niesel (1992) and Müller-Kuhrt and Boesel (1993). Niesel found hypericin and pseudohypericin in tea preparations to be stable at 60 °C for 3 hours, but not at 95 °C revealing 50% degradation within the same time. Müller-Kuhrt and Boesel got similar results, examining methanol - water (1:1) solutions of a Hyperici herba extract for 8 hours. The two compounds were stable at 60 °C, but only 40% of the initial amount were left at 95 °C after 8 hours.

Monosubstance solutions: Sattler (1997) examined the stability of hypericin as monosubstance in the solvent-system Krebs-Ringer-buffer / methanol (9:1), in which only 40% of the initial amount could be found after 5 hours. Liebes *et al.* (1991) showed hypericin calibration solutions to give the same HPLC response factors for periods up to 6 months, when protected from evaporation, excluded from light and subjected to resonication. Maisenbacher (1991) and Maisenbacher and Kovar (1992) examined a solution of hypericin in acetone after irradiation with a daylight lamp for 40 h. The generation of lipophilic hypericin-derivatives by photolysis was discussed. Preparative TLC led to the isolation of one of the derivatives, which was analyzed by UV/VIS, IR and ^{1}H NMR spectroscopy. The spectra allowed the assumption of a basic structure that is depicted in Figure 3.4 (Maisenbacher, 1991).

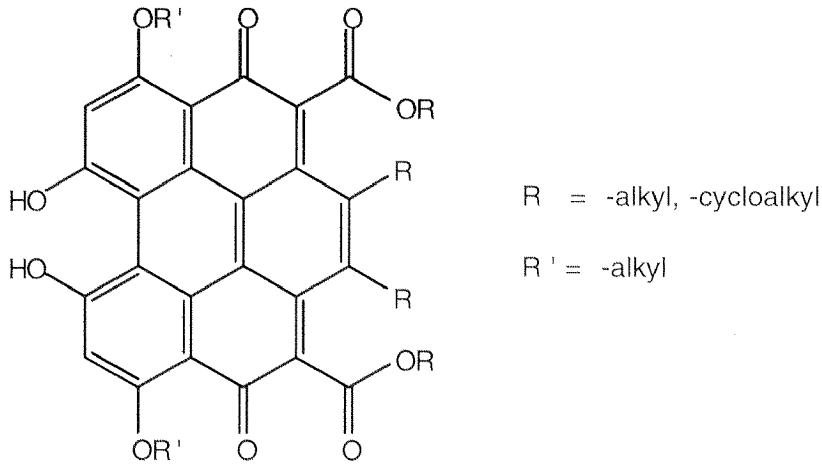


Figure 3.4 Assumed basic structure of lipophilic hypericin-derivatives generated by photolysis of hypericin (Maisenbacher, 1991)

This study dealt with the solubility of hypericin in methanol. It was investigated as well, in which degree the addition of 1% pyridine improved the solubility and whether it influenced the absorbance data, as it was preferred to prepare extract solutions of Hyperici herba without pyridine. Influences on the absorption data would make it necessary to add the same concentration of pyridine to the extracts.
As it was reported for hypericin that increasing percentages of water in ethanol - water mixtures went along with a decrease of its extinction coefficient (see above), it was investigated if it was the same for pseudohypericin. Differing data would implicit that the homoassociation behavior of the two substances were not alike.
Stability tests over a longer period were a further scope of this investigation to gain some information about best storage conditions of standard solutions. The influence of light and temperature were examined as well.

Table 3.1A Overview of absorbance data of hypericin described in literature

solvent	λnm (ε)	c [mol/l]	reference
acetone	596/551/514/477		Wynn and Cotton, 1995
acetone	596(51190)/552(23550)/514(8190)/481(11260)	$1x10^{-5}$	Falk and Meyer, 1994
acetone	597		Yamazaki *et al.*, 1993
acetone	596(36790)/350(17640)		Maisenbacher, 1991
acetone	592		Walker *et al.*, 1979
acetone	599/583/554/542/513/475		Pace and Mackinney, 1939
acetonitrile	594/550		Xia *et al.*, 1998
acetonitrile	594/550/512/478/384/336/286		Wynn and Cotton, 1995
acetonitrile	594(39560)/550(18990)/512(6730)/478(9890)	$1x10^{-5}$	Falk and Meyer, 1994
acetonitrile	594		Gai *et al.*, 1994
benzyl alcohol	596/551/515/480/390/335		Wynn and Cotton, 1995
butanol	592/546/511/476/385/333/286		Wynn and Cotton, 1995
carbon tetrachloride	609/568/479/395/330/286		Wynn and Cotton, 1995
chloroform	595/570/484/397/330/286		Wynn and Cotton, 1995
cyclohexane	613/570/484/332/289		Wynn and Cotton, 1995
dimethylformamide	598/554	$1.9x10^{-5}$	Papageorgiou *et al.*, 1996
dimethylformamide	598/554/515/482/386		Wynn and Cotton, 1995
dimethylformamide	600(46060)/554(20730)/516(6910)/482(10870)	$1x10^{-5}$	Falk and Meyer, 1994
dimethylsulfoxide	600/550/516/481/454/388/335		Kapinus *et al.*, 1999
dimethylsulfoxide	598(39500)/554(17600)/514(5600)/480(9400)/ 450(7700)/388(10800)/343(25900)/334(26100)		Vandenbogaerde *et al.*, 1998a
dimethylsulfoxide	598(39500)		Vandenbogaerde *et al.*, 1998b
dimethylsulfoxide	600(48950)/555(22030)/516(7340)/482(11260)	$1x10^{-5}$	Falk and Meyer, 1994
dimethylsulfoxide	598		Gai *et al.*, 1994
dimethylsulfoxide	600		Yamazaki *et al.*, 1993
dimethylsulfoxide	599(43000)/555/514/480/384/343/333/281		Raser *et al.*, 1992
dioxane	590/547/510/471/330/283		Wynn and Cotton, 1995

solvent	λnm (ε)	c [mol/l]	reference
ethanol	592/549	$1.9x10^{-5}$	Papageorgiou *et al.*, 1996
ethanol	590(52000)/545		Lavie *et al.*, 1995
ethanol	591/547/510/475/382/332/285		Wynn and Cotton, 1995
ethanol	591(37410)/549(22800)/510(7760)/476(12130)	$1x10^{-5}$	Falk and Meyer, 1994
ethanol	591		Yamazaki *et al.*, 1993
ethanol	592(41000)/548(20400)/511(7600)/476(10700)/ 379(10700)/327(29300)		Falk and Schoppel, 1992
ethanol	591/578/547/510/477/383/337/326/284		Raser *et al.*, 1992
ethanol	590(46000)/547(22000)/510/474/383/340/285/ 234	4.4x 10^{-5}	Liebes *et al.*, 1991
ethanol	590(40000)/510(9500)/308(21200)		Jardon *et al.*, 1986
ethanol	586		Walker *et al.*, 1979
ethanol	590(41600)/548(23500)/509(8700)/473(13000)/ 375(83000)/331(26200)/284(36800)	6.31x 10^{-5}	Scheibe and Schöntag, 1942
ether	580/569/540/507/452/428/326/280/230		Wynn and Cotton, 1995
ether	582/569/541/530/506/455		Pace and Mackinney, 1939
ethyl acetate	592/549/511/477/384/334/285		Wynn and Cotton, 1995
ethyl acetate	592(43450)/548(17580)/510(5990)/475(6700)	$1x10^{-5}$	Falk and Meyer, 1994
ethyl acetate	597/554/516	A	Brockmann *et al.*, 1942
hexane	613/570/486/333/292		Wynn and Cotton, 1995
methanol	590(45650)		Butterweck, 1997
methanol	588/545/509/471/384/328/282		Wynn and Cotton, 1995
methanol	588		Gai *et al.*, 1994
methanol	589(43450)/546(20860)/509(7390)/472(12170)	$1x10^{-5}$	Falk and Meyer, 1994
methanol	630(24000)/588(22800)/547(13400)	$1x10^{-2}$ B	Falk and Schmitzberger, 1992
methanol	589(43600)/546(24700)/509(10000)/472(13800)	$2x10^{-5}$ B	Falk and Schmitzberger, 1992
methanol	590(36260)/548(17980)		Maisenbacher, 1991

solvent	λnm (ε)	c [mol/l]	reference
pyridine	602(49410)/557(22230)/518(7410)/484(10870)	$1x10^{-5}$	Falk and Meyer, 1994
pyridine	601/556		Freytag, 1984
pyridine	601/557		Cameron and Raverty, 1976
pyridine	600/557/518/484/455/388/335		Sevenants, 1965
pyridine	603/559/520		Brockmann *et al.*, 1942
pyridine	603/558/520/483	A	Pace and Mackinney, 1939
tetrahydrofuran	600/555/516/482/385/339/286		Wynn and Cotton, 1995
tetrahydrofuran	601(55150)/556(23160)/516(8280)/481(13800)	$1x10^{-5}$	Falk and Meyer, 1994
toluene	609/570/481/331		Wynn and Cotton, 1995
water	598(6000)/558(10000)	$4.4x10^{-5}$	Liebes *et al.*, 1991
water	598/560/324/285		Wynn and Cotton, 1995
acetone-water (95:5)	593/551/512/480/450/387/350		Sevenants, 1965
acetone-water (9:1)	596/551/514/477		Pace and Mackinney, 1939
acetone-ether (33:67)	599/584/555/542/455		Pace and Mackinney, 1939
acetone-ether (25:75)	599/583/569/542/529/454		Pace and Mackinney, 1939
ether + trace pyridine	589/573/546		Pace and Mackinney, 1939
ether-pyridine (8:2)	599/555/517		Pace and Mackinney, 1939
chloroform-hexan (8:2)	601/563/525/489/388/332		Raser *et al.*, 1992
ethanol-water (6:94)	586		Yamazaki *et al.*, 1993
ethanol- water (8:2), pH 0.6	580(40000)/535(22000)/460(25000)/320(33000)		Freeman *et al.*, 1994
ethanol-water (8:2), pH 8.6	590(50000)/550(24000)/470(16000)/340(35000)	B	Freeman *et al.*, 1994
ethanol-water (8:2), pH 13.5	470(18000)/365(41500)	C	Freeman *et al.*, 1994
dimethylsulfoxide-acetic acid (9:1)	587/545/461/437/322	$5x10^{-6}$	Kapinus *et al.*, 1999
dimethylsulfoxide-water (8:2), pH 0.45	586(2900)/544 (1600)/507(700)/461(2000)/320(3000)		Falk and Mayr, 1995
dimethylsulfoxide-water (8:2), pH 8.8	599(3700)/555(1800)/545(1300)/516(800)/ 388(1200)/333(2500)		Falk and Mayr, 1995

solvent	λnm (ε)	c [mol/l]	reference
acetic acid	579/567/538/503/453/430/320/279		Wynn and Cotton, 1995
acetic anhydride	593/552/512/475		Sevenants, 1965
ethanol + H^+	581/569/539/504/454/430/321/280		Wynn and Cotton, 1995
formic acid	578/537/501/454/432/321/279		Wynn and Cotton, 1995
lactic acid	581/569/540/504/445/433/320		Wynn and Cotton, 1995
methanol-10 mM HCl	580		Gai *et al.*, 1994
methanol-0.1 N KOH (1:1)	625/460		Maisenbacher, 1991
conc sulphuric acid	650/597/500/325/238/220	A	Wynn and Cotton, 1995
conc sulphuric acid	658		Gai *et al.*, 1994
conc sulphuric acid	645/598/506		Brockmann *et al.*, 1942
water, pH 13	650		Gai *et al.*, 1994

A: compound not defined properly; B: monoanion of hypericin; C: dianion of hypericin

Table 3.1B Overview of absorbance data of pseudohypericin described in literature

solvent	λnm (ε)	c [mol/l]	reference
dimethylsulfoxide	598(42100)		Vandenbogaerde *et al.*, 1998b
methanol	590(43100)/546(24800)/509(10300)/ 470(13600)/446(12700)/384(13300)	2×10^{-5} B	Falk and Schmitzberger, 1992
methanol	634(24200)/589(23200)/465(13700)	1×10^{-2} B	Falk and Schmitzberger, 1992

B: monoanion of pseudohypericin

3.2 Experimental

3.2.1 Materials

The ethanol-water (1:1, m/m) dry extract (Ze117) from Hyperici herba was obtained from the Zeller Company (CH-Romanshorn). Hypericin and pseudohypericin were isolated from the same extract (see section 2). The commercial sample of hypericin was from Roth Company (D-Karlsruhe); it was hypericin ROTICHROM® and of TLC quality (lot 31628333). Methanol, ethyl acetate and tetrahydrofuran were of HPLC quality (Romil Chemicals, GB-Shepshed). *Ortho*-phosphoric acid 85% (Ph. Helv. VI / Ph. Eur.) was purchased from Hänseler (CH-Herisau). Pyridine (p.a.) was from Fluka (CH-Buchs) and sodium dihydrogen phosphate (z.A.) from Merck (CH-Dietikon). De-ionized water was obtained using an NANO-pure cartridge system (Skan, CH-Basel). For stability tests, an air-conditioned room (23 °C) and cold-storage chambers of 4 °C and -20 °C were used. For the experiments with light exposure a blended light lamp (Philips MLR 160W, 230-240V) was used.

3.2.2 Instrumentation and Quantification

UV/VIS spectroscopy. UV/VIS spectra were measured on a Kontron-Uvikon 930 spectrophotometer (Kontron Instruments, CH-Zürich).

HPLC. HPLC analyses were performed, using a Hewlett Packard instrument (Model 79994A analytical workstation, model 1090 liquid chromatograph, model 1040 diode-array detector). For chromatographic separations a Knauer (D-Berlin) prepacked cartridge column (250 x 4 mm I.D., 5 µm) filled with Spherisorb S ODS2 and a guard column (10 x 4 mm I.D.) of the same material was used.

Quantification by HPLC. The quantitative determination of hypericin and pseudohypericin was performed using the external standard method. The standard solutions of hypericin were used for the quantification of both hypericin and pseudohypericin. The calculation graphs were generated by a least squares regression method. All calculations were based on area counts. Over the selected concentration range of the standard solutions, the calibration curve showed a linear detector response. The correlation coefficient was 1.000. The external standard solutions were injected three times into the HPLC system for analysis.

3.2.3 Procedure

3.2.3.1 Absorbance Data of Hypericin and Pseudohypericin

Standard solutions of hypericin. Six stock solutions were prepared for each solvent (methanol and methanol-pyridine (99:1, v/v)). 0.500 mg hypericin were dissolved in the corresponding solvent in a 100.0 ml volumetric flask, sonicated 3 times for 10 min and stored for 4 days at room temperature. Five different dilutions were prepared from each stock solution. The concentration range for hypericin in methanol and methanol-pyridine (99:1, v/v) was 1.27 - 5.56 µg/ml and 1.30 - 6.30 µg/ml, respectively. The molar and specific coefficient of absorbance of hypericin was determined in methanol and methanol-pyridine (99:1, v/v).

Standard solutions of pseudohypericin. Six stock solutions were prepared using methanol-pyridine (99:1, v/v) as solvent. 0.500 mg pseudohypericin were dissolved in 100.0 ml solvent in a 100.0 ml volumetric flask and sonicated 3 times for 5 min. As the solubility of pseudohypericin in methanol is better than that one of hypericin, but its stability at room temperature is worse (see section 3.3.4), the dilution of the stock solutions was done after 18 hours. The concentration range was 1.20 - 5.90 µg/ml.

UV/VIS. UV/VIS spectra were recorded from 200 to 600 nm.

3.2.3.2 Influence of Water Addition to Methanol Solutions on the Molar Coefficient of Absorbance of Hypericin and Pseudohypericin

Test solutions. 1.99 mg hypericin were dissolved in methanol-pyridine (99:1, v/v) in a 100.0 ml volumetric flask. The stock solution was stored three days at room temperature and sonicated three times 10 min. Solutions of 20, 40, 60 and 80% aqueous methanol were prepared by diluting 10.0 ml of the stock solution to 50.0 ml with the corresponding amount of methanol and water (v/v). The 100% methanol solution was prepared by diluting 5 ml stock solution to 25.0 ml. The final hypericin concentration was 3.98 µg/ml.
0.99 mg pseudohypericin were dissolved in methanol-pyridine (99:1, v/v) in a 50.0 ml volumetric flask. The solution was sonicated for 15 min and stored over night at room temperature. The further procedure was

according to the preparation of the hypericin solutions. The final pseudohypericin concentration was 3.96 µg/ml.

Standard solutions. To determine the hypericin and pseudohypericin content of the test solutions five standard solutions of each reference compound in methanol-pyridine (99:1, v/v) were prepared. The concentrations of the standard solutions were between 1.39 µg/ml and 5.54 µg/ml. They were used both for HPLC and UV/VIS spectroscopy.

UV/VIS. UV/VIS spectra were recorded from 200 nm to 600 nm.

HPLC. The applied HPLC method was the method developed by Krämer and Wiartalla (1992). The composition of the mobile phase was methanol - ethyl acetate - buffer (1893.4:526:618.4, m/m). The buffer consisted of 13.8 g sodium dihydrogen phosphate in 1 litre adjusted to pH 2.1 with *ortho*-phosphoric acid 85%. The run time was 15 min, the flow rate 0.8 ml/min, the column temperature 25 °C and the injection volume 20 µl. The detection wavelength of the DAD was set at 590 nm.

3.2.3.3 Determination of the Solubility of Hypericin in Methanol and Methanol-Pyridine (99:1, v/v) and the Range of Linearity

Standard solutions. 2.496 mg hypericin were dissolved in methanol-pyridine (99:1, v/v) in a 25.0 ml volumetric flask. The solution was stored at room temperature for five days, shaken and sonicated 5 min each day.
From this stock solution, eight standard solutions were prepared, their concentrations ranging from 0.9984 to 99.84 µg/ml.

Test sample in methanol. To prepare a saturated solution, 0.464 mg hypericin were distributed in methanol in a 5.0 ml volumetric flask. The sample was kept at room temperature for five days, shaken and sonicated 5 min each day. Then, the sample was centrifuged (5 min at 2000 rpm) and the supernatant (A) pipetted off. 1 ml of the supernatant was diluted with methanol to 5.0 ml (B).

Test sample in methanol-pyridine (99:1, v/v). To prepare a saturated solution, 3.494 mg hypericin were distributed in methanol-pyridine (99:1, v/v) in a 5.0 ml volumetric flask. The sample was kept at room temperature for five days, shaken and sonicated 5 min each day. The

sample was centrifuged (5 min at 2000 rpm) afterwards and the supernatant (C) pipetted off. 1 ml of the supernatant was diluted with methanol-pyridine (99:1, v/v) to 50.0 ml (D).

VIS spectroscopy. Absorptions were measured at 589 and 590 nm.

HPLC. The modified method of Kerb *et al.* (1996) was applied. Solvents used were solvent A (methanol) and solvent B (methanol - tetrahydrofuran - buffer (9:6:5, v/v)). The buffer consisted of 13.8 g sodium dihydrogen phosphate in 1 litre water adjusted to pH 4.0 with *ortho*-phosphoric acid 85%. The elution profile was: 0-12 min 100% B, 12-17 min 100% A and 17-27 min 100% B. The flow rate was 0.8 ml/min for solvent B and 1 ml/min for solvent A. The column temperature was set at 25 °C and the injection volume at 25 µl. The detection wavelength of the DAD was set at 590 nm.

3.2.3.4 Stability of Hypericin and Pseudohypericin in Solution

Standard solutions of hypericin. 2.450 mg hypericin were dissolved in 250 ml methanol-pyridine (99:1, v/v) in a 250 ml volumetric flask (9.8 µg/ml). The solution was stored at room temperature for 4 days and sonicated 5 min each day. From this stock solution, different dilutions were prepared, their concentrations ranging from 0.98 to 4.9 µg/ml. The standard solutions were kept at -20 °C at darkness. The area counts and the absorption of the standard solutions did not decrease over the investigated time period.

Test samples of hypericin, pseudohypericin and extracts

Hypericin. Four solutions with 1.0 mg hypericin dissolved in 200.0 ml methanol and one solution with 1.0 mg hypericin in 200.0 ml methanol-pyridine (99:1, v/v) were prepared and sonicated two times 10 min.
Pseudohypericin. Four solutions with 0.5 mg pseudohypericin dissolved in 100.0 ml methanol and one solution with 1 mg pseudohypericin in 100.0 ml methanol-pyridine (99:1, v/v) were prepared and sonicated two times 10 min.
Extracts. 0.8 g of the extract Ze117 were distributed in 200.0 ml methanol, sonicated for 15 min and centrifuged at 2000 rpm for 2 min. 50 ml were filtered through a regenerated cellulose syringe filter.

All the solutions were stored at room temperature for 24 h. Then, their hypericin and pseudohypericin content was determined by VIS spectroscopy and HPLC. After the first measurement, the solutions were stored under the following conditions: room temperature at darkness, room temperature under blended light lamp, 4 °C at darkness, -20 °C at darkness. The solutions prepared with methanol-pyridine (99:1, v/v) were stored at -20 °C in the dark. The solutions were analyzed over a time period of 140 days.

VIS spectroscopy. Absorptions were measured at 546, 555 and 589 nm.

HPLC. The modified method of Kerb *et al.* (1996) was applied (see 3.2.3.3). The detection wavelength of the DAD was set at 590 and 254 nm. All the vials were kept in the dark during HPLC analysis.

3.3 Results and Discussion

3.3.1 Absorbance data of Hypericin and Pseudohypericin

Absorbance data of hypericin were recorded in methanol and methanol-pyridine (99:1, v/v). The absorbance of pseudohypericin was measured in methanol-pyridine (99:1, v/v). As most HPLC / DAD methods and VIS spectroscopy make use of the maximum of the longest wavelength absorption band of hypericin for quantification, most attention was given to the absorption maximum and the molar coefficient of absorbance of this band.
The recorded position of the *absorption maxima* of hypericin (588 nm) and pseudohypericin (589 nm) were in accordance with literature data of methanolic solutions (Table 3.1, Wynn and Cotton, 1995). However, the *molar coefficient of absorbance (ε)* of the isolated hypericin in methanol was higher (ε = 51712 at 588 nm) than those described in literature (Table 3.1 and 3.2; Falk and Meyer, 1994: ε = 43450 at 589 nm). The commercially available hypericin from Roth Company revealed a smaller ε-value as well (ε = 33603 at 588 nm, Table 3.2). Differing molar coefficients of absorbance could be caused by varying degrees of polymerization and degradation or other impurities. The isolated hypericin was assumed purer than the hypericins with lower ε-values, as the presence of impurities with higher ε-values than hypericin seemed to be less likely. Experiences of one's own showed hypericin to be only sparingly soluble in methanol, sonication and time (up to four days)

being necessary for full dissolution (see section 3.3.3.2). Not completely dissolved hypericin could be another reason for low ε–values.

The results presented in Tables 3.3 and 3.4 show that the addition of 1% pyridine to methanol does not significantly influence the molar coefficient of absorbance (ε) of hypericin at 588 nm. The solubility, however, can be enhanced considerably adding small amounts of pyridine (see section 3.3.3). Consequently, if VIS spectroscopy is used as quantification method for hypericin in extracts of Hyperici herba, standard solutions of hypericin can be prepared with methanol-pyridine (99:1, v/v), also if the extracts are made with pure methanol.

Hypericin and pseudohypericin revealed tiny differences in the location of their absorption maxima (Tables 3.3 to 3.5). Furthermore, they showed different ε-values in the maximum of their longest wavelength, being 51936 and 43486 for hypericin and pseudohypericin, respectively, in methanol-pyridine (99:1, v/v). Most quantification methods make use of the calibration curve of hypericin to quantify pseudohypericin. Consequently, calibrated contents are too low, as far as detection is done by absorption measurement (for further discussion see 3.4).

Table 3.2 Absorbance data of hypericin in methanol

λnm (ε)	Reference
588 (51712) / 545 (24393) / 472 (13544)	hypericin from isolation
589 (43450) / 546 (20860) / 472 (12170)	Falk and Meyer (1994)
588 (33603)	hypericin from Roth Company

ε molar coefficient of absorbance

Table 3.3 Absorbance data of hypericin in methanol determined using six calibration curves with six concentration levels each

λ_{max} [nm]	$A^{1\%}$	ε [l mol^{-1} cm^{-1}]	s_{rel} [%]
588	1025	51712	1.22
545	484	24393	1.22
472	269	13544	1.01
328	578	29173	1.31
285	755	38082	1.47

$A^{1\%}$ coefficient of absorbance for 1% (w/v) solution

ε molar coefficient of absorbance

Table 3.4 Absorbance of hypericin at 588 nm in methanol-pyridine (99:1, v/v) determined using six calibration curves with six concentration levels each

λ_{max} [nm]	$A^{1\%}$	ε [l mol^{-1} cm^{-1}]	s_{rel} [%]
588	1030	51936	1.08

$A^{1\%}$ coefficient of absorbance for 1% (w/v) solution

ε molar coefficient of absorbance

Table 3.5 Absorbance data of pseudohypericin in methanol-pyridine (99:1, v/v) determined using six calibration curves with six concentration levels each

λ_{max} [nm]	$A^{1\%}$	ε [l mol^{-1} cm^{-1}]	s_{rel} [%]
589	836	43486	0.47
546	412	21468	0.93
473	250	13025	6.30
327	518	26817	1.74

$A^{1\%}$ coefficient of absorbance for 1% (w/v) solution

ε molar coefficient of absorbance

3.3.2 Influence of Water Addition to Methanol Solutions on the Molar Coefficient of Absorbance of Hypericin and Pseudohypericin

The molar coefficients of absorbance (ε) of hypericin and pseudohypericin decreased with increasing amounts of water (Tables 3.6 and 3.7 and Figure 3.5). In 80%, 60% and 40% aqueous methanol the ε-values for the long wavelength absorption band were 90%, 65% and 31%, respectively, of the ε-value in pure methanol. The decrease of the intensity caused by water addition was more pronounced at the longest wavelength than at 546 nm. The ratios of the absorption at 588 nm to the absorption at 546 nm were 2.1, 2.0 and 1.6 (1.5 for pseudohypericin) for the solvents 100% methanol, 80% and 60% aqueous methanol, respectively. The absorption maxima of the hypericin/pseudohypericin spectra in 20% aqueous methanol were shifted to 594/595 nm and 555/555 nm. In 40% aqueous methanol, only the absorption band at 546 nm was shifted to 554 nm. As results were the same for hypericin and pseudohypericin, it was concluded that they did not differ in their tendency for homoassociation. Therefore, different association behavior of hypericin and pseudohypericin was not the explanation for the differences of VIS spectroscopic and HPLC results in the quantification of hypericin and pseudohypericin.

Table 3.6 Molar coefficient of absorbance (ε) of hypericin (c = $8x10^{-6}$ mol/l) in aqueous methanol at different wavelengths

% MeOH	ε			
	588 nm	546 nm	470 nm	327 nm
40%	16185			
60%	30140	18353	10964	26262
80%	46706	22979	13334	29595
100%	52017	24538	13460	29811

Table 3.7 Molar coefficient of absorbance (ε) of pseudohypericin (c = $8x10^{-6}$ mol/l) in aqueous methanol at different wavelengths

% MeOH	ε		
	589 nm	546 nm	473 nm
40%	13721		
60%	25668	16678	9949
80%	39205	20069	11828
100%	43765	20805	11250

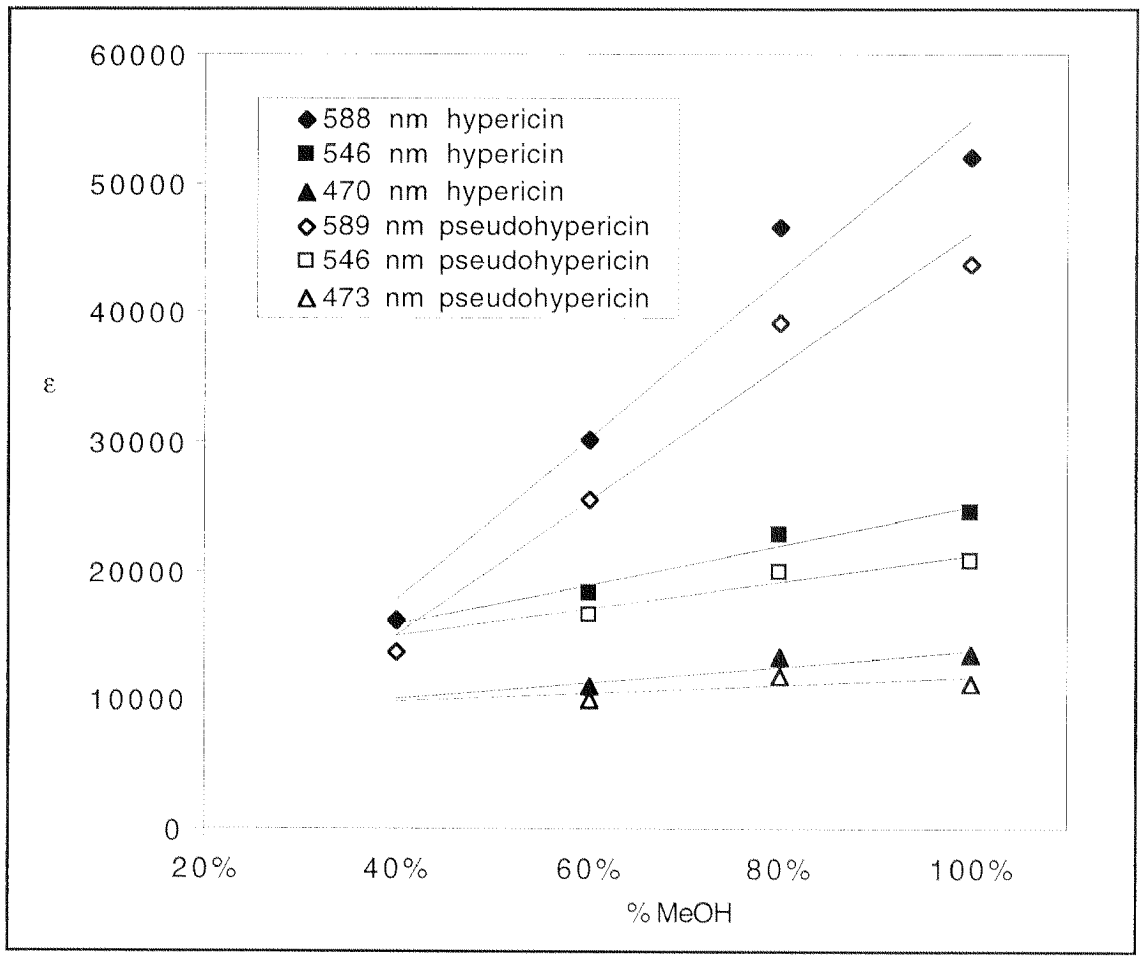


Figure 3.5 Molar coefficient of absorbance (ε) of hypericin (c = $8x10^{-6}$ mol/l) and pseudohypericin (c = $8x10^{-6}$ mol/l) in aqueous methanol at different wavelengths

It was investigated, if the water-methanol ratio of the test solutions influenced the results determined by HPLC. The HPLC method of Krämer and Wiartalla (1992) was applied to determine the concentration of hypericin and pseudohypericin. The test solution 100% methanol was examined by VIS spectroscopy as well. The calibration curves used were $y = 86.321x - 75.557$ (HPLC) and $y = 1017.2x + 0.0049261$ (VIS spectroscopy) for hypericin and $y = 58.236x - 6.5581$ (HPLC) and $y = 867.38x + 0.0011542$ (VIS spectroscopy) for pseudohypericin.
The results indicated that the water-methanol ratio of the test solutions did not influence the HPLC results (Table 3.8).

Table 3.8 Determination of the hypericin (H) and pseudohypericin (P) concentrations of the test solutions by HPLC and VIS spectroscopy (Theoretical concentrations were 3.98 µg/ml hypericin and 3.96 µg/ml pseudohypericin.)

Test solution	HPLC		VIS spectroscopy	
	H [µg/ml]	P [µg/ml]	H (588 nm) [µg/ml]	P (589 nm) [µg/ml]
100% MeOH	3.98	3.79	3.99	3.83
80% MeOH	4.05	4.03		
60% MeOH	4.04	3.84		
40% MeOH	4.01	3.84		
20% MeOH	3.99	3.89		

Identical results of HPLC and VIS spectroscopy indicated the reference compounds not to be responsible for differing results of the two methods, analyzing extracts of Hyperici herba.

3.3.3 Determination of the Solubility of Hypericin in Methanol and Methanol-Pyridine (99:1, v/v) and the Range of Linearity

3.3.3.1 Determination of the Range of Linearity

The standard solutions were used to determine the range of linearity. In VIS spectroscopy at 589 nm and 590 nm, solutions obeyed *Lambert-Beer's* law in the concentration range of 0.9984 to 14.98 µg/ml; the detector itself was the limiting factor. In HPLC, linear detector response was given in the whole concentration interval examined from 0.9984 to 99.84 µg/ml. Correlation coefficients were 0.99999 and 1.00000 (Table 3.9).

Table 3.9 Determination of linearity in VIS spectroscopy and HPLC

Detection mode		Regression curve	Correlation coefficient (r)
VIS spectroscopy	589 nm	y = 1008x - 0.002326	0.99999
	590 nm	y = 971.5x - 0.002601	0.99999
HPLC	590 nm	y = 930326x - 10.38	1.00000

x being the concentration in [g/100 ml]

3.3.3.2 Determination of the Solubility of Hypericin in Methanol and Methanol-Pyridine (99:1, v/v)

The solubility of hypericin in methanol and in methanol-pyridine (99:1, v/v) was examined. It could be shown that the solubility of hypericin in methanol-pyridine (99:1, v/v) is about nine times better than in pure methanol.

Solubility of Hypericin in Methanol

HPLC could be used for the determination of the concentration of the undiluted supernatant (A) and the five times diluted sample (B). A was not in the linear range of VIS spectroscopy. The detection wavelength was 589 nm.

Table 3.10 Determination of the solubility of hypericin in methanol

Detection method	Sample	Absorption at 589 nm	Peak area	Sample concentration [µg/ml]	Solubility of hypericin in methanol [µg/ml]	[µmol/l]
VIS spectroscopy	A	3.1767		-	-	-
	B	0.7657		7.571	37.85	75.03
HPLC	A		3388.152	36.53	36.53	72.42
	B		680.416	7.425	37.13	73.60

The mean value of the solubility of hypericin in methanol was 37.17 µg/ml (Table 3.10). This finding is not consistent with the result of Sattler (1997). She determined the solubility of hypericin to be 500 µg/ml in pure methanol. About the causes of the differences can only be speculated. Polymerization can diminish solubility. The high molar coefficient of absorbance of the isolated hypericin (see section 3.3.1) speaks against this hypothesis, as association would decrease the coefficient. Salt formation and coisolated solubilizers from the extract have been discussed to enhance solubility. The ^{1}H NMR of Sattler did not reveal any impurities, or a resonance at 18.4 ppm, which can be typical for ionized hypericin (see section 2.3.2). Stock (1992) assumed varying amounts of water of crystallization to influence solubility.

Solubility of Hypericin in Methanol-Pyridine (99:1, v/v)

HPLC could be used for the determination of the concentration of the undiluted supernatant (C) and the fifty times diluted sample (D). C was not in the linear range of VIS spectroscopy.

Table 3.11 Determination of the solubility of hypericin in methanol-pyridine (99:1, v/v)

Detection method	Sample	Absorption at 589 nm	Peak area	Sample concentration [µg/ml]	Solubility of hypericin in methanol-pyridine (99:1, v/v) [µg/ml]	[µmol/l]
VIS spectroscopy	C	3.7849		-	-	-
	D	0.6514		7.571	321.89	638.10
HPLC	C		29465.796	36.53	321.67	637.66
	D		552.596	7.425	319.16	632.69

The mean value of the solubility of hypericin in methanol-pyridine (99:1, v/v) was 320.91 µg/ml (Table 3.11).

The results showed that the solubility of hypericin in methanol-pyridine (99:1, v/v) is about nine times better than in pure methanol.
The improved solubility of hypericin in the presence of pyridine may be explained by the formation of hypericin-pyridine-complexes. As absorption spectra of hypericin in methanol and methanol-pyridine (99:1, v/v) were the same, the finding could not be explained by ionization of hypericin or the accelerated break down of homoassociates by pyridine due to enhanced solvatization (Kapinus *et al.*, 1999).

3.3.4 Stability of Hypericin and Pseudohypericin in Solution

Solutions of hypericin, pseudohypericin and extracts of *Hypericum perforatum* in methanol and methanol-pyridine (99:1, v/v) were stored at -20 °C and 4 °C (both in darkness), at room temperature in the dark and in the light. Monitoring of the transformation and degradation was done by HPLC-VIS / DAD and VIS spectroscopy. Results of both methods were comparable. HPLC, however, indicated the decomposition to be faster in some cases, explainable by its better selectivity.

The degradation of the naphthodianthrones roughly followed a first order kinetic. The semi-logarithmic plots of *% initial amount* vs. *time* yielded straight lines. The linear regression revealed correlation coefficients between 0.92 and 1.00. Exceptions with lower coefficients were VIS spectroscopic results of the pseudohypericin samples and the extract solutions stored at -20 °C with and without pyridine (correlation coefficients being between 0.72 and 0.73).

3.3.4.1 Results of VIS Spectroscopy

Hypericin (Figure 3.6). Hypericin was stable over a time period of 140 days under all storage conditions, except at room temperature with light exposure. Light exposure led to the loss of 21% of the initial hypericin amount during the investigated time period ($t_{1/2}$=384 days). The addition of 1% pyridine to the solvent did not influence the stability.

Pseudohypericin (Figure 3.7). The degradation of pseudohypericin could only be prevented by storage at -20 °C in the dark. Light exposure at

room temperature left only about 20% of the initial amount unchanged after 140 days ($t_{1/2}$=58 days). The solutions containing pyridine decomposed slightly faster ($t_{1/2}$=718 days) than the methanol solutions also stored at -20 °C ($t_{1/2}$=4491 days). Significant decomposition ($t_{1/2}$=158 days) could also be observed at room temperature with exclusion of light. Consequently, the stability of pseudohypericin is influenced by light and temperature.

Extract solutions (Figure 3.8). In the extract solutions, decomposition of the naphthodianthrones mainly occurred at room temperature: $t_{1/2}$ was 935 days with exclusion of light and 71 days with light exposure. Storage conditions at reduced temperature in the dark largely prevented degradation, $t_{1/2}$ being 14956, 4338 and 10689 days for the conditions -20 °C, 4 °C and -20 °C/pyridine, respectively.

3.3.4.2 Results of HPLC-VIS / DAD

Hypericin (Figure 3.9). Hypericin showed good stability when stored in the dark. Light exposure at room temperature, however, led to the degradation of 29% of the initial amount within 140 days ($t_{1/2}$=271 days), being only 21% determined with VIS spectroscopy ($t_{1/2}$=384 days). Differing results of the two methods may be explained by better selectivity of HPLC determination. Transformation and degradation products of hypericin may still show absorbance at 589 nm. While they contribute to the results in VIS spectroscopy and falsify the findings, HPLC allows the separation of the degradation products from hypericin.
Hypericin in extract solutions (Figure 3.11). Decomposition of hypericin in the extract solution at room temperature in the light was more pronounced ($t_{1/2}$=50 days) than in the monosubstance solution ($t_{1/2}$=271 days). The extracts may contain substances accelerating the degradation of hypericin under light exposure.

Pseudohypericin (Figure 3.10). The HPLC and VIS spectrophotometric results of the pseudohypericin degradation were fairly in accordance, referring to the solutions stored at room temperature, $t_{1/2}$ being 54 days for the solutions exposed to light and 162 days for the one kept in the dark. For the solutions stored at reduced temperature, VIS spectroscopy results indicated a slightly faster degradation than HPLC data.
Pseudohypericin in extract solutions (Figure 3.12). At room temperature with light exposure degradation was slightly faster in the extract solution ($t_{1/2}$=41 days) than in the monosubstance solution ($t_{1/2}$=54 days). At

room temperature in darkness, it was vice versa: pseudohypericin revealed a $t_{1/2}$ of 338 days in the extract solution and a $t_{1/2}$ of 162 days in the monosubstance solution. Under all the other conditions, pseudohypericin was fairly stable in the extract solutions.

Sum of hypericin and pseudohypericin in extract solutions (Figure 3.13). HPLC results of hypericin and pseudohypericin were summed up and compared with the spectrophotometric data. Results were about the same, except for the solution stored at room temperature in the light. HPLC results indicated the degradation to be slightly faster ($t_{1/2}$=44 days vs. 71 days). Here again, degradation products are probably separated from hypericin and pseudohypericin in HPLC, while they falsify the spectrophotometric results.

3.3.4.3 Products of Degradation and Transformation

Degradation and transformation products could not be definitely identified. One of the transformation products of pseudohypericin was assumed to be cyclopseudohypericin, which was in accordance with literature data (Häberlein *et al.*, 1992). Association and polymerization of hypericin and pseudohypericin has also to be considered.
HPLC. HPLC results of the pseudohypericin solutions stored in the dark at room temperature showed the appearance of a new peak at 4.5 min. Its absorption spectrum was compared with literature data (Häberlein *et al.*, 1992) and found similar to the spectrum of cyclopseudohypericin. The increase of the area counts of cyclopseudohypericin was most prominent in the pseudohypericin solutions stored at room temperature in the dark (Figure 3.14). The detection wavelength was set at 254 nm, because the detection limit was lower there than at 590 nm. The extract solutions (Figure 3.15) contained small amounts of cyclopseudohypericin already in the beginning, its peak area also increasing most in the solution stored at room temperature in the dark. 590 nm was chosen for detection of cyclopseudohypericin in the extract solutions, as at 254 nm the cyclopseudohypericin peak was not well separated from other compounds of the extract. The HPLC chromatograms of the hypericin test solutions did not reveal any cyclopseudohypericin signal. It was assumed that no significant amount of cyclopseudohypericin was generated or at least, that the generated amount was to low to be detected in all the hypericin solutions and the pseudohypericin solutions stored at -20 °C. There was no cyclopseudohypericin detectable in the

solution stored in the light, indicating that cyclopseudohypericin itself is decomposing, when exposed to light.
Polymerization of hypericin and pseudohypericin is to be considered leading to inhomogeneous higher molecular weight compounds, which do not elute at all or not as peaks with defined retention time.

VIS spectroscopy. Cyclopseudohypericin and pseudohypericin are differing in their absorption spectrum. At 555 nm cyclopseudohypericin reveals an absorption maximum, pseudohypericin does not. At 589 nm it is vice versa, pseudohypericin shows a maximum, which is missing in the cyclopseudohypericin spectrum. Growing amounts of cyclopseudohypericin would increase absorption at 555 nm. At the same time, absorption at 589 nm would decrease as a result of the transformation of pseudohypericin. Therefore, the ratio absorbance at 589 nm to absorbance at 555 nm is expected to decrease over time. It could be observed that the decrease of the ratio (Figures 3.16 to 3.18) was nearly proportional to the degradation of the hypericin derivatives. An exception was the solution of pseudohypericin stored at room temperature in the light; at the beginning, its ratio changed slower than the ratio of the solution stored at room temperature in the dark.
According to these results, the transformation product revealed the same absorption spectrum as cyclopseudohypericin. Its chromatographic behavior in HPLC on reversed phase material (Kerb *et al.*, 1996) corresponded to that of cyclopseudohypericin described in literature (Häberlein *et al.*, 1992; Krämer and Wiartalla, 1992), as it was eluted after pseudohypericin but before hypericin. Therefore, it was concluded cyclopseudohypericin to be one of the transformation products of pseudohypericin.

To monitor decomposition of hypericin and pseudohypericin, VIS spectroscopy turned out to be the less selective method than HPLC. For extract solutions, HPLC allows separate analysis of the naphthodianthrones, while VIS spectroscopy only detects their sum.

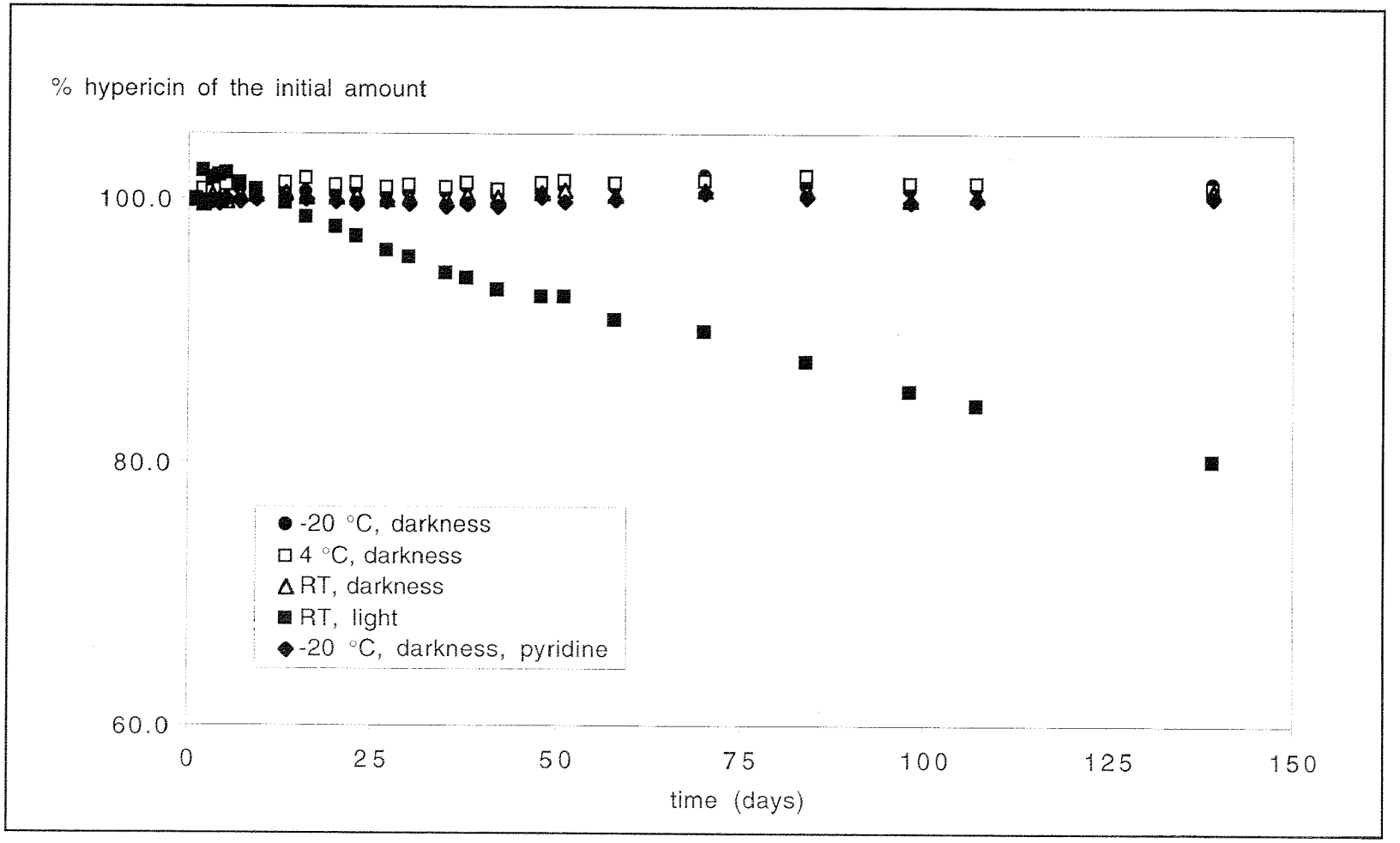


Figure 3.6 Degradation of hypericin under different conditions determined by VIS spectroscopy

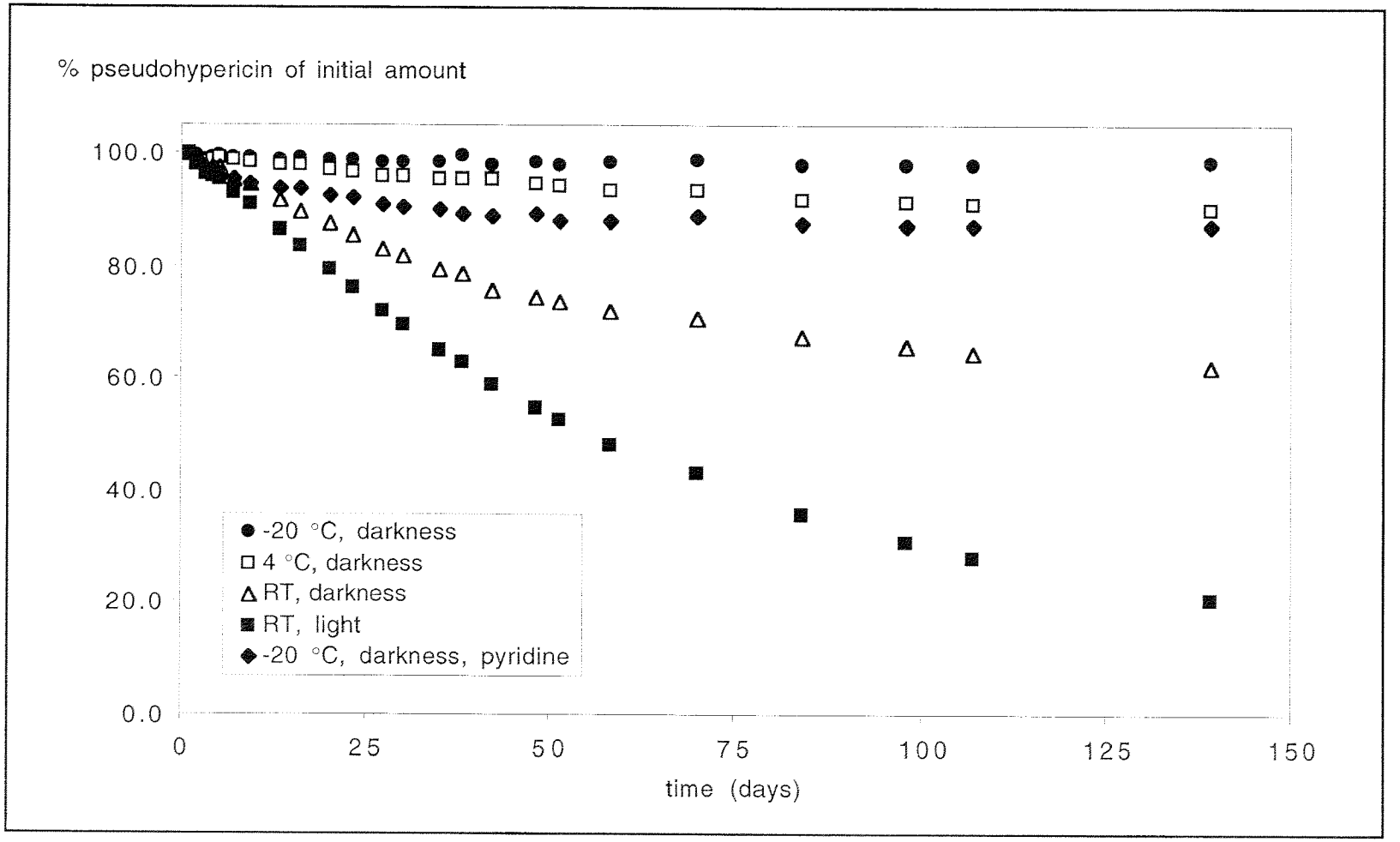


Figure 3.7 Degradation of pseudohypericin under different conditions determined by VIS spectroscopy

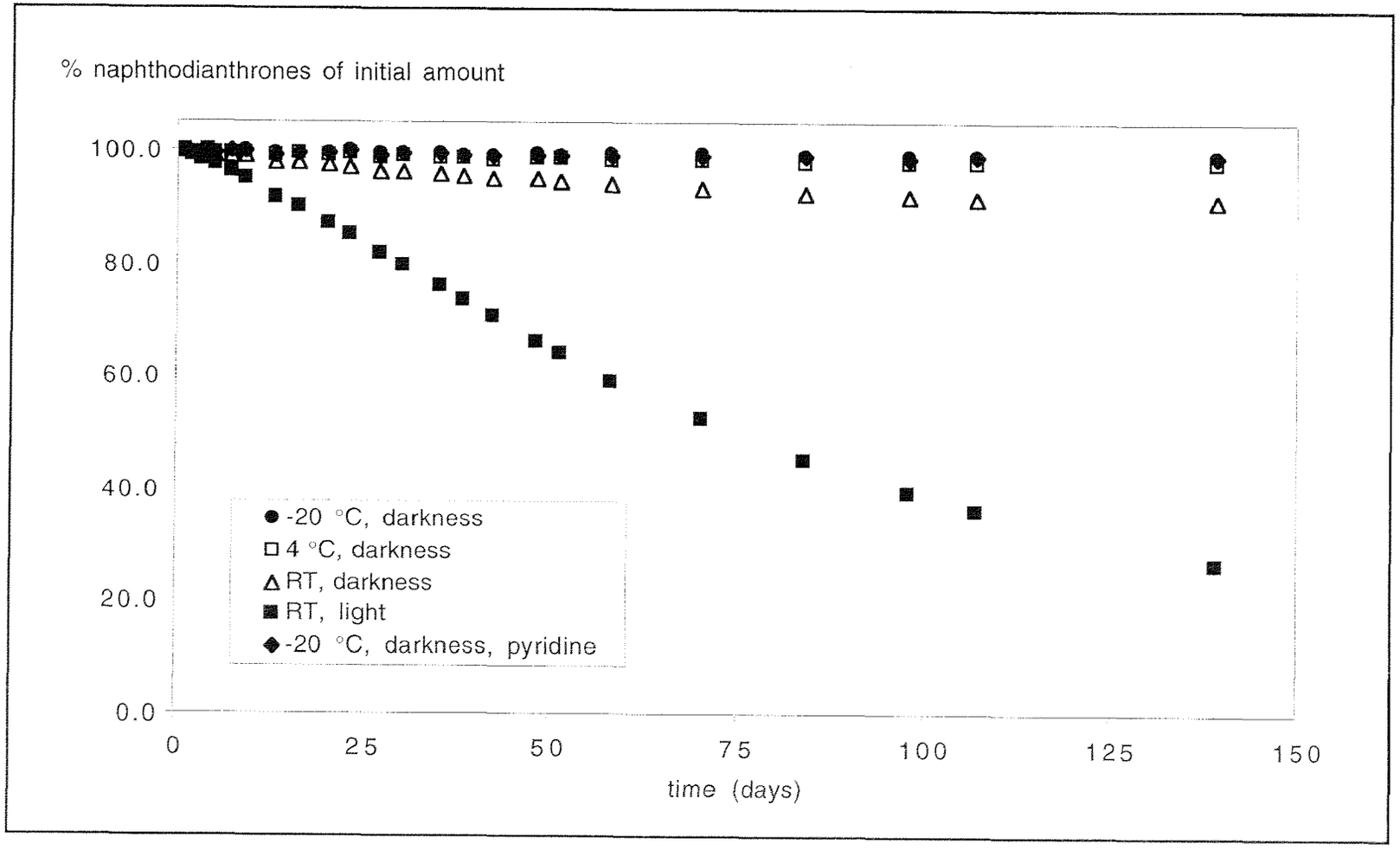


Figure 3.8 Degradation of naphthodianthrones under different conditions determined by VIS spectroscopy

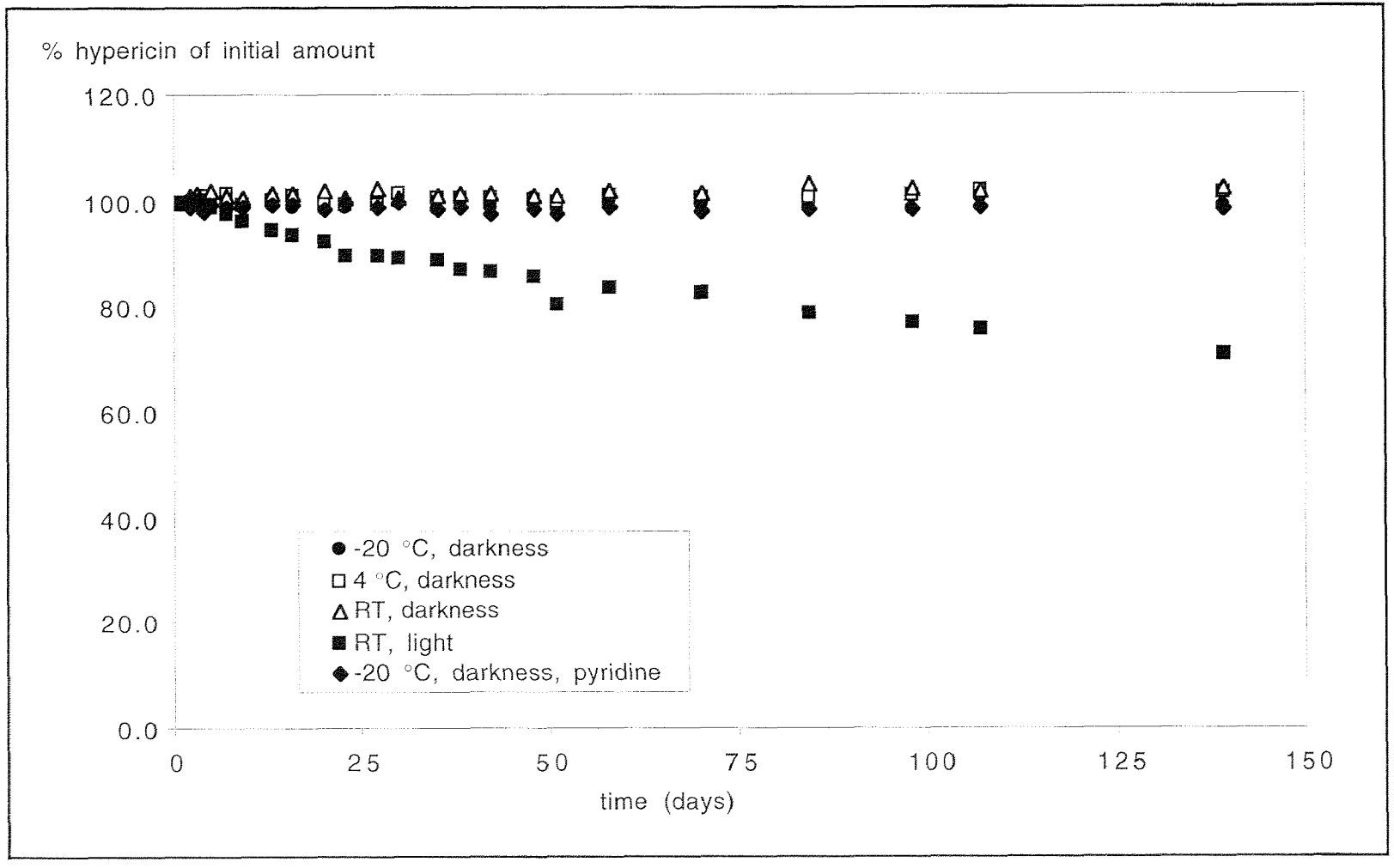


Figure 3.9 Degradation of hypericin under different conditions determined by HPLC

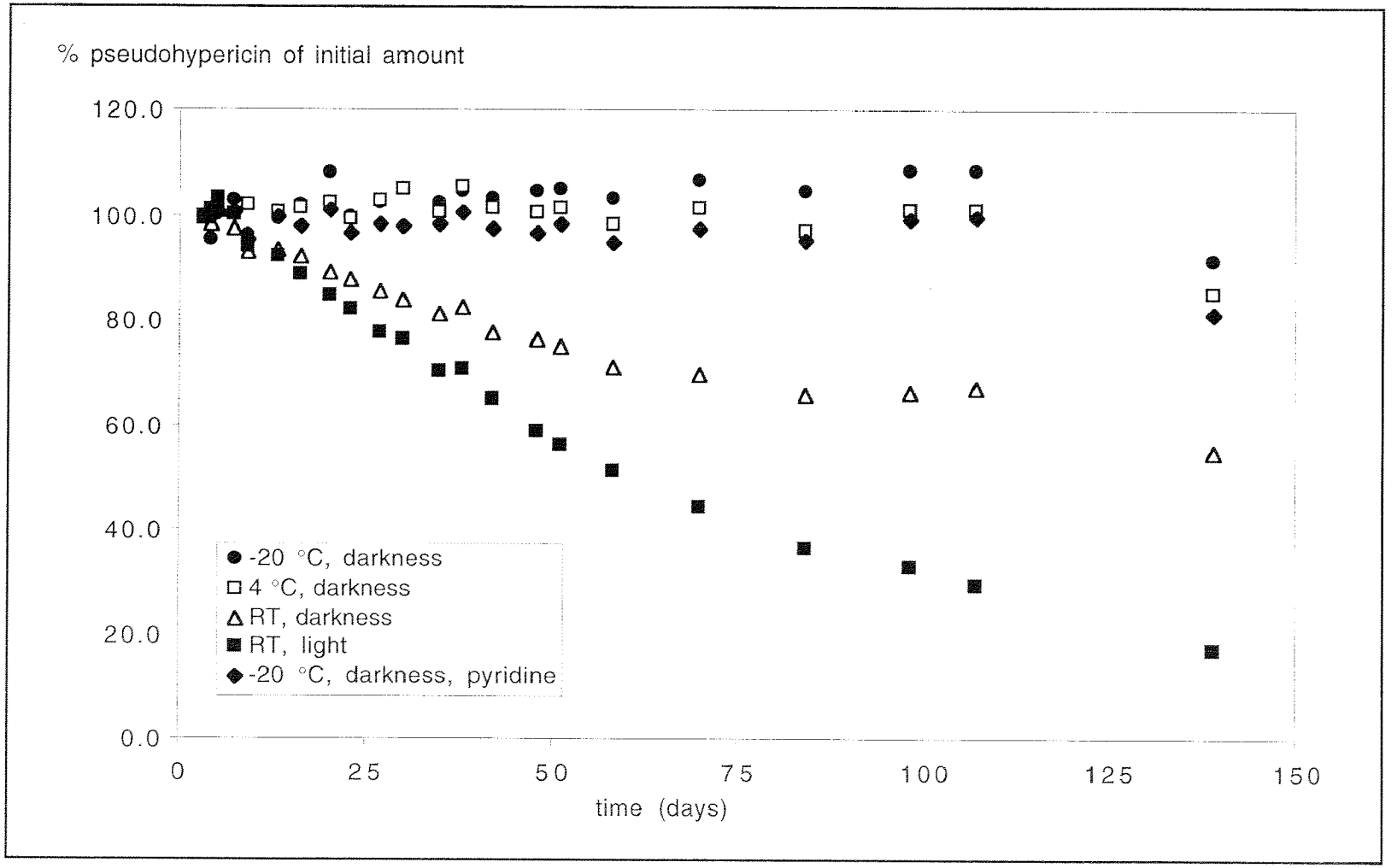


Figure 3.10 Degradation of pseudohypericin under different conditions determined by HPLC

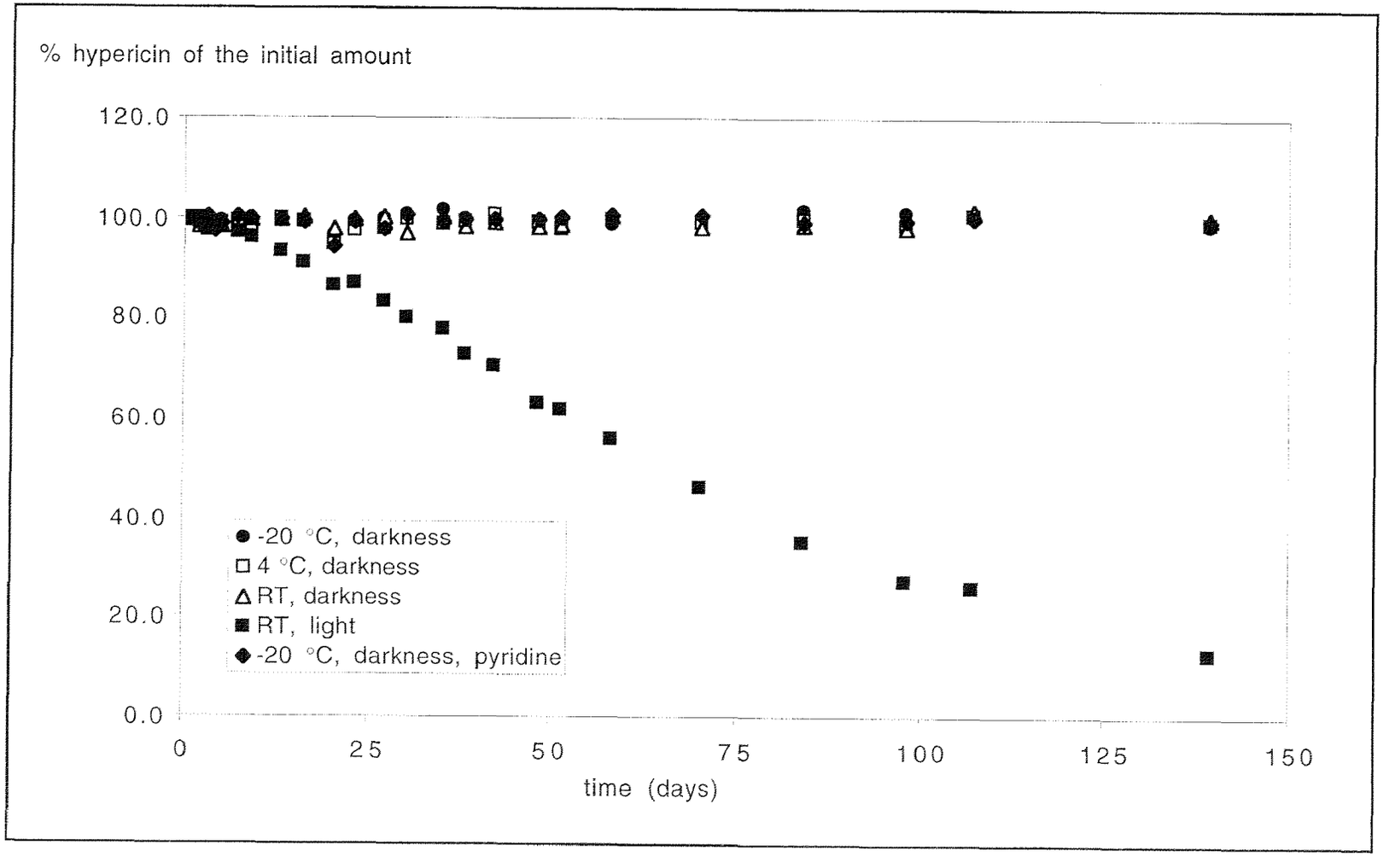


Figure 3.11 Degradation of hypericin in the extract solutions under different conditions determined by HPLC

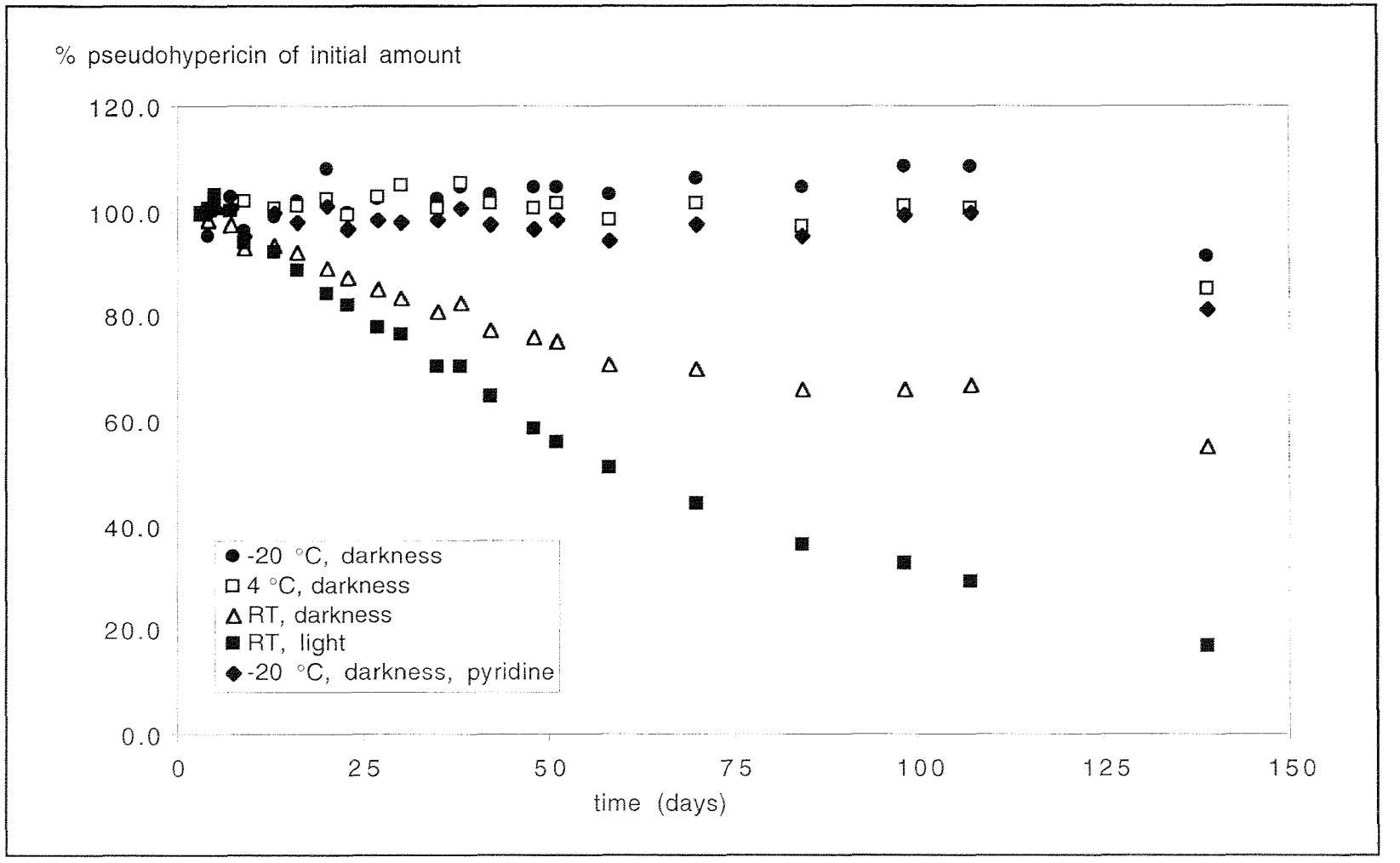


Figure 3.12 Degradation of pseudohypericin in the extract solutions under different conditions determined by HPLC

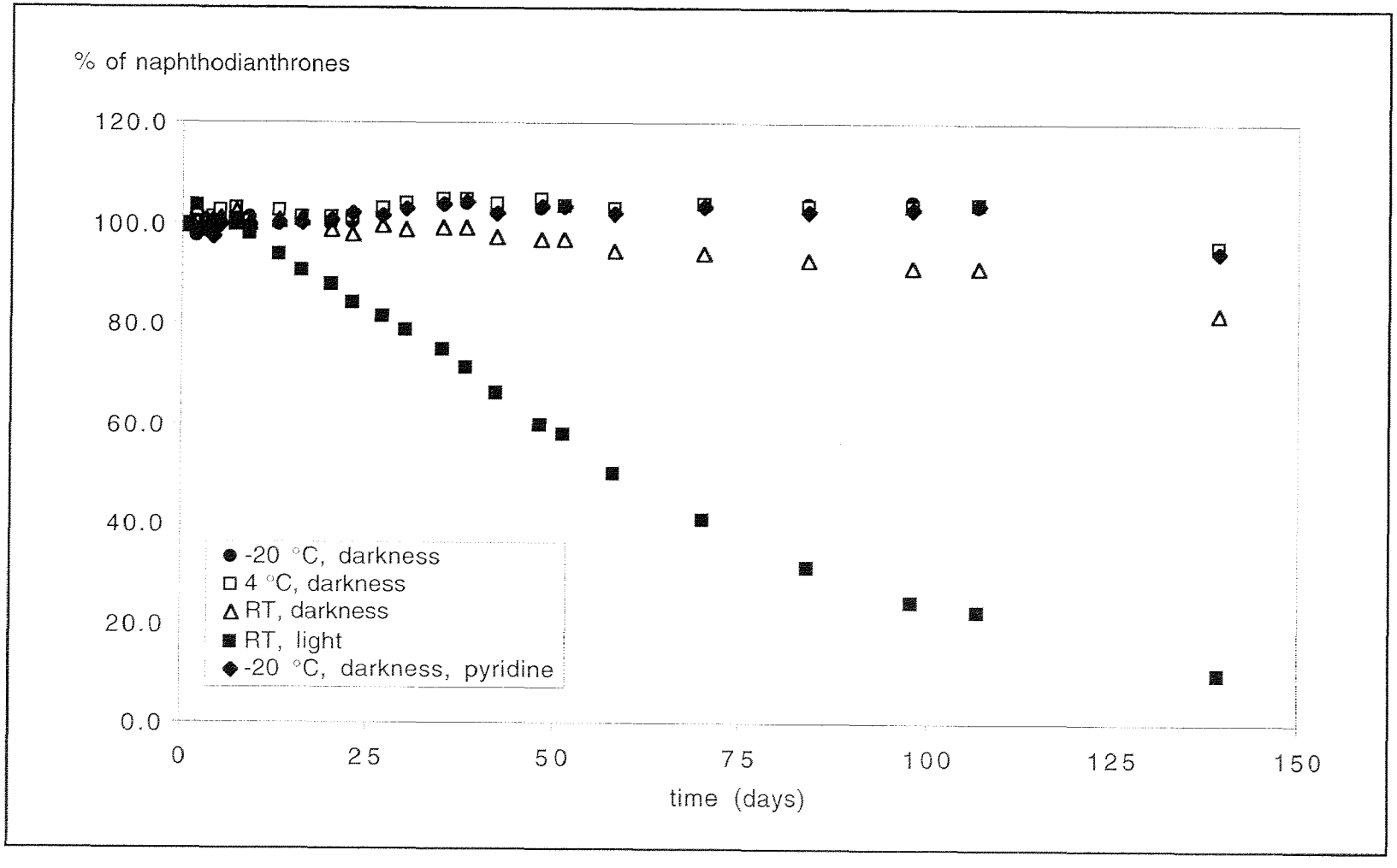


Figure 3.13 Degradation of naphthodianthrones in the extract solutions under different conditions determined by HPLC

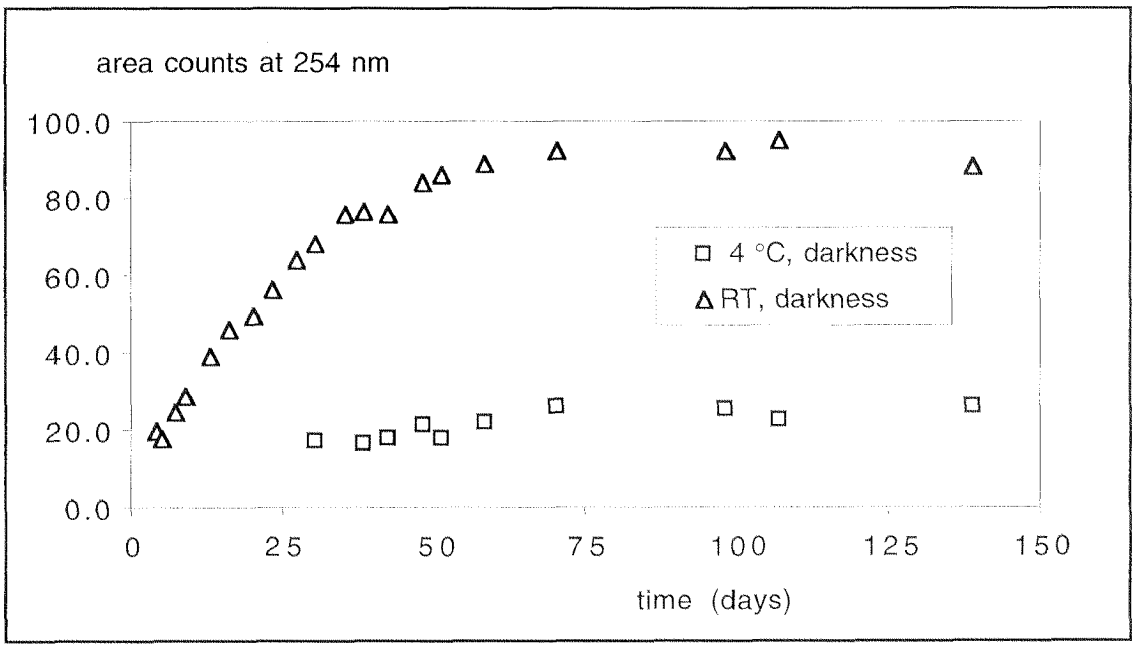


Figure 3.14 Appearance of the degradation product cyclopseudohypericin in pseudohypericin solutions determined by HPLC

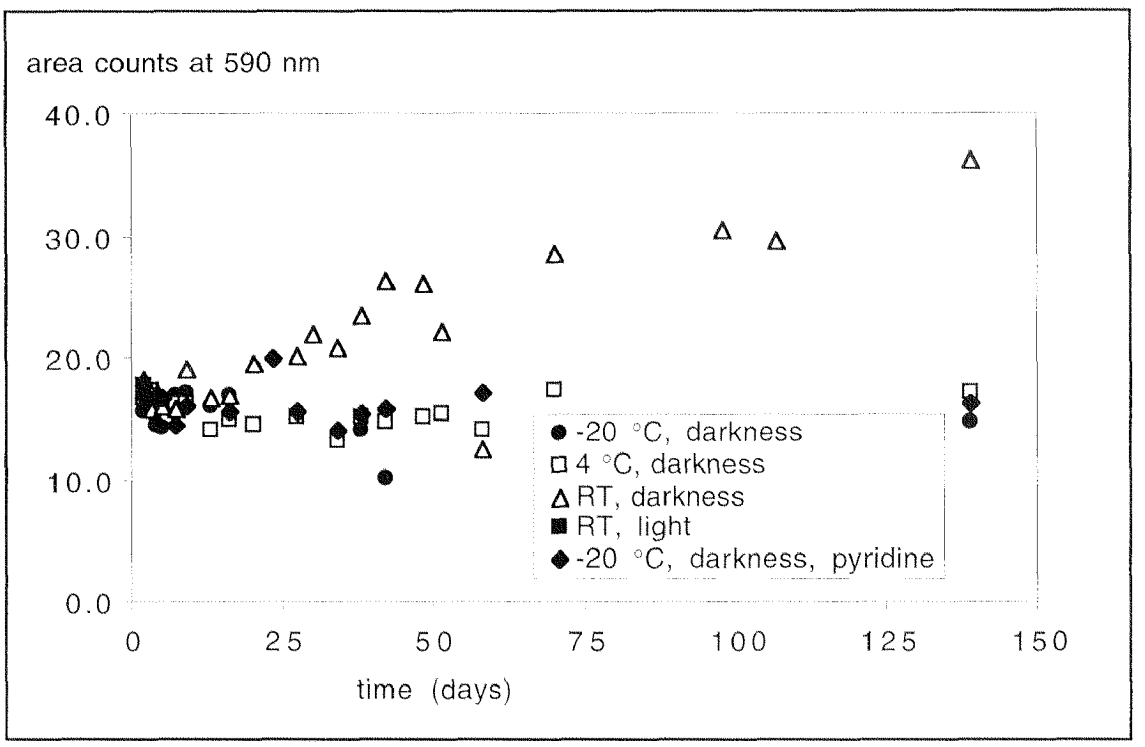


Figure 3.15 Appearance of the degradation product cyclopseudohypericin in extract solutions determined by HPLC

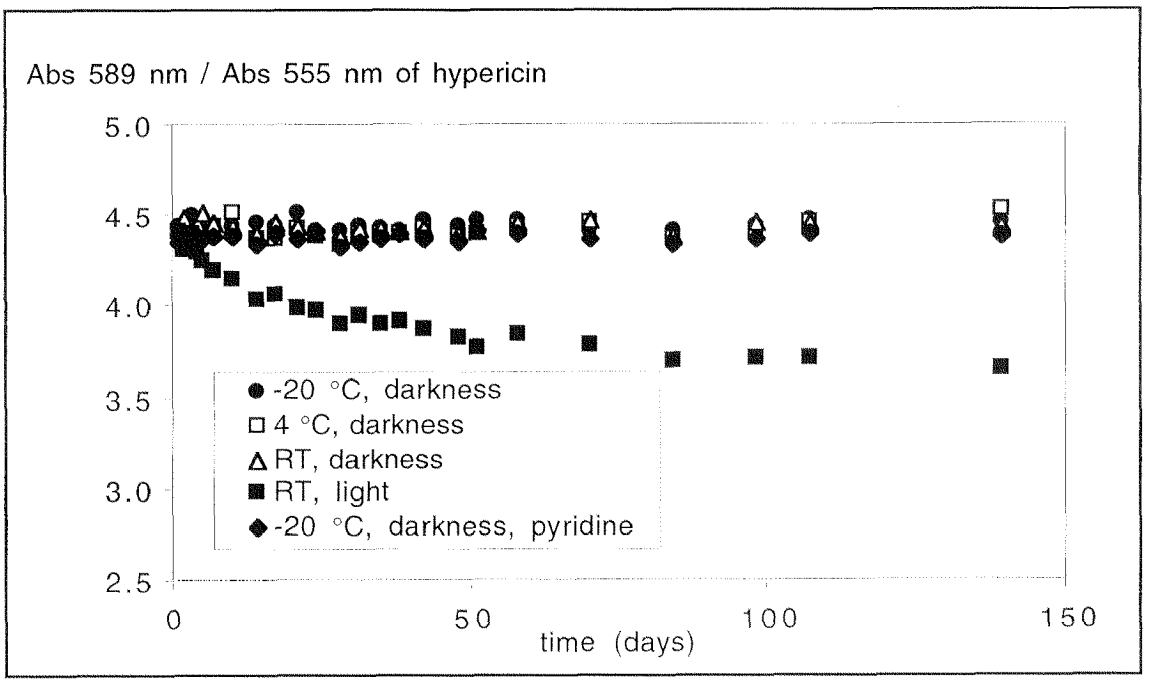


Figure 3.16 Changing of the ratio Abs 555 nm / Abs 589 nm of hypericin solutions under different conditions

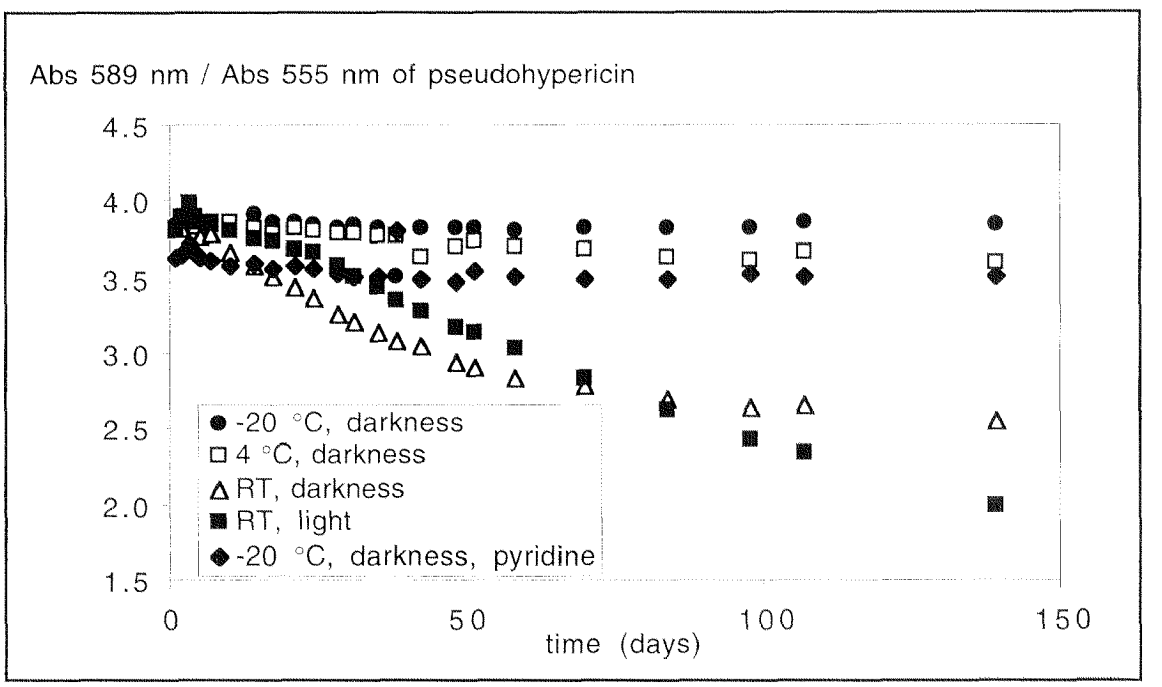


Figure 3.17 Changing of the ratio Abs 555 nm / Abs 589 nm of pseudohypericin solutions under different conditions

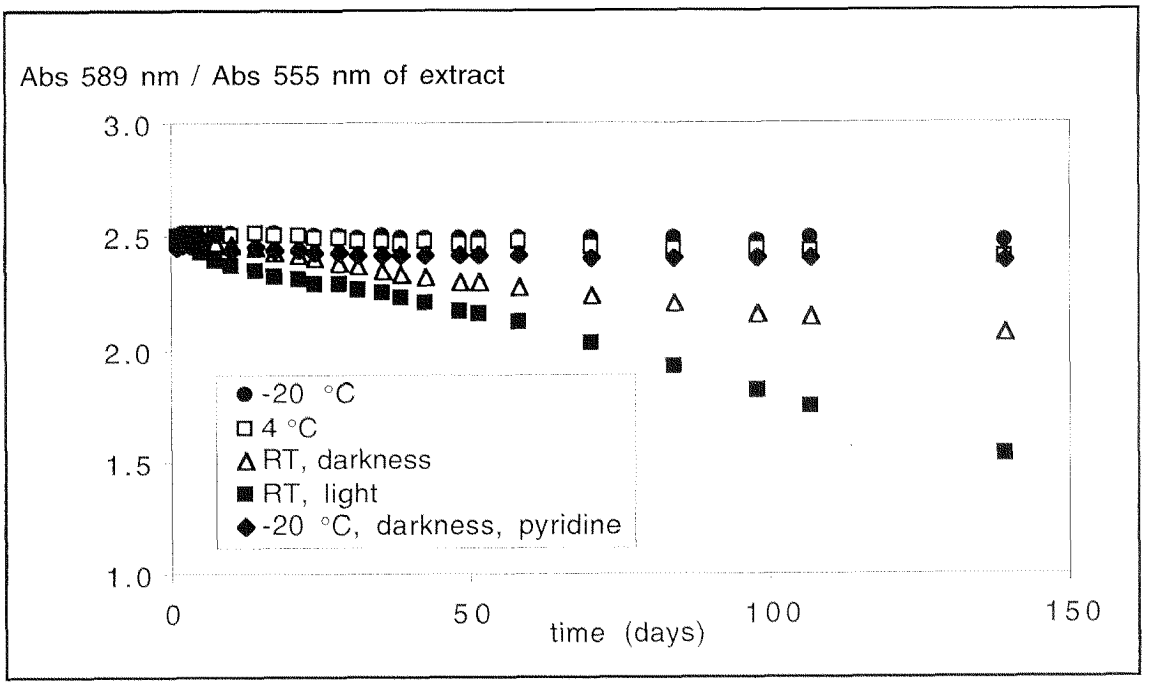


Figure 3.18 Changing of the ratio Abs 555 nm / Abs 589 nm of extract solutions under different conditions

3.4 Conclusions

Based on the results of this investigation, hypericin reveals a higher molar coefficient of absorbance (51936) than pseudohypericin (43486). As the quantification of pseudohypericin is generally done with the calibration graph of hypericin, evaluated contents are too low. Therefore, it should be worked with standard solutions of hypericin *and* pseudohypericin. However, as the availability of pure pseudohypericin is limited, the use of a conversion factor is reasonable as far as HPLC is applied for quantification. Referring to the calibration graphs of hypericin and pseudohypericin of section 3.3.2, the conversion factor (F) is 0.8 (F = Area pseudohypericin per 10 ng / Area hypericin per 10 ng). The factor is valid for the HPLC method of Krämer and Wiartalla (1992).
The ratio of pseudohypericin to hypericin in extracts of Hyperici herba is not always the same, depending on the extracting solvent (see section 4 and Kurth and Spreemann, 1998) and the origin of the drug material (see section 4). Schütt (1996) found the ratio to vary from 1.5 to 7.5, depending on the origin of the drug material. As separate determination of hypericin and pseudohypericin can not be done with VIS spectroscopy, it is not possible to determine a conversion factor for VIS spectroscopy. Results will always contain some uncertainty and therefore, HPLC / DAD is the more precise method.
The specific coefficient of absorbance ($A^{1\%}$=870) used for quantification by the German Drug Codex (1986, 3rd suppl.), the Ph. Helv. 8 and the new monograph of the European Pharmacopoeia (Ph. Eur. III, suppl. 2000) is lower than that of hypericin in methanol-pyridine (99:1, v/v) determined in this study ($A^{1\%}$=1030), but slightly higher than the value of pseudohypericin ($A^{1\%}$=836). As the drug generally contains more pseudohypericin than hypericin, the applied value can be used as approximation.
Hypericin is only sparingly soluble in methanol and other common solvents. Addition of 1% pyridine, sonication and waiting time before dilution (about four days) are means to prevent incomplete dissolution leading to wrong calibration graphs. Stability tests proved that the addition of 1% pyridine and a waiting time of four days do not affect hypericin solutions, when excluded from light. The degradation of pseudohypericin, however, is slightly accelerated by pyridine and therefore not recommendable for standard solutions of pseudohypericin. Solubility is less a problem with pseudohypericin, as the additional hydroxyl group makes it better soluble in common solvents. It could be shown that the absorbance properties of hypericin are not influenced by the addition of 1% pyridine. Therefore, it is possible to prepare standard

solutions of hypericin with methanol-pyridine (99:1, v/v) and extract solutions with pure methanol.
The results of the investigation on stability indicate that light exposure should be reduced during extraction and manipulation with naphthodianthrones. This stands in conflict with the necessity to irradiate the extract solutions of Hyperici herba with light to convert the proto compounds to hypericin and pseudohypericin. Gaedcke (1997) showed the transformation to be completed after 15 min, using artificial light (Lumilux Daylight 36W/11) for irradiation. According to her results, no degradation took place within 30 min. Consequently, extracts should be exposed to artificial light at least 15 min, but not longer than 30 to 45 min. Furthermore, standard solutions of hypericin and pseudohypericin and also extract solutions should be stored at -20 °C to prevent degradation.

3.5 References

Ahrer, W., Falk, H. and Tran, H. T. N.: Concerning the Dissociation of Hypericin: An Atmospheric Pressure Ionization Mass Spectrometric Study. *Monatshefte für Chemie* **129**: 643-647 (1998)

Altmann, R. and Falk, H.: The Deprotonation and Protonation Equilibria of a Hypericin Derivative in Aqueous Solution. *Monatshefte für Chemie* **128**: 571-583 (1997)

Amer, A. M., Falk, H. and Tran, H. T. N.: The Dissociation and Tautomerization Equilibria of Hypericin: Alkyl Protected Hydroxyl Derivatives. *Monatshefte für Chemie* **129**: 1237-1244 (1998)

Brockmann, H., Pohl, F., Maier, K. and Haschad, M. N.: Über das Hypericin, den photodynamischen Farbstoff des Johanniskrautes (*Hypericum perforatum*). *Annalen der Chemie* **553**: 52 (1942)

Brolis, M., Gabetta, B., Fuzzati, N., Pace, R., Panzeri, F. and Peterlongo, F.: Identification by High-Performance Liquid Chromatography-Diode Array Detection-Mass Spectrometry and Quantification by High-Performance Liquid Chromatography-UV Absorbance Detection of Active Constituents of *Hypericum perforatum*. *J. Chromatogr. A* **825**: 9-16 (1998)

Burel, L. and Jardon, P.: Homo-association de l'hypéricine dans l'eau et conséquences sur ses propriétés photodynamiques. *J. Chim. Phys.* **93**: 300-316 (1996)

Burel, L., Jardon, P. and Lepretre, J.-C.: Electrochemical Behavior of Hypericin in Aqueous Liposome and Dimethylsulfoxide Media. *New J. Chem.* **21**: 399-403 (1997)

Butterweck, V.: Beitrag zur Pharmakologie und Wirkstoff-Findung von *Hypericum perforatum* L. Thesis. Mathematisch-Naturwissenschaftliche Fakultät. Westfälische Wilhelms-Universität Münster, 1997

Cameron, D. W. and Raverty, W. D.: Pseudohypericin and Other Phenanthroperylene Quinones. *Aust. J. Chem.* **29**: 1523-1533 (1976)

Eloy, D., Le Pellec, A. and Jardon, P.: Protonation et déprotonation dans l'état fondamental et le premier état singulet excité de l'hypéricine en milieu micellaire non ionique. *J. Chim. Phys.* **93**: 442-457 (1996)

Etzlstorfer, C., Falk, H. and Oberreiter, M.: On the Tautomerism of Hypericin: The 1,6-Dioxo Tautomer. *Monatshefte für Chemie* **124**: 923-929 (1993)

Etzlstorfer, C., Falk, H., Müller, N. and Tran, T. N. H.: Structural Aspects and Electronic Absorption of the Hydroxyphenanthroperylene Quinones Fringlit D, Hypericin, and Stentorin. *Monatshefte für Chemie* **127**: 659-668 (1996)

European Pharmacopoeia (Ph. Eur. III, suppl. 2000), Hypericum Monograph, Strasbourg, Cedex, Council of Europe

Falk, H. and Mayr, E.: Synthesis and Properties of Fringelite D (1,3,4,6,8,10,11,13-octahyrdoxyphenanthro[1,10,9,8,o,p,q,r,a] perylene-7,14-dione). *Monatshefte für Chemie* **126**: 699-710 (1995)

Falk, H. and Meyer, J.: On the Homo- and Heteroassociation of Hypericin. *Monatshefte für Chemie* **125**: 753-762 (1994)

Falk, H. and Schmitzberger, W.: On the Nature of "Soluble" Hypericin in Hypericum Species. *Monatshefte für Chemie* **123**: 731-739 (1992)

Falk, H. and Schoppel, G.: On the Synthesis of Hypericin by Oxidative Trimethylemodin Anthrone and Emodin Anthrone Dimerization: Isohypericin. *Monatshefte für Chemie* **123**: 931-938 (1992)

Freeman, D., Frolow, F., Kapinus, E., Lavie, D., Lavie, G., Meruelo, D. and Mazur, Y.: Acidic Properties of Hypericin and its Octahydroxy Analogue in the Ground and Excited States. *J. Chem. Soc., Chem. Commun.*: 891-892 (1994)

Freytag, W. E.: Bestimmung von Hypericin und Nachweis von Pseudohypericin in *Hypericum perforatum* L. durch HPLC. *Dtsch. Apoth. Ztg.* **124**: 2383-2386 (1984)

Gaedcke, F.: Johanniskraut und dessen Zubereitungen. Qualitätsbeurteilung über ein selektives, reproduzierbares HPLC-Verfahren. *Dtsch. Apoth. Ztg.* **137**: 3753-3757 (1997)

Gai, F., Fehr, J. and Petrich, J. W.: Observation of Excited-State Tautomerization in the Antiviral Agent Hypericin and Identification of Its Fluorescent Species. *J. Phys. Chem.* **98**: 5784-5795 (1994)

German Drug Codex, St. John's Wort Monograph. DAC 1986 (3rd suppl.) Eschborn, Stuttgart: Govi- and Deutscher Apotheker-Verlag, 1991

Häberlein, H., Tschiersch, K.-P., Stock, S. and Hölzl, J.: Johanniskraut (*Hypericum perforatum* L.). *Pharm. Ztg. Wiss.* **137**: 169-173 (1992)

Jardon, P., Lazorchak, N. and Gautron, R.: Propriétés du premier état triplet de l'hypericine étude par spectroscopie laser. *Journal de chimie physique* **83**: 311-315 (1986)

Kapinus, E. I., Falk, H. and Tran, H. T. N.: Spectroscopic Investigation of the Molecular Structure of Hypericin and its Salts. *Monatshefte für Chemie* **130**: 623-635 (1999)

Kerb, R., Brockmöller, J., Staffeldt, B., Ploch, M. and Roots, I.: Single-Dose and Steady-State Pharmacokinetics of Hypericin and Pseudohypericin. *Antimicrobial Agents and Chemotherapy* **40**: 2087-2093 (1996)

Krämer, W. and Wiartalla, R.: Bestimmung von Naphthodianthronen (Gesamthypericin) in Johanniskraut (*Hypericum perforatum* L.). *Pharm. Ztg. Wiss.* **137**: 202-207 (1992)

Kurth, H. and Spreemann, R.: Phytochemical Characterization of Various St. John's Wort. *Advances in Therapy* **15**: 117-128 (1998)

Lavie, D., Freeman, D., Bock, H., Fleischer, J., van Kranenburg, K., Ittah, Y., Mazur, Y., Lavie, G., Liebes, L. and Meruelo, D.: Hypericin, a Potential Anti-AIDS Drug. *Proc. Xth Int. Nat. Symp. on Medicinal Chemistry (IUPAC)*. Jerusalem, 321-327, 1990

Lavie, G., Mazur, Y., Lavie, D. and Meruelo, D.: The Chemical and Biological Properties of Hypericin - A Compound with a Broad Spectrum of Biological Activities. *Medicinal Research Reviews* **15**: 111-118 (1995)

Liebes, L., Mazur, Y., Freeman, D., Lavie, D., Lavie, G., Kudler, N., Mendoza, S., Levin, B., Hochster, H. and Meruelo, D.: A Method for the Quantitation of Hypericin, an Antiviral Agent, in Biological Fluids by High-Performance Liquid Chromatography. *Analytical Biochemistry* **195**: 77-85 (1991)

Maisenbacher, P.: Untersuchungen zur Analytik von Johanniskrautöl. Thesis. Fakultät für Chemie und Pharmazie. Eberhard-Karls-Universtität Tübingen, 1991

Maisenbacher, P. and Kovar, K.-A.: Analysis and Stability of Hyperici Oleum. *Planta Medica* **58**: 351-354 (1992)

Müller-Kuhrt, L. and Boesel, R.: Analytik der Hypericine im Hypericum-Extrakt. *Nervenheilkunde* **12**: 359-361 (1993)

Niesel, S.: Untersuchungen zum Freisetzungsverhalten und zur Stabilität ausgewählter wertbestimmender Pflanzeninhaltsstoffe unter besonderer Berücksichtigung moderner phytochemischer Analysenverfahren. Thesis. Pharmazie. Freie Universität Berlin, 1992

Ostrowski, E.: Untersuchungen zur Analytik, ^{14}C-Markierung und Pharmakokinetik phenolischer Inhaltsstoffe von *Hypericum perforatum* L. Thesis. Pharmazie und Lebensmittelchemie. Philipps-Universität Marburg, 1988

Pace, N. and Mackinney, G.: On the Absorption Spectrum of Hypericin. *J. Am. Chem. Soc.* **61**: 3594-3595 (1939)

Papageorgiou, G. C., Alygizaki-Zorba, A., Loukas, S. and Brody, S. S.: Photodynamic Effects of Hypericin on Photosynthetic Electron Transport and Fluorescence of *Anacystis nidulans* (*Synechococcus* 6301). *Photosynthesis Research* **48**: 221-226 (1996)

Pharmacopoeia Helvetica (Ph. Helv. 8), St. John's Wort Monograph, Bern, Eidgenössische Drucksachen- und Materialzentrale

Raser, L. N., Kolaczkowski, S. V. and Cotton, T. M.: Resonance Raman and Surface-Enhanced Resonance Raman Spectroscopy of Hypericin. *Photochemistry and Photobiology* **56**: 157-162 (1992)

Sattler, S.: Naphthodianthrone aus *Hypericum perforatum* L.: Isolierung, Pharmakokinetik, Löslichkeitsverbesserung und Absorptionsstudien am Caco-2 Zellkulturmodell. Thesis. Pharmazie und Lebensmittelchemie. Philipps-Universität Marburg/Lahn, 1997

Scheibe, G. and Schöntag, A.: Lichtabsorption und Fluorescenz des Hypericins. *Chem. Ber.* **75**: 2019-2026 (1942)

Schütt, H.: Morphologische, phytochemische und botanische Untersuchungen zur Selektion hypericin-, pseudohypericin- und flavonoidreicher *Hypericum perforatum* L. Stämme. Thesis. *Dissertationes Botanicae* **263**. Cramer, J. Berlin, Stuttgart, 1996

Sevenants, M. R.: Pigments of *Blepharisma undulans* Compared with Hypericin. *J. Protozool.* **12**: 240-245 (1965)

Stock, S.: ^{14}C-Markierung und Pharmakokinetik von Naphthodianthronen. Thesis. Pharmazie und Lebensmittelchemie. Philipps-Universität Marburg/Lahn, 1992

Vandenbogaerde, A. L., Delaey, E. M., Vantieghem, A. M., Himpens, B. E., Merlevede, W. J. and de Witte, P. A.: Cytotoxicity and Antiproliferative Effect of Hypericin and Derivatives after Photosensitization. *Photochemistry and Photobiology* **67**: 119-125 (1998a)

Vandenbogaerde, A. L., Kamuhabwa, A., Delaey, E., Himpens, B. E., Merlevede, W. J. and de Witte, P. A.: Photocytotoxic Effect of Pseudohypericin versus Hypericin. *Journal of Photochemistry and Photobiology B: Biology* **45**: 87-94 (1998b)

Walker, E. B., Lee, T. Y. and Song, P.-S.: Spectroscopic Characterization of the Stentor Photoreceptor. *Biochim. Biophys. Acta* **587**: 129-144 (1979)

Wynn, J. L. and Cotton, T. M.: Spectroscopic Properties of Hypericin in Solution and at Surfaces. *J. Phys. Chem.* **99**: 4317-4323 (1995)

Xia, G., He, X., Zhou, Y., Zhang, M. and Shen, T.: A Study of Photoinduced Electron Transfer and Redox Properties of Hypericin. *Journal of Photochemistry and Photobiology A: Chemistry* **114**: 31-35 (1998)

Yamazaki, T., Ohta, N. and Yamazaki, I.: Excited-State Properties of Hypericin: Electronic Spectra and Fluorescence Decay Kinetics. *J. Phys. Chem.* **97**: 7870-7875 (1993)

4 Study of some Factors influencing the Quantification of Naphthodianthrones

Most of the commercial extracts of Hyperici herba are standardized on a certain content of naphthodianthrones. The evaluation of this content is affected by various factors, as the quality of the reference compounds, the extraction and the quantification method. Comparison of commercial extracts in respect to the content of naphthodianthrones is only possible, if these factors are well known.

4.1 Comparison of HPLC-DAD Methods, VIS Spectroscopy and TLC-Densitometry for the Quantification of Naphthodianthrones in the Dry Extract Ze117 of Hyperici herba

4.1.1 Introduction

There are existing various methods for the quantification of hypericin and pseudohypericin, making use of **HPLC** with UV/VIS or fluorimetric detection (Table 4.1.1 A-C), **VIS spectroscopy** (German Drug Codex 1986; Ph. Helv. 8), **fluorimetry** (Klein-Bischoff and Klumpp, 1993) and **TLC-densitometry** (Mulinacci *et al.*, 1999; Kartnig and Göbel, 1992; Maisenbacher, 1991).

HPLC Methods

Some of the developed HPLC methods allow the evaluation of the whole variety of constituents of extracts from Hyperici herba. These methods are efficient tools for the comparison of extracts and products and for the validation of extraction procedures (Meier, 1999). However, most of them are less suited for the quantification of hypericin and pseudohypericin due to long retention times of the naphthodianthrones (Table 4.1.1C, e.g. Butterweck, 1997; Häberlein *et al.*, 1992; Hölzl and Ostrowski, 1987).

Besides the methods for qualitative analysis, various methods were elaborated for specific quantification of hypericin and pseudohypericin in

extracts of Hyperici herba and in alcoholic beverages (Table 4.1.1A) and in plasma samples (Table 4.1.1B). Most of them reveal short run times. Methods only considering hypericin, not mentioning pseudohypericin, were developed by Chi and Franklin (1999), Micali *et al.* (1996) and Liebes *et al.* (1991).

Most of the HPLC methods make use of reversed-phase material (C-18) of medium grain size (4-5 µm). Liebes *et al.* (1991) worked with less hydrophobic phenyl reversed-phase material to reduce the retention time of hypericin. Chi and Franklin (1999) applied C-8 material.
Peak broadening and tailing can be diminished by shortening the column length. Short columns combined with short retention times (Gaedcke, 1997; Schütt *et al.*, 1994) improve the sharpness and the symmetry of the peaks. Schütt (1996) reported variations of the flow to reduce peak tailing of hypericin as well. Some methods run at increased temperature generally improving peak shape and shortening retention time (Gaedcke, 1997; Kerb *et al.*, 1996; Micali *et al.*, 1996).
Neutral and acidic systems are employed as eluents. Phosphoric acid, acidic phosphate buffers and trifluoroacetic acid are used for acidification. Piperopoulos *et al.* (1997) showed a triethylammonium acetate buffer (pH 7) to give good results for combined HPLC-ESI (electrospray mass spectrometry) experiments. Brolis *et al.* (1998) replaced the phosphoric acid with citric acid for ESI detection.
Isocratic and gradient elution are both used. As long as pseudohypericin is calculated as hypericin and detection is done by absorption measurement, isocratic elution is to be preferred (Niesel, 1992). Various factors are influencing the molar coefficient of absorbance of hypericin and pseudohypericin, as for example the kind of solvent and the formation of homoassociates in the presence of water (see section 3). Using gradient elution, the composition of the eluent at the detection time of the two compounds can be different, causing hypericin and pseudohypericin to reveal not the same coefficients of absorbance and leading to wrong results. Methods with isocratic elution were developed by Gaedcke (1997), Kerb *et al.* (1996), Krämer and Wiartalla (1992), Niesel (1992), Schütt and Hölzl (1994), Stock (1992) and Zevakov *et al.* (1991).
The toxicity of the solvents is a further point of concern. Considering acetonitrile and tetrahydrofuran as quite toxic solvents, methods using eluents of low toxicity were elaborated by Freytag (1984), Gaedcke (1997), Krämer and Wiartalla (1992) and Schütt *et al.* (1994). UV and VIS absorption is the detection mode generally applied. VIS absorption at 590 nm, the long wavelength band of hypericin and pseudohypericin, is

Table 4.1.1A HPLC methods described in literature for the quantification of hypericin (H) and pseudohypericin (P) in extracts of Hyperici herba and in alcoholic beverages

Reference	Column[a]	Eluent[b] and Flow	°C	Detection	H [min]	P [min]
Freytag (1984)	LiChrosorb RP-18 (250x4 mm I.D., 5 µm)	MeOH-EtOAc-0.1 M NaH_2PO_4 (pH 2.1) (5.6:1.6:1, m/m) Flow: 0.8 ml/min	RT	VIS absorption (590 nm)	8	-
Freytag (1984)	LiChrospher 100 CH-18/2 (250x4 mm I.D., 5 µm)	A: MeOH, B[d]: aqueous H_3PO_4 (pH 2.0) 0-15 min A-B (25:75) to A-B (5:95) Flow: 1 ml/min	RT	VIS absorption (590 nm)	9	18
Gaedcke (1997)	Kromasil RP-18 (125x4 mm I.D., 5 µm) (MZ-Analytical)	MeOH-EtOAc-NaH_2PO_4/H_3PO_4 (pH 2.1) (3.6:1:1.2, m/m) Flow: 1 ml/min	40	VIS absorption (590 nm)	5	1.8
Krämer and Wiartalla (1992)	LiChrosorb RP-18 (250x4 mm I.D., 5 µm) (Merck)	MeOH-EtOAc-0.1 M NaH_2PO_4/H_3PO_4 (pH 2.1) (3.6:1:1.2, m/m) Flow: 0.8 ml/min	25-28	VIS absorption (590 nm)/Fluorimetric emission (excitation 254 nm, emission >530 nm)	14.0	5.3
Maisen-bacher (1991)	LiChrospher 100 RP-18 (125x4 mm I.D., 5 µm) (Merck)	A: MeOH-THF (315:185) B: 0.01 M $NH_4H_2PO_4/H_3PO_4$ (pH 2.2-2.7) Elution profile: 0-5 min A-B (50:50) to A-B (80:20), 5.01-15 min A-B (80:20) Flow: 0.6 ml/min	30	VIS absorption (588 nm)	14	11.9
Micali *et al.* (1996[e])	HS RP-18 (83x4 mm I.D., 3 µm) (Perkin Elmer)	A: MeOH-0.05 M Na_2HPO_4/KH_2PO_4 (pH 7) (7:3), B: H_2O-MeOH (3:7) Elution profile: 0-3 min 100% A, 3.01-5 min 100% A to 100% B, 5.01-18.0 100% B Flow: 1.5 ml/min	50	Fluorimetric emission (excitation 470 nm, emission 590 nm)	9	-

Reference	Column[a]	Eluent[b] and Flow	°C	Detection	H [min]	P [min]
Niesel (1992)	MN Nucleosil 5 100 RP-18, (250x4 mm I.D.)	MeOH-0.01 M NaH_2PO_4/NaOH (75:25) Flow: 0.8 ml/min	35	VIS absorption (589 nm)	19.5	6.5
Ostrowski (1988)	Supersphere RP-18 (250 mm, 4 µm) (Merck)	MeOH-ACN-THF-H_2O-H_3PO_4 (19.8:48.3:11.4:19.5:1) Flow profile: 0-17 min 0.3 ml/min, 17.01-50 min 1.3 ml/min	c	UV absorption (254 nm)	23	14.4
Pipero-poulos *et al.* (1997)	LiChrosorb RP-18 (125x4 mm I.D., 5 µm) (Merck)	A: MeOH-ACN (5:4), B: 0.1 M triethylammonium acetate (pH 7) Elution profile: 0-8 min A-B (70:30) to A-B (90:10), 8.01-13 min A-B (90:10) to A-B (70:30) Flow: 0.6 ml/min	c	(1) VIS absorption (590 nm) (2) Electrospray mass spectrometry for qualitative analysis	11.9	8.2
Schütt and Hölzl (1994)	LiChrospher 100 RP-18 (125 mm, 5 µm) (Merck)	MeOH-EtOAc-0.1 M NaH_2PO_4/H_3PO_4 (pH 2.1) (3.6:1:1.2) Flow: 1 ml/min	c	VIS absorption (590 nm)	7.6	2.5
Schütt (1996)	LiChrospher 100 RP-18 (250x4 mm I.D., 5 µm)	MeOH-ACN-THF-H_2O-H_3PO_4 (18.5:49:4:28:0.5) Flow profile: 0-2 min 0.5 to 1 ml/min, 2.01-5 min 1 ml/min, 5.01-8 min 1 to 2 ml/min, 8.01-10 min 2 to 2.5 ml/min, 10.01-15 min 2.5 ml/min, 15.01-20 min 2.5 to 0.5 ml/min	RT	VIS absorption (590 nm)	12.3	4.8
Stock (1992[f])	LiChrosorb 100 RP-18 (250x4 mm I.D., 5 µm) (Merck)	MeOH-ACN-H_3PO_4 85% (69.5:30:0.5) Flow: 1 ml/min	c	UV absorption (254 nm)/Fluorimetric emission (excitation 580 nm, emission 600 nm)	13.9	3.4

Reference	Column[a]	Eluent[b] and Flow	°C	Detection	H [min]	P [min]
Tateo *et al.* (1998[e])	Techsphere RP-18 (150x4.6 mm I.D., 3 µm)	A: ACN-MeOH-H_3PO_4 (59:40:1) B: ACN-H_2O-H_3PO_4 (19:80:1) Elution profile: 0-8 min 100% B, 8.01-28 min 100% B to 100% A, 28.01-55 min 100% A	c	VIS absorption (590 nm)	33.5	27
Zeller Company (1992)	Spherisorb ODS II (250x4 mm I.D., 5 µm) (Bischoff)	A: ACN-THF (6:4), B: (1.2% H_3PO_4 in H_2O) Elution profile: 0-3 min A-B (50:50), 3.01-10 min A-B (50:50) to A-B (70:30), 10.01-13 min A-B (70:30)	30	VIS absorption (590 nm)	10	4
Zevakov *et al.* (1991)	Silasorb (250x4 mm I.D., 7 µm)	H_2O-EtOAc-MeOH-ACN-H_3PO_4 (41:25:22:9:3)	c	VIS absorption (590 nm)	16.0	13.7

a Precolumns are not listed in this table.
b Wash out and reequilibration are not considered.
c not mentioned in the reference
d not clearly defined
e analysis of alcoholic beverages
f The method was used for analysis of plasma samples as well (Table 4.1.1B)

Table 4.1.1B HPLC methods described in literature for the quantification of hypericin (H) in plasma samples. Some of the methods also include the analysis of pseudohypericin (P).

Reference	Column[a]	Eluent[b] and Flow	°C	Detection	H [min]	P [min]
Chi and Franklin (1999)	RP-8 (150x4.6 mm I.D., 5 µm) (Captital HPLC)	MeOH-0.03 M KH_2PO_4/K_2HPO_4 (pH 7) (7:3) Flow: 1 ml/min	60	Fluorimetric emission (excitation 315 nm, emission 519 nm)	3.8	-
Kerb *et al.* (1996)	LiChrospher 60 RP select B (250x4 mm I.D.) (Merck)	MeOH-THF-0.1 M NaH_2PO_4 (pH 4) (45:30:25) Flow: 0.8 ml/min	60	Fluorimetric emission (excitation 315 nm, emission 590 nm)	8.6	5.5
Liebes *et al.* (1991)	RP-phenyl (100x4.5 mm I.D., 4 µm) (Waters Associates)	A: ACN-0.1% $(NH_4)_3PO_4$/NaOH (pH 7) (3:7), B: ACN-H_2O (7:3) Elution profile: 0-15 min 100% A to 100% B, 15.01-19 min 100% B Flow: 1.2 ml/min	c	VIS absorption (590 nm)/Fluorimetric emission (excitation 470 nm, emission >550 nm)	12.9	-
Stock (1992[d])	LiChrosorb 100 RP-18 (250x4 mm I.D., 5 µm) (Merck)	MeOH-ACN-H_3PO_4 85% (69.5:30:0.5) Flow: 1 ml/min	c	UV absorption (254 nm)/Fluorimetric emission (excitation 580 nm, emission 600 nm)	13.9	3.4

a Precolumns are not listed in this table.
b Wash out and reequilibration are not considered.
c not mentioned in the reference
d The method was also applied to analyse extracts from Hyperici herba (4.1.1A).

Table 4.1.1C HPLC methods described in literature for the qualitative analysis of extracts of Hyperici herba including the determination of hypericin (H) and pseudohypericin (P)

Reference	Column[a]	Eluent[b] and Flow	°C	Detection	H [min]	P [min]
Brantner *et al.* (1994)	Superspher RP-18 (250x4 mm I.D.) (Merck)	A: H_2O, B: H_3PO_4, C: ACN, D: MeOH Elution profile: 0-30 min A-B-C (82:1:17), 30.01-40 min A-B-C (67:1:32), 40.01-45 min A-B-C-D (30:1:49:20), 45.01-55 min A-B-C-D (30:1:49:20), 55.01-75 min A-B-C-D (10:1:49:40) Flow profile: 0-40 min 1 ml/min, 40.01-75 min 0.6 ml/min	c	UV absorption (254 nm)	c	c
Brolis *et al.* (1998)	201 TP 54 RP-18 (250x4.6 mm I.D., 5 µm) (Vydac Separation Group Hesperia)	A1 (Detection 1): H_2O-85% H_3PO_4 (99.7:0.3), A2 (Detection 2): H_2O-HCOOH (99.7:0.3), B: ACN, C: MeOH Elution profile: 0-10 min 100% A to A-B (85:15), 10.01-30 min A-B (85:15) to A-B-C (70:20:10), 30.01-40 min A-B-C (70:20:10) to A-B-C (10:75:15), 40.01-55 min A-B-C (10:75:15) to A-B-C (5:80:15) Flow: 1 ml/min	30	(1) UV absorption (270 nm) (2) Electrospray mass spectrometry for qualitative analysis	43.4	40.9
Butterweck (1997)	Prosep RP-18 (150x4 mm I.D., 5 µm) (Latek)	A: ACN, B: 0.5% TFA in H_2O, C: MeOH-ACN-0.5% TFA (60:39.5:0.5) Elution profile: 0-5 min A-B (1:9) to A-B (2:8), 5.01-7.0 min A-B (2:8) to B-C (7:3), 7.01-10 B-C (7:3) to B-C (5:5), 10.01-20 min B-C (5:5) to 100% C, 20.01-45 min 100% C Flow: 1 ml/min	25	UV absorption (284 nm)	30.7	24.1

Reference	Column[a]	Eluent[b] and Flow	°C	Detection	H [min]	P [min]
Häberlein *et al.* (1992)	LiChrospher RP-18 (250x4 mm I.D., 5 µm) (Merck)	A: ACN-H_2O-H_3PO_4 (19:80:1) B: ACN-MeOH-H_3PO_4 (59:40:1) Elution profile: 0-8 min 100% A, 8.01-30 min 100% A to A-B (50:50), 30.01-40 min A-B (50:50) to 100% B, 40.01-60 min 100% B Flow profile: 0.6 to 0.8 ml/min (linear)	c	UV absorption (254 nm)	60.1	47.4
Hölzl and Ostrowski (1987)	Supersphere RP-18 (250x4 mm I.D.) (Merck)	A: ACN-H_2O-H_3PO_4 (19:80:1), B: ACN-MeOH-H_3PO_4 (59:40:1) Elution profile: 0-8 min 100% A, 8.01-30 min 100% A to A-B (50:50), 30.01-45 min A-B (50:50) to 100% B, 45.01-80 min 100% B Flow: 0.6 ml/min	RT	UV absorption (254 nm)	75.5	59.3
Mulinacci *et al.* (1999)	LiChrosorb RP-18 (250x4.6 mm I.D., 5 µm) (Merck)	A: H_2O/H_3PO_4 (pH 3.2), B: MeOH, C: ACN Elution profile: 0-10 min A-C (88:12), 10.01-15 min A-C (82:18), 15.01-30 min A-C (82:18) to A-C (55:45), 30.01-35 min A-C (55:45) to B-C (55:45), 35.01-42 min B-C (55:45) Flow: 1 ml/min	26	VIS absorption (590 nm)	36.2	37.1
Ostrowski (1988)	Supersphere RP-18 (250 mm, 4 µm) (Merck)	A: ACN-MeOH-H_2O-H_3PO_4 (36:20:44:1), B: ACN-MeOH-H_3PO_4 (59:40:1) Elution profile: 0-3 min 100% A, 3.01-6 min 100% A to 100% B, 6.01-45 min 100% B Flow: 0.6 ml/min	RT	UV absorption (254 nm)	40	24

a Precolumns are not listed in this table.
b Wash out and reequilibration are not considered.
c not mentioned in the reference

more selective than UV absorption. The sensitivity of VIS absorption is high, as the two compounds reveal high molar coefficients of absorption at 590 nm (see section 3.3.1). Even better sensitivity is achieved by fluorimetric detection (Chi and Franklin, 1999; Kerb *et al.*, 1996; Krämer and Wiartalla, 1992; Liebes *et al.*, 1991; Micali *et al.*, 1996; Stock, 1996). Brolis *et al.* (1998) and Piperopoulos *et al.* (1997) employed combined HPLC - electrospray mass spectrometry for the qualitative analysis of extracts of Hyperici herba.

VIS Spectroscopy and Fluorimetry

VIS spectroscopy with absorption measurement at 590 nm was applied by the German Drug Codex (1986) to evaluate the content of naphthodianthrones in extracts of Hyperici herba. The detection wavelength 590 nm is not in full accordance with the absorbance maxima of hypericin and pseudohypericin in methanol, which are 588 nm and 589 nm, respectively (see section 3). However, effects on the results are only marginal.

VIS spectroscopy does not allow the selective detection of hypericin and pseudohypericin. Furthermore, compounds absorbing at 590 nm, for example chlorophyll, are falsifying the spectrophotometric result, while they can be chromatographically separated from hypericin and pseudohypericin in HPLC (Krämer and Wiartalla, 1992). Therefore, VIS spectroscopy reveals a lower selectivity than HPLC methods causing the spectrophotometric results to be higher. Removal of interfering substances by preextraction is a mean to lower the results obtained by spectrophotometry. Preextraction with dichloromethane takes away chlorophyll diminishing differences but not equalizing the results of the two methods. It was assumed that the degradation products of chlorophyll and naphthodianthrones could not be removed by preextraction with dichloromethane (Schütt and Hölzl, 1994). Klein-Bischoff and Klumpp (1993) elaborated an additional process to remove chlorophyll from the extracts using a pre-column extraction on Sephadex LH 20.

Cyclopseudohypericin, protohypericin and protopseudohypericin are further compounds probably contributing to the differing results. The maxima of their longest wavelength absorption band are 555 nm for cyclopseudohypericin and 546 nm for the proto components (Gaedcke, 1997). Although these compounds do not have an absorption maximum at 590 nm, they still slightly increase VIS spectroscopic results, while they are separated from hypericin and pseudohypericin in most HPLC

methods. Cyclopseudohypericin is only a minor component of *Hypericum perforatum*. It is a transformation product of pseudohypericin as well (Häberlein *et al.*, 1992; see section 3.3.4). The proto components can be converted to hypericin and pseudohypericin by irradiation with light (Gaedcke, 1997). Therefore, irradiation with light diminishes differences between HPLC and VIS spectrophotometric results. It improves the reproducibility of both methods as well.
The fluorimetric method developed by Klein-Bischoff and Klumpp (1993) is more selective than VIS spectroscopy (DAC 1986), as only fluorescent compounds are detected. The VIS spectroscopic and the fluorimetric method convince by their simple equipment and procedure. However, they do not allow the separate detection of hypericin and pseudohypericin and the sample preparation is time consuming.

TLC-Densitometry

TLC-densitometry includes like HPLC a chromatographic separation of the compounds followed by VIS spectroscopic or fluorimetric detection. Mulinacci *et al.* (1999) compared a direct TLC-densitometric method with an HPLC method and found the accuracy and reproducibility of the two methods to be in good agreement. In contrast to fluorimetric detection in TLC-densitometry, spectrophotometric detection was used in the HPLC method. TLC-densitometry turned out to be more sensitive resulting from the application of different detection modes. The main advantage of TLC-densitometry is its low solvent consumption.
Kartnig and Göbel (1992) and Maisenbacher (1991) described further TLC-densitometry methods applying fluorimetric detection as well. They used dipping reagents increasing fluorescence intensity and improving stability, but complicating the process.

In this study, techniques for the quantification of hypericin and pseudohypericin in extracts of Hyperici herba were evaluated. HPLC, VIS spectroscopy and TLC densitometry were compared. Main reasons for the study were the lack of a comparison of different HPLC methods and the still open question why HPLC results are not consistent with VIS spectroscopic data. Limited reproducibility of the results applying the HPLC method from Zeller Company (1992) gave the final impetus to do further investigations on the factors influencing quantification. An aim was to improve the Zeller method and to find an assay with good selectivity, reproducibility, precision and short run time. Equipment and

procedure were considered as well. The selection of the HPLC techniques compared in this study was done at the beginning of 1997. The method of Krämer and Wiartalla (1992) was included in the investigation, as its eluent contains ethyl acetate, an unconventional solvent for HPLC analysis revealing high absorption up to 260 nm. Unlike the other methods, the Ostrowski method (Ostrowski, 1988) was elaborated applying the "PRISMA" model (Nyiredy *et al.*, 1985). The eluent consists of three solvents (methanol, acetonitrile and tetrahydrofuran), which derive from different selectivity groups, and water modifying the solvent strength. The method of Stock (1992) seemed interesting, as only traces of water are present in the solvent system. The method from Kerb *et al.* (1996) makes use of a two-solvent system together with an acidic water phase, as the method from Zeller Company does. The Zeller Company works with acetonitrile and tetrahydrofuran, while Kerb *et al.* (1996) replaced acetonitrile with the less toxic solvent methanol. Among the methods investigated, the Zeller method was the only one applying gradient elution. All the methods were modified, for example by employing the same column for all the methods and not making use of the flow gradient in the Ostrowski method.
The dry extract Ze117 of Hyperici herba was used to exclude influences deriving from the not manufactured drug material. It was assumed that in the dry extract most transformation processes were completed.

4.1.2 Experimental

Materials

The ethanol-water (1:1, m/m) dry extract (Ze117, batch EX-24-482-95) from Hyperici herba was obtained from Zeller Company (CH-Romanshorn). Hypericin was isolated from the same extract (see section 2): UV-VIS (MeOH) λnm (ε) 588 (51712), 545 (24393), 472 (13544); EIMS *m/z* 504.2; ^{1}H-NMR (DMSO-d_6, δ, 600.13 MHz) 14.70 (OH-1,6), 14.06 (OH-8,13), 7.44 (CH-9,12), 6.58 (CH-2,5), 2.74 (CH_3-10,11); ^{13}C-NMR (DMSO-d_6, δ, 150.92 MHz) 183.56 (C=O-7,14), 174.12 (C-3,4), 168.04 (C-1,6), 161.35 (C-8,13), 143.81 (C-10,11), 126.94 (C-3a,3b), 126.05 (C-7b,13b), 121.38 (C-7c,14c), 120.81 (C-10a,10b), 119.12 (C-6b,14b), 118.85 (C-9,12), 108.36 (C-7a,13a), 105.49 (C-2,5), 102.04 (C-6a,14a), 23.58 (CH_3-10,11). HPLC purity (Hölzl and Ostrowski, 1987) was 100% at the wavelengths 254, 366, 450, 555 and 590 nm. At 290 nm occurred an additional signal at 48 min, revealing an UV spectrum with absorption maxima at about 220 and 310

nm. Referring to the area integration values, the HPLC purity at 290 nm was 93% for hypericin.
Ethyl acetate, methanol, tetrahydrofuran, acetonitrile and dichloromethane were of HPLC quality (Romil Chemicals, GB-Shepshed). Pyridine p.a. and sodium chloride p.a. were purchased from Fluka (CH-Buchs). *Ortho*-phosphoric acid 85% (Ph. Helv. VI / Ph. Eur.) was from Hänseler (CH-Herisau) and sodium dihydrogen phosphate from Merck (CH-Dietikon). De-ionized water was obtained using an NANO-pure cartridge system (Skan, CH-Basel). Regenerated cellulose syringe filters (0.2 µm, 13 mm) were from Schleicher&Schuell (D-Dassel). For irradiation of the extracts with light a blended light lamp (Philips MLR 160W, 230-240V) was used.

Instrumentation and Column

HPLC. All HPLC analyses were performed using a Hewlett Packard instrument (Model 79994A analytical workstation, model 1090 liquid chromatograph, model 1040 diode-array detector). For chromatographic separations a Knauer (D-Berlin) prepacked cartridge column (250 x 4 mm I.D., 5 µm) filled with Spherisorb S ODS2 and a guard column (10 x 4 mm I.D.) of the same material was used.

VIS spectroscopy. Absorptions were measured on a Kontron-Uvikon 930 spectrophotometer (Kontron Instruments, CH-Zürich) at 589 and 590 nm.

TLC-Densitometry. Densitometric evaluations were performed by using a Camag TLC Scanner II (CH-Muttenz).

Chromatographic Conditions

HPLC-DAD.

The methods from literature were partly modified.
The injection volume of 25 µl was the same for all the methods. The detection wavelength of the DAD was set at 590 nm.

Kerb method (Kerb *et al.*, 1996). Solvents used were solvent A (methanol) and solvent B (methanol - tetrahydrofuran - buffer (9:6:5, v/v)). The buffer consisted of 13.8 g sodium dihydrogen phosphate in

1 litre water adjusted to pH 4.0 with *ortho*-phosphoric acid 85%. The elution profile was: 0-12 min 100% B, 12.01-17 min 100% A and 17.01-27 min 100% B. The flow rate was 0.8 ml/min for solvent B and 1 ml/min for solvent A. The column temperature was set at 25 °C.

Krämer method (Krämer and Wiartalla, 1992). Solvents used were solvent A (methanol) and solvent B (methanol - ethyl acetate - buffer (1893.4:526:618.4, m/m)). The buffer consisted of 13.8 g sodium dihydrogen phosphate in 1 litre water adjusted to pH 2.1 with *ortho*-phosphoric acid 85%. The elution profile was: 0-14 min 100% B, 14.01-19 min 100% A and 19.01-29 min 100% B. The flow rate was 0.8 ml/min for solvent B and 1 ml/min for solvent A. The column temperature was set at 25 °C.

Zeller method (Zeller Company, 1992 (internal method)). Solvents used were A (methanol), B (acetonitrile - tetrahydrofuran (6:4, v/v)) and C (1.2% *ortho*-phosphoric acid in water). The elution profile was: 0-3 min 50% B and 50% C, 3.01-10 min 50% B and 50% C to 70% B and 30% C, 10.01-15 min 70% B and 30% C, 15.01-20 min 100% A, 20.01-30 min 50% B and 50% C. The flow rate was 1 ml/min. The column temperature was set at 30 °C.

Ostrowski method (Ostrowski, 1988). Solvents used were solvent A (methanol) and solvent B (methanol - acetonitrile - tetrahydrofuran - water - *ortho*-phosphoric acid 85% (19.8:48.3:11.4:19.5:1, v/v)). The elution profile was: 0-25 min 100% B, 25.01-30 min 100% A and 30.01-40 min 100% B. The flow rate was 0.5 ml/min for solvent B and 1 ml/min for solvent A. The column temperature was set at 25 °C.

Stock method (Stock, 1992). Solvents used were solvent A (methanol) and solvent B (methanol - acetonitrile - *ortho*-phosphoric acid 85% (69.5:30:0.5, v/v)). The elution profile was: 0-11 min 100% B, 11.01-16 min 100% A and 16.01-26 min 100% B. The flow rate was 1 ml/min. The column temperature was set at 25 °C.

TLC-Densitometry.

The method applied based on an own development. TLC was performed on a HPTLC plate RP-18 $WF_{254}s$ (10 cm x 20 cm) precoated with concentrating zone (2.5 cm x 20 cm), which was obtained from Merck (D-Darmstadt). The TLC plate was pre-washed with methanol -

dichloromethane (2:8, v/v) and dried at 100 °C. The six extracts and five standard solutions were sprayed onto the plate by means of a Camag (CH-Muttenz) Linomat Model IV sample application device. The samples were applied twice a plate. The band length was 4 mm and the distance between the lanes was 5 mm. The applied volume was 10 µl. The TLC plate was developed with the mobile phase tetrahydrofuran - methanol - water - sodium chloride - *ortho*-phosphoric acid 85% (380:380:240:5:5, v/v/v/m/v) in a saturated chamber over a distance of 32 mm. Rf values of hypericin and pseudohypericin were 0.31 and 0.44, respectively. Densitometric evaluation was performed in reflectance mode, measuring the absorption of hypericin and pseudohypericin at 590 nm. A tungsten lamp was applied. No spray or dipping reagents were used.

Procedures

Extract solutions. 0.4 g dry extract (Ze117) were weighed in a 100.0 ml volumetric flask. 80.0 ml methanol were added and put in an ultrasonic bath for 15 min. After cooling to room temperature, it was made up to volume and centrifuged at 2000 rpm for 2 min. 20 ml of the supernatant were filtered through a regenerated cellulose syringe filter and irradiated with a blended light lamp in a distance of 25 cm for 45 min. For VIS spectroscopy and each HPLC method ten new extracts were prepared. The extracts used for VIS spectroscopy measurements were diluted after irradiation with light: 3.0 ml extract were transferred into a 10.0 ml volumetric flask, which was filled up to the mark with methanol. In a separate experiment, TLC-densitometry and HPLC (Kerb method) were compared. Six samples were extracted for this task.
To examine the pyridine influence on the HPLC method Stock, two extracts were prepared as described above using methanol and methanol-pyridine (99:1, v/v), respectively, as extracting solvent. For further investigations of the influence of pyridine on the extraction of hypericin and pseudohypericin by the Kerb HPLC method, another twelve extracts were prepared (six with methanol and six with methanol-pyridine (99:1, v/v)).

Reference solutions. 2.004 mg hypericin were dissolved in 250.0 ml methanol-pyridine (99:1, v/v) in a 250.0 ml volumetric flask. The solution was stored at room temperature in the dark for four days and sonicated 5 min each day. From this stock solution (8.0 µg/ml), five different dilutions were prepared, their concentrations ranging from

1.6 µg/ml to 6.4 µg/ml. The same reference solutions were used for all the HPLC methods and VIS spectroscopy. The solutions were stored at -20 °C in the dark. Stability tests proofed them to be stable under these conditions over a time period of 140 days (see section 3.3.4).
Another calibration graph was prepared for the comparison of TLC-densitometry and HPLC. The concentrations of these reference solutions ranged from 2.4 µg/ml to 12 µg/ml.

Quantification by HPLC. The quantitative determination of hypericin and pseudohypericin in the extracts was performed using the external standard method. The calibration curve of hypericin was used for the quantification of both hypericin and pseudohypericin. The calculation graphs were generated by a least squares regression method. All calculations were based on area counts. Over the selected concentration range of the standard solutions, the calibration curves showed a linear detector response. The correlation coefficients were between 0.9902 and 1.000 (Tables 4.1.2 and 4.1.3). The external standard solutions were injected three times into the HPLC system for analysis. The average of ten extracts was used to calculate the content of hypericin and pseudohypericin.

Table 4.1.2 Regression curves of hypericin used for the comparison of the HPLC methods

HPLC method	Regression curve x: [µg/ml] y: area counts	Correlation coefficient (r)
Kerb	74.700x - 3.759	1.000
Krämer	74.372x - 13.50	1.000
Zeller	59.663x - 32.21	0.9902
Ostrowski	97.518x - 39.67	0.9995
Stock	47.785x - 63.08	0.9958

Table 4.1.3 Regression curves applied investigating the influence of pyridine (Tables 4.1.11 and 4.1.12)

HPLC method	Regression curve x: [µg/ml] y: area counts	Correlation coefficient (r)
Kerb	74.676x - 2.791	1.000
Stock	40.210x - 37.27	0.9934

Quantification by VIS spectroscopy. The content of naphthodianthrones was evaluated applying the calibration curve of hypericin. The calculation graphs were generated by a least squares regression method. Over the concentration range of the standard solutions, the calibration curves showed a linear detector response. The correlation coefficient was 1.000 (Table 4.1.4). The average of ten extracts was used to determine the content of naphthodianthrones.

Table 4.1.4 Regression curves of VIS spectroscopy:
(A) was used for the comparison of VIS spectroscopic and HPLC results (Tables 4.1.14 and 4.1.15), (C) and (D) were applied determining the influence of pyridine on the extraction (Table 4.1.13)

Detection wavelength	Regression curve x: [g/100 ml] y: absorbance	Correlation coefficient (r)	ε [l mol^{-1} cm^{-1}]
590 nm	(A) 984.49x - 9.395*10^{-4}	1.000	49663
589 nm	(B) 1021.4x - 1.225*10^{-3}	1.000	51504
590 nm	(C) 991.05x + 6.790*10^{-4}	1.000	49994
589 nm	(D) 1027.3x + 5.559*10^{-4}	1.000	51822

ε molar coefficient of absorbance

Quantification by TLC-Densitometry. The calibration curve of hypericin was used for the quantification of both hypericin and pseudohypericin. Calculations were based on the relationship between peak area and the amount of hypericin standard applied. Over the selected concentration range of 2.4 µg/ml to 12 µg/ml, the calibration curve was linear. The correlation coefficient was 1.000 (Table 4.1.5). The average of six extracts was used to determine the content of hypericin and pseudohypericin.

Table 4.1.5 Regression curves of hypericin used for the comparison of TLC-densitometry and HPLC

Method	Regression curve x: [µg/ml] y: area counts	Correlation coefficient (r)
TLC-Densitometry	42.721x + 69.01	1.000
HPLC (Kerb method)	85.843x - 39.72	0.997

Precision. The precision was determined by measuring ten times the same reference solution. The concentration of the standard applied was 4.008 µg/ml hypericin for HPLC and VIS spectroscopy and 4.80 µg/ml for TLC-densitometry.

4.1.3 Results and Discussion

Quantification of Hypericin and Pseudohypericin by HPLC

Among the methods investigated, the Kerb method turned out to be the HPLC method with the best reproducibility, precision and the shortest run time (Table 4.1.6 to 4.1.9). The Ostrowski method had the longest run time. The Zeller and the Stock method showed the least good reproducibility. The evaluated contents of hypericin and pseudohypericin were highest applying the Stock method (Tables 4.1.6 to 4.1.8).

Table 4.1.6 Quantification of hypericin by different HPLC methods

Method	Content of hypericin n = 10			Retention time of hypericin	
	mean [%]	s [%]	s_{rel} [%]	min	s_{rel} [%]
Kerb	0.0711	0.000420	0.591	6.5	0.15
Krämer	0.0738	0.000797	1.08	8.8	0.20
Zeller	0.0716	0.00168	2.34	9.2	0.21
Ostrowski	0.0737	0.00127	1.72	16.9	0.18
Stock	0.0900	0.00203	2.25	9.1	0.30
Mean of all methods	0.0760				

Table 4.1.7 Quantification of pseudohypericin by different HPLC methods

Method	Content of pseudohypericin n = 10			Retention time of pseudohypericin	
	mean [%]	s [%]	s_{rel} [%]	min	s_{rel} [%]
Kerb	0.143	0.000493	0.345	3.4	0.086
Krämer	0.153	0.000643	0.420	3.8	0.091
Zeller	0.142	0.00166	1.17	4.7	0.37
Ostrowski	0.168	0.00110	0.653	5.7	0.10
Stock	0.174	0.00194	1.12	3.0	0.17
Mean of all methods	0.156				

Table 4.1.8 Sum of hypericin and pseudohypericin content determined by different HPLC methods

Method	Sum of hypericin and pseudohypericin content n = 10		
	mean [%]	s [%]	s_{rel} [%]
Kerb	0.214	0.000580	0.271
Krämer	0.227	0.00103	0.456
Zeller	0.214	0.00300	1.40
Ostrowski	0.242	0.00155	0.639
Stock	0.264	0.00226	0.854
Mean of all methods	0.232		

Table 4.1.9 The precision was determined by injecting the reference solution containing 4.008 µg/ml hypericin 10 times.

Method	s_{rel} [%]
Kerb	0.757
Krämer	0.887
Zeller	4.21
Ostrowski	2.28
Stock	6.46

It is striking that repeated injection of a hypericin standard solution led to higher relative standard deviations (Table 4.1.9) than the analysis of hypericin in extract solutions (Table 4.1.6). Only with the Krämer method, it was opposite. As the standard solutions, unlike the extract solutions, contained pyridine, it can be speculated that pyridine interfered with the chromatographic separation of hypericin. Later in this section, further investigations on the influence of pyridine were done. It could be shown that results from extracts containing pyridine were slightly more deviating than the results from extracts prepared with pure methanol (Tables 4.1.12 and 4.1.13). The discrepancy between the reproducibility of the results from standard solutions and extracts could also be caused by other constituents of the extracts influencing the chromatographic behavior of hypericin.

The ratio of pseudohypericin to hypericin results was not greatly influenced by the HPLC method, only the Ostrowski method revealing a slightly higher ratio (Table 4.1.10).

Table 4.1.10 Dependence of the ratio pseudohypericin to hypericin on the HPLC method applied

Method	Ratio pseudohypericin / hypericin
Kerb	2.01
Krämer	2.07
Zeller	1.98
Ostrowski	2.28
Stock	1.92

Pyridine and its Influence on Retention Time, Extraction and Quantification of Hypericin and Pseudohypericin

The Stock method revealed different retention times for hypericin in the extracts (RT 9.1 min) and reference solutions (RT 6.3). This was caused by pyridine, which was used for the preparation of the reference solutions but not for extraction. For further examination of this problem, two extracts were prepared: one with methanol and one with methanol-pyridine (99:1, v/v). Both extracts were injected ten times. It could be shown that pyridine influenced the retention time *and* the evaluated content of hypericin and pseudohypericin (Table 4.1.11). The result for the hypericin content was higher in the extract without pyridine, for pseudohypericin it was vice versa. Retention times were shorter in the extracts prepared with pyridine.
The shortening of the retention time indicates that the solubility of hypericin in the HPLC eluent is improved by the addition of pyridine. This is in accordance with results from section 3.3.3. In contrast to the other methods, the eluent of the Stock method contains hardly any water possibly facilitating the formation of hypericin-pyridine-complexes.

Table 4.1.11 Influence of pyridine on the quantification of hypericin (H) and pseudohypericin (P) and their retention time, applying the Stock method, examined by injecting one extract without pyridine and one with pyridine 10 times each

Compound	Content determined by the Stock method			Retention time	
	mean [%]	s [%]	s_{rel} [%]	[min]	s_{rel} [%]
H (extract *without* pyridine)	0.0870	0.00170	1.95	8.5	0.87
H (extract with pyridine)	0.0782	0.00464	5.93	6.0	1.3
P (extract *without* pyridine)	0.187	0.00650	3.48	2.9	0.44
P (extract with pyridine)	0.253	0.00370	1.46	2.0	0.066
H + P (extract *without* pyridine)	0.274	0.00706	2.58		
H + P (extract with pyridine)	0.331	0.00572	1.73		

To make sure that the different results of the two extracts really derived from applying the Stock method and not from influences of pyridine on the extraction of hypericin and pseudohypericin, twelve extracts were prepared (six with methanol and six with methanol-pyridine (99:1, v/v)).

The quantification of hypericin and pseudohypericin was done by the Kerb method and VIS spectroscopy. It could be shown that the addition of 1% pyridine to the extracting solvent methanol did not influence the extracted amount of hypericin and pseudohypericin (Tables 4.1.12 and 4.1.13). Consequently, it is possible to prepare standard solutions with pyridine and doing extraction without, as far as the Kerb method is applied for quantification. The reproducibility of the quantification is slightly worsened by the addition of pyridine to the extracting solvent (Tables 4.1.12 and 4.1.13).

Table 4.1.12 Influence of pyridine on the extracted amount of hypericin (H) and pseudohypericin (P) determined by the Kerb method, examining six extracts without pyridine and six extracts with pyridine

Compound	Content determined by the Kerb method n = 6			Retention time	
	mean [%]	s [%]	s_{rel} [%]	[min]	s_{rel} [%]
H (extracts *without* pyridine)	0.0716	0.000518	0.724	6.5	0.43
H (extracts with pyridine)	0.0716	0.000719	1.00	6.4	0.39
P (extracts *without* pyridine)	0.141	0.00189	1.34	3.4	0.20
P (extracts with pyridine)	0.141	0.00238	1.68	3.4	0.39
H + P (extracts *without* pyridine)	0.213	0.00203	0.953		
H + P (extracts with pyridine)	0.213	0.00262	1.23		

Table 4.1.13 Influence of pyridine on the extracted amount of naphthodianthrones determined by VIS spectroscopy at 589 and 590 nm

Extracts	Content of naphthodianthrones determined by VIS spectroscopy n = 6			Detection wavelength [nm]
	mean [%]	s [%]	s_{rel} [%]	
extracts *without* pyridine	0.292	0.00304	1.04	590
extracts with pyridine	0.291	0.00447	1.54	
extracts *without* pyridine	0.287	0.00294	1.02	589
extracts with pyridine	0.284	0.00436	1.54	

Comparison of the Results obtained by HPLC and VIS Spectroscopy

An advantage of the spectrophotometric method is the simple equipment and procedure. Measurements were done at 590 and 589 nm. Results at both wavelengths were only marginally differing (Table 4.1.14; see Table 4.1.13 as well). The German Drug Codex (1986, 3rd suppl.) and most HPLC methods make use of the wavelength 590 nm; therefore it was used in this study for the comparison of the methods. The precision of VIS spectroscopy was high. It was determined by measuring the reference solution containing 4.008 µg/ml hypericin 10 times at 590 nm, revealing a relative standard deviation of 0.0539%. However, the spectrophotometric method allows only the determination of total naphthodianthrones (Table 4.1.14).

Table 4.1.14 Content of naphthodianthrones determined by VIS spectroscopy: (A) Quantification was done using the calibration graph of hypericin. (B) Calculation was carried out with the specific coefficient of absorbance ($A^{1\%}$=870) applied by the German Drug Codex (1986, 3rd suppl.).

Detection Wavelength	Content of naphthodianthrones n = 10					
	(A)			(B)		
	mean [%]	s [%]	s_{rel} [%]	mean [%]	s [%]	s_{rel} [%]
590 nm	0.294	0.00290	0.987	0.332	0.00331	0.981
589 nm	0.289	0.00282	0.977			

In addition, other compounds that are absorbing at 590 nm can falsify the result. HPLC methods are much more selective, making possible the separate quantification of hypericin and pseudohypericin. Results obtained by VIS spectroscopy were 1.11 to 1.37 times higher than HPLC results, depending on the HPLC method used (Table 4.1.15).

Table 4.1.15 Comparison of the quantification of naphthodianthrones by VIS spectroscopy at 590 nm (0.294%) with the results (sum of hypericin and pseudohypericin content) obtained by the HPLC methods (Table 4.1.8).

Method	Ratio VIS spectroscopy / HPLC results
Kerb	1.37
Krämer	1.30
Zeller	1.37
Ostrowski	1.21
Stock	1.11
Mean of all HPLC methods	1.27

Causes for these differences have been widely discussed (Schütt and Hölzl, 1994). Chlorophyll and other lipophilic compounds, degradation products of chlorophyll and of naphthodianthrones, contributing to the absorption at 590 nm, were factors that were made responsible for differing results of the two techniques. It was assumed that the degradation products could not be removed by preextraction with dichloromethane (Schütt and Hölzl, 1994). As the dry extract Ze117 does contain hardly any chlorophyll and as preextraction of Ze117 with dichloromethane did not change the ratio of VIS spectroscopic to HPLC results (see section 4.4, Table 4.4.2, method A and B), degradation, polymerization and association products were assumed to be the reason for differing results.
The application of the specific coefficient of absorbance ($A^{1\%}$=870) used for quantification by the German Drug Codex (1986, 3rd suppl.) and the Ph. Helv. 8 leads to higher contents of naphthodianthrones (Table 4.1.14) and therefore causes even bigger differences between VIS spectroscopic and HPLC results.

In section 4.3, drug samples of Hyperici herba were examined. It could be shown that chlorophyll increases the VIS spectroscopic results, but differently from the dry extract, chlorophyll removal nearly equalized the results obtained by VIS spectroscopy and HPLC (Table 4.3.6, method A). This indicated the dry extract Ze117 to contain more of the unidentified products mentioned than the drug samples.
Kurth and Spreemann (1998) proposed the use of a factor to convert results obtained by VIS spectroscopy to HPLC results. Referring to the results obtained in this section, this converting factor would be 1.37

(VIS spectroscopy/HPLC) for the extract Ze117 performing HPLC with the Kerb method (Table 4.1.15).
Schütt and Hölzl (1994) showed that the converting factor is depending on the commercial extract itself as well, not making it possible to determine a factor, which is valid for all extracts (see section 4.4.3 as well).

Comparison of the Results obtained by TLC-Densitometry and HPLC

The developed TLC-densitometry method did not turn out to be suitable for quantification of naphthodianthrones regarding its reproducibility. Results obtained by TLC-densitometry were higher than HPLC results (Table 4.1.17) and deviating much more, as can be seen in the high relative standard deviations (Table 4.1.16).

Table 4.1.16 Content of hypericin (H) and pseudohypericin (P) determined by TLC-densitometry and the HPLC method Kerb

Compound	Content determined by TLC-Densitometry n=6			Content determined by the HPLC method Kerb n=6		
	mean [%]	s [%]	s_{rel} [%]	mean [%]	s [%]	s_{rel} [%]
H	0.109	0.0105	9.59	0.0750	0.00102	1.35
P	0.290	0.0228	7.87	0.152	0.000943	0.620
H + P	0.399	0.00880	2.20	0.227	0.000873	0.380

Table 4.1.17 Comparison of TLC-densitometric and HPLC (Kerb method) results, quantifying hypericin (H) and pseudohypericin (P)

Compound	Ratio TLC-Densitometry / HPLC (Kerb method)
H	1.45
P	1.91
H + P	1.76

The precision was determined by applying, developing and measuring the reference solution containing 4.80 µg/ml hypericin 10 times. A relative standard deviation of 3.42% was obtained, being higher compared to

VIS spectroscopy (s_{rel} [%] = 0.0539%) and HPLC (Kerb method: s_{rel} [%] = 0.757%).

Mulinacci *et al.* (1999) described a TLC-densitometric method, which showed similar results and reproducibility as the applied HPLC method. However, it has to be taken into account that they used fluorimetric detection in TLC-densitometry and absorbance measurement in HPLC. They did not consider hypericin and pseudohypericin separately, but used the sum of both compounds for comparison.
The TLC-densitometric method of this study revealed the relative standard deviation to be lower when hypericin and pseudohypericin were summed up (Table 4.1.16). Nonetheless, contents determined by TLC-densitometry were still significantly higher than those of HPLC.

4.1.4 Conclusions

Based on the results of this study, HPLC is the most selective and therefore the most reliable method. VIS spectroscopy does not allow the separate determination of hypericin and pseudohypericin and results of TLC-densitometry were much more deviating than those of HPLC.

Among the HPLC methods investigated, the Kerb method turned out to be the method most suited for the quantification of naphthodianthrones in commercial extracts of Hyperici herba, as it showed the shortest runtime and best reproducibility. Applying this method, pyridine did not influence the retention time and evaluated content of naphthodianthrones, allowing preparing standard solutions with pyridine and doing extraction without. Reproducibility was slightly higher for extracts prepared without pyridine.

Among the published HPLC methods, the methods of Gaedcke (1997) and Schütt and Hölzl (1994) fulfill most of the criteria mentioned in the introduction, as short runtime and the application of isocratic elution, short columns and solvents of low toxicity. Gaedcke applied the same eluent as Schütt and Hölzl. Comparisons with the Kerb method evaluating reproducibility and precision could be a subject of further investigations.

In summary, this study showed that the results obtained by different quantification methods are not in accordance. VIS spectroscopic results are higher than HPLC results. Not even all the HPLC methods come up with the same content of naphthodianthrones. Therefore, it is essential

to declare the quantification method. It is one of the factors that has to be known to make possible the comparison of commercial extracts that are standardized on a certain content of naphthodianthrones.

The quantification results are also affected by the non-uniform quality of hypericin standards. Hypericins revealing different coefficients of absorbance lead to differing results (see section 3). Therefore, consistent quality and availability of hypericin standards are of importance.

4.2 Influence of the Extraction Method on the Evaluated Content of Naphthodianthrones in a Drug Sample of Hyperici herba determined by VIS Spectroscopy and HPLC

4.2.1 Introduction

Various methods for quantitative extraction of hypericin and pseudohypericin from Hyperici herba have been applied. The German Drug Codex (DAC 1986) and the Pharmacopoeia Helvetica 8 (Ph. Helv. 8) make use of different methods. The DAC 1986 did a Soxhlet extraction with acetone after chlorophyll removal with dichloromethane. The Ph. Helv. 8 refluxes the drug sample with tetrahydrofuran - water (8:2) without foregoing dichloromethane extraction. Soxhlet extraction and refluxing with methanol were also described (Brolis *et al.*, 1998; Kurth and Spreemann, 1998; Krämer and Wiartalla, 1992). Krämer and Wiartalla (1992) adapted the extraction method to the following quantification method. They refluxed with methanol for HPLC measurements and followed the instruction of the German Drug Codex (1986) for VIS spectroscopic quantification. They also compared the efficiency of acetone and methanol using a Soxhlet apparatus and HPLC measurement. Methanol was found more efficient. Maisenbacher (1991) used Soxhlet extraction with acetone - methanol (1:1). Stirring with ethanol 80% followed by defatting the extract with hexane was reported by Mulinacci *et al.* (1999). As extracting solvents do not always reveal the same efficiency for hypericin and pseudohypericin, the ratio of hypericin to pseudohypericin is not consistent as was shown by Kurth and Spreemann (1998). Section 5 deals with further aspects of the extracting solvent.

This study examined the extraction and quantification of the naphthodianthrones in a drug sample of Hyperici herba. It was investigated how the extraction method and the extracting solvent influenced the evaluated contents of hypericin and pseudohypericin and their ratio. As some factors only affect the result in combination with a specific quantification method, as chlorophyll removal combined with VIS spectroscopy for example, HPLC and VIS spectroscopy were both used for quantification.

4.2.2 Experimental

Material

The drug sample analyzed was Hyperici herba, HOO 687, provided by the Pharmacopoeia laboratory (Strasbourg) for an interlaboratoy test. Acetone was of HPLC quality (Romil Chemicals, GB-Shepshed). Further material is described in section 4.1.2.

Instrumentation and Chromatographic Conditions

Mill. The plant material was pulverized with a centrifugal mill model ZM1 (Retsch, Schieritz and Hauenstein AG, CH-Arlesheim) with a 1.0 mm sieve.

HPLC. The Kerb method was applied, see section 4.1.2.

VIS spectroscopy. Absorptions were measured on a Kontron-Uvikon 930 spectrophotometer (Kontron Instruments, CH-Zürich) at 590 nm.

Procedures

Extract solutions.

Six extracts were prepared with each method.
Differences between method A and B are bold printed.

Method A: *Soxhlet extraction with dichloromethane (to remove chlorophyll) and acetone (according to the German Drug codex, slightly modified).* 1.000 g Hyperici herba were weighed in a Soxhlet thimble, covered with glass marbles and **extracted with 130 ml dichloromethane in a Soxhlet apparatus (30 ml) at 60 °C for 5 h**. The thimble was dried at room temperature over night and extracted with 130 ml acetone at 70 °C for 3 h afterwards. The acetone extract was concentrated to dryness with reduced pressure below 35 °C. The residue was dissolved in 10 ml methanol, transferred into a 25.0 ml volumetric flask, which was made up to volume with methanol. The sample was filtered through a regenerated cellulose syringe filter; the

first 2 ml were discarded. 5.0 ml of the filtrate were diluted to 25.0 ml with methanol in a volumetric flask.

Method B: *Soxhlet extraction with acetone.* 1.000 g Hyperici herba was weighed in a Soxhlet thimble, covered with glass marbles and extracted with 130 ml acetone at 70 °C for 3 h. The acetone extract was concentrated to dryness with reduced pressure below 35 °C. The residue was dissolved in 10 ml methanol, transferred into a 25.0 ml volumetric flask, which was made up to volume with methanol. The residue was taken up in 10 ml of methanol **with the help of sonication** and transferred into a 25.0 ml volumetric flask, which was made up to volume. The sample was filtered through a regenerated cellulose syringe filter; the first 2 ml were discarded. 5.0 ml of the filtrate were diluted to 25.0 ml with methanol in a volumetric flask.

Method C *(according to Ph. Helv. 8, slightly modified)*:
0.800 g of the powdered drug were weighed in a 100 ml round-bottomed flask. 60 ml of a mixture of 20 volumes of water, 80 volumes of tetrahydrofuran and a magnetic stirrer were added. The mixture was boiled in a water bath at 70 °C under a reflux condenser for 30 min. The mixture was centrifuged (2 min at 2000 rpm) and the supernatant decanted into a 250 ml flask. 60 ml of a mixture of 20 volumes of water and 80 volumes of tetrahydrofuran were added to the residue and the flask heated again under a reflux condenser for 30 min. The content was centrifuged (2 min at 2000 rpm) and the supernatant decanted. The combined extracts were evaporated to dryness. The residue was taken up in 15 ml of methanol with the help of sonication and transferred into a 25.0 ml volumetric flask, which was made up to volume. The sample was centrifuged again. 10 ml were filtrated through a syringe filter (0.2 μm); the first two milliliters of the filtrate were discarded. 5.00 ml of the filtrate were introduced into a graduated flask and diluted to 25.0 ml with methanol.

Method D: The procedure was the same as described for method C with the difference that the extracting solvent of method C was exchanged to acetone.

Reference solutions. 2.004 mg hypericin were dissolved in 250.0 ml methanol-pyridine (99:1, v/v) in a 250.0 ml volumetric flask. The solution was stored at room temperature in the dark for four days and sonicated 5 min each day. From this stock solution (8.02 μg/ml), six different dilutions were prepared, their concentration ranging from

1.60 µg/ml to 8.02 µg/ml. The same reference solutions were used for all the HPLC methods and VIS spectroscopy. The solutions were stored at -20 °C in the dark. Stability tests proofed them to be stable under these conditions over a time period of 140 days (see section 3.3.4).

Quantification by HPLC. See section 4.1.2. The average of six extracts was used to calculate the content of hypericin and pseudohypericin.

Quantification by VIS spectroscopy. See section 4.1.2. The average of six extracts was used to determine the content of naphthodianthrones.

4.2.3 Results and Discussion

A drug sample of Hyperici herba was subjected to various extraction methods. Quantification of the naphthodianthrones was done with VIS spectroscopy and HPLC.

Evaluated contents of the naphthodianthrones were not much affected by the exchange of Soxhlet extraction for refluxing. Method B, which represents a Soxhlet extraction with acetone, gave similar HPLC results as method D that refluxes the drug with acetone (Table 4.2.1). However, the result was clearly dependent on the extracting solvent, tetrahydrofuran - water (8:2, v/v) extracting about twice as much naphthodianthrones as acetone. This was shown by comparison of method C and D, which only differed in their extracting solvent (Table 4.2.1).

Table 4.2.1 Influences of the extraction and quantification methods on the evaluation of the content of naphthodianthrones

Method	VIS spectroscopy Content of naphthodianthrones n=6			HPLC Content of hypericin and pseudohypericin n=6		
	mean [%]	s [%]	s_{rel} [%]	mean [%]	s [%]	s_{rel} [%]
A	**0.0385**	0.00231	6.00	**0.0324**	0.00292	9.01
B	**0.0488**	0.00211	4.33	**0.0314**	0.00296	9.43
C	**0.0862**	0.00259	3.01	**0.0625**	0.00410	6.55
D	**0.0438**	0.00194	4.43	**0.0291**	0.00123	4.22

Method A: chlorophyll removed, B: chlorophyll not removed, C: Ph. Helv. 8, D: Ph. Helv. 8 / acetone

The extracting solvent influenced the ratio of pseudohypericin to hypericin content determined by HPLC analysis. Tetrahydrofuran - water (8:2, v/v) (method C) was more effective than acetone (method D) in the extraction of both compounds (Table 4.2.2), but due to the larger polarity of pseudohypericin, this difference in effectiveness was even more pronounced for pseudohypericin (Table 4.2.3).

Table 4.2.2 Influence of the extraction method on the extracted amount of hypericin and pseudohypericin determined by HPLC

Method	HPLC					
	Content of hypericin n=6			Content of pseudohypericin n=6		
	mean [%]	s [%]	s_{rel} [%]	mean [%]	s [%]	s_{rel} [%]
A	**0.0105**	0.000938	8.91	**0.0219**	0.00201	9.18
B	**0.00958**	0.00116	12.1	**0.0218**	0.00203	9.28
C	**0.0180**	0.00167	9.24	**0.0445**	0.00262	5.89
D	**0.00929**	0.000561	6.04	**0.0198**	0.00163	8.22

Method A: chlorophyll removed, B: chlorophyll not removed, C: Ph. Helv. 8, D: Ph. Helv. 8 / acetone

Table 4.2.3 Influence of the extraction method on the ratio of pseudohypericin to hypericin determined by HPLC analysis

Method	Ratio pseudohypericin / hypericin
A (chlorophyll removed)	2.09
B (chlorophyll not removed)	2.28
C (Ph. Helv. 8)	2.47
D (Ph. Helv. 8 / acetone)	2.13

Method A: chlorophyll removed, B: chlorophyll not removed, C: Ph. Helv. 8, D: Ph. Helv. 8 / acetone

The influence of the chosen quantification method was immanent in all the extraction methods A to D. VIS spectroscopic results were generally higher than HPLC results (Table 4.2.4), probably caused by differing selectivity of the two techniques (see section 4.1). The ratio VIS spectroscopic to HPLC results was 1.55 applying Method B (chlorophyll not removed) and 1.18 for method A (chlorophyll removed).

Table 4.2.4 Comparison of the quantification of naphthodianthrones by VIS spectroscopy with the results (sum of hypericin and pseudohypericin content) obtained by HPLC analysis (Table 4.2.1)

Method	Ratio VIS spectroscopy / HPLC
A (chlorophyll removed)	1.18
B (chlorophyll not removed)	1.55
C (Ph. Helv. 8)	1.38
D (Ph. Helv. 8 / acetone)	1.51

The differing selectivity of the two quantification methods is obvious comparing the results from method A and B. HPLC results are almost the same for both extraction methods (Table 4.2.1, method A and B). VIS spectroscopic analysis, however, revealed a 1.27 times higher content of naphthodianthrones for method B than for method A, probably caused by chlorophyll, which is not removed in method B and is absorbing at 590 nm as well.

Further investigations on the influence of chlorophyll removal are done in section 4.3.

4.2.4 Conclusions

Among the methods investigated, refluxing with tetrahydrofuran - water (8:2) (method C according to Ph. Helv. 8) turned out to be most efficient in extracting naphthodianthrones from drug samples of Hyperici herba.
Quantification results show again the poor selectivity of VIS spectroscopy. Compounds absorbing at 590 nm, chlorophyll and other constituents not definitely defined yet, are made responsible for the differences between VIS spectroscopic and HPLC results. Polarity and selectivity of the extracting solvent influence the extracted amount of these compounds. Consequently, if quantification is done by VIS spectroscopy, the extraction process must include a preextraction removing these compounds. Preextraction with hexane and dichloromethane has been shown to remove at least the chlorophyll portion.

4.3 Influence of Chlorophyll Removal on Evaluated Contents of Naphthodianthrones in Drug Samples of Hyperici herba determined by VIS Spectroscopy and HPLC Analysis

4.3.1 Introduction

The ratio of the constituents of the herbal drug from *Hypericum perforatum* is depending on various factors (see section 1), for example genetic features (Büter *et al.*, 1998), environmental influences as location and temperature (Jensen *et al.*, 1995), harvesting period, drying process (Brantner *et al.*, 1994), storage and ratio of flowers to leaves. The ratio of flowers to leaves is important, as hypericin levels are higher in blossoms than in leaves (Southwell and Campbell, 1991). It also affects the amount of chlorophyll present in the drug sample.
In this study, the connection of drug sample and differences between VIS spectroscopic and HPLC results, determining the content of naphthodianthrones, was examined. The effects of chlorophyll removal were investigated, testing four drug samples. In contrast to section 4.2, the extracts in this section were irradiated with light to convert proto components into hypericin and pseudohypericin.

4.3.2 Experimental

Material

The samples analyzed were Hyperici herba (lot 50279L (1995)) and Hyperici herba Ph. Helv. 8 (lot: 50279L (1998)) from DIXA (CH-St. Gallen), Hyperici herba cum flores DAC 86 (lot: 1998110819 (1998)) from Hänseler (CH-Herisau) and Hyperici herba (lot: HPS2-S) from Vita Plant (CH-Witterswil). Further material is described in section 4.1.2.

Instrumentation and Chromatographic Conditions

See section 4.2.2

Procedures

Extract solutions.

Method A: *Soxhlet extraction with dichloromethane (to remove chlorophyll) and acetone.* 1.000 g Hyperici herba was weighed in a Soxhlet thimble, covered with glass marbles and extracted with 130 ml dichloromethane in a Soxhlet apparatus (30 ml) at 60 °C for 5 h. The thimble was dried at room temperature over night and extracted with 130 ml acetone at 70 °C for 3 h afterwards. The acetone extract was concentrated to dryness with reduced pressure below 35 °C. The residue was dissolved in 10 ml methanol, transferred into a 20.0 ml volumetric flask, which was made up to volume with methanol. The sample was filtered through a regenerated cellulose syringe filter; the first 2 ml were discarded. 5.0 ml of the filtrate were diluted to 20.0 ml with methanol in a volumetric flask. The flask was irradiated with a blended light lamp in a distance of 25 cm for 45 min. Six extracts were prepared for each of the four drug samples.

Method B: *Soxhlet extraction with acetone.* 1.000 g Hyperici herba was weighed in a Soxhlet thimble, covered with glass marbles and extracted with 130 ml acetone at 70 °C for 3 h. The further procedure was the same as described in method A. Six extracts were prepared for each of the four drug samples.

Loss on drying. 1.000 g of drug sample was dried at 100 °C for two hours. The loss on drying was performed three times.

Reference solutions. 2.492 mg hypericin were dissolved in 250.0 ml methanol-pyridine (99:1, v/v) in a 250.0 ml volumetric flask. The solution was stored at room temperature in the dark for four days and sonicated 5 min each day. From this stock solution (9.97 µg/ml) six different dilutions were prepared, their concentration ranging from 0.8 µg/ml to 4.99 µg/ml. The same reference solutions were used for all the HPLC methods and VIS spectroscopy. The solutions were stored at -20 °C in the dark. Stability test proofed them to be stable under these conditions over a time period of 140 days (see section 3.3.4).

Quantification by HPLC (Kerb method). See section 4.1.2. The average of six extracts was used to calculate the content of hypericin and pseudohypericin. The loss on drying of the drug samples was included in the calculations.

Quantification by VIS spectroscopy. See 4.1.2 The average of six extracts was used to determine the content of naphthodianthrones. The loss on drying of the drug samples was included in the calculations.

4.3.3 Results and Discussion

Four drug samples were analyzed with method A and B to get more information about the influence of chlorophyll on the evaluated content of naphthodianthrones, using VIS spectroscopy and HPLC as quantification methods.

VIS spectroscopy revealed 1.10 to 1.23 times higher contents of naphthodianthrones in the extracts that still contain chlorophyll than in the extracts that were obtained after preextraction with dichloromethane (Table 4.3.1). The results of method A and B determined by HPLC analysis were less differing (Tables 4.3.2 to 4.3.4), as HPLC is more selective, chromatographically separating hypericin and pseudohypericin from chlorophyll.
The ratio VIS spectroscopic to HPLC results is affected by the proportion of blossoms to leaves in the drug sample. Drug samples with high portions of leaves contain more chlorophyll than samples rich in blossoms. High chlorophyll levels lead to bigger differences between VIS spectroscopic and HPLC results, because chlorophyll increases absorption values measured with VIS spectroscopy, but does not influence HPLC results. As blossoms contain more hypericin than leaves (Southwell and Champbell, 1991) the final content of hypericin in the drug sample depends on the ratio of the flowering parts to leaves as well. Therefore, high levels of hypericin should correlate with low levels of chlorophyll and consequently with small differences between VIS spectroscopic and HPLC results. This is true for the drug sample from VitaPlant (Table 4.3.3; Table 4.3.6, method B). The plant material from VitaPlant consists only of the top flowering parts of the plant being especially rich in blossoms. The sample DIXA 98 contained the lowest amount of hypericin among the samples investigated and results from the two quantification methods differed most, indicating the presence of high amounts of chlorophyll.

The ratio pseudohypericin to hypericin was depending on the drug sample as well (Table 4.3.5). Referring to the results of method A, drug samples from DIXA and Hänseler revealed 1.5 to 3.1 times higher

amounts of pseudohypericin than of hypericin. In contrast, the sample from VitaPlant contained 1.33 times more hypericin than pseudohypericin. Schütt (1996) investigated drug samples from different origins and found the ratio pseudohypericin to hypericin to vary from 1.5 to 7.5.

VIS Spectroscopy

Table 4.3.1 Quantification of naphthodianthrones in different drug samples of Hyperici herba by VIS spectroscopy, using method A and B for extraction

Sample	Content of naphthodianthrones Method **A** (chlorophyll removed) n=6			Content of naphthodianthrones Method **B** (chlorophyll **not** removed) n=6			Ratio mean **B** / mean **A**
	mean [%]	s [%]	s_{rel} [%]	mean [%]	s [%]	s_{rel} [%]	
DIXA 95	**0.0548**	0.00266	4.85	**0.0619**	0.00463	7.48	**1.13**
DIXA 98	**0.0312**	0.00123	3.95	**0.0370**	0.00253	6.85	**1.18**
Hänseler	**0.0359**	0.00335	9.33	**0.0440**	0.00243	5.52	**1.23**
VitaPlant	**0.138**	0.0118	8.55	**0.152**	0.00753	4.97	**1.10**

HPLC-DAD (Kerb method)

Table 4.3.2 Quantification of hypericin and pseudohypericin (sum) in different drug samples of Hyperici herba by HPLC, using method A and B for extraction

Sample	Content of hypericin and pseudohypericin Method **A** (chlorophyll removed) n=6			Content of hypericin and pseudohypericin Method **B** (chlorophyll **not** removed) n=6			Ratio mean **B** / mean **A**
	mean [%]	s [%]	s_{rel} [%]	**mean** [%]	s [%]	s_{rel} [%]	
DIXA 95	**0.0495**	0.00231	4.67	**0.0486**	0.00393	8.08	**0.982**
DIXA 98	**0.0293**	0.00117	3.99	**0.0284**	0.00189	6.67	**0.969**
Hänseler	**0.0330**	0.00325	9.86	**0.0349**	0.00218	6.26	**1.06**
VitaPlant	**0.127**	0.0120	9.50	**0.123**	0.00676	5.48	**0.969**

Table 4.3.3 Quantification of hypericin in different drug samples of Hyperici herba by HPLC, using method A and B for extraction

Sample	Content of hypericin Method **A** (chlorophyll removed) n=6			Content of hypericin Method **B** (chlorophyll **not** removed) n=6			Ratio mean **B** / mean **A**
	mean [%]	s [%]	s_{rel} [%]	mean [%]	s [%]	s_{rel} [%]	
DIXA 95	**0.0198**	0.00133	6.74	**0.0182**	0.00156	8.61	**0.919**
DIXA 98	**0.00713**	0.000228	3.20	**0.00687**	0.000695	10.1	**0.964**
Hänseler	**0.0113**	0.001027	9.10	**0.0116**	0.000725	6.25	**1.03**
VitaPlant	**0.0723**	0.00745	10.3	**0.0690**	0.0036	5.22	**0.954**

Table 4.3.4 Quantification of pseudohypericin in different samples of *Hyperici herba* by HPLC, using method A and B for extraction

Sample	Content of pseudohypericin Method **A** (chlorophyll removed) n=6			Content of pseudohypericin Method **B** (chlorophyll **not** removed) n=6			Ratio mean **B** / mean **A**
	mean [%]	s [%]	s_{rel} [%]	mean [%]	s [%]	s_{rel} [%]	
DIXA 95	**0.0298**	0.00119	3.99	**0.0305**	0.00251	8.25	**1.02**
DIXA 98	**0.0221**	0.00104	4.69	**0.0215**	0.00131	6.11	**0.973**
Hänseler	**0.0217**	0.00224	10.3	**0.0233**	0.00148	6.34	**1.07**
VitaPlant	**0.0543**	0.00468	8.61	**0.0544**	0.00324	5.96	**1.00**

Table 4.3.5 Determination of the ratio pseudohypericin to hypericin evaluated by HPLC

Sample	Ratio pseudohypericin /hypericin	
	Method **A**	Method **B**
DIXA 95	1.5	1.7
DIXA 98	3.1	3.1
Hänseler	1.9	2.0
VitaPlant	0.75	0.79

Comparison of the Results obtained by VIS Spectroscopy and HPLC

Chlorophyll removal nearly equalized results obtained by VIS spectroscopy and HPLC (Table 4.3.6, method A). VIS spectroscopic results were still slightly higher than HPLC results, maybe caused by cyclopseudohypericin. Cyclopseudohypericin is another naphthodianthrone, having its absorption maximum at 555 nm, increasing VIS spectroscopic results and being chromatographically separated from the other compounds in HPLC (Häberlein *et al.*, 1992). Its genuine content in *Hypericum perforatum* is small. Further amounts are generated by the transformation of pseudohypericin (see section 3.3.4.3).

Table 4.3.6 Comparison of the quantification of naphthodianthrones by VIS spectroscopy with HPLC results (sum of hypericin and pseudohypericin content) for method A and B

Sample	Ratio VIS spectroscopy / HPLC	
	Method **A** (chlorophyll removed)	Method **B** (chlorophyll **not** removed)
DIXA 95	1.11	1.27
DIXA 98	1.06	1.30
Hänseler	1.09	1.26
VitaPlant	1.09	1.24

Results of the ratio VIS spectroscopy to HPLC were differing more in section 4.2 (Table 4.2.4, method A (1.18) and B (1.55)), lack of irradiation with light in section 4.2 being probably one of the causes. The extracts of this section have been irradiated with light, unlike the extract from section 4.2. Irradiation with light converts protopseudohypericin and protohypericin to pseudohypericin and hypericin (Krämer and Wiartalla, 1992). This is important as the proto components show another absorption maximum (546 nm) than pseudohypericin and hypericin (590 nm) (Kurth and Spreemann, 1998; Gaedcke, 1997). While the proto components are chromatographically separated from hypericin and pseudohypericin in most HPLC methods and therefore do not influence their quantification results, they show some absorption at 590 nm, increasing the VIS spectroscopic results (Müller-Kuhrt and Boesel, 1993).

The loss on drying was included into the calculations and did not exceed the limit required by the Ph. Helv. 8 (at most 10%, Table 4.3.7).

Table 4.3.7 Loss on drying

Sample	Loss on drying		
	mean [%]	s [%]	s_{rel} [%]
DIXA 95	9.50	0.0329	0.346
DIXA 98	9.37	0.00890	0.090
Hänseler	8.30	0.0192	0.231
VitaPlant	7.48	0.0105	0.140

4.3.4 Conclusions

The ratio of VIS spectroscopic to HPLC results is not always the same. One of the modifying factors is the quality of the genuine drug sample. Therefore, the declaration of a commercial extract should include specifications of the applied plant material. Consistent quality would be desirable, cultivation being one of the tools to get more homogenous row material.

Irradiation of the extracts with light diminishes the difference between the results of VIS spectroscopy and HPLC. For this reason and to enhance reproducibility, irradiation with light is recommended. As light exposure is accelerating decomposition of naphthodianthrones, extracts should be illuminated no longer than 45 min, but at least 15 min with artificial light (see section 3.4).

The calibration curve of hypericin is used for the evaluation of the content of all naphthodianthrones, although they do not reveal the same molar coefficient of absorbance (see section 3.3.1). This is affecting the results, as the ratio of pseudohypericin to hypericin content is varying, depending on the drug sample (Table 4.3.5) and the extracting solvent (see sections 4.2.3 and 5). Therefore, pseudohypericin should be used as second reference substance or a conversion factor should be determined as far as HPLC is applied.

4.4 Influence of the Extraction Method on the Evaluated Content of Naphthodianthrones in the Dry Extract Ze117 of Hyperici herba determined by VIS Spectroscopy and HPLC

4.4.1 Introduction

Besides the quantification of naphthodianthrones in drug samples of Hyperici herba, their determination in commercial extracts is a further point of concern. Schütt and Hölzl (1994) investigated the content of naphthodianthrones in pharmaceutical preparations. They adapted the extraction procedure to the quantification method applied. When VIS spectroscopy was used, the commercial extracts were treated with petroleum ether to remove residues of chlorophyll and then extracted with methanol - pyridine (97:3) by sonication. Preextraction with petroleum ether was omitted when HPLC was used for quantification. Schütt and Hölzl (1994) obtained higher contents of naphthodianthrones with VIS spectroscopy in spite of the removal of chlorophyll. Degradation products of chlorophyll and naphthodianthrones were assumed to increase VIS spectroscopic results. Gaedcke (1997) extracted commercial extracts of Hyperici herba with methanol by sonication, applying the same extraction procedure for VIS spectroscopic and HPLC measurement. She found VIS spectroscopy to give 1.11 to 1.56 times higher contents of naphthodianthrones than HPLC. Both authors irradiated the extracts with light to convert protohypericin and protopseudohypericin into hypericin and pseudohypericin to increase reproducibility.

To clarify if the factors affecting the quantification of naphthodianthrones were different for drug material and commercial extracts, additional investigations were done in this study. The influences of the extraction method, the extracting solvent, irradiation of the extracts with light and removal of chlorophyll on the evaluated content of naphthodianthrones in a commercial dry extract (Ze117) were examined.

4.4.2 Experimental

Material

The ethanol-water (1:1, m/m) dry extract (Ze117) from Hyperici herba was obtained from Zeller Company (CH-Romanshorn). Hypericin was isolated from the same extract (see section 4.1.2). Extrelut® 20 was purchased from Merck (D-Darmstadt). Further material is described in section 4.1.2.

Instrumentation and Chromatographic Conditions

See section 4.2.2

Procedures

Extract solutions.

***Method A**: Soxhlet extraction with dichloromethane and acetone.*
0.40 g dry extract (Ze117) were placed on 1 g Extrelut® in a mortar and covered with 3 g Extrelut®. Altogether was mixed with a pistil, transferred into a Soxhlet thimble, covered with 1 cm Extrelut® and extracted with 150 ml dichloromethane in a Soxhlet apparatus (30 ml) at 70 °C for 5 h. The thimble was dried at room temperature over night, followed by extraction with 150 ml acetone at 70 °C for 3 h. The acetone extract was concentrated to dryness with reduced pressure below 35 °C. The residue was dissolved in 10 ml methanol, transferred into a 25.0 ml volumetric flask, which was made up to volume with methanol. The sample was filtered through a regenerated cellulose syringe filter; the first 2 ml were discarded. 5.0 ml of this stock solution were diluted to 50.0 ml with methanol in a volumetric flask for VIS spectroscopy. For HPLC analysis, 3.0 ml of the stock solution were diluted to 10.0 ml with methanol. Six extracts were prepared.

***Method B**: Soxhlet extraction with acetone.*
0.40 g dry extract (Ze117) were placed on 1 g Extrelut® in a mortar and covered with 3 g Extrelut®. Altogether was mixed, transferred into a Soxhlet thimble and covered with 1 cm Extrelut®. It was extracted

with 150 ml acetone in a Soxhlet apparatus (30 ml) at 70 °C for 5 h. The further procedure was the same as described for method A.

***Method C**: Soxhlet extraction with methanol.*
0.40 g dry extract (Ze117) were placed on 1 g Extrelut® in a mortar and covered with 3 g Extrelut®. Altogether was mixed, transferred into a Soxhlet thimble and covered with 1 cm Extrelut®. It was extracted with 130 ml methanol in a Soxhlet apparatus (30 ml) at 75 °C for 3.5 h. The methanol extract was concentrated with reduced pressure below 35 °C and transferred into a 25.0 ml volumetric flask, which was made up to volume with methanol. The sample was centrifuged and filtered through a regenerated cellulose syringe filter; the first 2 ml were discarded. 5.0 ml of this stock solution were diluted to 50.0 ml with methanol in a volumetric flask for VIS spectroscopy. For HPLC analysis 3.0 ml of the stock solution were diluted to 10.0 ml with methanol. Six extracts were prepared.
The samples were analyzed by HPLC and VIS spectroscopy before and after irradiation with light (45 min with a blended light lamp in a distance of 25 cm).

***Method D**: Ultrasonic extraction with methanol.*
0.400 g dry extract (Ze117) were transferred into a 100.0 ml volumetric flask. 80 ml methanol were added and put into an ultrasonic bath for 15 min. After cooling to room temperature, the flask was made up to volume, centrifuged at 2000 rpm for 2 min and filtered through a regenerated cellulose syringe filter; the first 2 ml were discarded. This stock solution was directly used for HPLC analysis. For VIS spectroscopy 3.0 ml of the stock solution were diluted to 10.0 ml in a volumetric flask. Six extracts were prepared.
The samples were analyzed by HPLC and VIS spectroscopy before and after irradiation with light (45 min with a blended light lamp in a distance of 25 cm).

Reference solutions. Isolated hypericin was used to prepare reference solutions in the concentration range of 1.2 µg/ml to 9.6 µg/ml. The same reference solutions were used for all the HPLC methods and VIS spectroscopy. The solutions were stored at -20 °C in the dark. Stability test proofed them to be stable under these conditions over a time period of 140 days (see section 3.3.4).

Quantification by HPLC (Kerb method). See section 4.1.2. The average of six extracts was used to calculate the content of hypericin and pseudohypericin.

Quantification by VIS spectroscopy. See 4.1.2. The average of six extracts was used to determine the content of naphthodianthrones.

4.4.3 Results and Discussion

Effects of the extraction method, the extracting solvent, irradiation of the extracts with light and removal of chlorophyll on the determined content of naphthodianthrones depended on the quantification technique as well.

VIS spectroscopy revealed higher results for the extracting solvent methanol than for acetone (Table 4.4.1, method B and C). Ultrasonic extraction led to slightly higher contents than Soxhlet extraction (Table 4.4.1, method D and C). Irradiation with light did not significantly influence the results. The removal of residual chlorophyll (method A) did not make a big difference (Table 4.4.1). As the dry extract Ze117 was already separated from chlorophyll during the manufacturing process, this came up to expectations. The ratio of VIS spectroscopic to HPLC results of method A was 1.21 (Table 4.4.2), which indicated that besides chlorophyll, there had to be an additional factor, which caused different results of VIS spectroscopy and HPLC (see section 4.1.3).

Table 4.4.1 Study of the influences of extraction methods and quantification techniques on evaluated contents of naphthodianthrones from the dry extract Ze117 of Hyperici herba

Method	VIS spectroscopy Content of naphthodianthrones n=6			HPLC Content of hypericin and pseudohypericin n=6		
	mean [%]	s [%]	s_{rel} [%]	mean [%]	s [%]	s_{rel} [%]
D: Ultrasonic extraction with MeOH *before* irradiation[a]	**0.301**	0.00163	0.542	**0.201**	0.00243	1.21
D: Ultrasonic extraction with MeOH *after* irradiation[a]	**0.299**	0.00195	0.654	**0.225**	0.00152	0.676
C: Soxhlet extraction with MeOH *before* irradiation[a]	**0.289**	0.0156	0.540	**0.215**	0.00124	0.576
C: Soxhlet extraction with MeOH *after* irradiation[a]	**0.289**	0.00233	0.808	**0.217**	0.00256	1.18
A: Soxhlet extraction with dichloromethane and acetone	**0.256**	0.00696	2.72	**0.211**	0.00655	3.10
B: Soxhlet extraction with acetone	**0.260**	0.00707	2.72	**0.213**	0.00590	2.77

[a] irradiation with light for 45 min in a distance of 25 cm

If *HPLC* results after irradiation with light were looked at, ultrasonic extraction was slightly more efficient than Soxhlet extraction (Table 4.4.1, method D and C).

Irradiation with light slightly influenced HPLC results (Tables 4.4.1 and 4.4.3). The results obtained by ultrasonic extraction showed higher contents of naphthodianthrones after irradiation with light. This effect was not observed for the Soxhlet extraction, probably because of the longer light exposure during the extraction procedure compared to ultrasonic extraction, which was sufficient to convert the residual proto components of the dry extract Ze117. The influence of irradiation with light is better seen with HPLC than with VIS spectroscopy, as the proto components are chromatographically separated from hypericin and pseudohypericin in HPLC, while they slightly contribute to the absorption in VIS spectroscopy (see section 4.1). This was consistent with the finding that the ratio of VIS spectroscopic to HPLC results (1.33) of method D and C was similar after irradiation with light (Table 4.4.2).

Differences of HPLC and VIS spectroscopic results were bigger for the extracting solvent methanol than for acetone. The ratio VIS spectroscopic to HPLC results of method D and C (using methanol as extracting solvent) was 1.3 after irradiation with light and 1.2 for method A and B (using acetone as extracting solvent) (Table 4.4.2). Methanol probably extracted more chlorophyll or other compounds absorbing at 590 nm and therefore increasing VIS spectroscopic results.

Table 4.4.2 Comparison of VIS spectrometric and HPLC results

Method	Ratio VIS spectroscopy / HPLC
D: Ultrasonic extraction with MeOH *before* irradiation [a]	1.50
D: Ultrasonic extraction with MeOH *after* irradiation [a]	1.33
C: Soxhlet extraction with MeOH *before* irradiation [a]	1.34
C: Soxhlet extraction with MeOH *after* irradiation [a]	1.33
A: Soxhlet extraction with dichloromethane and acetone	1.21
B: Soxhlet extraction with acetone	1.22

[a] irradiation with light for 45 min in a distance of 25 cm

Table 4.4.3 Influences of extraction methods on extraction of hypericin and pseudohypericin from the dry extract Ze117 of Hyperici herba

Method	HPLC					
	Content of hypericin (H) n=6			Content of pseudohypericin (P) n=6		
	mean [%]	s [%]	s_{rel} [%]	mean [%]	s [%]	s_{rel} [%]
D: Ultrasonic extraction with MeOH *before* irradiation [a]	**0.0715**	0.000770	1.08	**0.130**	0.00219	1.69
D: Ultrasonic extraction with MeOH *after* irradiation [a]	**0.0757**	0.000995	1.31	**0.149**	0.00114	0.763
C: Soxhlet extraction with MeOH *before* irradiation [a]	**0.0727**	0.000276	0.379	**0.142**	0.00103	0.725
C: Soxhlet extraction with MeOH *after* irradiation [a]	**0.0738**	0.00268	3.62	**0.144**	0.000832	0.580
A: Soxhlet extraction with dichloromethane and acetone	**0.0689**	0.00366	5.31	**0.142**	0.00320	2.25
B: Soxhlet extraction with acetone	**0.0709**	0.00180	2.53	**0.142**	0.00420	2.96

[a] irradiation with light for 45 min in a distance of 25 cm

If HPLC results of hypericin after irradiation with light were looked at, ultrasonic extraction showed best reproducibility (Table 4.4.3, method D). The relative standard deviation of the evaluated content of hypericin was highest for Soxhlet extraction with dichloromethane and acetone (Table 4.4.3, method A). Extraction of drug samples, instead of the dry extract Ze117, with similar methods led to results considerably more deviating (Tables 4.3.1 to 4.3.4). The higher homogeneity of the commercial extract compared to the drug sample is probably one of the causes. Moreover, it was assumed that transformation processes were still going on in the drug sample, while they have been nearly completed in the extract Ze117, as commercial dry extracts have been exposed to various manufacturing processes. However, irradiation of the extracts from Ze117 with light still altered the results indicating that the transformation of the proto components to hypericin and pseudohypericin has not been fully completed (Table 4.4.3, method D). Nonetheless, it can be speculated that further transformation processes,

not known yet, are taking place. Influences of the extracting solvent and the extraction method in general on these processes could be various. There are open questions still waiting for clarification. Is it really only the difference in polarity of the extracting solvents tetrahydrofuran - water (8:2) and acetone, which led to significantly higher contents of naphthodianthrones applying tetrahydrofuran - water (8:2) (Table 4.2.1, method C and D)? Why does prolonged extraction with acetone not give similar results as tetrahydrofuran - water (8:2)? The Soxhlet extraction of a drug sample with acetone is apparently completed after 3 hours. Why does resumed extraction the day after provide additional hypericin? Does tetrahydrofuran accelerate some transformation processes in contrast to acetone? Have reactions been completed that were assumed to happen during biosynthesis (Cameron *et al.*, 1976; Gill and Giménez, 1991), or is the transformation of austroventin to penicillopsin and the conversion of penicillopsin to protohypericin still going on when the drug is extracted? How do the transformation and degradation products of hypericin and pseudohypericin look like? Is pseudohypericin synthesized in a separate biosynthetic pathway or is it generated from hypericin by oxidation? Considering these questions it is probable that the content of hypericin in drug samples of *Hypericum perforatum* is not stable but part of a dynamic system (Figure 4.1).

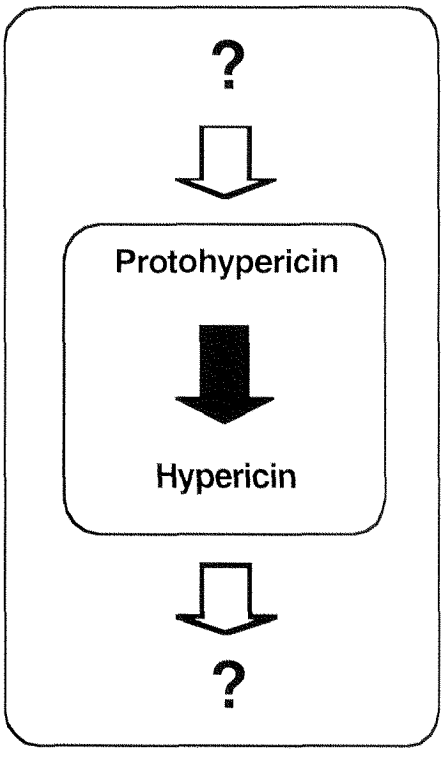


Figure 4.1 Content of hypericin - a dynamic system

At least, the ratio pseudohypericin to hypericin seemed not to be influenced by the extraction method. It was nearly the same for all the methods, only extracts obtained by ultrasonic extraction showed a smaller ratio before irradiation with light (Table 4.4.4).

Table 4.4.4 Influence of the extraction method on the ratio pseudohypericin to hypericin results determined by HPLC analysis

Method	Ratio pseudohypericin / hypericin
D: Ultrasonic extraction with MeOH *before* irradiation [a]	1.8
D: Ultrasonic extraction with MeOH *after* irradiation [a]	2.0
C: Soxhlet extraction with MeOH *before* irradiation [a]	2.0
C: Soxhlet extraction with MeOH *after* irradiation [a]	2.0
A: Soxhlet extraction with dichloromethane and acetone	2.1
B: Soxhlet extraction with acetone	2.0

[a] irradiation with light for 45 min in a distance of 25 cm

4.4.4 Conclusions

HPLC results revealed ultrasonic extraction to be the fastest, most efficient and precise method for the extraction of the naphthodianthrones from the dry extract Ze117. Methanol was more efficient than acetone in Soxhlet extraction. Preextraction of Ze117 with dichloromethane did not equalize quantification results from VIS spectroscopy and HPLC. Schütt and Hölzl, 1994 made similar observations. They found the ratio of VIS spectroscopic to HPLC results not to be the same for all the commercial extracts. This indicates that the commercial extracts contain, besides the naphthodianthrones, differing portions of further compounds absorbing at 590 nm. Therefore, it is recommended to use HPLC for quantification if commercial extracts are compared, as HPLC allows the selective determination of hypericin and pseudohypericin.

4.5 References

Brantner, A., Kartnig, Th. and Quehenberger, F.: Vergleichende phytochemische Untersuchungen an *Hypericum perforatum* L. und *Hypericum maculatum* Crantz. *Sci. Pharm.* **62**: 261-276 (1994)

Brolis, M., Gabetta, B., Fuzzati, N., Pace, R., Panzeri, F. and Peterlongo, F.: Identification by High-Performance Liquid Chromatography-Diode Array Detection-Mass Spectrometry and Quantification by High-Performance Liquid Chromatography-UV Absorbance Detection of Active Constituents of *Hypericum perforatum*. *J. Chromatogr. A* **825**: 9-16 (1998)

Büter, B., Orlacchio, C., Soldati, A. and Berger, K.: Significance of Generic and Environmental Aspects in the Field Cultivation of *Hypericum perforatum*. *Planta Med.* **64**: 431-437 (1998)

Butterweck, V.: Beitrag zur Pharmakologie und Wirkstoff-Findung von *Hypericum perforatum* L. Thesis. Mathematisch-Naturwissenschaftliche Fakultät. Westfälische Wilhelms-Universität Münster, 1997

Cameron, D.W., Edmonds J.S. and Raverty, W.D.: Oxidation of Emodin Anthrone and Stereochemistry of Emodin Bianthrone. *Aust. J. Chem.* **29**: 1535-1548 (1976)

Chi, J. D. and Franklin, M.: Determination of Hypericin in Plasma by High-Performance Liquid Chromatography. *J. Chromatogr. B* **724**: 195-198 (1999)

Freytag, W. E.: Bestimmung von Hypericin und Nachweis von Pseudohypericin in *Hypericum perforatum* L. durch HPLC. *Dtsch. Apoth. Ztg.* **124**: 2383-2386 (1984)

Gaedcke, F.: Johanniskraut und dessen Zubereitungen. Qualitätsbeurteilung über ein selektives, reproduzierbares HPLC-Verfahren. *Dtsch. Apoth. Ztg.* **137**: 3753-3757 (1997)

German Drug Codex, St. John's Wort Monograph. DAC 1986 (3rd suppl.) Eschborn, Stuttgart: Govi- and Deutscher Apotheker-Verlag, 1991

Gill, M. and Giménez, A.: Austrovenetin, the Principal Pigment of the Toadstool *Dermocybe Austroveneta. Phytochemistry* **30**: 951-955 (1991)

Häberlein, H., Tschiersch, K.-P., Stock, S. and Hölzl, J.: Johanniskraut (*Hypericum perforatum* L.). Teil 1: Nachweis eines weiteren Naphthodianthrones. *Pharm. Ztg. Wiss.* **137**: 169-173 (1992)

Hölzl, J. and Ostrowski, E.: Johanniskraut (*Hypericum perforatum* L.). HPLC-Analyse der wichtigen Inhaltsstoffe und deren Variabilität in einer Population. *Dtsch. Apoth. Ztg.* **127**: 1227-1230 (1987)

Jensen, K. I. N., Gaul, S. O., Specht, E. G. and Doohan, D. J.: Hypericin Content of Nova Scotia Biotypes of *Hypericum perforatum* L. *Can. J. Plant Sci.* **75**: 923-926 (1995)

Kartnig, Th. and Göbel, I.: Determination of Hypericin and Pseudohypericin by Thin-Layer Chromatography-Densitometry. *J. Chromatogr.* **609**: 423-426 (1992)

Kerb, R., Brockmöller, J., Staffeldt, B., Ploch, M. and Roots, I.: Single-Dose and Steady-State Pharmacokinetics of Hypericin and Pseudohypericin. *Antimicrob. Agents Chemother.* **40**: 2087-2093 (1996)

Klein-Bischoff, U. and Klumpp, U.: Hypericin und Fluoreszenz, eine quantitative fluorimetrische Bestimmungsmethode. *Pharm. Ztg. Wiss.* **138**: 55-58 (1993)

Krämer, W. and Wiartalla, R.: Bestimmung von Naphthodianthronen (Gesamthypericin) in Johanniskraut (*Hypericum perforatum* L.). *Pharm. Ztg. Wiss.* **137**: 202-207 (1992)

Kurth, H. and Spreemann, R.: Phytochemical Characterization of Various St. John's Wort. *Advances in Therapy* **15**: 117-128 (1998)

Liebes, L., Mazur, Y., Freeman, D., Lavie, D., Lavie, G., Kudler, N., Mendoza, S., Levin, B., Hochster, H. and Meruelo, D.: A Method for the Quantitation of Hypericin, an Antiviral Agent, in Biological Fluids by High-Performance Liquid Chromatography. *Analytical Biochemistry* **195**: 77-85 (1991)

Maisenbacher, P.: Untersuchungen zur Analytik von Johanniskrautöl. Thesis. Fakultät für Chemie und Pharmazie. Eberhard-Karls-Universtität Tübingen, 1991

Meier, B.: The Science Behind *Hypericum*. *Advances In Therapy* **16**: 135-147 (1999)

Micali, G., Lanuzza, F. and Currò, P.: High-Performance Liquid Chromatographic Determination of the Biologically Active Principle Hypericin in Phytotherapeutic Vegetable Extracts and Alcoholic Beverages. *J. Chromatogr. A* **731**: 336-339 (1996)

Mulinacci, N., Bardazzi, C., Romani, A., Pinelli, P., Vincieri, F. F. and Costantini, A.: HPLC-DAD and TLC-Densitometry for Quantification of Hypericin in *Hypericum perforatum* L. Extracts. *Chromatographia* **49**: 197-201 (1999)

Müller-Kuhrt, L. and Boesel, R.: Analytik der Hypericine im Hypericum-Extrakt. *Nervenheilkunde* **12**: 359-361 (1993)

Niesel, S.: Untersuchungen zum Freisetzungsverhalten und zur Stabilität ausgewählter wertbestimmender Pflanzeninhaltsstoffe unter besonderer Berücksichtigung moderner phytochemischer Analysenverfahren. Thesis. Pharmazie. Freie Universität Berlin, 1992

Nyiredy, Sz., Meier, B., Erdelmeier, C. A. J. and Sticher, O.: "PRISMA": A Geometrical Design for Solvent Optimization in HPLC. *J. of HRC and CC* **8**: 186-188 (1985)

Ostrowski, E.: Untersuchungen zur Analytik, ^{14}C-Markierung und Pharmakokinetik phenolischer Inhaltsstoffe von *Hypericum perforatum* L. Thesis. Pharmazie und Lebensmittelchemie. Philipps-Universität Marburg, 1988

Papageorgiou, G. C., Alygizaki-Zorba, A., Loukas, S. and Brody, S. S.: Photodynamic Effects of Hypericin on Photosynthetic Electron Transport and Fluorescence of *Anacystis nidulans* (*Synechococcus* 6301). *Photosynthesis Research* **48**: 221-226 (1996)

Pharmacopoeia Helvetica (Ph. Helv. 8), St. John's Wort Monograph, Bern, Eidgenössische Drucksachen- und Materialzentrale

Piperopoulos, G., Lotz, R., Wixforth, A., Schmierer, T. and Zeller, K.-P.: Determination of Naphthodianthrones in Plant Extracts from *Hypericum perforatum* L. by Liquid Chromatography-Electrospray Mass Spectrometry. *J. Chromatogr B* **695**: 309-316 (1997)

Schütt, H.: Morphologische, phytochemische und botanische Untersuchungen zur Selektion hypericin-, pseudohypericin- und flavonoidreicher *Hypericum perforatum* L. Stämme. Thesis. *Dissertationes Botanicae* **263**. Cramer, J. Berlin, Stuttgart, 1996

Schütt, H. and Hölzl, J.: Vergleichende Qualitätsuntersuchung von Johanniskraut-Fertigarzneimitteln unter Verwendung verschiedener quantitativer Bestimmungsmethoden. *Pharmazie* **49**: 206-209 (1994)

Southwell, I. A. and Campbell, M. H.: Hypericin Content Variation in *Hypericum perforatum* in Australia. *Phytochemistry* **30**: 475-478 (1991)

Stock, S.: ^{14}C-Markierung und Pharmakokinetik von Naphthodianthronen aus *Hypericum perforatum* L. sowie Untersuchungen zur Analytik von Oleum Hyperici. Thesis. Pharmazie und Lebensmittelchemie. Philipps-Universität Marburg/Lahn, 1992

Tateo, F., Martello, S., Lubian, E. and Bononi, M.: Hypericin and Hypericin-Like Substances: Analytical Problems. In: *Food Flavors: Formation, Analysis and Packaging Influences. Developments in Food Science 40.* Contis, E. T., Ho, C.-T., Mussinan, C. J., Parliment, T. H., Shahidi, F. and Spanier, A. M. (Ed.); Elsevier: Amsterdam, 1998, pp 143-157

Zeller Company (CH-Romanshorn), internal method (1992)

Zevakov, V. A., Glyzin, V. I., Shemeryankina, T. B. and Patudin, A. V.: Quantitative Evaluation of some Species of *St. John's Wort* for their Level of Hypericins by the HPLC Method. *Chemistry of Natural Compounds* **27**: 122 (1991)

5 Investigations on some Factors influencing the Extractability of Hyperforin, Adhyperforin, Hypericin and Pseudohypericin

5.1. Introduction

Hyperforin and Adhyperforin

The acylphloroglucinol derivative hyperforin (Figure 5.1) is the major non-nitrogenous secondary metabolite of *Hypericum perforatum* and has only been isolated from this plant (Chatterjee *et al.*, 1998). Adhyperforin (Figure 5.1) is a homologous compound containing one additional methylen group. Both compounds occur in flowers and fruits, but not in leaves (Berghöfer, 1987). Flowers contain 1.9% hyperforin and 0.17% adhyperforin, fruits 4.4% and 1.9%, respectively, according to Maisenbacher (1991). Hölzl and Ostrowski (1987) found the concentration of hyperforin in flowers to be 2.8%. Consequently, the content of hyperforin and adhyperforin in extracts of Hyperici herba strongly depends on the ratio of flowers and fruits to leaves in the genuine drug sample. Extracts with high levels of hyperforin are obtained, when the herb is collected *after* flowering time, when portions of fruits are high. However, this proceeding contradicts the monograph in the European Pharmacopoeia (Ph. Eur. III, suppl. 2000), which prescribes harvesting *during* flowering time.

Hyperforin has recently gained importance as some evidence for its contribution to the antidepressant activity of *Hypericum perforatum* has been found (Chatterjee *et al.*, 1998; Müller *et al.*, 1998; Dimpfel *et al.*, 1998; Schellenberg *et al.*, 1998; Laakmann *et al.*, 1998). Still, hyperforin is at best one active principle among others, as also products with low levels of hyperforin showed activity in clinical trials (Schrader, in press; Schrader *et al.*, 1998).
Only few methods were published for the quantification of hyperforin and adhyperforin all making use of HPLC (Table 5.1; Maisenbacher and Kovar, 1992; Melzer *et al.*, 1998; Orth, 1999). Reversed phase material combined with acetonitrile-phosphate buffer mixtures seem to be well suited for this task. UV absorption around 272 nm is generally used for detection, as hyperforin reveals an UV absorption maximum at 272 nm derived from a diode-array-UV-spectrum (Orth, 1999). Fingerprint chromatography allows qualitative evaluations, but is less useful for

Table 5.1 HPLC methods described in literature for the qualitative and quantitative evaluation of hyperforin (HF) and adhyperforin (A)

Reference[a]	Column[b]	Eluent and Flow	°C	Detection	HF [min]	A [min]
Biber *et al.* (1998)	Nucleosil RP-18 (125x3 mm, 5 µm) (Macherey+Nagel)	ACN-0.26 % H_3PO_4 in H_2O (75:25) Flow: 1 ml/min	c	UV absorption (273 nm)	c	c
Biber *et al.* (1998)	Intersil RP-8 (60x4 mm) (M&W)	MeOH-0.02 M NH_4COOCH_3 (6:4)	c	MS/MS	c	c
Butterweck (1997)	Prosep RP-18 (150x4 mm I.D., 5 µm) (Latek)	A: ACN, B: 0.5% TFA in H_2O, C: MeOH-ACN-0.5% TFA (60:39.5:0.5) Elution profile: 0-5 min A-B (1:9) to A-B (2:8), 5.01-7.0 min A-B (2:8) to B-C (7:3), 7.01-10 B-C (7:3) to B-C (5:5), 10.01-20 min B-C (5:5) to 100% C, 20.01-45 min 100% C Flow: 1 ml/min	25	UV absorption (284 nm)	24.9	c
Erdelmeier (1998)	Spherisorb ODS (250x4 mm I.D., 2.5 µm) (Knauer)	A: H_2O-H_3PO_4-triethylamine (995:3:2) B: ACN-H_3PO_4-triethylamine-H_2O (935:3:2:60) Elution profile: 0-5 min A-B (99:1), 5.01-55 min A-B (99:1) to A-B (60:40), 55.01-90 min A-B (60:40) to A-B (1:99) Flow: 1.2 ml/min	c	UV absorption (254 nm)	92	93
Hölzl and Ostrowski (1987)	Supersphere RP-18 (250x4 mm I.D.) (Merck)	A: ACN-H_2O-H_3PO_4 (19:80:1), B: ACN-MeOH-H_3PO_4 (59:40:1) Elution profile: 0-8 min 100% A, 8.01-30 min 100% A to A-B (50:50), 30.01-45 min A-B (50:50) to 100% B, 45.01-80 min 100% B Flow: 0.6 ml/min	RT	UV absorption (254 nm)	65	c

Reference[a]	Column[b]	Eluent and Flow	°C	Detection	HF [min]	A [min]
Maisenbacher (1991)	LiChrospher 100 RP-8 (125x4 mm I.D., 5 µm) (Merck)	ACN-0.01 M $NH_4H_2PO_4/H_3PO_4$ (pH 2.2-2.7) (8:2) Flow: 0.8 ml/min	30	UV absorption (270 nm)	≈**10**	≈**12**
Melzer *et al.* (1998)	LiChrospher 100 RP-18 (250x4 mm I.D., 5 µm) (Merck)	ACN-0.01 M $NH_4H_2PO_4$ (pH 2.5) (85:15) Flow: 2 ml/min	30	UV absorption (270 nm)	**10.1**	**12.2**
Orth (1999)	Nucleosil 100 RP-18 (250x4 mm I.D., 5 µm) (Macherey + Nagel)	ACN-H_2O/H_3PO_4 (pH 4.5) (89.5:10.5) Flow: 1.2 ml/min	c	UV absorption (272 nm)	**9.2**	**10.5**
Ostrowski (1988)	Supersphere RP-18 (250 mm, 4 µm) (Merck)	MeOH-ACN-THF-H_2O-H_3PO_4 (19.8:48.3:11.4:19.5:1) Flow profile: 0-17 min 0.3 ml/min, 17.01-50 min 1.3 ml/min	c	UV absorption (254 nm)	≈47	c

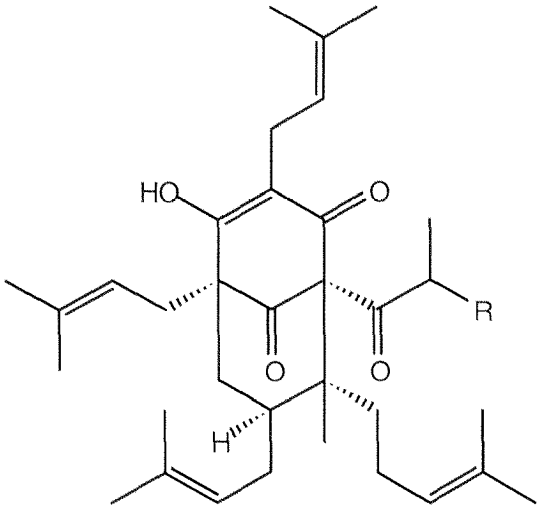
a References in bold print are suited for the quantification of hyperforin as far as retention time is the decisive factor.
b Precolumns are not listed in this table.
c not mentioned in the reference

quantification due to the long retention time of hyperforin (Table 5.1, e.g. Hölzl and Ostrowski, 1987; Erdelmeier, 1998). Biber *et al.* (1998) employed MS-MS detection for qualitative analysis. LC-(+)ESI-MS-MS technique was chosen by Orth *et al.* (1999b) to investigate the purity of isolated hyperforin.

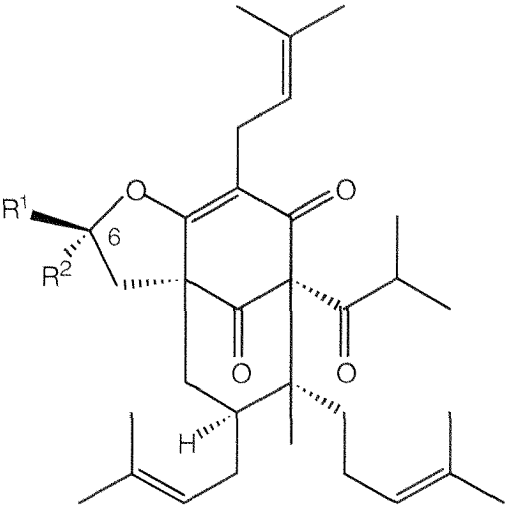
The stability of hyperforin has been discussed controversially. Known as a labile, photo- and oxygen-sensitive compound, its contribution to the antidepressant activity of *Hypericum perforatum* has not been considered first. Hyperforin was observed to decompose completely within weeks in Hyperici oleum (Maisenbacher and Kovar, 1992) and also in extracts of the dry herb (Berghöfer, 1987). However, Chatterjee *et al.* (1998) recently reported that avoiding light, air and higher temperatures during extraction and extraction with supercritical carbon dioxide led to extracts, in which hyperforin was stable for prolonged periods at room temperature in dark bottles. Furthermore, Melzer *et al.* (1998) reported commercial dry extracts to contain up to 6% hyperforin. Even in plasma samples it was possible to detect hyperforin and this 3 days after a single dose administration, demanding some stability of hyperforin (Biber *et al.*, 1998). Berghöfer (1987) observed hyperforin to be relatively stable in extracts of fresh plant material.

Orth (1999) studied the stability of hyperforin in methanol, heptane, cyclohexane, hexane and petroleum ether. In methanol only 1.8% degraded within 24 hours, while in the more lipophilic solvents degradation up to 97.9% occurred. Light exposure was shown to destabilize hyperforin and adhyperforin. This effect was more pronounced in acetone than in methanol. Degradation under light exposure could be diminished in methanol by acidification (pH 2). In contrast, light exposure at pH 12 led to total decomposition within 30 days. When kept in the dark, both substances were as stable in acidic as in alkaline methanolic solutions; the stability was slightly higher than that of acidic solutions exposed to light. The use of L-(+)-ascorbic acid and flushing of solvents with nitrogen or helium to expel oxygen were reported to improve the stability of hyperforin (Orth *et al.*, 1999b). The importance of the absence of peroxides has been discussed by Orth (1999) and Maisenbacher (1991). Further studies on the stability of hyperforin in the solid state have been done by Orth (1999). He found solid hyperforin only to be sufficiently stable at temperatures of -70 °C and below. Storage under nitrogen, argon and helium improved stability. The stabilizing effect of several additives was tested. β1,8-methyl-cyclodextrin turned out to give best results restricting degradation of hyperforin stored at 20 °C for 6 months to 3%. Ascorbic acid was less efficient allowing degradation up to 79%. The stabilizing effect of

ascorbic acid was improved when applied together with the synergist citric acid, but still results were not satisfactory showing 57% degradation. The antioxidants sodium sulphite and DL-α-tocopherol turned out to be least suited.



Hyperforin (**1**) (R = CH_3)
Adhyperforin (**2**) (R = CH_2CH_3)



3 (R^1 = $C(CH_3)_2OH$, R^2 = H)
4a (R^1 = H, R^2 = OH)
4b (R^1 = OH, R^2 = H)

Figure 5.1 Structural formula of hyperforin and its derivatives

Trifunovic *et al.* (1998), Orth *et al.* (1999a) and Verotta *et al.* (1999) described a degradation product of hyperforin, the new structural element being a cyclic ether probably resulting from oxidation (**3**, see Figure 5.1). Orth *et al.* (1999a) showed it to be an intermediate product undergoing further oxidation. Trifunovic *et al.* (1998) isolated a second compound, bearing a hydroxy function in position 6 instead the

1-methyl-1-hydroxyethyl group of the first compound. It was a mixture of 6α- and 6β-hydroxy epimers (**4a** and **4b**, see Figure 5.1). If these hyperforin analogues are degradation products of hyperforin or if they are genuinely present in the drug has to be further investigated. Maisenbacher (1991) assumed 2-methyl-3-buten-2-ol to be a degradation product of hyperforin, generated by oxidative cleavage of the isoprenyl side chains. 2-methyl-3-buten-2-ol is a component of the essential oil of Hyperici herba (Roth, 1990). The presence of other minor hyperforin analogues has been reported by Fuzzati *et al.* (1999). They used HPLC-DAD-UV analysis and HPLC-thermospray-mass spectrometry for identification.

This study dealt with the extractability of hyperforin and adhyperforin from Hyperici herba, as only few data have been published on this subject. Kurth and Spreemann (1998) found the solvents ethanol 60%, methanol 80%, pure ethanol and pure acetone to be best suited for the extraction of hyperforins. Further investigations on this subject seemed necessary.

Hypericin and Pseudohypericin

The extraction procedure is of interest in production and also quality control, as it is one of various factors influencing quantification results (see section 3). Extraction methods have been examined by various authors.
Wagner and Bladt (1993) studied the extractability of hypericin and pseudohypericin from Hyperici herba by various solvents, extracting 10 min at 80 °C. They applied a drug to solvent ratio of 1:50 (m/v). Highest yields were obtained by methanol (75% hypericin, 80% pseudohypericin), followed by ethanol (34% hypericin, 37% pseudohypericin), acetone (20% of both compounds) and propan-2-ol (10% of both compounds). Ethyl acetate is not suited as extracting solvent. Among methanol-water mixtures, **methanol 80%** gave best results for hypericin (83%) and **methanol 60%** for pseudohypericin (94%), extracting 10 min at the boiling point. Methanol 80% allowed nearly quantitative extraction of both compounds within 30 min. Kurth and Spreemann (1998) found **ethanol 60%** to give better results than methanol 80%, pure ethanol, pure acetone, ethanol 30% and water. Tateo *et al.* (1998) macerated drug samples of Hyperici herba with ethanol-water mixtures of varying proportions for three days, applying a drug to solvent ratio of 2:10 (m/v). They found that the ethanolic

portion had to be at least 40% to make hypericin extraction possible. Pseudohypericin was already extractable with 30% ethanol. Increasing alcohol concentrations improved the extractability of the naphthodianthrones. Niesel (1992) did extractions with aqueous methanol 20, 40, 60, 80% and 100% methanol at 80 °C. The drug to solvent ratio was 1.5:150 (m/v). After 20 min, **methanol** concentrations between **40%** and **80%** gave best results for pseudohypericin, while hypericin was more efficiently extracted by **pure methanol,** not being in full agreement with the results of Wagner and Bladt (1993). Further extracting solvents were examined: water, methanol, ethanol, 2-propanol, ethyl acetate, acetone and chloroform. Methanol and ethanol provided highest yields. Methanol extracted about four times more pseudohypericin and twelve times more hypericin than water. Acetone and 2-propanol gave similar results as water, while extracts with the lowest content were obtained with ethyl acetate and chloroform.

Niesel (1992) also studied the extracted amount of naphthodianthrones in tea preparations. He applied a drug to water ratio of 1.5:200 (m/v). After ten mininutes 5.7% hypericin and about 22% pseudohypericin of the total amount were extracted by water decoction. The influence of temperature was studied. Highest yields were obtained with water temperatures of 60 °C (23.5% pseudohypericin, 9.5% hypericin) to 80 °C (21.8% pseudohypericin, 8.2% hypericin). Higher temperatures led to the degradation of the naphthodianthrones. Temperatures below 40 °C did not allow the extraction of hypericin with water. Wagner and Bladt (1993) found the solubility of hypericin and pseudohypericin in water to be generally higher than reported by Niesel (1992). Results of water decoction (with a drug to water ratio of 1.5:200 (m/v)) were two and three times higher for pseudohypericin (about 38%) and hypericin (about 16%), respectively. Extraction at 60 °C for 10 min yielded about 24% of pseudohypericin and 10% of hypericin. In contrast to Niesel, extraction at 100 °C for 10 min provided higher hypericin (14%) and pseudohypericin (38%) yields than extraction at 60 °C. Longer heating decreased the hypericin content, but not considerably the pseudohypericin content.

It was shown by Kurth and Spreemann (1998) that also the ratio of pseudohypericin to hypericin is affected by the extracting solvent. They found ratios of 5:1 for water, 3:1 for ethanol 30%, 1.5:1 for both ethanol 60% and methanol 80% and about 1:1 for pure ethanol and pure acetone. The additional hydroxyl group of pseudohypericin explains its better solubility in polar solvents.

Reports about the extractability of hypericin and pseudohypericin from Hyperici herba were not fully consistent. Therefore, additional experiments on this subject were done in this study.

5.2 Experimental

Materials

The drug sample analyzed was Hyperici herba (lot 50279L (1995)) from Dixa (CH-St. Gallen). Hypericin was isolated (see section 2) from an ethanol-water (1:1, m/m) dry extract (Ze117) of Hyperici herba, which was obtained from Zeller Company (CH-Romanshorn). Hyperforin (HPLC purity 92%, batch: RHF 00498) was from Addipharma GmbH (D-Hamburg). Methanol, ethanol absolute, tetrahydrofuran and acetonitrile were of HPLC quality (Romil Chemicals, GB-Shepshed). Pyridine p.a. and ascorbic acid p.a. were purchased from Fluka (CH-Buchs). *Ortho*-phosphoric acid 85% (Ph. Helv. VI / Ph. Eur.) was from Hänseler (CH-Herisau) and sodium dihydrogen phosphate from Merck (CH-Dietikon). De-ionized water was obtained using an NANO-pure cartridge system (Skan, CH-Basel). Regenerated cellulose syringe filters (0.2 µm, 13 mm) were from Schleicher&Schuell (D-Dassel). For irradiation of the extracts a blended light lamp (Philips MLR 160W, 230-240V) was used.

Instrumentation and Column

HPLC. All HPLC analyses were performed using a Hewlett Packard instrument (Model 79994A analytical workstation, model 1090 liquid chromatograph, model 1040 diode-array detector). For chromatographic separations a Knauer (D-Berlin) prepacked cartridge column (250 x 4 mm I.D., 5 µm) filled with Spherisorb S5 ODS2 and a guard column (10 x 4 mm I.D.) of the same material was used.

Chromatographic Conditions

Hyperforin. The method applied was an own development. Solvents used were solvent A (methanol) and solvent B (acetonitrile - tetrahydrofuran - water - *ortho*-phosphosphoric acid 85% (105:45:50:1.2, v/v). The

elution profile was: 0-20 min 100% B, 20.01-25 min 100% A and 25.01-35 min 100% B. The flow rate was 1.6 ml/min for solvent B and 1 ml/min for solvent A. The column temperature was set at 25 °C and the injection volume was 25 µl. The detection wavelength of the DAD was set at 274 nm. Retention times were 14.5 min and 17.3 min for hyperforin and adhyperforin, respectively.

Hypericin and pseudohypericin. The partly modified method of Kerb *et al.* (1996) was applied. Solvents used were solvent A (methanol) and solvent B (methanol - tetrahydrofuran - buffer (9:6:5, v/v)). The buffer consisted of 13.8 g sodium dihydrogen phosphate in 1 litre water adjusted to pH 4.0 with *ortho*-phosphoric acid 85%. The elution profile was: 0-12 min 100% B, 12.01-17 min 100% A and 17.01-27 min 100% B. The flow rate was 0.8 ml/min for solvent B and 1 ml/min for solvent A. The column temperature was set at 25 °C and the injection volume was 25 µl. Retention times were 6.5 min and 3.5 min for hypericin and pseudohypericin, respectively.

Procedures

Extract solutions. 1.000 g of Hyperici herba was macerated with 5 g extracting solvent in a 50 ml round-bottom flask for an hour, stirring with a magnetic stirrer. The extract was filtrated under vacuum and transferred to a 20.0 ml volumetric flask, which was made up to volume with the same solvent. Additionally, a reflux condenser and a water bath with contact thermometer were used for extraction at 40, 50 and 60 °C. The preparation of the solvent mixtures was done m/m.
The solvent mixtures aqueous ethanol 40%, 50%, 60%, 70%, 80% and ethanol 100% and aqueous methanol 60%, 70%, 80%, 90% and methanol 100% were examined at room temperature. Ethanol 100% was used for extraction at 40, 50 and 60 °C.
For the quantitative analysis of hyperforin, extracts were directly subjected to HPLC analysis after filtration over a 0.2 µm regenerated cellulose syringe filter. For the quantification of hypericin and pseudohypericin, 2 ml of the filtered samples were transferred into HPLC vials, irradiated with a blended light lamp for 15 min and then analyzed by HPLC.

Tea preparation. 100 ml water of 95 °C were poured on 1.000 g Hyperici herba and let stand for 10 min. The suspension was filtrated under vacuum, and the extract lyophilized over night. The residual was

dispersed in aqueous ethanol 50% and transferred to a 20.0 ml volumetric flask, which was made up to volume with the same solvent.

Exhaustive extraction of hyperforin. 6.2 ml ethanol 100% were added to 1.000 g Hyperici herba, stirred 1 h at room temperature and filtered under vacuum. Once more, 6.2 ml ethanol 100% were added to the extracted drug material, stirred 1 h at room temperature and filtered under vacuum. The extracts were combined and transferred to a 20.0 ml volumetric flask, which was made up to volume with ethanol 100%. The extract was filtered over a 0.2 µm regenerated cellulose syringe filter and directly subjected to HPLC analysis.

Exhaustive extraction of hypericin and pseudohypericin. 1.000 g of Hyperici herba was weighed in a Soxhlet thimble, covered with glass beads and extracted with 130 ml methanol at 75 °C for 5 h. The methanol solution was concentrated under vacuum and transferred to a 20.0 ml volumetric flask, which was filled up to the mark with methanol. 2 ml of the filtered sample was transferred into a HPLC vial, irradiated with a blended light lamp for 15 min and analyzed by HPLC.

Extracts prepared with exclusion of light and oxygen. The procedure was the same as described for *Extract solutions* (see above) with the difference that light and oxygen was excluded by working in a dark room and gassing with argon. Extractions were done with ethanol 100% at room temperature and 60 °C and with aqueous ethanol 80% at room temperature. The content of hyperforin and adhyperforin was determined.

Stability tests. The extract solutions prepared without exclusion of light and oxygen were stored at -20 °C and subjected to HPLC analysis for a second time after 14 days.
The samples prepared with exclusion of light were stored at room temperature. 1% ascorbic acid was added to half of the extract prepared with ethanol 100% at room temperature. The extracts were analyzed by HPLC, after preparation and after 14 days.

Precision.
Hyperforin. The precision of injection was determined by injecting the reference solution 0.2022 mg/ml 10 times.

Reference solutions.

Hyperforin. Reference solutions were prepared with hyperforin in methanol. Their concentrations ranged from 0.005054 mg/ml to 0.5054 mg/ml. The solutions were stored at -20 °C in the dark. They were stable during the investigated time period (60 days), as their area counts did not change.
Additional dilutions were prepared to determine the detection limit.

Hypericin. Six reference solutions were prepared with hypericin in methanol-pyridine (99:1, v/v) their concentrations ranging from 0.9984 µg/ml to 14.98 µg/ml. The solutions were stored at -20 °C in the dark. Stability tests proofed them to be stable under these conditions over a time period of 140 days (see section 3.3.4).

Quantification by HPLC.

Hyperforin and adhyperforin. Quantification was carried out by the external standard method on the basis of area counts at 274 nm. Calibration curves were generated by a least squares regression method, using six calibration solutions. Over the selected concentration range of the standard solutions, the calibration curve showed a linear detector response. The correlation coefficient was 0.9999. The external standard solutions were injected three times into the HPLC system for analysis. The calibration curve of hyperforin was used for the quantification of both hyperforin and adhyperforin. The purity of the reference substance was determined by HPLC to be 92%, which was included into the calculation. The average of six extracts was used to calculate the content of hyperforin and adhyperforin.

Hypericin and pseudohypericin. Quantification was carried out by the external standard method on the basis of area counts at 590 nm. Calibration curves were generated by a least squares regression method, using six calibration solutions. All the calibration graphs were linear and showed correlation coefficients between 0.9997 and 1.000. The external standard solutions were injected three times into the HPLC system for analysis. The calibration curve of hypericin was used for the quantification of both hypericin and pseudohypericin. The average of six extracts was used to calculate the content of the two naphthodianthrones.

5.3 Results and Discussion

HPLC Method for the Quantification of Hyperforin and Adhyperforin

The developed HPLC method allowed baseline separation of hyperforin and adhyperforin within a reasonable run time. Retention times were 14.5 min and 17.3 min for hyperforin and adhyperforin, respectively. Acidification of the eluent turned out to be necessary. Lack of acid led to a shortening of the retention times of hyperforin and adhyperforin after some runs.
The detection wavelength was set at 274 nm, because the UV absorption spectrum of hyperforin revealed a maximum at 274 nm in the eluent employed. This was derived from the diode-array-UV-spectrum.
The detection limit of hyperforin in the applied HPLC system was 0.005054 mg/ml. The determination of the precision with the reference solution 0.2022 mg/ml revealed a relative standard deviation of 0.181%.

Effects of the Extracting Solvent and Temperature on the Yield of Hyperforin and Adhyperforin

Yields of hyperforin and adhyperforin were expressed as percentage of the total amount, which was defined as the results of the exhaustive extraction. The exhaustive extraction revealed contents of hyperforin and adhyperforin of 0.776% and 0.0816%, respectively, for the drug sample investigated. Therefore, the ratio of hyperforin to adhyperforin content was 9.51.

Aqueous ethanol 70% to ethanol 100% turned out to be the most effective solvents among those investigated. Extraction at higher temperature decreased the yield (Figure 5.2). Among the methanol-water mixtures, the highest yields were achieved with methanol 90% (Figure 5.3). No hyperforin could be detected in the tea preparation. Light exclusion did not significantly influence the results (Figure 5.6). The polarity of the extracting solvent did not shift the ratio of the hyperforin to adhyperforin yield to a defined direction. The ratio of the yields was between 11.4:1 and 16.2:1 (Table 5.2).

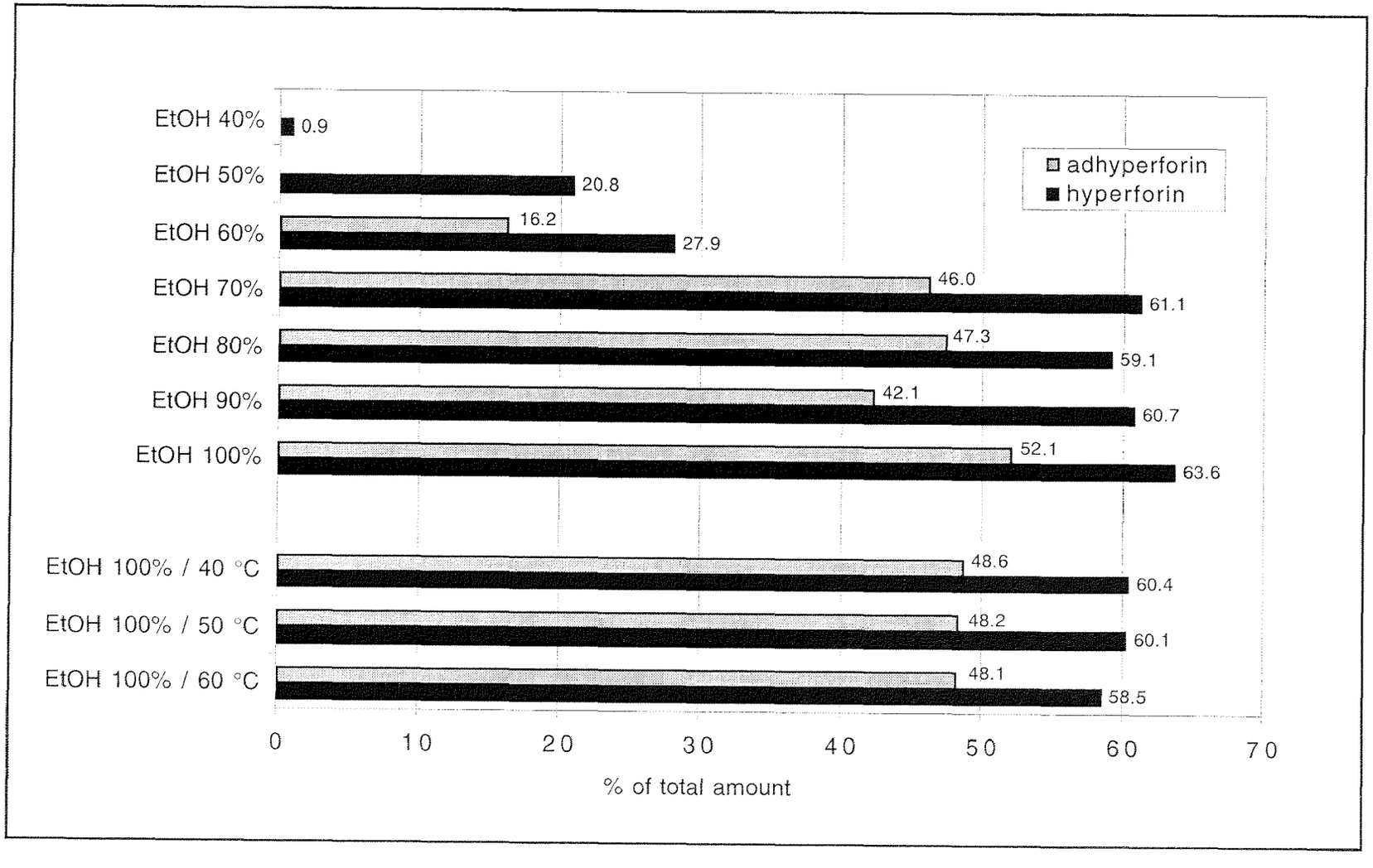


Figure 5.2 Influence of the extracting solvent and temperature on the yield of hyperforin and adhyperforin

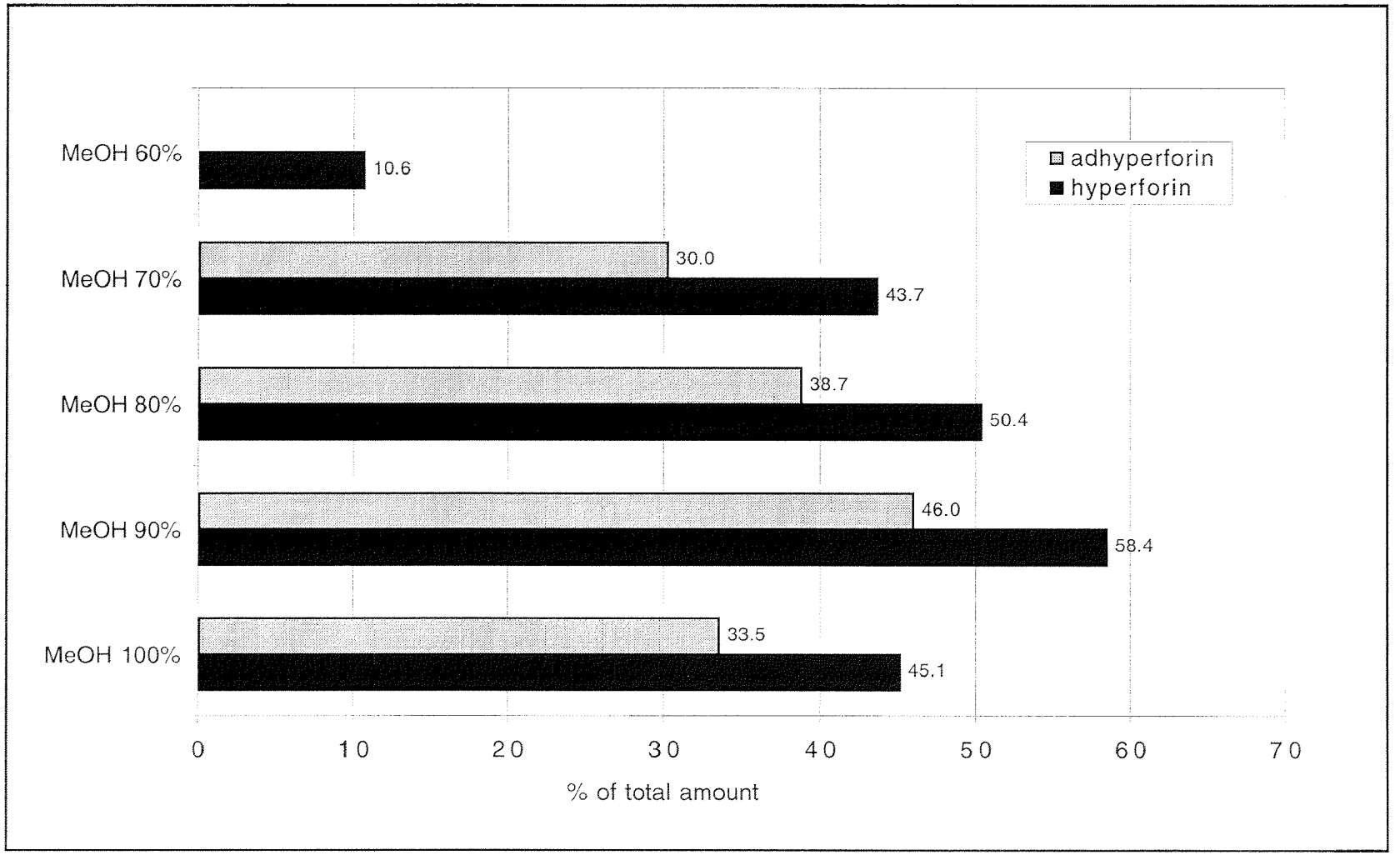


Figure 5.3 Influence of the extracting solvent on the yield of hyperforin and adhyperforin

Table 5.2 Influence of the extracting solvent on the ratio of hyperforin to adhyperforin yields

Extracting solvent	Ratio hyperforin / adhyperforin
EtOH 40%	a
EtOH 50%	a
EtOH 60%	16.2
EtOH 70%	12.4
EtOH 80%	11.7
EtOH 90%	13.5
EtOH 100%	11.4
EtOH 100% / 40 °C	11.6
EtOH 100% / 50 °C	11.6
EtOH 100% / 60 °C	11.4
MeOH 60%	a
MeOH 70%	13.6
MeOH 80%	12.2
MeOH 90%	11.9
MeOH 100%	12.6

a The amount of adhyperforin was below the detection limit.

Stability of Hyperforin and Adhyperforin

The content of hyperforin in the extracts prepared without light exclusion decreased up to 80% within 14 days, although they were stored at -20 °C. Fastest degradation of hyperforin and adhyperforin could be observed in the solvent ethanol 100%, when extraction was done at room temperature (Figure 5.4). Methanol 100% provided least stability of the methanolic extract solutions for hyperforin (Figure 5.5). Extraction at increased temperature led to extracts with improved stability (Figure 5.4). Best results regarding stability were achieved when light and oxygen were excluded during extraction. The contents of those extracts did not change significantly within 14 days in spite being stored at 20 °C. The addition of ascorbic acid turned out not to be necessary (Table 5.3).

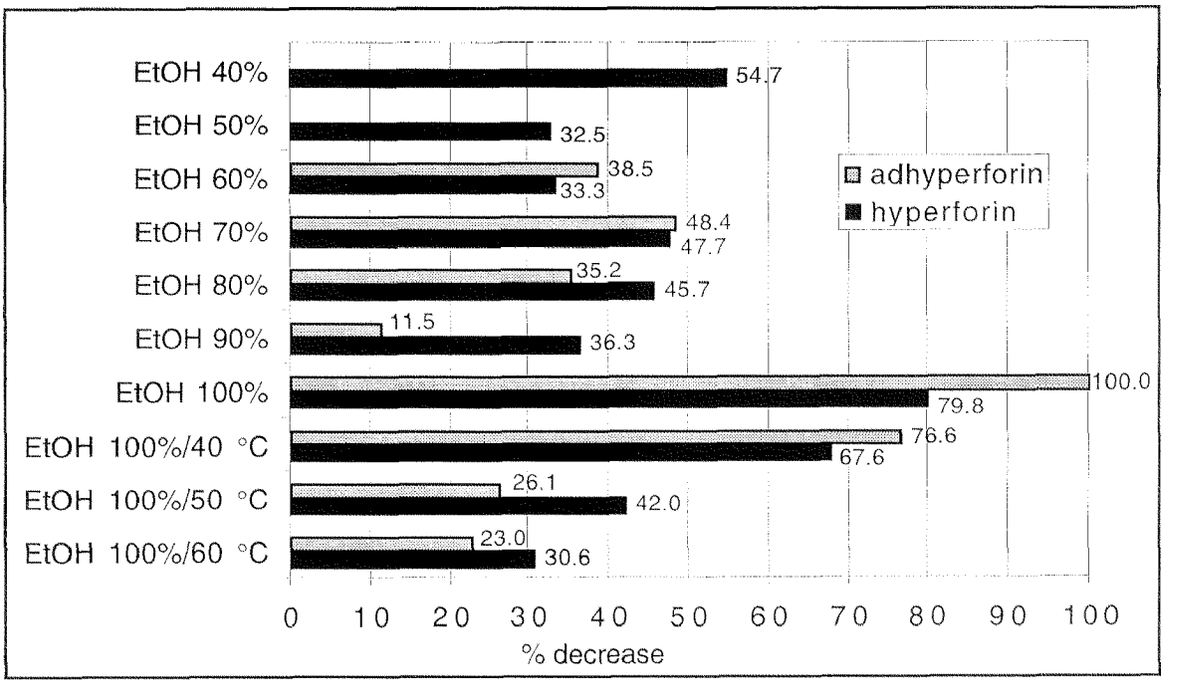


Figure 5.4 Decrease of the content of hyperforin and adhyperforin within 14 days at -20 °C

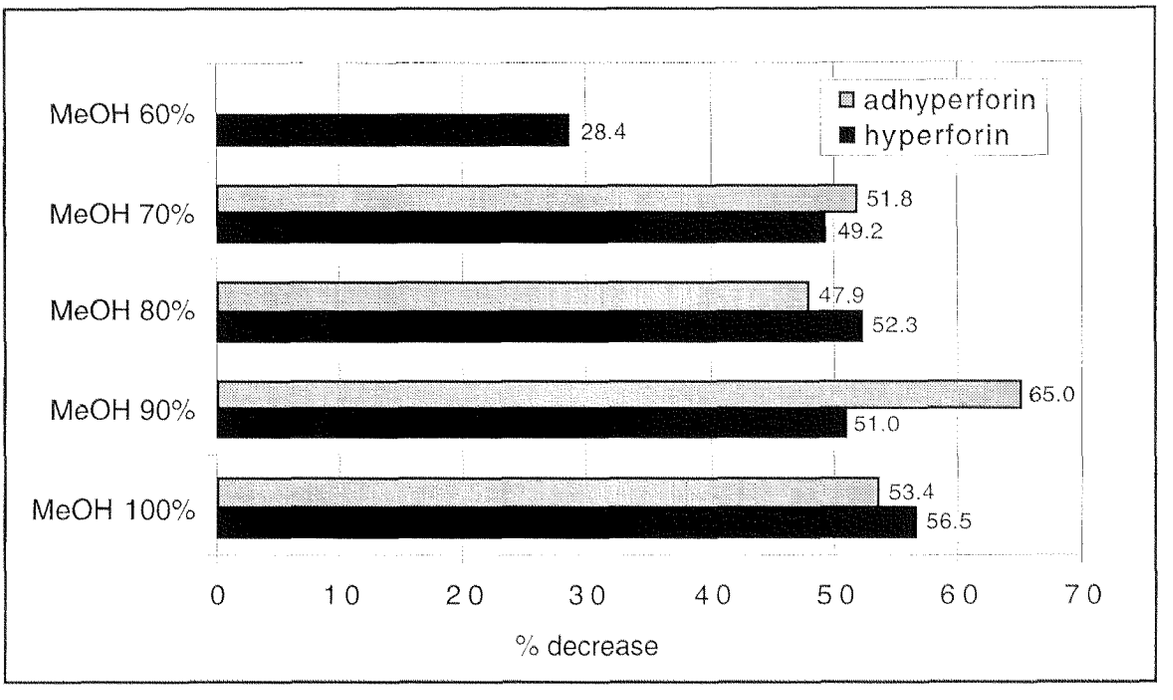


Figure 5.5 Decrease of the content of hyperforin and adhyperforin within 14 days at -20 °C

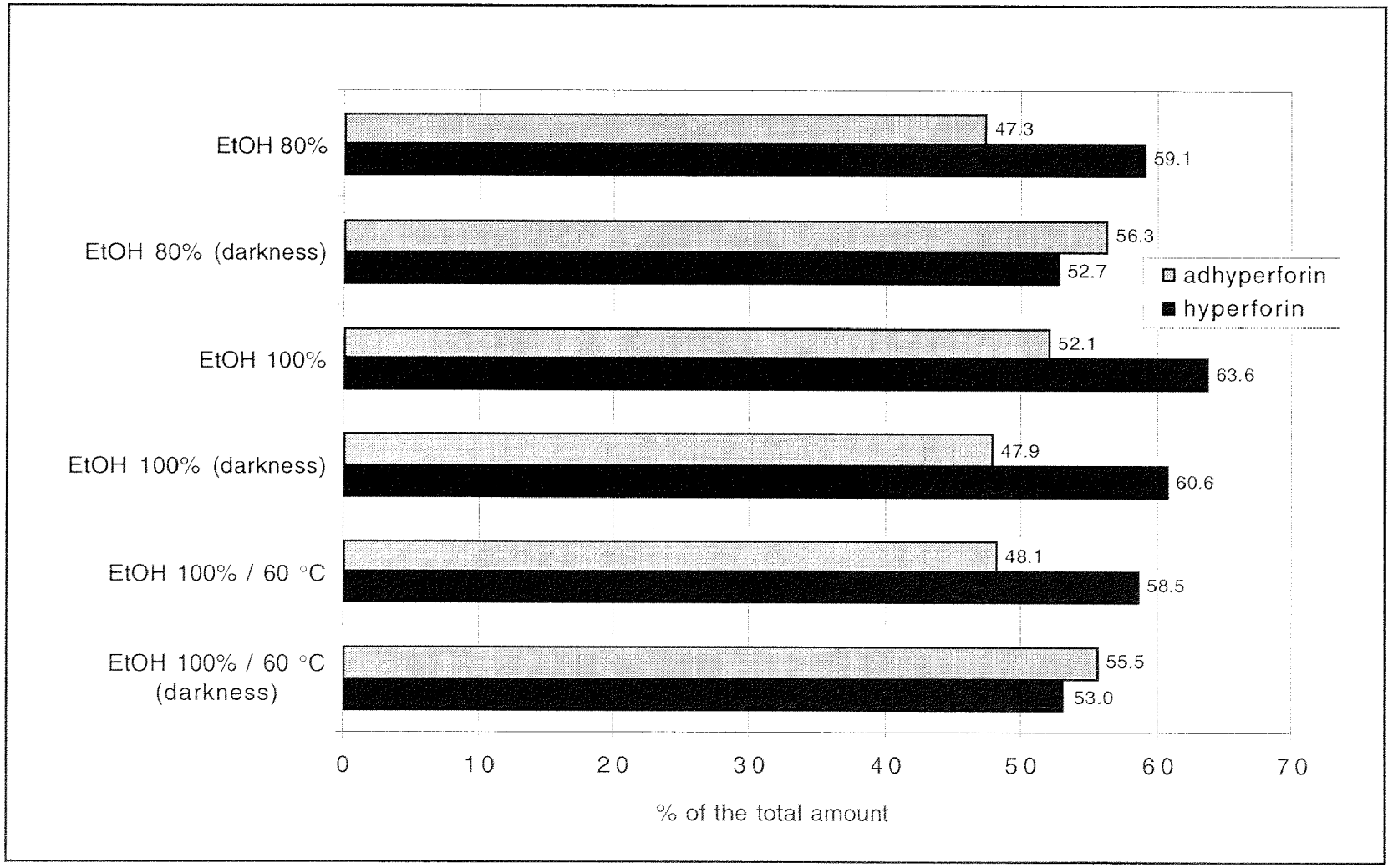


Figure 5.6 Influence of the exclusion of light and oxygen on the yield of hyperforin and adhyperforin

Table 5.3 Stability of hyperforin (HF) and adhyperforin (A) in the extract solutions that were prepared with exclusion of light and oxygen

Extracting solvent	concentration [mg/ml] n=6				% decrease / increase (from day 1 to 15)	
	day 1 HF	A	day 15 HF	A	HF	A
EtOH 100%	0.464	0.0392	0.467	0.0390	+1	-1
EtOH 100% / ascorbic acid	0.464	0.0392	0.460	0.0388	-1	-1
EtOH 100% / 60 °C	0.406	0.0455	0.428	0.0431	+5	-5
EtOH 80%	0.404	0.0461	0.409	0.0438	+1	-5

Methanolic reference solutions of hyperforin were stable over the investigated time period (60 days), not revealing any changes in their area counts. This indicates that the degradation of hyperforin is initiated by components of the extract. Preparation of the extracts at increased temperature and exclusion of light and oxygen led to extracts with better stability, may inactivating or preventing the generation of the hyperforin destroying agents. The result that exclusion of light and oxygen during extraction improved the stability of the extracts is consistent with the finding of Chatterjee *et al.* (1998).

Effects of the Extracting Solvent and Temperature on the Yield of Hypericin and Pseudohypericin

Yields of hypericin and pseudohypericin were expressed as percentage of the total amount. The total amount was defined as the results of the exhaustive Soxhlet extraction of the drug with methanol. The contents of hypericin and pseudohypericin determined by the exhaustive extraction were 0.0224% and 0.0302%, respectively. The ratio of pseudohypericin to hypericin content was 1.35.
Aqueous ethanol 70% provided the highest yields of hypericin and pseudohypericin (Figure 5.7) among the extracting solvents investigated. Of the extracting solvents methanol 100%, methanol-water 90%, 80% and 60%, methanol 100% turned out to be most effective in the extraction of the naphthodianthrones (Figure 5.9). The increase of temperature from room temperature to 60 °C led to higher yields (Figure 5.7) examined with the extracting solvent ethanol 100%.

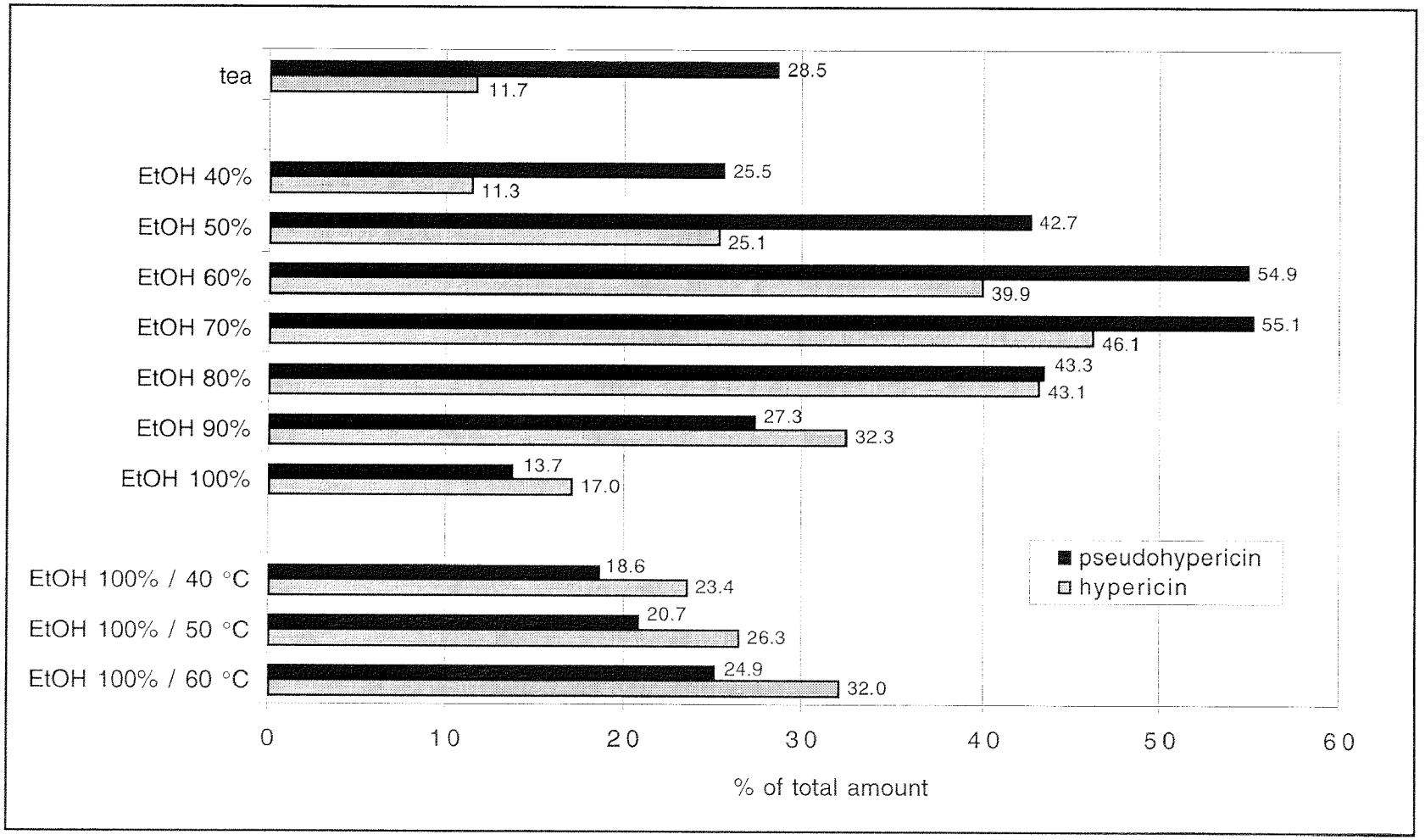


Figure 5.7 Influence of the extracting solvent on the yield of hypericin and pseudohypericin

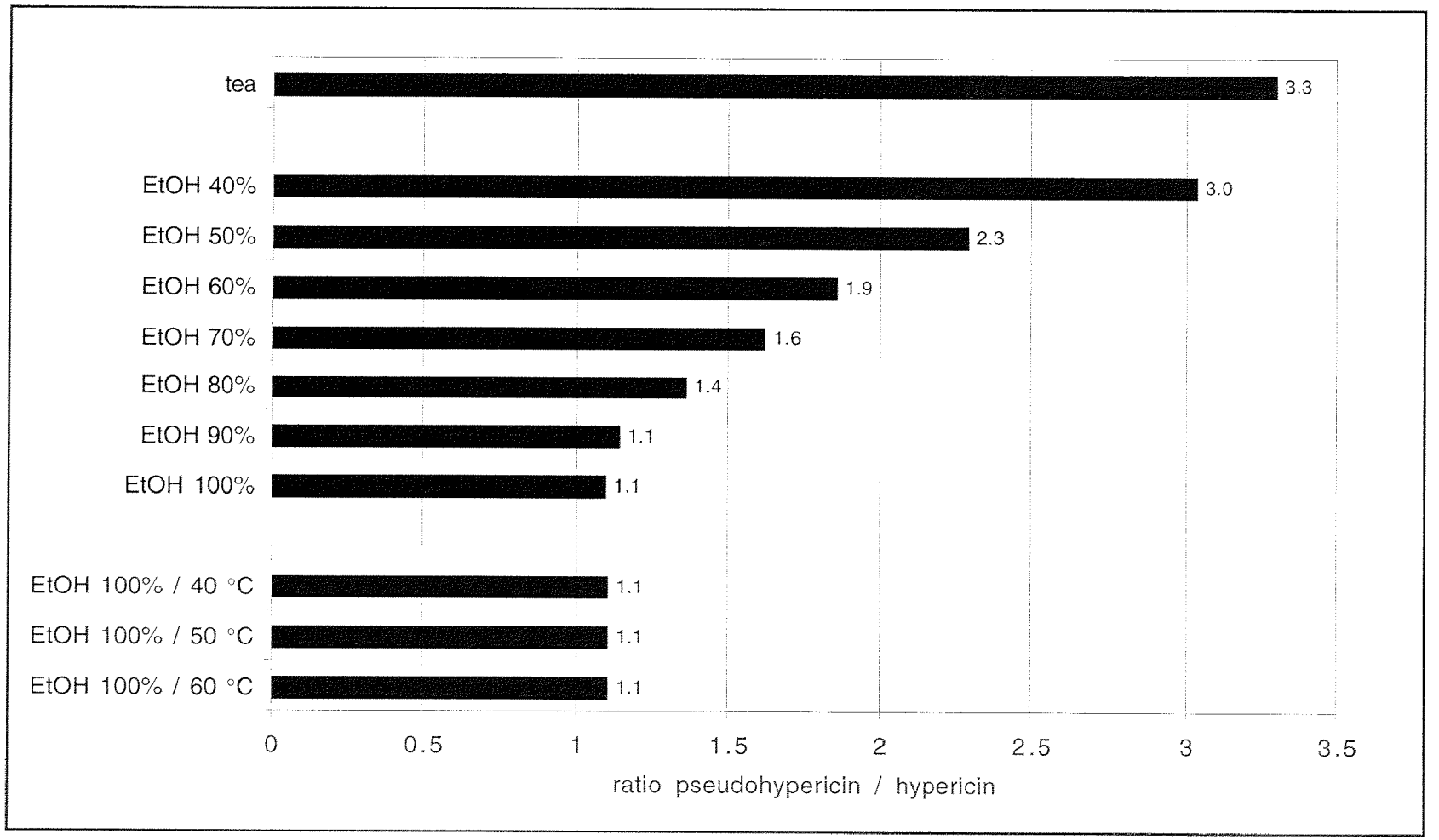


Figure 5.8 Effects of the extracting solvent on the ratio of the yields of pseudohypericin and hypericin

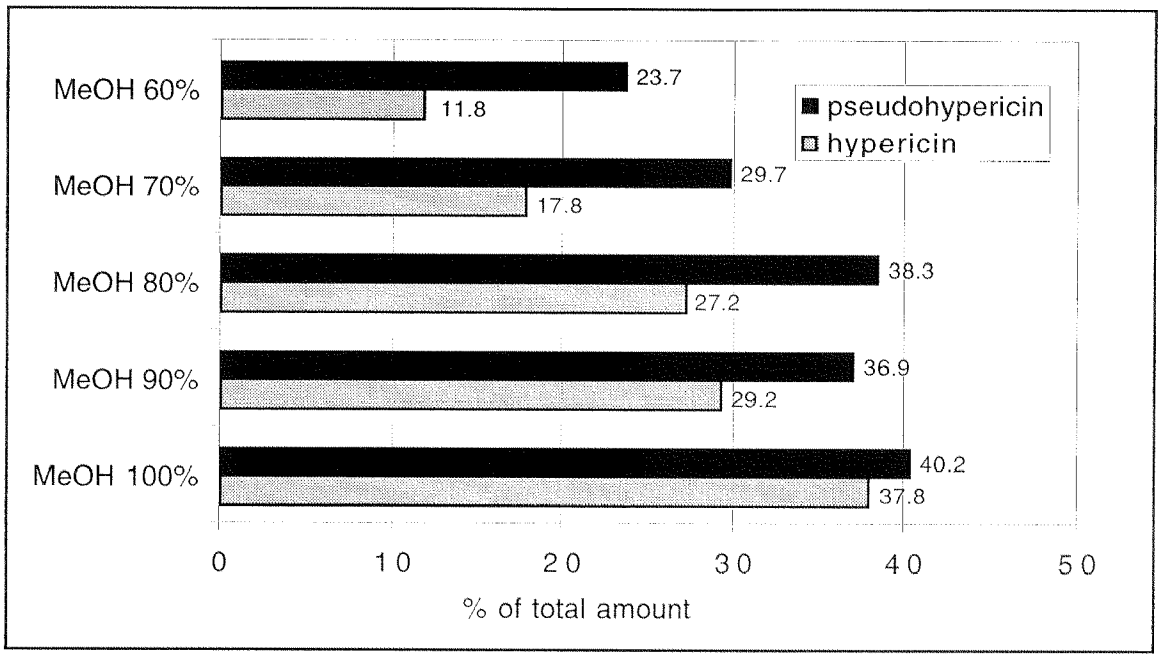


Figure 5.9 Influence of the extracting solvent on the yield of hypericin and pseudohypericin

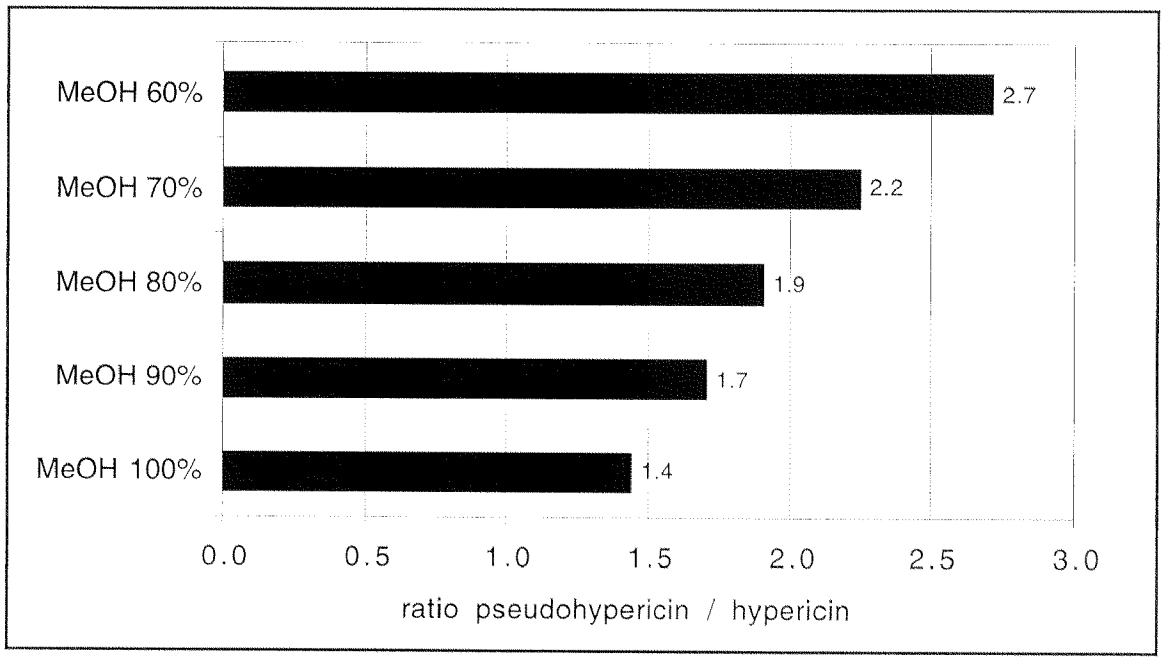


Figure 5.10 Influence of the extracting solvent on the ratio of the yields of pseudohypericin and hypericin

12% of total hypericin and 29% of total pseudohypericin could be extracted by the tea preparation, results of Wagner and Bladt (1993) being higher and results of Niesel (1992) lower. The investigated drug sample revealed a content of hypericin and pseudohypericin of 0.0224% and 0.0302%, respectively (see above). Therefore, a cup of tea (1.8 g Hyperici herba in 180 ml water) contains about 48 µg hypericin and 158 µg pseudohypericin. Two cups of tea would correspond to the dosage of 2 - 4 g herb recommended by the European Scientific Cooperative on Phytotherapy or by the Commission E. For comparison, 230 - 2000 µg naphthodianthrones (determined by HPLC) are generally contained in the daily dose of commercial products of Hyperici herba (Meier, 1999). The intake of naphthodianthrones by two cups of tea lies within this limit.
The ratio of pseudohypericin to hypericin was strongly affected by the polarity of the solvent (Figures 5.8 and 5.10). This came up to expectations, as the polarity of pseudohypericin is larger, due to its additional hydroxyl group. The highest ratio (3.3) revealed the tea preparation with water, decreasing from ethanol 40% (3.0) to ethanol 100% (1.1) and from methanol 60% (2.7) to methanol 100% (1.4). Extraction at higher temperature did not influence the ratio. The results of Kurth and Spreemann (1998) were slightly different (Table 5.4).

Table 5.4 Influence of the solvent strength on the yield of hypericin and pseudohypericin

Extracting solvent	Ratio of pseudohypericin to hypericin yields	
	Measured[a]	Kurth and Spreemann (1998)
Tea preparation	3.3	5
Ethanol 60%	1.9	1.5
Ethanol 100%	1.1	1
Methanol 80%	1.9	1.5

[a] The ratio of pseudohypericin to hypericin in the drug sample was 1.35, determined by exhaustive extraction.

5.4 Conclusions

Hyperforin and Adhyperforin

Referring to the results above, highest yields of hyperforin and adhyperforin were achieved with the extracting solvents aqueous

ethanol 70% to ethanol 100%. Exclusion of light and oxygen during the extraction process is most important in favor of getting extracts with good stability; this is consistent with the results of Chatterjee *et al.* (1998).
Methanol was suited for the preparation of standard solutions of hyperforin, as the solutions were stable during the investigated time period (60 days). Orth (1999) also reported the stability of hyperforin in methanolic solutions to be better than in more lipophilic solvents.

Hypericin and Pseudohypericin

Ethanol 70% turned out to be the solvent best suited for the extraction of hypericin and pseudohypericin from Hyperici herba at room temperature. As an increase in temperature leads to higher yields with ethanol 100%, it is probable that it is the same with ethanol 70%. However, further experiments would be necessary to proof this assumption and to make sure that the stability of hypericin and pseudohypericin at higher temperature is not affected by the water addition. A decrease in stability could compensate the extraction of higher amounts.
Among the solvents methanol 100%, methanol-water 90%, 80% and 60%, methanol 100% was most effective extracting hypericin and pseudohypericin. This was not in agreement with Wagner and Bladt (1993), who reported methanol 80% and methanol 60% to give best results for hypericin and pseudohypericin, respectively. Niesel (1992) also found methanol 100% to give highest yields for hypericin. However, his investigations revealed methanol concentrations between 40% and 80% to be best suited for pseudohypericin. Both authors worked at elevated temperature, while this study generally used room temperature.

5.5 References

Berghöfer, R.: Analytik und Isolierung phenolischer Inhaltsstoffe von *Hypericum perforatum* L. aus Anbau und Wildvorkommen. Thesis. *Dissertationes Botanicae* **106**. Cramer, J. Berlin, Stuttgart, 1987

Biber, A., Fischer, A., Römer, A. and Chatterjee, S.S.: Oral Bioavailability of Hyperforin from Hypericum Extracts in Rats and Human Volunteers. *Pharmacopsychiat.* **31** (suppl. 1): 36-43 (1998)

Butterweck, V.: Beitrag zur Pharmakologie und Wirkstoff-Findung von *Hypericum perforatum* L. Thesis. Mathematisch-Naturwissenschaftliche Fakultät. Westfälische Wilhelms-Universität Münster, 1997

Chatterjee, S. S., Nöldner, M., Koch, E. and Erdelmeier, C.: Antidepressant Activity of *Hypericum perforatum* and Hyperforin: the Neglected Possibility. *Pharmacopsychiat.* **31** (suppl. 1): 7-15 (1998)

Dimpfel, W., Schober, F. and Mannel, M.: Effects of a Methanolic Extract and a Hyperforin-Enriched CO_2 Extract of *St. John's Wort* (*Hypericum perforatum*) on Intracerebral Field Potentials in the Freely Moving Rat (Tele-Stereo-EEG). *Pharmacopsychiat.* **31** (suppl. 1): 30-35 (1998)

Erdelmeier, C.A.J.: Hyperforin, Possible the Major Non-Nitrogenous Secondary Metabolite of *Hypericum perforatum* L. *Pharmacopsychiat.* **31** (suppl. 1): 2-6 (1998)

European Pharmacopoeia (Ph. Eur. III, suppl. 2000), Hypericum Monograph, Strasbourg, Cedex, Council of Europe

Fuzzati, N., Gabetta, F., Peterlongo, F. and Strepponi, I.: HPLC-TSP-MS Characterization of Hyperforin-Like Phloroglucinols from *Hypericum perforatum*. Poster at the Joint Meeting of ASP, AFERP, GA and PSE. Amsterdam, 1999. Abstract Book (abstract 590)

Hölzl, J. and Ostrowski, E.: Johanniskraut (*Hypericum perforatum* L.). HPLC-Analyse der wichtigen Inhaltsstoffe und deren Variabilität in einer Population. *Dtsch. Apoth. Ztg.* **127**: 1227-1230 (1987)

Kerb, R., Brockmöller, J., Staffeldt, B., Ploch, M. and Roots, I.: Single-Dose and Steady-State Pharmacokinetics of Hypericin and Pseudohypericin. *Antimicrob. Agents Chemother.* **40**: 2087-2093 (1996)

Kurth, H. and Spreemann, R.: Phytochemical Characterization of Various St. John's Wort Extracts. *Advances in Therapy* **15**: 117-128 (1998)

Laakmann, G., Schüle, C., Baghai, T. and Kieser, M.: St. John's Wort in Mild to Moderate Depression: The Relevance of Hyperforin for the Clinical Efficacy. *Pharmacopsychiat.* **31** (suppl. 1): 54-59 (1998)

Maisenbacher, P.: Untersuchungen zur Analytik von Johanniskrautöl. Thesis. Fakultät für Chemie und Pharmazie. Eberhard-Karls-Universtität Tübingen, 1991

Maisenbacher, P. and Kovar, K.-A.: Analysis and Stability of Hyperici Oleum. *Planta Medica* **58**: 351-354 (1992)

Meier, B.: Vergleichbarkeit von Phytopharmaka am Beispiel von Johanniskraut. In: *Phytopharmaka V.* Loew, D., Blume, H. and Dingermann, T. (Ed.); Steinkopff: Darmstadt, 1999 pp 51-61

Melzer, M., Fuhrken, D. and Kolkmann, R.: Hyperforin im Johanniskraut. Hauptwirkstoff oder nur Leitsubstanz? *Dtsch. Apoth. Ztg.* **138**: 4754-4760 (1998)

Müller, W. E., Singer, A., Wonnemann, M., Hafner, U., Rolli, M. and Schäfer, C.: Hyperforin Represents the Neurotransmitter Reuptake Inhibiting Constituent of Hypericum Extract. *Pharmacopsychiat.* **31** (suppl. 1): 16-21 (1998)

Niesel, S.: Untersuchungen zum Freisetzungsverhalten und zur Stabilität ausgewählter wertbestimmender Pflanzeninhalsstoffe unter besonderer Berücksichtigung moderner phytochemischer Analysenverfahren. Thesis. Pharmazie. Freie Universität Berlin, 1992

Orth, H. C. J.: Isolierung, Stabilität und Stabilisierung von Hyperforin und Identifizierung seines Hauptabbauproduktes aus *Hypericum perforatum* L. Thesis. Chemie und Pharmazie. Eberhard-Karls-Universität Tübingen, 1999

Orth, H. C. J., Hauer, H., Erdelmeier, C. A. J. and Schmidt, P. C.: Orthoforin: The Main Degradation Product of Hyperforin from *Hypericum perforatum* L. *Pharmazie* **54**: 76-77 (1999a)

Orth, H. C. J., Rentel, C. and Schmidt, P. C.: Isolation, Purity Analysis and Stability of Hyperforin as a Standard Material from *Hypericum perforatum* L. *J. Pharm. Pharmacol.* **51**: 193-200 (1999b)

Ostrowski, E.: Untersuchungen zur Analytik, ^{14}C-Markierung und Pharmakokinetik phenolischer Inhaltsstoffe von *Hypericum perforatum* L. Thesis. Pharmazie und Lebensmittelchemie. Philipps-Universität Marburg, 1988

Roth, L.: *Hypericum*, Hypericin: Botanik, Inhaltsstoffe, Wirkung. Landsberg, Lech: Ecomed, 1990

Schellenberg, R., Sauer, S. and Dimpfel, W.: Pharmacodynamic Effects of two Different Hypericum Extracts in Healthy Volunteers Measured by Quantitative EEG. *Pharmacopsychiat.* **31** (suppl. 1): 44-53 (1998)

Schrader, E.: Equivalence of St John's Wort Extract Ze117 and Fluoxetine: A Randomised, Controlled Study in Mild-Moderate Depression. *International Clinical Psychopharmacology*, in press

Schrader, E., Meier, B. and Brattström, A.: Hypericum Treatment of Mild-Moderate Depression in a Placebo-Controlled Study. A Prospective, Double-Blind, Randomized, Placebo-Controlled Multicentre Study. *Human Psychopharmacology* **13**: 163-169 (1998)

Tateo, F., Martello, S., Lubian, E. and Bononi, M.: Hypericin and Hypericin-Like Substances: Analytical Problems. In: *Food Flavors: Formation, Analysis and Packaging Influences. Developments in Food Science 40.* Contis, E. T., Ho, C.-T., Mussinan, C. J., Parliment, T. H., Shahidi, F. and Spanier, A. M. (Ed.); Elsevier: Amsterdam, 1998, pp 143-157

Trifunovic, S., Vajs, V., Macura, S., Juranic, N., Djarmati, Z., Jankov, R. and Milosavljevic, S.: Oxidation Products of Hyperforin from *Hypericum perforatum*. *Phytochemistry* **49**: 1305-1310 (1998)

Verotta, L., Appendino, G., Belloro, E., Jakupovic, J. and Bombardelli, E.: Furohyperforin, a Prenylated Phloroglucionol from St. John's Wort (*Hypericum perforatum*). *J. Nat. Prod.* **62**: 770-772 (1999)

Wagner, H. and Bladt, S.: Pharmazeutische Qualität der Hypericum-Extrakte. *Nervenheilkunde* **12**: 362-366 (1993)

6 Isolation and CRH-1 Binding Affinity of Bisanthraquinone Glycosides of *Hypericum perforatum*

6.1 Introduction

Hypericum perforatum is widely used for the treatment of mild depression. The pharmacological activity of its extracts has been established in several extensive clinical studies (for references see Linde and Mulrow, 1999; Volz, 1997; Linde *et al.*, 1996), but still there are questions about the active principle(s) and the mode of action. It has been assumed that the antidepressant effect results from a synergism of several active constituents as hypericin, pseudohypericin, amentoflavone, procyanidins, hyperforin and others (Simmen *et al.*, 1999; Butterweck, 1997; Baureithel *et al.*, 1997). On the search for further potentially active compounds, the four new bisanthraquinone glycosides **1** to **4** were isolated from an ethanol-water dry extract (1:1, m/m, Ze117, Zeller Company). The aglycone of the four glycosides is skyrin. It has been found in various fungi in pure culture (e.g. *Penicillium islandicum* and *P. rugulosum)*, in fruit bodies of *Cortinarius atrovirens* and *Dermocybe austroveneta*, in lichens (e.g. *Acroscyphus sphaerophoroides*) and in the insect *Pseudococcus albizziae* (Thomson, 1971, 1987 and 1997). Its occurrence in *Hypericum perforatum* has been assumed (Berghöfer, 1987).

The presence of both skyrin and hypericin in *Hypericum perforatum* is not surprising since their biosynthesis was proposed to follow a similar pathway (Gill and Giménez, 1991).
The biosynthesis of skyrin and hypericin follows the acetate/malonate pathway starting with the assemblance of an octaketide chain (Figure 6.1A). Condensation, followed by decarboxylation, leads to atrochrysone (3,4-dihydro-3,6,8,9-tetrahydroxyanthracen-1-on). This is the key compound of the anthraquinone biosynthesis. Oxidation of atrochrysone provides a radical existing in several mesomeric structures.
Dimeric pre-anthraquinones result from the coupling of two dihydroanthracenone units. Depending on the position of coupling, the linkage is formed between 5,5'-, 7,7'-, 5,10'- 10,10'- or 8-O,10'. Further enzymatic and chemical modification can follow (Billen *et al.*, 1988; Jägers, 1980; Gill and Steglich, 1987; Gill and Giménez, 1991; Steglich and Oertel, 1984).

Atrovirin, a 5,5'-linked dimeric pre-anthraquinone, is transformed into austrovenetin by loss of water (Figure 6.1B). Austrovenetin is proposed to be the precursor of both skyrin and hypericin. Penicillopsin is an intermediate on the pathway leading to hypericin, which is formed by water elimination and isomerisation from austrovenetin. It is converted to protohypericin (Figure 6.1C) on oxidation and then on exposure to light, to hypericin (Cameron *et al.*, 1976; Gill and Giménez, 1991).

It has been shown that extracts of Hyperici herba contain various substances, which reveal different mechanisms of antidepressant action. It can be assumed that the efficiency of each substance on its own is moderate, but taken all together, they reveal an antidepressant effect, which is comparable to synthetic antidepressants. This also explains the lower incidence of undesirable side effects.
Information about the mechanisms probably involved in the therapeutic effects of *Hypericum perforatum* were provided by experimental pharmacology. Modes of action, which were found to play a role, were inhibition of serotonin, norepinephrine and dopamine reuptake, down-regulation of beta-adrenergic receptors, upregulation of serotonin receptors, dopaminergic activation and reduced cytokine expression. The inhibition of monoamine oxidase (MAO) seems to be less significant (Meier, 1999). Furthermore, extracts of *Hypericum perforatum* showed considerable binding affinity in receptor-binding-studies at the receptors gamma aminobutyric acid $(GABA)_A$, $GABA_B$, benzodiazepine, adenosine and inositol triphosphate (Cott, 1997). Effects on estrogen, μ-, kappa- and delta-opioid receptors have been reported (Simmen *et al.*, 1999).

As aberrations in the hypothalamic-pituitary-adrenocortical system (hypersecretion of ACTH and cortisol) have also been associated with depression (Reul *et al.*, 1993), compound **1** and **2** were tested for their potency to inhibit the binding to corticotropin releasing hormone-1 (CRH-1) receptors in this study.

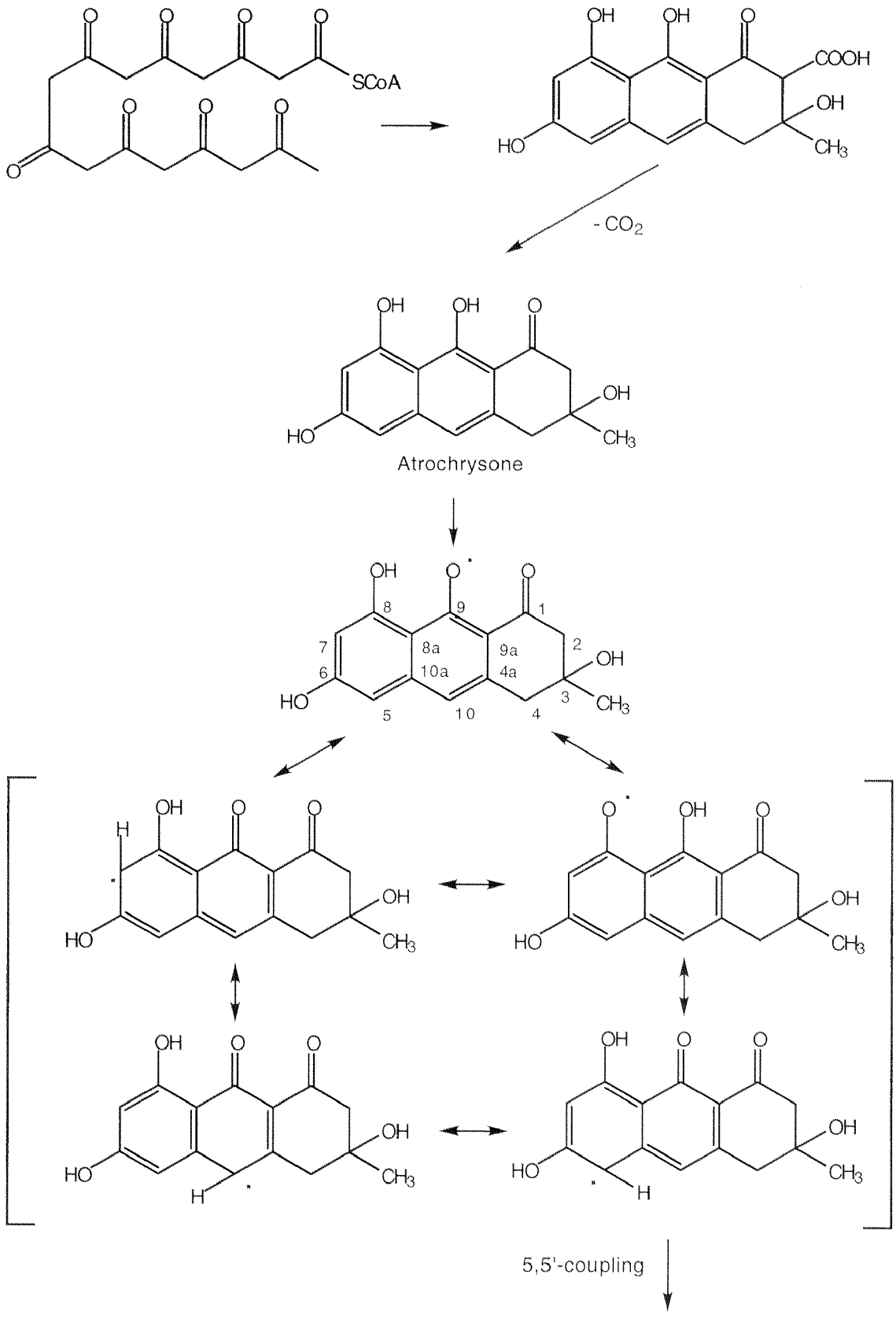


Figure 6.1A Biosynthesis of skyrin and hypericin. Part I.

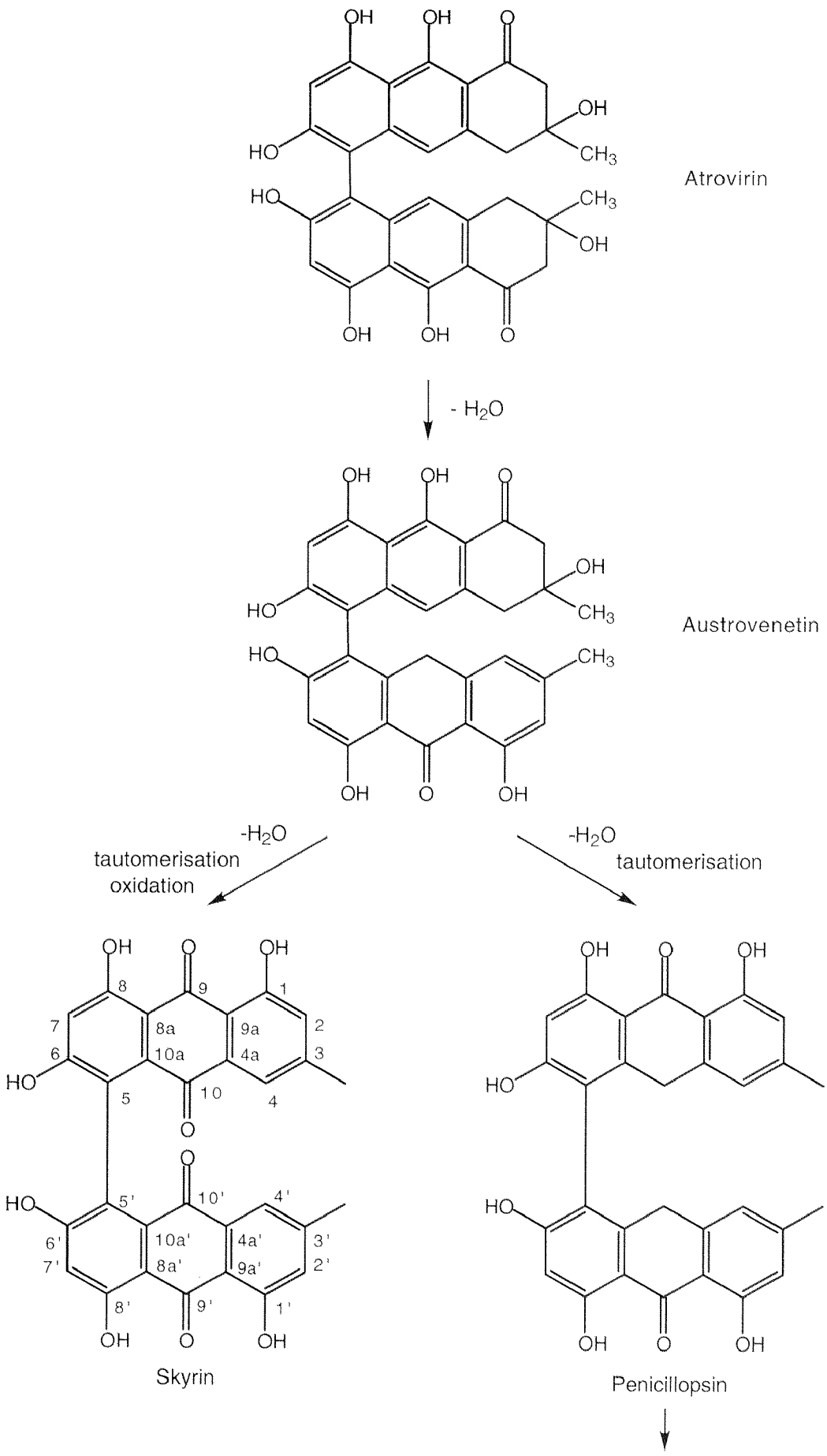


Figure 6.1B Biosynthesis of skyrin and hypericin. Part II.

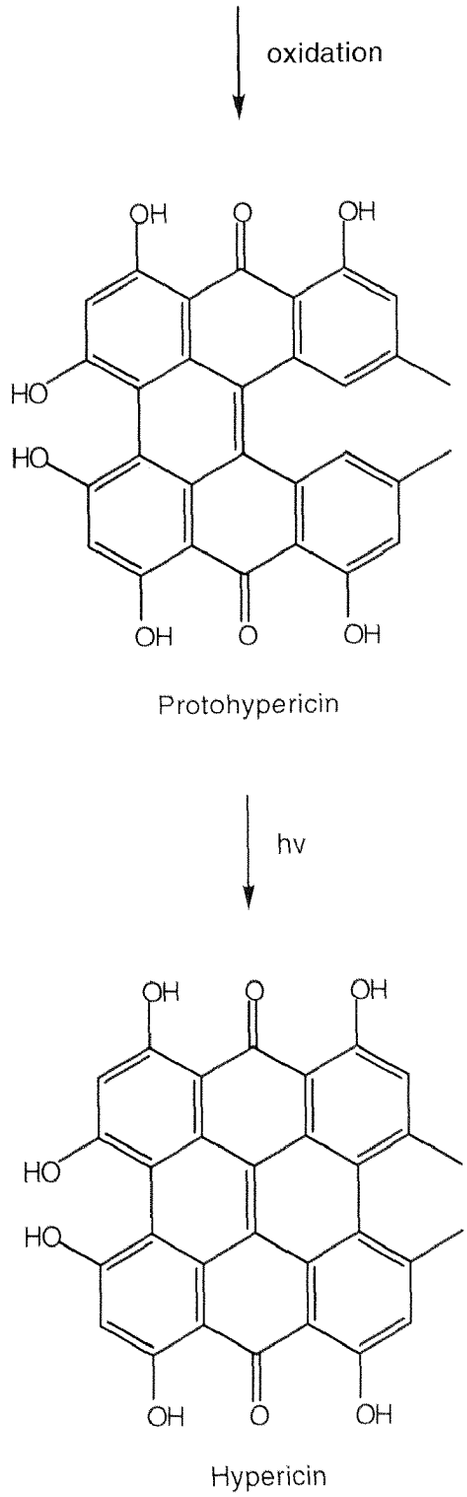


Figure 6.1C Biosynthesis of skyrin and hypericin. Part III.

6.2 Experimental

6.2.3 Isolation and Structural Elucidation

Materials

The source material was an ethanol-water (1:1, m/m) dry extract (Ze117 from Zeller Company, CH-Romanshorn) of Hyperici herba.
Ethanol absolute, hexane, ethyl acetate, methanol, acetonitrile, tetrahydrofuran, chloroform and acetone were of HPLC quality (Romil Chemicals, GB-Shepshed). Toluene (p.a.) was purchased from Scharlau (EGT Chemie, CH-Tägerig). Formic acid (p.a.), acetic anhydride (p.a.), acetic acid glacial 100% (p.a.) and potassium bromide for spectroscopy were from Merck (CH-Dietikon). Trifluoroacetic acid (purum, ≥98%), pyridine (p.a.), benzol (p.a.) and magnesium acetate (purum) were obtained from Fluka (CH-Buchs). Ammonia 25% (purum) was from Hänseler (CH-Herisau). De-ionized water was obtained using an NANO-pure cartridge system (Skan, CH-Basel). The NMR solvents methanol-d_4 and chloroform-d_1 (isotopic purity: >99.5%) were purchased from Dr. Glaser AG (CH-Basel). Regenerated cellulose syringe filters (0.2 µm, 13 mm) were from Schleicher&Schuell (D-Dassel). Sep-Pak® tC18 solid phase extraction columns (3 ml, 500 mg) were bought from Waters (USA-Milford, MA). RP material (C-Gel C 18 HL, particle size 0.04-0.063 mm) was obtained from Chemie Uetikon AG (CH-Uetikon). Silica gel 60 A C.C (particle size 35-70 µm) was from SDS (F-Peypin). RP-18 F_{254}s pre-coated plates (layer thickness 0.25 mm) and silica gel 60 F_{254} pre-coated aluminium sheets (layer thickness 0.2 mm) were obtained from Merck (CH-Dietikon).

Instrumentation and Methods

NMR spectroscopy

NMR spectra were recorded on Bruker AMX-300 (operating at 300.13 MHz for ^1H and 75.47 MHz for ^{13}C), Bruker AMX-500 (operating at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C) and Bruker AMX-600 spectrometers (operating at 600.13 MHz for ^1H and 150.92 MHz for ^{13}C) (Spectrospin, CH-Fällanden). The skyrin glycosides were measured in methanol-d_4, their acetylated derivatives in chloroform-d_1. All spectra were referenced to residual hydrogen or carbon resonances of the respective solvents.

MS spectrometry

ESIMS spectra were measured on a Finnigan TSQ 7000 mass spectrometer in the negative-ion mode. FAB mass spectra were obtained on a ZAB 2-SEQ (VG) spectrometer at 8.3 keV either in the positive-ion mode or in the negative-ion mode, using 3-NOBA (3-nitrobenzyl alcohol) as a matrix.

Infrared spectroscopy

IR spectra were recorded from **1** and **2** on a Perkin-Elmer system 2000 FT infrared spectrophotometer (CH-Rotkreuz) using KBr pellets (1 mg compound/300 mg KBr).

Ultraviolet spectroscopy

UV/VIS spectra were measured in methanol from 200-500 nm on a Kontron-Uvikon 930 spectrophotometer (Kontron Instruments, CH-Zürich). The concentrations of the solutes were 14.8 µg/ml (**1**), 16.2 µg/ml (**2**), 17.2 µg/ml (**3**) and 16.2 µg/ml (**4**).

Circular dichroism

CD spectra were recorded in methanol from 200-400 nm on a Jasco J-710/720 spectropolarimeter (Jasco Corporation, J-Tokyo) at 23 °C using a 0.1 cm cell. Measurements were performed with 14.8 µg/ml of **1**, 16.2 µg/ml of **2**, 17.2 µg/ml of **3** and 16.2 µg/ml of **4**. Molecular circular dichroisms, $[\Delta\varepsilon]$, are reported of the most important extrema in deg · cm^2 · $decimole^{-1}$.

Isolation procedure

The isolation procedure is summarized in Figure 6.2 as a flow chart.
20 g dry extract of Hyperici herba were partitioned in the solvent system hexane - toluene - water - ethyl acetate - formic acid (75:225:135:120:15, v/v). The lower level was shaken once more with a fresh upper level. The upper levels were combined, extracted with a fresh lower level, washed three times with 200 ml water and evaporated

below 35 °C under reduced pressure. The residue of the upper levels was dissolved in 20 ml methanol and stored at 8 °C. After 2 hours the formed precipitate was removed by filtration. Filtrate A was evaporated under vacuum to yield 320.0 mg residue. The procedure was repeated several times. 4.32 g of the collected residues were dissolved in 50 ml acetonitrile - water - trifluoroacetic acid (5:4.9:0.1, v/v/v) and stored at 8 °C for two hours. Again, the precipitate (quercetin) was removed by filtration. The residue of filtrate B was dissolved in 10 ml acetonitrile - water - trifluoroacetic acid (5:4.9:0.1, v/v/v) and applied to RP-18 VLC employing the same eluent. The fraction size was between 50 and 100 ml. After TLC (RP-18) control with the eluent acetonitrile - water - trifluoroacetic acid (5:4.9:0.1, v/v/v), the fractions containing **1** and **2** (C2: 340-500 ml) and the fractions enriched with **3** and **4** (C4: 615-815 ml) were combined. Evaporation of the solvent left 382.3 mg of C2 and 111.0 mg of C4. The samples were dissolved in ethanol absolute, filtered throughout regenerated cellulose syringe filters and partly subjected to TLC (silica gel) over a distance of approximately 15 cm employing ethyl acetate - acetonitrile - water (10:1:1, v/v/v) as eluent. The solutions were sprayed onto the plates using a Linomat TLC application device. The Rf values of the compounds were 0.20 (**1**), 0.31 (**2**), 0.38 (**3**) and 0.51 (**4**). The relevant bands were cut out and macerated twice with ethanol absolute for 20 min. After filtration over a sintered glass filter (porosity number 4) the ethanol was evaporated under reduced pressure below 30 °C. This led to the isolation of **1** (18.1 mg), **2** (29.8 mg), **3** (3.4 mg) and **4** (7.3 mg).

*Acetylation of compound **1***

3.2 mg of **1**, anhydrous pyridine (0.5 ml) and acetic anhydride (0.5 ml) were kept in the dark at room temperature for 18 hours. The reaction mixture was diluted with 2 ml water and stored at 8 °C for 1 hour. It was then applied to a Sep-Pak® tC18 cartridge, which had been washed with 10 ml of methanol and pre-conditioned with 10 ml water. Pyridine, acetic anhydride and not acetylated **1** were eluted with water, followed by the elution of the acetylated compound with chloroform. Evaporation of chloroform under reduced pressure below 30 °C left 3.1 mg of 1,1',8,8',2",3",4",6"-octaacetyl-S-(+)-skyrin-6-*O*-β-glucopyranoside (**1a**).

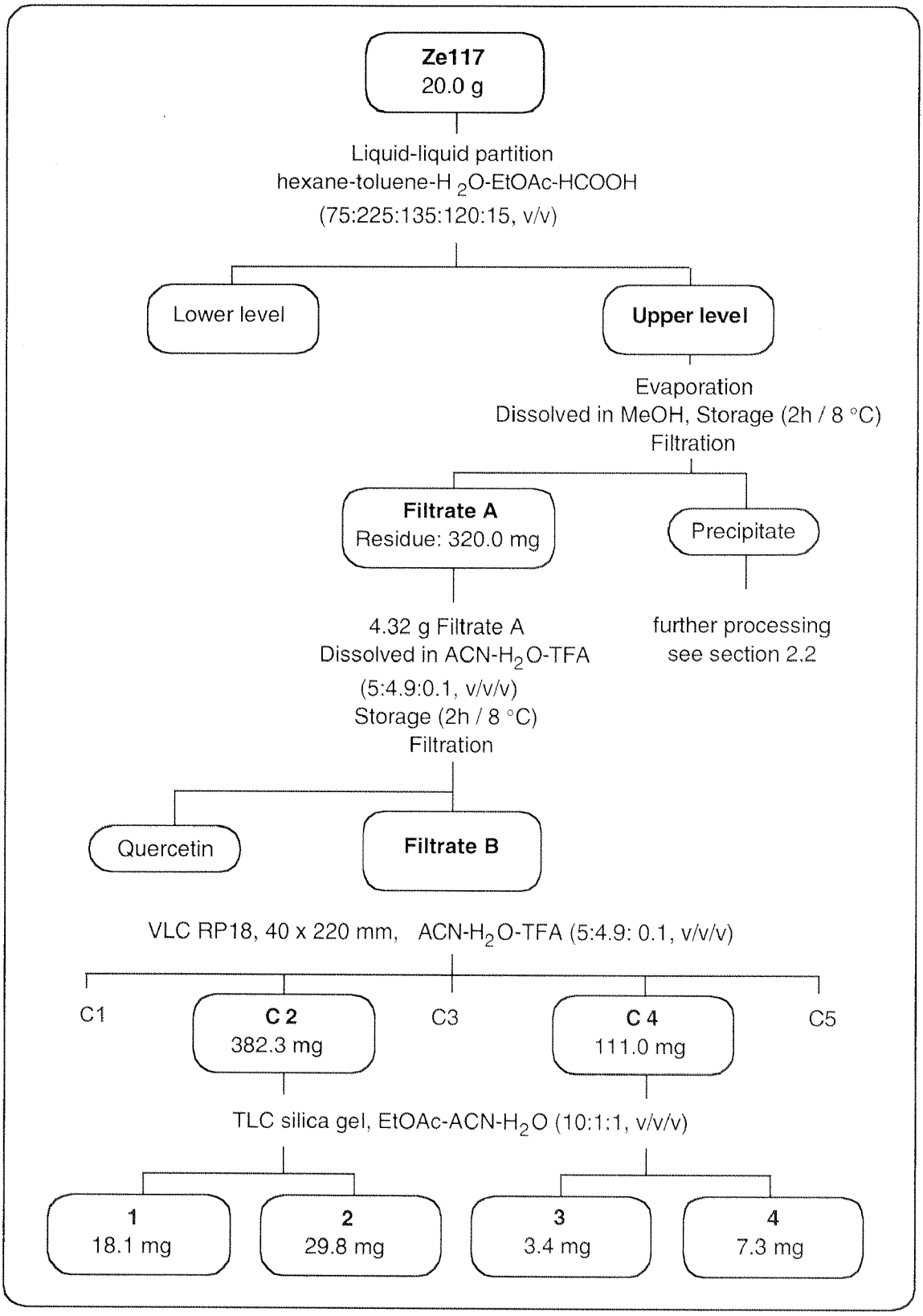


Figure 6.2 Isolation procedure of the bisanthraquinone glycosides **1-4**

*Acetylation of compound **2***

6.5 mg of **2** was treated as described above. Examination of the residue by TLC on silica gel with chloroform - methanol (60:3) showed several reaction products with different acetylation degrees. Consequently, the residue was subjected to the acetylation procedure again. The reaction time was elongated to 64 hours to give 9.38 mg of **2a**, which was still not pure. For further purification, the product was chromatographed on a silica gel column (320 x 10 mm I.D.) with benzol - acetone (9:1) as eluent. This led to the isolation of 3.5 mg pure 1,1',8,8',2",3",4",6"-octaacetyl-R-(-)-skyrin-6-*O*-β-glucopyranoside (**2a**).

*Hydrolysis of **1-3** on a TLC plate*

To confirm the sugar moiety of **1-3**, the compounds were hydrolyzed on a TLC plate (Kartnig and Wegschaider, 1971). Methanolic solutions of **1-3** were applied on a silica gel plate together with the reference substances glucose and xylose. The TLC plate was put into a double TLC chamber saturated with hydrochloric acid gas for 10 min at 100 °C. The plate was cooled down for 30 min in the chamber, dried for 60 min at room temperature afterwards and then 20 min at 80 °C. The plate was developed in ethyl acetate - methanol - acetic acid - water (60:15:15:10) over 8 cm. Detection was done with 0.5 g thymol and 5 ml sulfuric acid in 95 ml ethanol. After spraying, the plate was heated for 10 min at 120 °C.

6.2.4 Semiquantitative Determination of 1 and 2

Materials and Instrumentation

The samples analyzed were Hyperici herba (lot 50279L (1995)) and Hyperici herba Ph. Helv. VIII (lot: 50279L (1998)) from Dixa (CH-St. Gallen), Hyperici herba cum flores DAC 86 (lot: 1998110819 (1998)) from Hänseler (CH-Herisau) and Hyperici herba (lot: HPS2-S) from Vita Plant (CH-Witterswil). HPLC analyses were performed using a Hewlett Packard instrument (Model 79994A analytical workstation, model 1090 liquid chromatograph, model 1040 diode-array detector). For chromatographic separations a Knauer (D-Berlin) prepacked cartridge

column (250 x 4 mm I.D., 5 µm) filled with Spherisorb S ODS2 and a guard column (10 x 4 mm I.D.) of the same material was used.

Chromatographic Conditions

The method developed by Hölzl and Ostrowski (1987) was slightly modified. The mobile phase consisted of solvent A (methanol), B (acetonitrile - water - *ortho*-phosphoric acid 85% (19:80:1, v/v)) and C (acetonitrile - methanol - *ortho*-phosphoric acid 85% (59:40:1, v/v)). The elution profile was: 0-8 min 100% B, 8-30 min 100% to 50% B in C, 30-45 min 50% B in C to 100% C, 45-70 min 100% C, 70-75 min 100% A (wash out), 75-85 min 100% B (re-conditioning). All gradient steps were linear.
The flow rate was 0.6 ml/min, the column temperature 25 °C and the injection volume 25 µl. The detection wavelength of the DAD was set at 450 nm.

Extraction

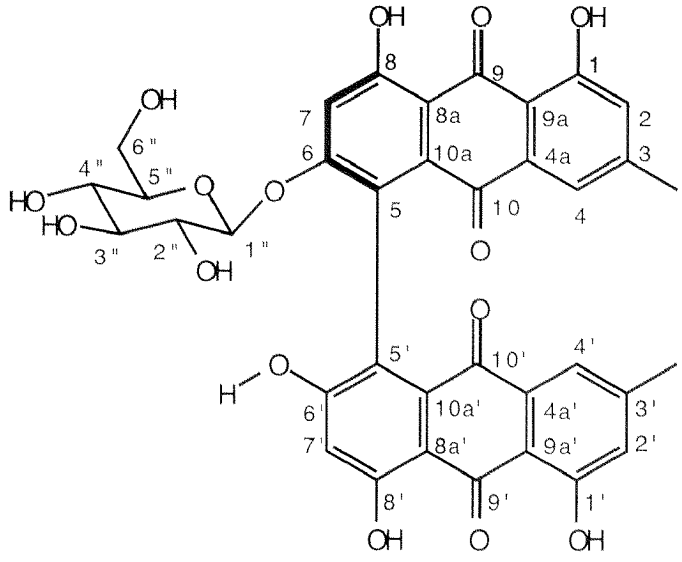
1.00 g dried and pulverized plant material (mesh 1 mm) was extracted with 130 ml methanol in a 30 ml Soxhlet for 4 hours. The extract was concentrated under reduced pressure to 3 ml, transferred into a 10 ml volumetric flask and filtered through a regenerated cellulose syringe filter. The solution was directly used for HPLC analysis.

Calibration Curve

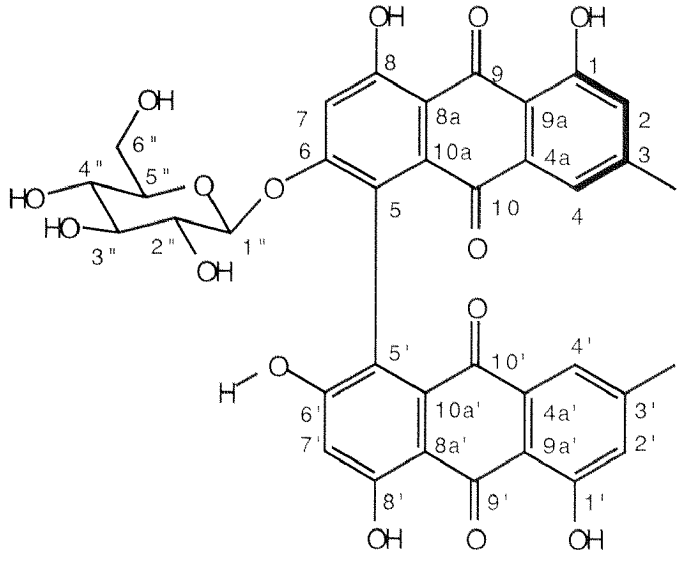
Five standard solutions of **2** in methanol were prepared with concentrations from 1 µg/ml to 14 µg/ml. The calibration graphs were generated by a least squares regression method. The calculations were based on area counts.

6.2.5 Corticotropin releasing hormone-1 (CRH-1) Receptor Binding Tests

Receptor binding tests were done according to Gottowik *et al.* (1997).

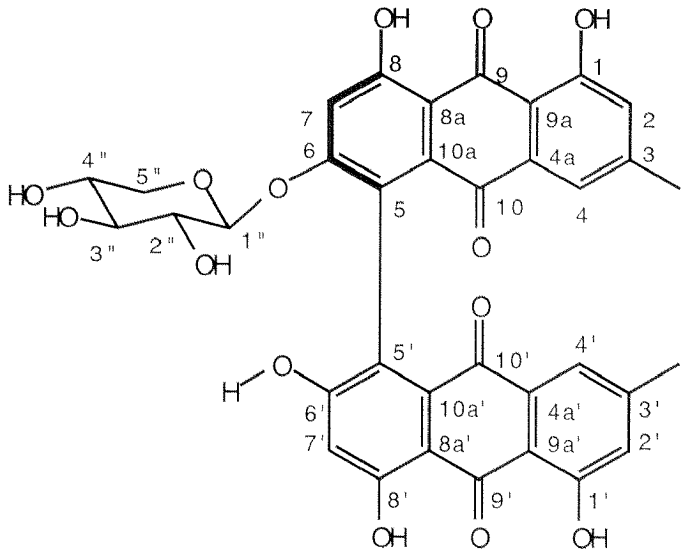


S-(+)-skyrin-6-*O*-β-glucopyranoside (**1**)

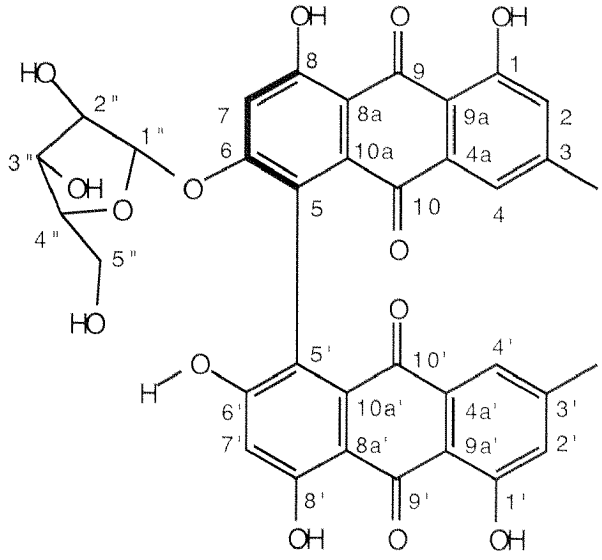


R-(-)-skyrin-6-*O*-β-glucopyranoside (**2**)

Figure 6.3A Formula of isolated compounds **1** and **2**



S-(+)-skyrin-6-*O*-β-xylopyranoside (**3**)



S-(+)-skyrin-6-*O*-α-arabinofuranoside (**4**)

Figure 6.3B Formula of isolated compounds **3** and **4**

6.3 Results and Discussion

NMR spectra important for the structural elucidation are displayed at the end of this section (Figures 6.5 to 6.21).

S-(+)-skyrin-6-*O*-β-glucopyranoside (**1**) was isolated as orange-red powder. The ^{1}H NMR of **1** (Table 6.1; Figure 6.5) revealed signals for 6 aromatic protons resonating as singlets at δ 7.32, 7.16, 7.06, 7.05, 6.95 and 6.28. It also showed the presence of a hexose and two signals for aromatic methyl protons at δ 2.30 and 2.35 (each 3H). The anomeric proton of the hexose was observed as a doublet at δ 5.06. The coupling constant of 7.5 Hz indicated the linkage to be β. The location of the biaryl bond followed from the absence of a signal arising from H-5 and H-5'.
The downfield shift of H-7 (δ 7.06) relative to H-7' (δ 6.28) could be explained by the deshielding influence of the sugar moiety (Banks *et al.*, 1976). However H-7' would be expected to resonate at lower field due to meta arylation (Antonowitz *et al.*, 1994). The H-7 shift in emodin, the monomeric counterpart of skyrin, is at δ 6.57 (Danielsen *et al.*, 1992). Compared to that value, H-7' in **1** was shifted to higher ($\Delta\delta$ -0.29) instead to lower field. H-7' (δ 6.84) of the skyrin aglycone showed the expected downfield shift (Sedmera *et al.*, 1978). It could be rationalized as an effect of intramolecular hydrogen bonding between the hydroxyl group at C-6' and the sugar moiety. This assumption is consistent with the significant downfield shift of the C-6' carbon signal in the ^{13}C NMR (δ 179.0) and its shift after acetylation (δ 157.8) resulting from rupture of the hydrogen bond. This is a strong hint for the binding site of the sugar to be OH-6; otherwise the shift value of C-6' could not be explained.
The ^{13}C NMR spectrum (Table 6.4; Figure 6.6) of **1** revealed 36 discrete signals that could be assigned to individual carbon atoms based on two-dimensional NMR techniques (HMQC and HMBC). The carbonyl carbons C-9 and C-10 resonated at δ 192.3 and δ 184.0, respectively. The significant downfield shift of C-9 results from strong intramolecular hydrogen bonding between OH-1 or OH-8 and the carbonyl oxygen. This was confirmed by the upfield shift after acetylation (δ 179.9). The carbonyl group of C-10 resonated at higher field due to the absence of a hydroxyl group in the near neighborhood (Höfle, 1977; Toma *et al.*, 1975). This finding was consistent with the IR spectrum showing absorptions at 1672 and 1625 cm^{-1} typical of free and chelated carbonyl groups, respectively, of an anthraquinone (Gill and Giménez, 1995).

Table 6.1 ^{1}H NMR spectral data of **1** and **2** (500.13 MHz; CD_3OD-d_4)

H	1		2	
	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)
2	7.05 *s*		7.05 *s*	
4	7.32 *s*		7.35 *s*	
7	7.06 *s*		7.09 *s*	
Me-3	2.35 *s*		2.36 *s*	
2'	6.95 *s*		6.93 *s*	
4'	7.16 *s*		7.21 *s*	
7'	6.28 *s*		6.25 *s*	
Me-3'	2.30 *s*		2.30 *s*	
1"	5.06 *d*	7.5	4.85 *d*	7.8
2"	3.16 *dd*	7.5,9.2	3.14 *dd*	7.9,8.9
3"	3.40 *dd*	9.2,9.2	3.28 *dd*	9.2,9.1
4"	3.24 *dd*	9.4,9.4	3.23 *dd*	9.3,9.2
5"	3.47 *ddd*	2.3,6.0,9.6	3.39 *ddd*	2.3,6.1,9.1
6A"	3.83 *dd*	2.3,12.1	3.84 *dd*	2.3,12.2
6B"	3.62 *dd*	5.8,12.5	3.63 *dd*	6.1,12.2

Table 6.2 ^{1}H NMR spectral data of **3** and **4** (600.13 MHz for **3**; 500.13 MHz for **4**; CD_3OD-d_4)

H	3		4	
	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)
2	7.03 *s*		7.04 *s*	
4	7.31 *s*		7.34 *s*	
7	6.99 *s*		7.01 *s*	
Me-3	2.34 *s*		2.35 *s*	
2'	6.94 *s*		6.93 *s*	
4'	7.17 *s*		7.20 *s*	
7'	6.25 s		6.23 *s*	
Me-3'	2.23 *s*		2.30 *s*	
1"	5.03 *d*	6.8	5.53 *d*	1.1
2"	3.17 *dd*	6.8,8.6	3.91 *dd*	1.5,4.1
3"	3.37-3.33 *m*		3.78 *dd*	4.1,6.7
4"	3.37-3.33 *m*		3.70 *m*	2.9,4.6,5.9
5A"	3.77 *dd*	2.9,9.9	3.67 *dd*	2.9,13.7
5B"	3.37-3.33 *m*		3.52 *dd*	4.7,12.2

Table 6.3 ^{1}H NMR spectral data of **1a** and **2a** (500.13 MHz, $CDCl_3$-d_1) and published data of hexaacetylskyrin ($CDCl_3$-d_1)

H	1a		2a		Takeda *et al.* (1973)	Huot, Brassard (1972)
	δ (ppm)	*J* (Hz)	δ (ppm)	*J* (Hz)	δ (ppm)	δ (ppm)
2	7.15 *s*		7.14 *s*		7.15	7.20
4	7.67 *s*		7.69 *s*		7.69	7.73
7	7.20 *s*		7.15 *s*		7.38	7.42
Me-3	2.40 *s*		2.37[a] *s*		2.42	2.38
2'	7.14 *s*		7.13 *s*		7.15	7.20
4'	7.65 *s*		7.64 *s*		7.69	7.69
7'	6.90 *s*		6.99 *s*		7.38	7.38
Me-3'	2.40 *s*		2.36[a] *s*		2.42	2.42
1"	5.14 *d*	7.6	5.32 *d*	6.0		
2"	4.89 *dd*	7.7,8.5	4.88 *dd*	6.1,6.5		
3"	5.17 *dd*	9.3,9.3	5.09 *dd*	7.0,9.1		
4"	4.98 *dd*	9.5,9.7	5.21 *dd*	9.3,9.6		
5"	3.88 *ddd*	2.9,5.7,9.9	3.92 *ddd*	2.6,5.1,10.1		
6A"; 6B"	4.14 *m*		4.19; 4.12 each *dd*	2.5,12.2; 5.1,12.4		
$COCH_3$-1	2.45 *s*		2.45 *s*		2.47	2.44
$COCH_3$-1'	2.45 *s*		2.44 *s*		2.47	2.44
$COCH_3$-8	2.50[a] *s*		2.49[b] *s*		2.50	2.46
$COCH_3$-8'	2.48[a] *s*		2.48[b] *s*		2.50	2.46
$COCH_3$-6					1.93	1.87
$COCH_3$-6'					1.93	1.87
$COCH_3$-2"	1.94 *s*		1.59 *s*			
$COCH_3$-3"	1.91 *s*		1.90 *s*			
$COCH_3$-4"	2.00 *s*		2.00 *s*			
$COCH_3$-6"	2.11 *s*		1.97 *s*			

[a,b] Assignments in the same column bearing the same superscript may be interchanged

The assignment of C-6, C-8, C-6' and C-8' involved some difficulties. The HMBC spectra of **1** in methanol-d_4 showed signals at δ 164.2 and δ166.2, which correlated both with H-7 (δ 7.06); the signal at δ 164.2 showed an additional correlation with the anomeric proton of the sugar H-1" (δ 5.06) (Figure 6.8). These data did not allow a definite assignment of C-6 and C-8. The signals at δ 179.0 and δ 168.1 showed both correlations with H-7' (δ 6.28) not making it clear which was C-6' and C-8'.

It was tried to get NMR spectra of **1** in DMSO-d_6 with sharp assignable OH-signals. However, there was only one sharp OH-signal (δ 12.45) which showed correlations with the signals of C-8a', C-7', C-9' and δ 166.6 (C-6' or C-8') in the HMBC spectrum, pointing the signal at δ 166.6 to be the resonance of OH-8'. Consequently, the signal resonating at δ 178.0 has to be C-6'.

The assignment of C-6 and C-8 has been done by comparison of the ^{1}H NMR data of the acetyl derivative of **1** (Table 6.3) with literature data of hexaacetylskyrin (Takeda *et al.*, 1973; Huot and Brassard, 1972). The acetylation of **1** was not complete; one OH-group of the skyrin backbone had not been acetylated. This finding was confirmed by the positive FABMS, which gave a $[M + 2H]^+$ peak at *m/z* 1038 being consistent with the molecular formula $C_{52}H_{44}O_{23}$. The literature data (Takeda *et al.*, 1973) showed signals at δ 2.50 and δ 2.47 for COC$\underline{H}_3$-8/8' and COC$\underline{H}_3$-1/1', respectively. Corresponding signals could be found in the ^{1}H NMR spectrum of **1a**: δ 2.50/δ 2.48 (3H each, *s*) and δ 2.45 (6H, *s*) (Figure 6.10). The resonances at δ 2.50/δ 2.48 were assigned to COC$\underline{H}_3$-8/8'. The HMBC experiment revealed correlations of COC$\underline{H}_3$-8/8' with the resonances at δ 152.0 and δ 151.95 (Figure 6.13); they were concluded to be the signals of C-8 and C-8'. δ 152.0 correlated with H-7 (δ 7.20) in the HMBC spectrum indicating to be the resonance of C-8. Correspondingly, δ 151.95 correlated with H-7' (δ 6.90). The protons of COC$\underline{H}_3$-6/6' showed resonances at δ 1.93 in the ^{1}H NMR spectrum described in literature (Takeda *et al.*, 1973). These signals were missing in the spectrum of **1a**. It was concluded that C-6' had not been acetylated. In the HMBC spectrum of **1a** C-6 (δ 158.5) revealed a correlation with the anomeric proton (δ 5.14, *d*, J = 7.6 Hz) confirming OH-6 to be the binding site of the sugar. The hydrogen bond between C-6' and the sugar moiety probably hindered the acetylation of OH-C-6'. Knowing now that OH-6 was the binding site of the sugar, the assignment could also be accomplished for the not acetylated compound. The signal at δ 164.2 correlating in the HMBC spectrum of **1** (Figure 6.8) with the anomeric proton of the sugar (δ 5.06) belonged to C-6.

Table 6.4 ^{13}C-NMR spectral data (δ ppm) of **1**, **2** (75.47 MHz, CD_3OD-d_4) and **3**, **4** (125.77 MHz, CD_3OD-d_4)

C	1		2	3	4
1	163.6	*s*	163.1	163.3	163.1
2	124.5	*d*	124.1	124.3	124.1
3	149.5	*s*	149.6	149.5	149.5
4	121.3	*d*	121.6	121.3	121.5
4a	135.7	*s*	135.7	135.8	135.8
5	129.0	*s*	130.4	129.5	130.2
6	164.2	*s*	164.4	164.1	164.0
7	108.9	*d*	110.3	109.0	108.6
8	166.2	*s*	165.8	166.1	165.9
8a	113.2	*s*	112.9	113.1	112.2
9	192.3	*s*	192.6	188.2	192.6
9a	115.0	*s*	114.8	115.0	114.9
10	184.0	*s*	183.6	183.8	183.8
10a	133.8[a]	*s*	133.0[a]	133.8[a]	133.1[a]
Me-3	22.0	*q*	22.1	22.0	22.0
1'	162.4	*s*	162.4	162.4	162.8
2'	124.0	*d*	123.7	123.9	123.9
3'	147.1	*s*	146.8	147.0	147.0
4'	120.6	*d*	120.8	120.5	120.6
4a'	135.4	*s*	135.5	135.5	135.5
5'	130.7	*s*	130.4	130.7	131.0
6'	179.0	*s*	178.1	179.2	178.7
7'	110.1	*d*	109.5	110.0	109.8
8'	168.1	*s*	168.1	168.2	168.2
8a'	106.4	*s*	106.4	106.2	106.2
9'	187.9	*s*	187.9	187.8	187.7
9a'	115.6	*s*	115.8	115.8	115.8
10'	186.3	*s*	186.1	186.4	186.2
10a'	132.3[a]	*s*	132.0[a]	132.3[a]	132.0[a]
Me-3'	21.9	*q*	21.9	21.8	21.9
1''	103.0	*d*	102.4	103.3	108.0
2''	75.1	*d*	74.3	74.4	83.4
3''	77.5	*d*	78.0	76.7	77.8
4''	70.8	*d*	71.1	70.5	86.2
5''	78.4	*d*	78.4	66.7	62.2
6''	62.4	*t*	62.6		

[a] Assignments in the same column bearing the same superscript may be interchanged

Table 6.5 ^{13}C-NMR spectral data (δ ppm) of **1a** and **2a** (125.77 MHz, $CDCl_3$-d_1)

C	1a		2a
1	149.6	*s*	149.6
2	130.2	*d*	130.88
3	146.2[a]	*s*	146.0[a]
4	126.1	*d*	126.1
4a	134.7[b]	*s*	134.8[b]
5	124.6	*s*	125.4
6	158.5	*s*	157.7
7	115.9	*d*	115.3
8	152.0	*s*	151.66[c]
8a	122.1	*s*	121.7
9	179.9	*s*	180.1
9a	122.7	*s*	122.7
10	182.4	*s*	182.4
10a	132.7[c]	*s*	132.7
Me-3	21.6	*q*	21.6
1'	149.7	*s*	149.7
2'	130.3	*d*	130.9
3'	145.4[a]	*s*	145.4[a]
4'	125.8	*d*	125.8
4a'	134.8[b]	*s*	134.7[b]
5'	121.9	*s*	122.9
6'	157.8	*s*	158.2
7'	116.6	*d*	116.9
8'	151.95	*s*	151.7[c]
8a'	120.8	*s*	120.6
9'	179.9	*s*	180.0
9a'	122.9	*s*	122.9
10'	183.0	*s*	183.2
10a'	134.2[c]	*s*	133.4
Me-3'	21.5	*q*	21.5
1"	98.4	*d*	96.6
2"	70.5	*d*	70.9
3"	72.3	*d*	72.5
4"	68.0	*d*	67.9
5"	72.3	*d*	72.1
6"	62.1	*t*	61.6

C	1a		2a
$\underline{C}OCH_3$-1	169.62	*s*	169.6
$\underline{C}OCH_3$-1'	169.55	*s*	169.6
$\underline{C}OCH_3$-8	170.1[d]	*s*	169.2[d]
$\underline{C}OCH_3$-8'	169.0[d]	*s*	168.9[d]
$\underline{C}OCH_3$-2"	168.4	*s*	168.2
$\underline{C}OCH_3$-3"	170.2	*s*	170.1
$\underline{C}OCH_3$-4"	169.2	*s*	170.0
$\underline{C}OCH_3$-6"	170.5	*s*	170.9
$CO\underline{C}H_3$-1	21.23[e]	*q*	21.2[e]
$CO\underline{C}H_3$-1'	21.20[e]	*q*	21.2[e]
$CO\underline{C}H_3$-8	21.14[e]	*q*	21.2[e]
$CO\underline{C}H_3$-8'	21.11[e]	*q*	21.1[e]
$CO\underline{C}H_3$-2"	20.6[f]	*q*	19.8
$CO\underline{C}H_3$-3"	20.5[f]	*q*	20.5
$CO\underline{C}H_3$-4"	20.4[f]	*q*	20.7
$CO\underline{C}H_3$-6"	20.3[f]	*q*	20.4
$CO\underline{C}H_3$-6"	20.3[f]	*q*	20.4

[a-f] Assignments in the same column bearing the same superscript may be interchanged

Literature data (Lin *et al.*, 1990) showed that the acetylation of an OH-group leads to a larger upfield shift of the adjacent carbon atom than the acetylation of the sugar moiety. This can also be seen in our data: C-6 and C-8 showed acetylation shifts of $\Delta\delta$ -5.7 and $\Delta\delta$ -14.2, respectively.
Compared to literature data of skyrin (Gill *et al.*, 1988) the resonances of C-5 (δ 129.0) and C-8a (δ 113.2) were slightly shifted downfield. The shift differences were $\Delta\delta$ +5.4 for C-5 and $\Delta\delta$ +4.3 for C-8a caused probably by their ortho- (C-5) and para-position (C-8a) to the sugar substitution (Sakamoto *et al.*, 1982).
The assignment of the carbons 4a (δ 135.7) and 9a (δ 115.0) has been done by comparison with literature data of other anthraquinone derivatives (Toma *et al.*, 1975; Rawat *et al.*, 1989; Coskun *et al.*, 1990).
The sugar functionality was identified as a β-glucopyranose by the ^{1}H and ^{13}C NMR spectral data supported by DQF-COSY (Figure 6.9) and ROESY experiments as well as published data (De Tommasi *et al.*, 1993).
It was described that skyrin forms a methylated or ethylated compound (dialkylpseudoskyrin) with methanol or ethanol on long standing in the alcoholic solution (Howard and Raistrick, 1954; Shibata *et al.*, 1955; Tanaka, 1958). This reaction could not be observed; the NMR data showed no signals of dimethylpseudoskyrin. The finding is consistent with the fact that the hydroxyl groups OH-6 and OH-6', involved in the reaction, are not available in **1**, because OH-6 is bearing the sugar moiety and OH-6' is forming a strong hydrogen bond.
The negative FABMS of **1** showed the molecular peak at *m/z* 700, being consistent with the molecular formula $C_{36}H_{28}O_{15}$. The $[M - H + Na]^-$ occurs at *m/z* 722. Loss of the sugar moiety produces *m/z* 537 from which a water fragment is cleaved leading to the fragment *m/z* 519.

R-(-)-skyrin-6-*O*-β-glucopyranoside (**2**) was isolated as orange-red powder. **1** and **2** showed different Rf values (**1**: 0.20, **2**: 0.31) on TLC (silica gel) employing ethyl acetate - acetonitrile - water (10:1:1) as eluent, small deviations in their ^{1}H and ^{13}C NMR spectral data and completely opposite CD curves (Figure 6.4). The two dimers gave almost identical UV absorption maxima, **2** showing slightly higher ε values. Their IR and MS spectra revealed hardly any difference.

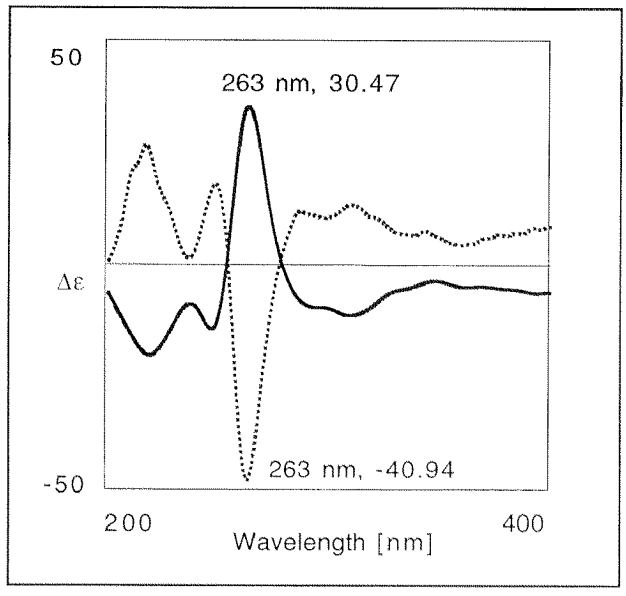


Figure 6.4 CD spectra of
——— (S)-(+)-skyrin-6-*O*-β-glucopyranoside (**1**) and
..... (R)-(-)-skyrin-6-*O*-β-glucopyranoside (**2**)

Consequently, **1** and **2** seem to differ in their axial stereochemistry. Skyrin has a chiral axis due to restricted rotation about the biaryl linkage. It exists in two atropisomeric forms, which show in the circular dichroism (CD) spectrum a strong Cotton effect around 255 nm (Koyama *et al.*, 1990; Takeda *et al.*, 1973). The CD of **1** and **2** showed antipodal split bands around 255 nm suggesting them to be atropisomers. The spectrum of **1** revealed strong positive first ([Δε] +30.47 (263 nm)) and negative second ([Δε] -10.12 (247 nm)) Cotton effects. On the other hand, the spectrum of **2** revealed strong negative first ([Δε] -40.94 (263 nm)) and positive second ([Δε] +12.90 (248 nm)) Cotton effects.

The absolute configuration was determined by comparison of the CD spectrum of **1** with that of (+)-skyrin (Jägers, 1980), to which S axial stereochemistry has been assigned by kinetic resolution studies (Billen *et al.*, 1988; Karl, 1988). The CD spectrum of **1** was similar to that of (+)-skyrin indicating S-configuration for **1** at the biaryl linkage. It has also been directly derived from the CD spectrum of (+)-skyrin that the two long axes of the anthraquinone chromophores are twisted in a clockwise manner, suggesting S-configuration of the compound (Koyama *et al.*, 1990). In **2**, the two long axes of the naphthalene chromophores are twisted in a counter-clockwise manner, contrary to that in **1**, indicating R-configuration of **2**.

There also exists a classification of the atropisomers into A- and B-type. Compounds that exhibit a negative first Cotton effect (that is the one to longer wavelength) and a positive second Cotton effect have been defined as A-type dimers. Type B dimers show Cotton effects with inverted signs (Steglich and Oertel, 1984). Consequently, **1** corresponded to the B-type and **2** to the A-type.
The NMR assignment of **2** has been done according to **1**. The ^{1}H and ^{13}C NMR spectral data of **1** and **2** revealed only small differences (Tables 6.1 and 6.4; Figures 6.14 and 6.15). The deviations (**2** - **1** ppm) in the ^{13}C NMR were biggest around the biaryl linkage at the carbons C-5 ($\Delta\delta$ 1.4), C-7 ($\Delta\delta$ 1.4), C-10a ($\Delta\delta$ -0.8), C-6' ($\Delta\delta$ -0.9) and C-7' ($\Delta\delta$ -0.6) and within the sugar moiety at the carbon 2" ($\Delta\delta$ -0.8) indicating different anisotropic effects in the two atropisomeric forms.
The molecular formula $C_{36}H_{28}O_{15}$ of **2** was consistent with the negative FABMS showing a $[M - H]^-$ peak at *m/z* 699. The $[M - H + Na]^-$ peak occurs at *m/z* 722. Loss of the sugar moiety produces *m/z* 537 from which a water fragment is cleaved leading to the fragment *m/z* 519. The ESIMS gave the $[M - H]^-$ ion peak at *m/z* 699.2. The positive FABMS of **2a** revealed a $[M + 2H]^+$ peak at *m/z* 1038 being consistent with the molecular formula $C_{52}H_{44}O_{23}$.
Hydrolysis of **1** and **2** on a TLC (silica gel) plate confirmed the finding that the sugar moiety of both compounds was glucose.

S-(+)-skyrin-6-*O*-β-xylopyranoside (**3**) was obtained as orange-red amorphous powder. The assignment of its NMR resonances has been done by comparison with NMR spectral data of **1** involving two-dimensional experiments (HMQC and HMBC).
The sugar moiety was determined as a β-xylopyranose by comparison of the ^{1}H and ^{13}C NMR spectral data (Tables 6.2 and 6.4; Figures 6.18 and 6.19) and ^{1}H-^{1}H spin coupling constants with published data (Fuchino *et al.*, 1995; Hirota *et al.*, 1990; Riccio *et al.*, 1986; Kusano *et al.*, 1995) and the interpretation of HMQC and HMBC spectra. COSY and off-resonance ROESY experiments confirmed the finding. As in the HMBC spectrum of **1** compound **3** displayed a long-range correlation between C-6 (δ 164.1) and the anomeric proton (δ 5.03, *d*, *J* = 6.8 Hz), revealing the site of glycosidation also to be 6-OH. The coupling constant of 6.8 Hz indicated the presence of a β-linkage. After hydrolysis on a TLC plate, the sugar moiety of **3** showed the same Rf value (0.51) and colour (blue) as xylose. The ESIMS was consistent with the molecular formula $C_{35}H_{36}O_{14}$ giving the $[M - H]^-$ ion peak at *m/z* 669. The CD spectrum of **3** revealed a B-type curve similar to the one of **1** indicating S chirality at the axis.

S-(+)-skyrin-6-*O*-α-arabinofuranoside (**4**) was isolated as orange-red powder. The assignment of the NMR spectral data was done in analogy to **3**. The sugar moiety was determined as α-arabinofuranose by ^{1}H and ^{13}C NMR spectral data (Tables 6.2 and 6.4; Figures 6.20 and 6.21) and ^{1}H-^{1}H spin coupling constants, being in agreement with reported data (Iorizzi *et al.*, 1996; D'Auria *et al.*, 1990). DQF-COSY and off-resonance ROESY spectra supported the result.
The HMBC spectrum displayed a long-range correlation between C-6 (δ 164.0) and the anomeric proton (δ 5.53, *d*, J = 1.1 Hz), revealing the site of glycosidation again to be 6-OH. The coupling constant of 1.1 Hz indicated the presence of a α-linkage. The ESIMS gave the $[M - H]^-$ ion peak at *m/z* 669.0 being consistent with the molecular formula $C_{35}H_{36}O_{14}$.
From the CD spectrum, it was derived that **4** has the same stereochemistry at the axis as **1** and **3**. In the ^{13}C NMR spectra of **1**, **3** and **4** the carbons of the sugar bearing moiety resonated with less relative intensity compared to the corresponding carbons in the other half of the molecule. For **2**, it was vice versa indicating that the stereochemistry influences the relaxation time.

To verify that **1**-**4** were not artefacts of the dry extract Ze117, the isolated compounds **1**-**4** and four commercial samples of Hyperici herba were examined by the HPLC method of Hölzl and Ostrowski, 1987. The retention times of **3** and **4** were 42.4 and 41.6 min, respectively. **1** and **2** coeluted at 40.5 min. These peaks could also be detected in the extract solutions of the dried plant material revealing the same UV/VIS spectrum. For comparison, the retention times of I3-II8-biapigenin, pseudohypericin and hypericin were 30.1, 43.9 and 57.8 min, respectively. The content of **1** and **2** was determined semiquantitatively using **2** as reference substance. The sum of **1** and **2** ranged from 0.0065% to 0.029% in the samples analyzed. The extract Ze117 contained 0.088% of the two substances. The content of **3** and **4** was even lower.

Aberrations in the hypothalamic-pituitary-adrenocortical system (hypersecretion of ACTH and cortisol) have been associated with depression (Reul *et al.*, 1993). Therefore, **1** and **2** were tested for their potency to inhibit the binding to CRH-1 (corticotropin releasing hormone) receptors. In our test system (according to Gottowik *et al.*, 1997) both compounds showed moderate activities with IC_{50} values of 1 and 4 µmol/l, respectively. IC_{50} values of hyperforin and hypericin were

10 and 6 µmol/l (unpublished data). So, **1**, **2** and skyrin derivatives in general may contribute to the antidepressant effect of *Hypericum perforatum* via the interaction with CRH-1 receptors. It has, however, to be taken into account that only small amounts of these compounds (0.0065% to 0.029%) are present in the dried plant.

Physical Data of the Isolates 1 to 4, 1a and 2a

*S-(+)-skyrin-6-O-β-glucopyranoside (**1**)*

Red-orange amorphous powder (18.1 mg), mp >300 °C, FABMS (negative) *m/z* 700 $[M]^-$, 722 $[M + Na - H]^-$, 537 $[M - C_6O_5H_{11}]^-$, 519 $[M - C_6O_5H_{11} - H_2O]^-$, ESIMS (negative) *m/z* 699 $[M - H]^-$, UV-Vis (MeOH) λ_{max} (logε): 219 (4.53), 258 (4.49), 298 (4.21), 455 (4.03) nm, CD (MeOH) λ_{max} ($\Delta\varepsilon$): 309 (-7.67), 263 (+30.47), 247 (-10.12), 219 (-14.85) nm, IR (KBr) ν_{max} 3396, 2967, 2926, 1672, 1625, 1603, 1552, 1487, 1453, 1385, 1361, 1272, 1244, 1194, 1134, 1118, 1072, 1039, 926, 913, 865, 849, 801, 784, 754, 657, 625, 584, 563 and 485 cm^{-1}, ^1H NMR spectral data see Table 6.1, ^{13}C NMR data see Table 6.4

*R-(-)-skyrin-6-O-β-glucopyranoside (**2**)*

Red-orange amorphous powder (29.8 mg), mp >300 °C, FABMS (negative) *m/z* 699 $[M - H]^-$, 722 $[M - H + Na]^-$, 537 $[M - C_6O_5H_{11}]^-$, 519 $[M - C_6O_5H_{11} - H_2O]^-$, ESIMS (negative) *m/z* 699 $[M - H]^-$, UV-Vis (MeOH) λ_{max} (logε): 222 (4.59), 258 (4.54), 296 (4.28), 455 (4.14) nm, CD (MeOH) λ_{max} ($\Delta\varepsilon$): 309 (+9.02), 263 (-40.94), 248 (+12.90) nm, IR (KBr) ν_{max} 3397, 2925, 1674, 1626, 1603, 1551, 1486, 1454, 1385, 1361, 1272, 1243, 1194, 1133, 1117, 1068, 1038, 925, 913, 865, 851, 831, 782, 753, 657, 626, 583, 561 and 484 cm^{-1}, ^1H NMR spectral data see Table 6.1, ^{13}C NMR data see Table 6.4

R-(-)-skyrin-6-O-β-xylopyranoside ***(3)***

Red-orange amorphous powder (3.4 mg), ESIMS (negative) *m/z* 669 [M - H]$^-$, UV-Vis (MeOH) λ_{max} (logε): 219 (4.41), 258 (4.37), 294 (4.10), 456 (3.87) nm, CD (MeOH) λ_{max} ($\Delta\varepsilon$): 310 (-5.42), 264 (+23.28), 251 (-7.25) nm, ^{1}H NMR spectral data see Table 6.2, ^{13}C NMR data in see Table 6.4, ^{13}C NMR spectral data of the β-xylopyranose moiety of **3** (pyridine-d_5): δ 105.2 (C-1"), 74.7 (C-2"), 77.7 (C-3"), 70.3 (C-4"), 67.4 (C-5")

R-(-)-skyrin-6-O-α-arabinofuranoside ***(4)***

Red-orange amorphous powder (7.3 mg), ESIMS (negative) *m/z* 669 [M - H]$^-$, UV-Vis (MeOH) λ_{max} (logε): 219 (4.44), 258 (4.39), 294 (4.15), 459 (3.92) nm, CD (MeOH) λ_{max} ($\Delta\varepsilon$): 310 (-7.82), 263 (+25.57), 250 (-7.71), ^{1}H NMR spectral data see Table 6.2, ^{13}C NMR data see Table 6.4

1,1',8,8',2",3",4",6"-octaacetyl-S-(+)-skyrin-6-O-β-glucopyranoside ***(1a)***

FABMS (positive) *m/z* 1038 [M + 2H]$^+$, ^{1}H NMR spectral data see Table 6.3, ^{13}C NMR data see Table 6.5

1,1',8,8',2",3",4",6"-octaacetyl-R-(-)-skyrin-6-O-β-glucopyranoside ***(2a)***

FABMS (positive) *m/z* 1038 [M + 2H]$^+$, ^{1}H NMR spectral data see Table 6.3, ^{13}C NMR data see Table 6.5

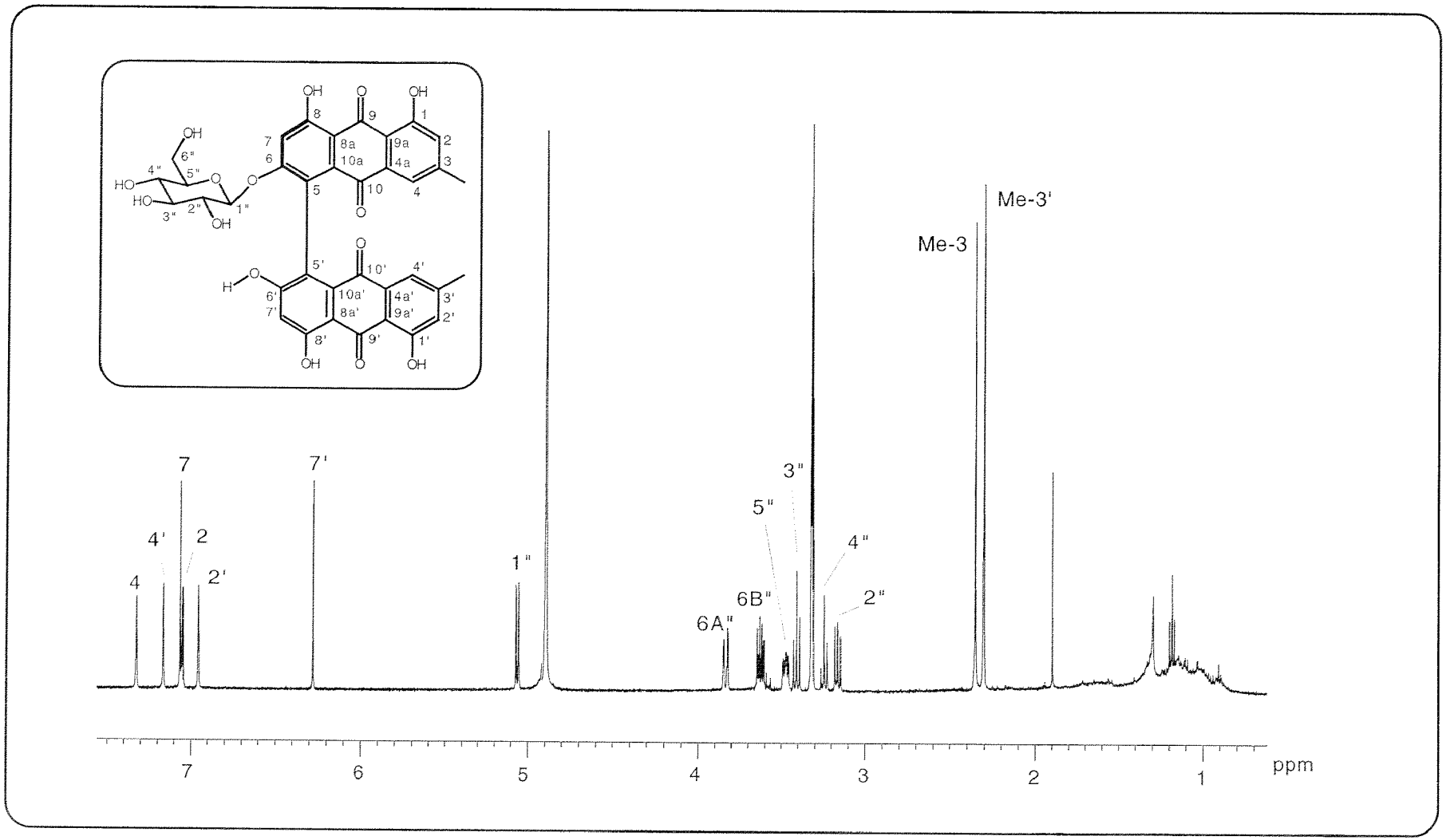


Figure 6.5 ^{1}H-NMR spectrum of **1** (500.13 MHz, methanol-d_4)

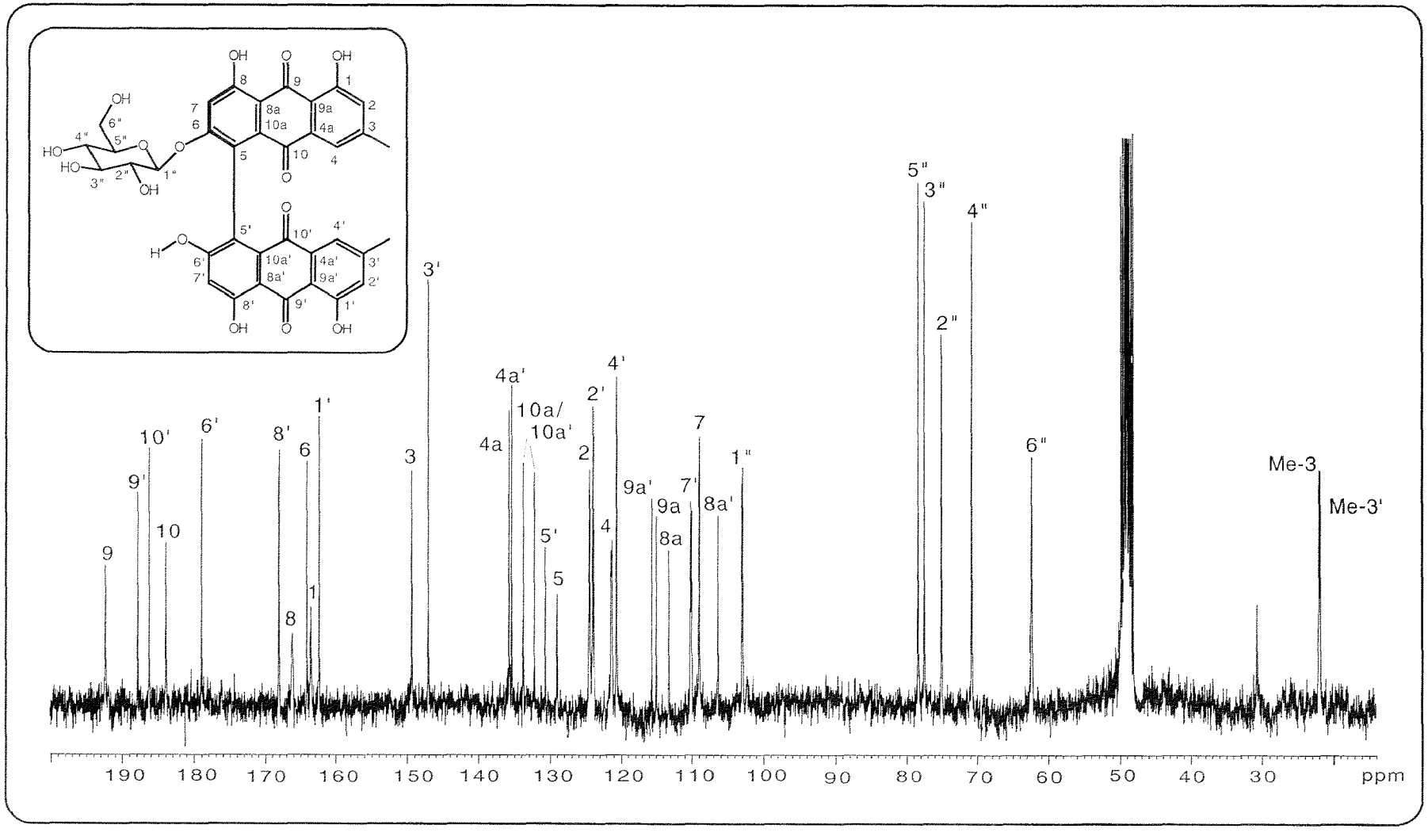


Figure 6.6 ^{13}C-NMR spectrum of **1** (75.47 MHz, methanol-d_4)

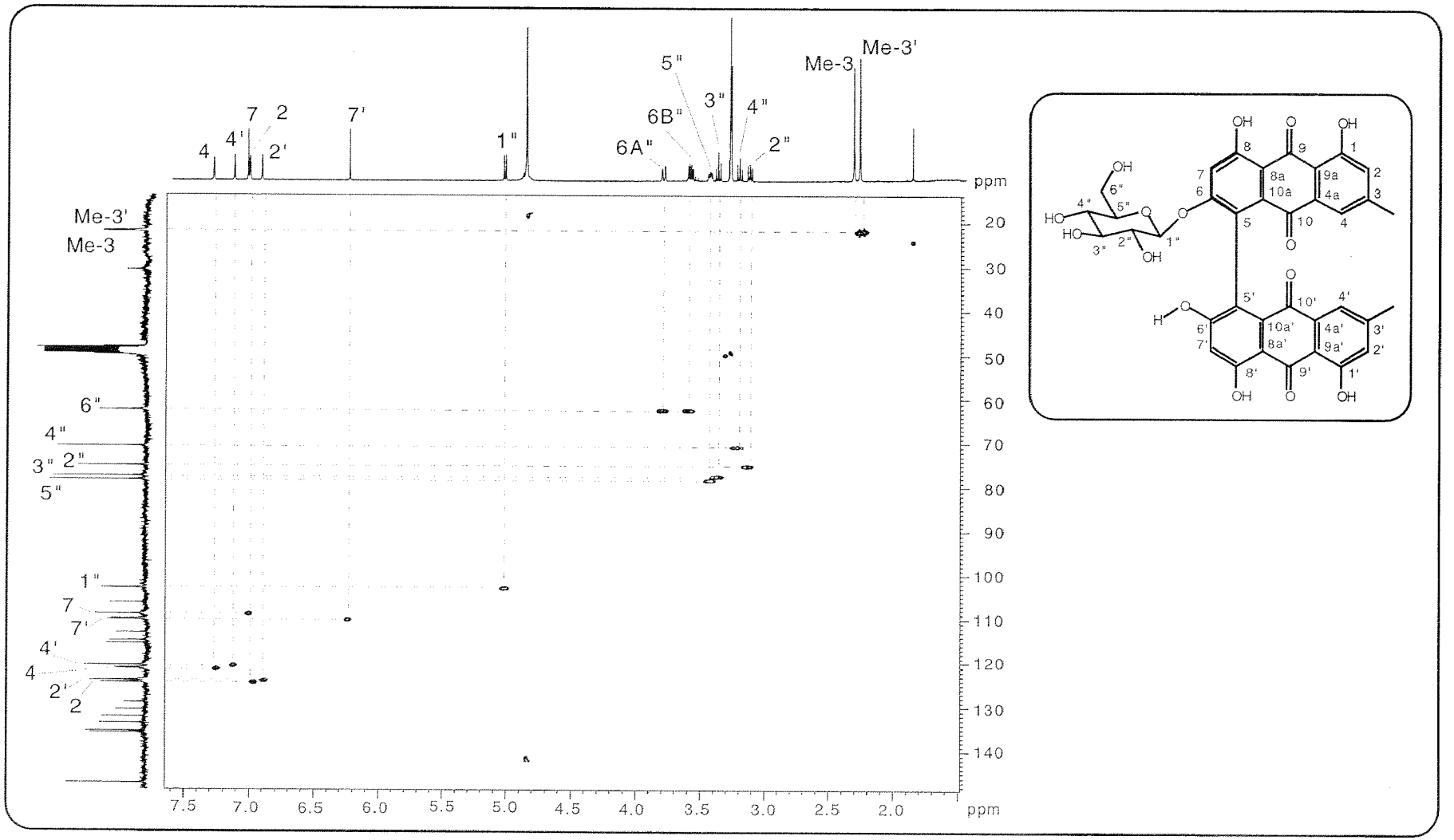


Figure 6.7 HMQC spectrum of **1** (500.13 MHz, methanol-d_4)

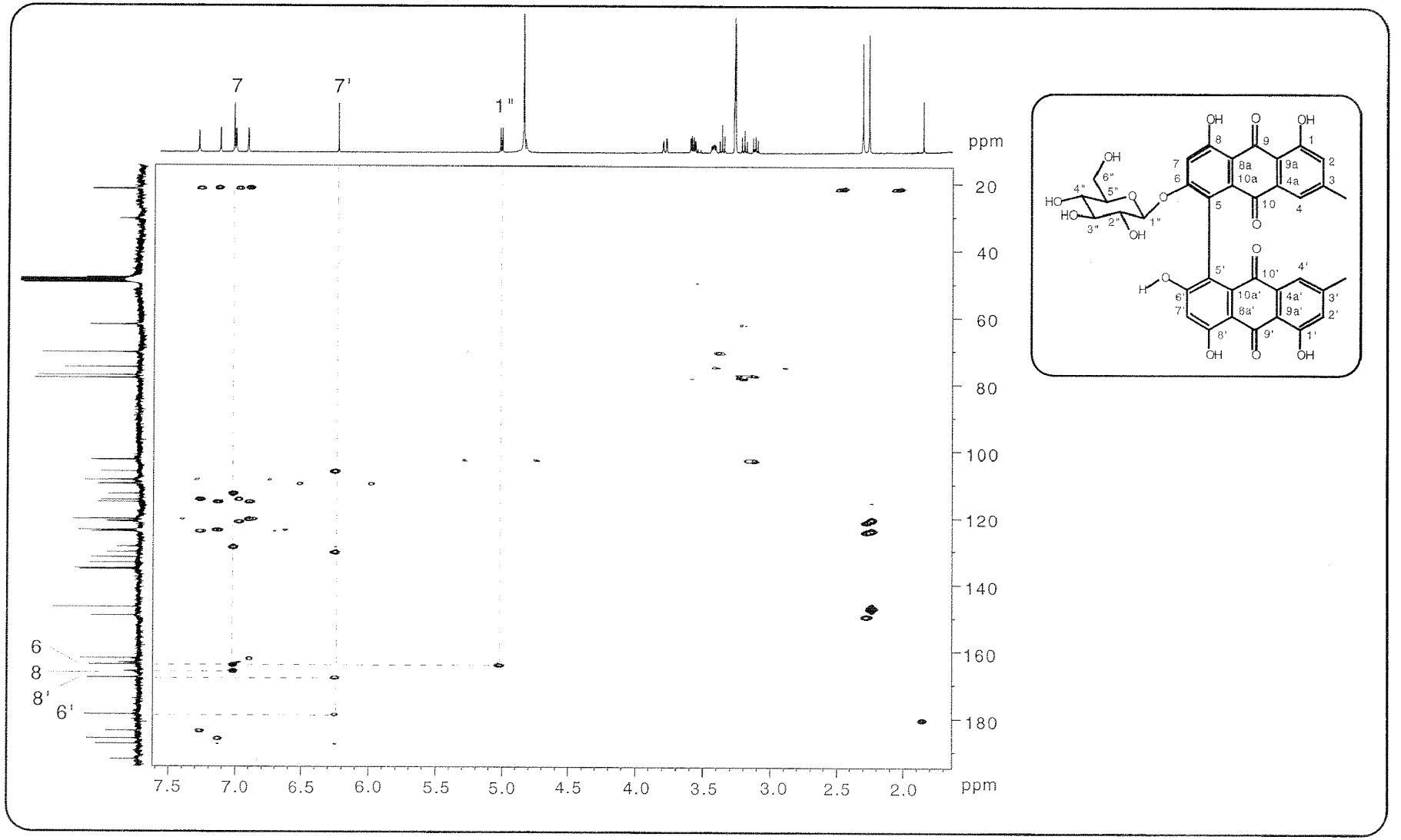


Figure 6.8 HMBC spectrum of **1** (500.13 MHz, methanol-d_4)

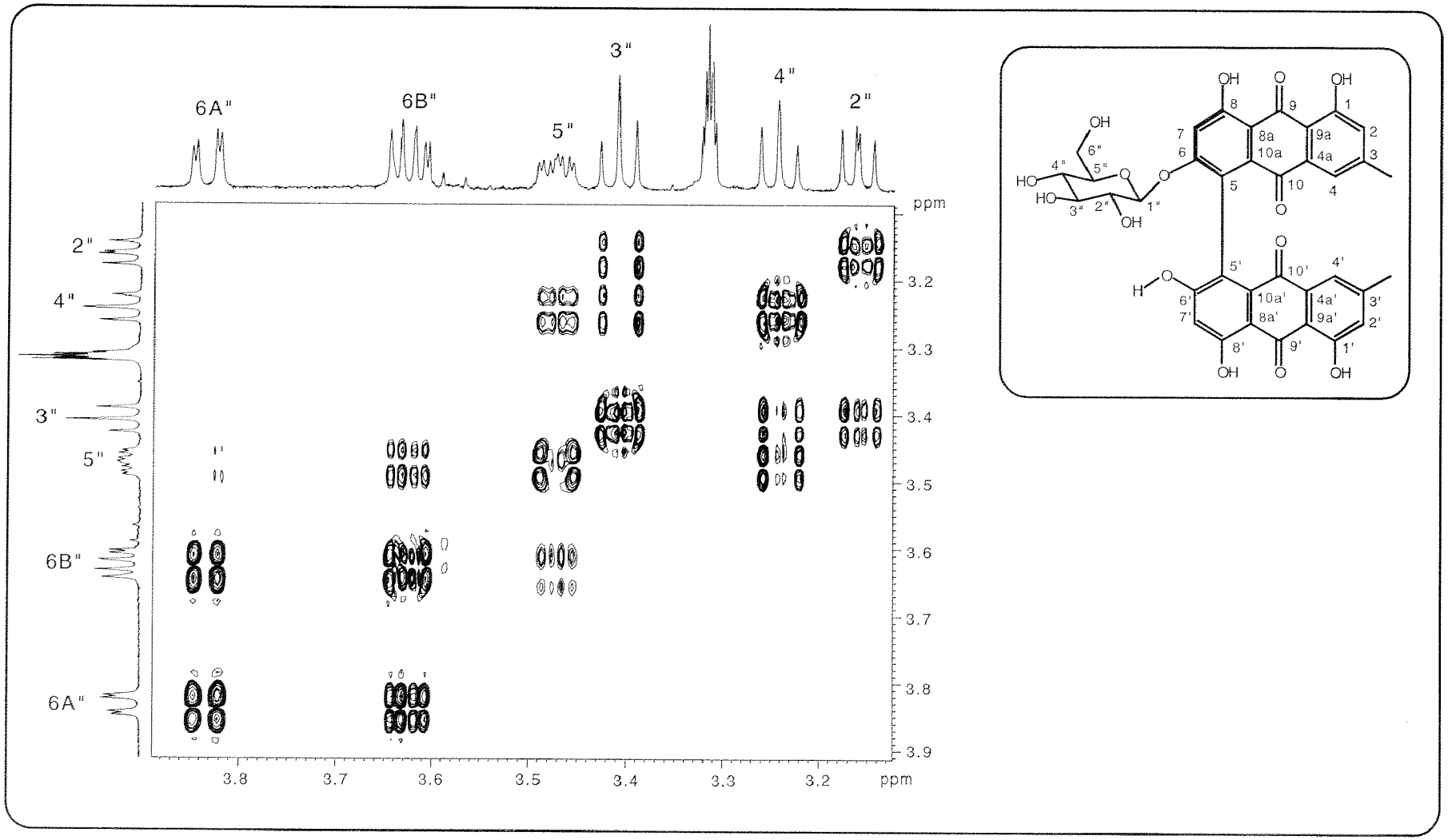


Figure 6.9 Part of the DQF-COSY spectrum of **1** (500.13 MHz, methanol-d_4)

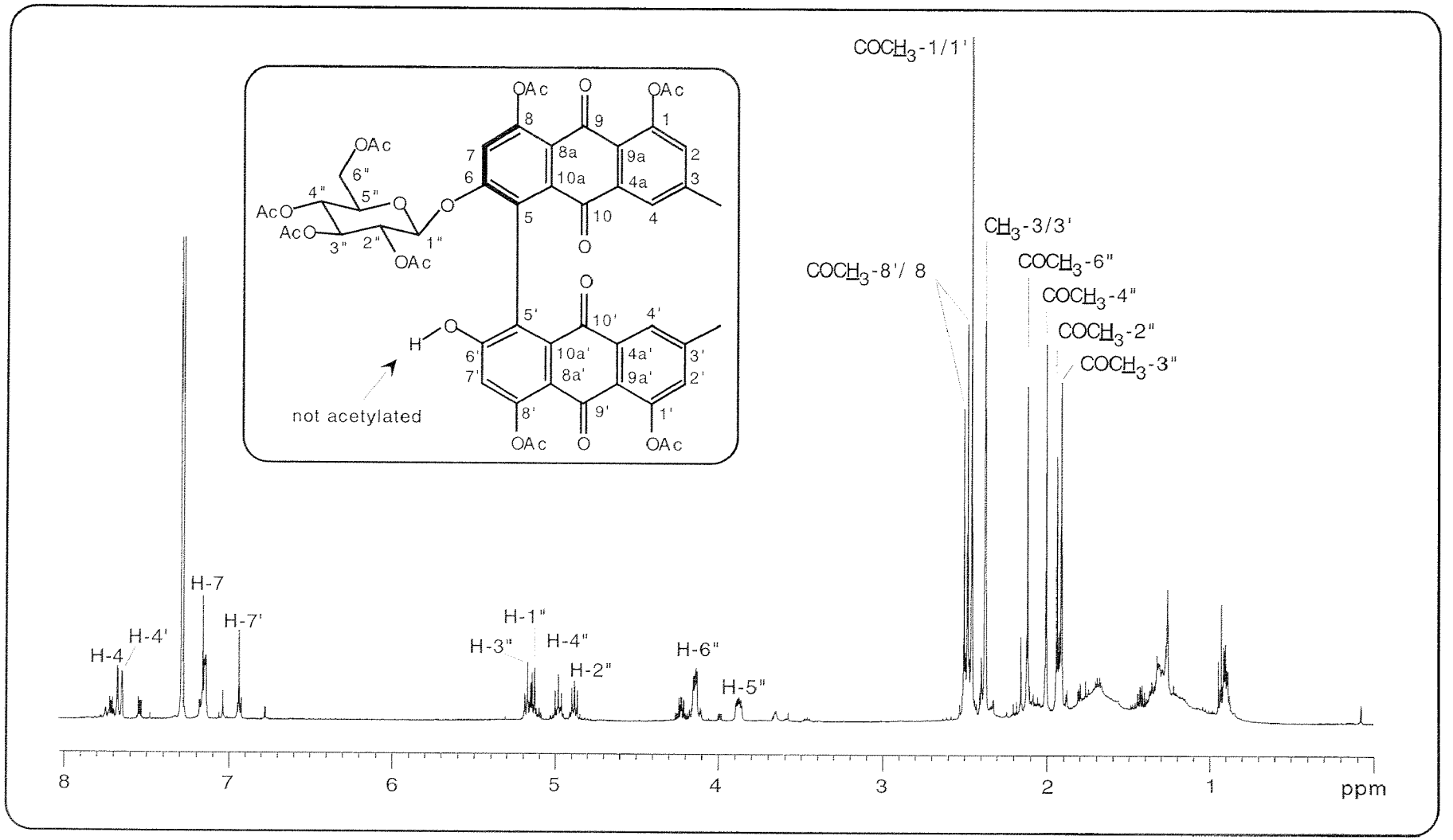


Figure 6.10 ^{1}H NMR spectrum of **1a** (500.13 MHz, chloroform-d_1)

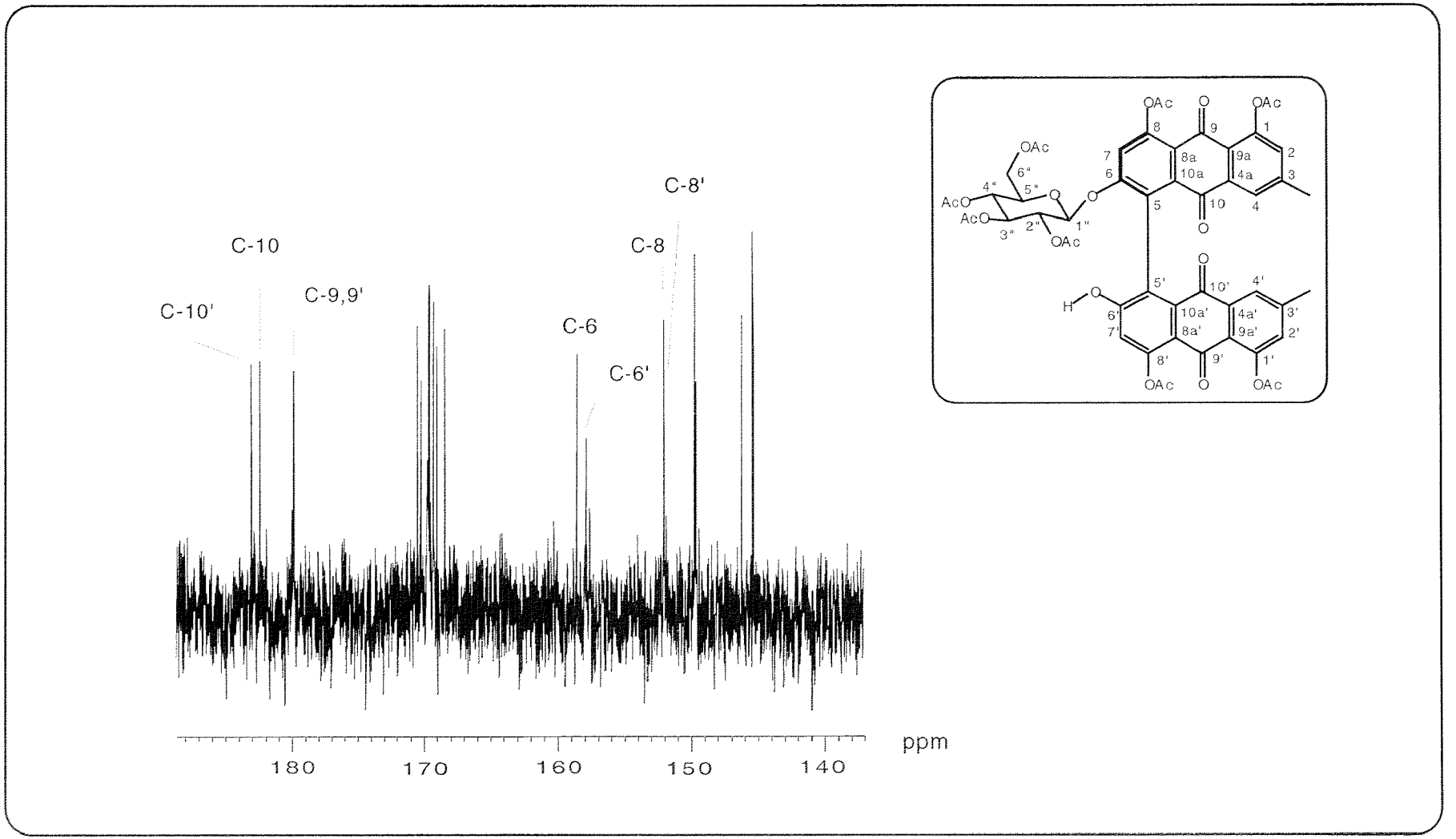


Figure 6.11 Part of the ^{13}C NMR spectrum of **1a** (125.77 MHz, chloroform-d_1)

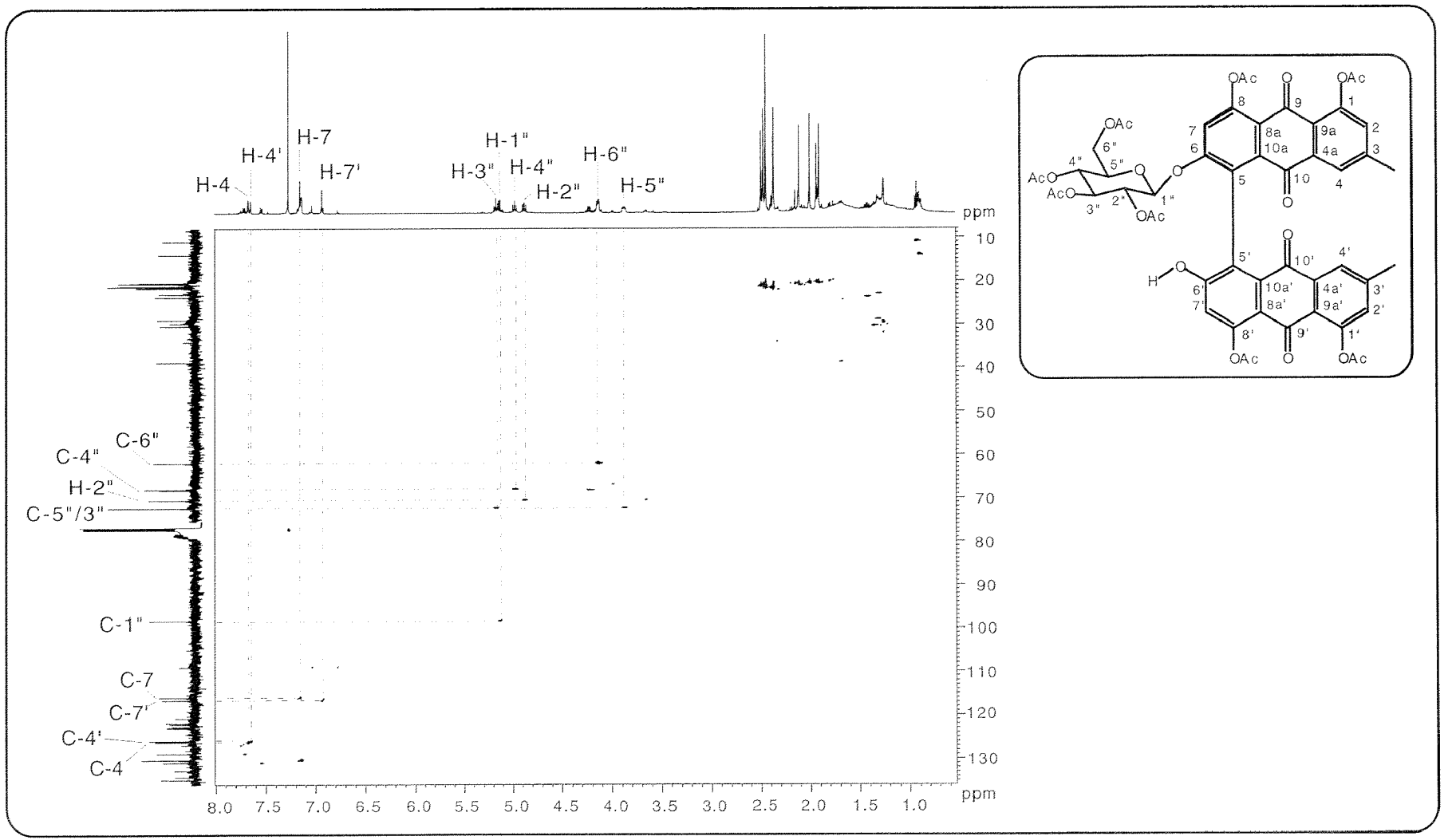


Figure 6.12 HMQC spectrum of **1a** (500.13 MHz, chloroform-d_1)

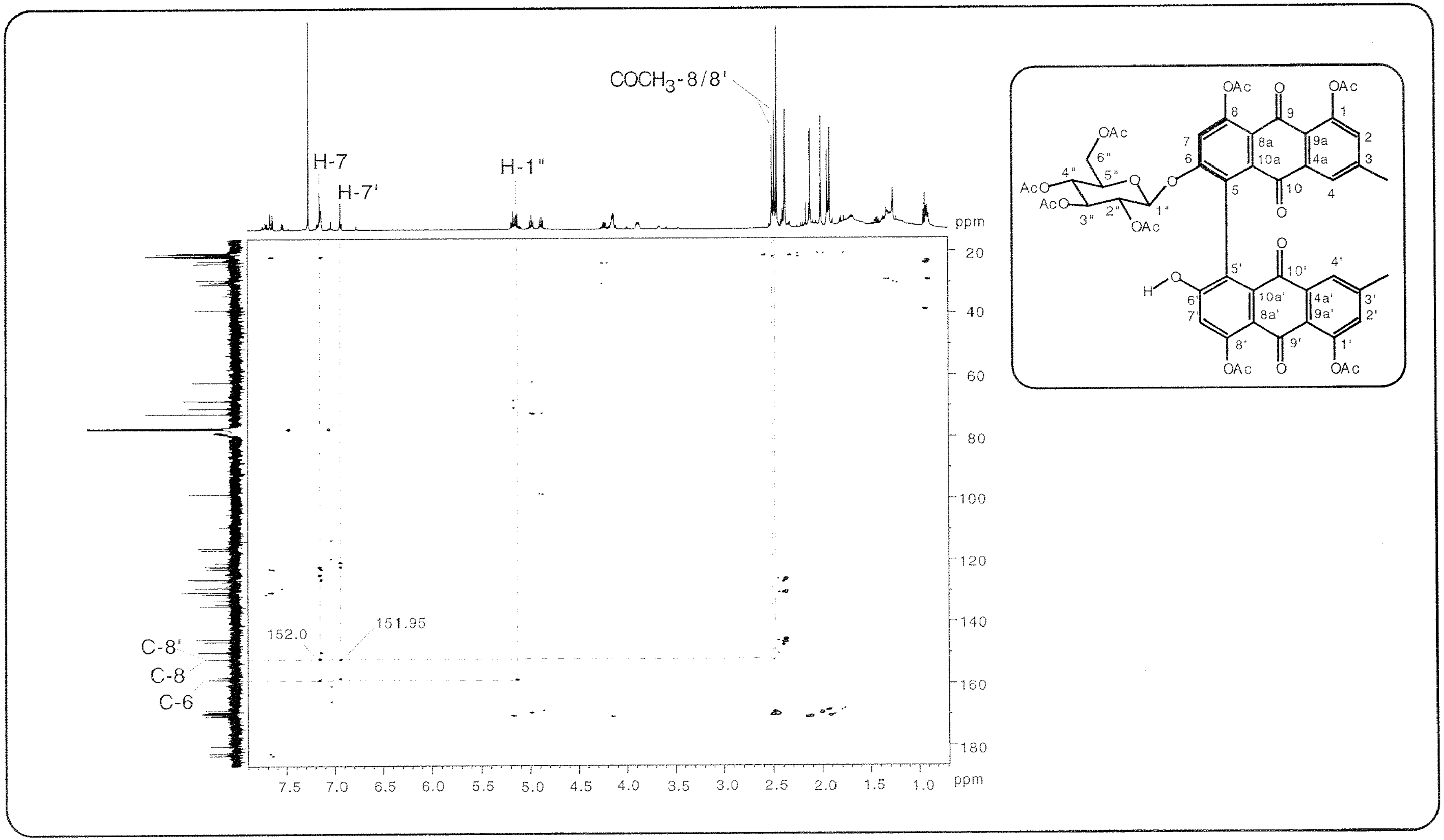


Figure 6.13 HMBC spectrum of **1a** (500.13 MHz, chloroform-d_1)

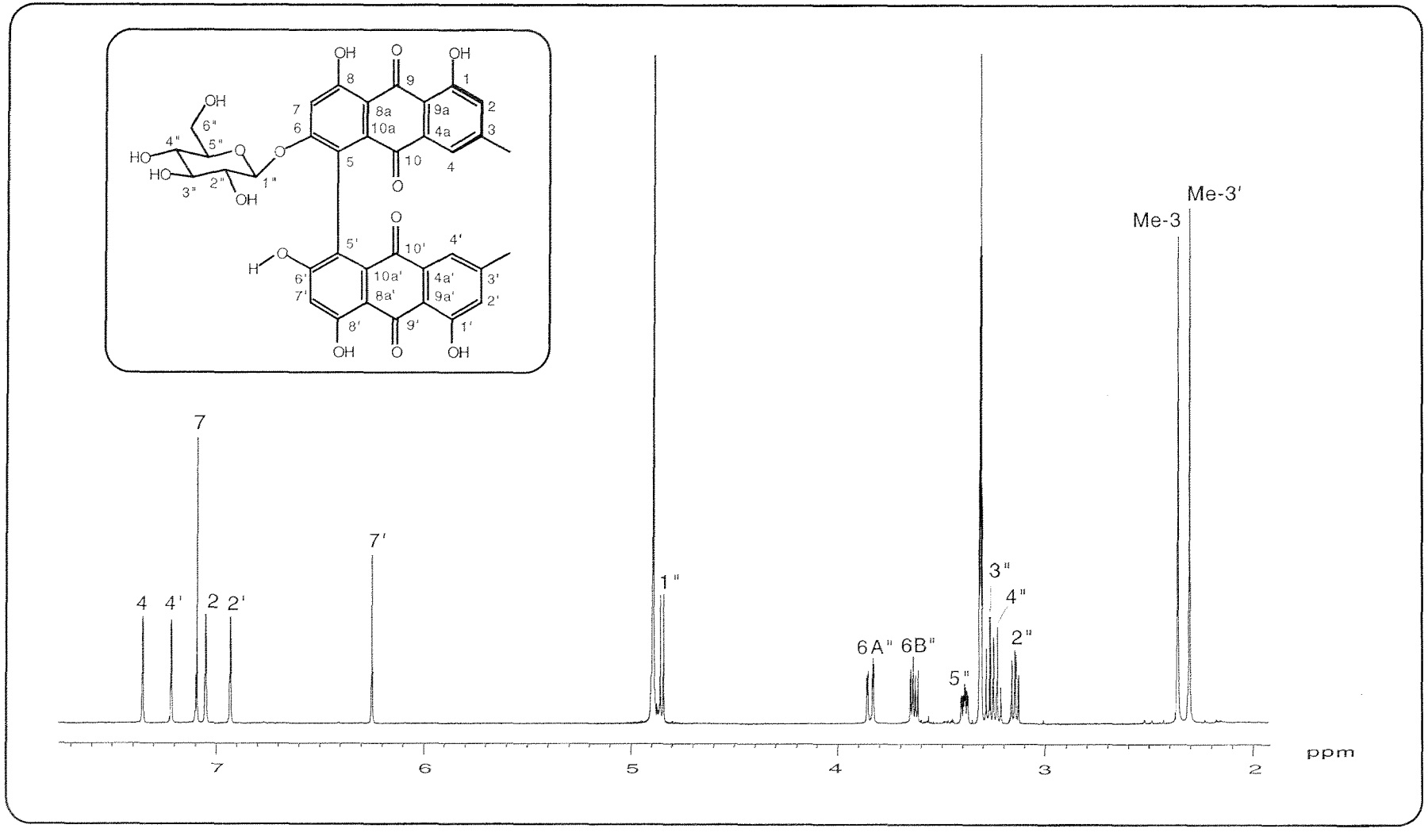


Figure 6.14 ^{1}H-NMR spectrum of **2** (500.13 MHz, methanol-d_4)

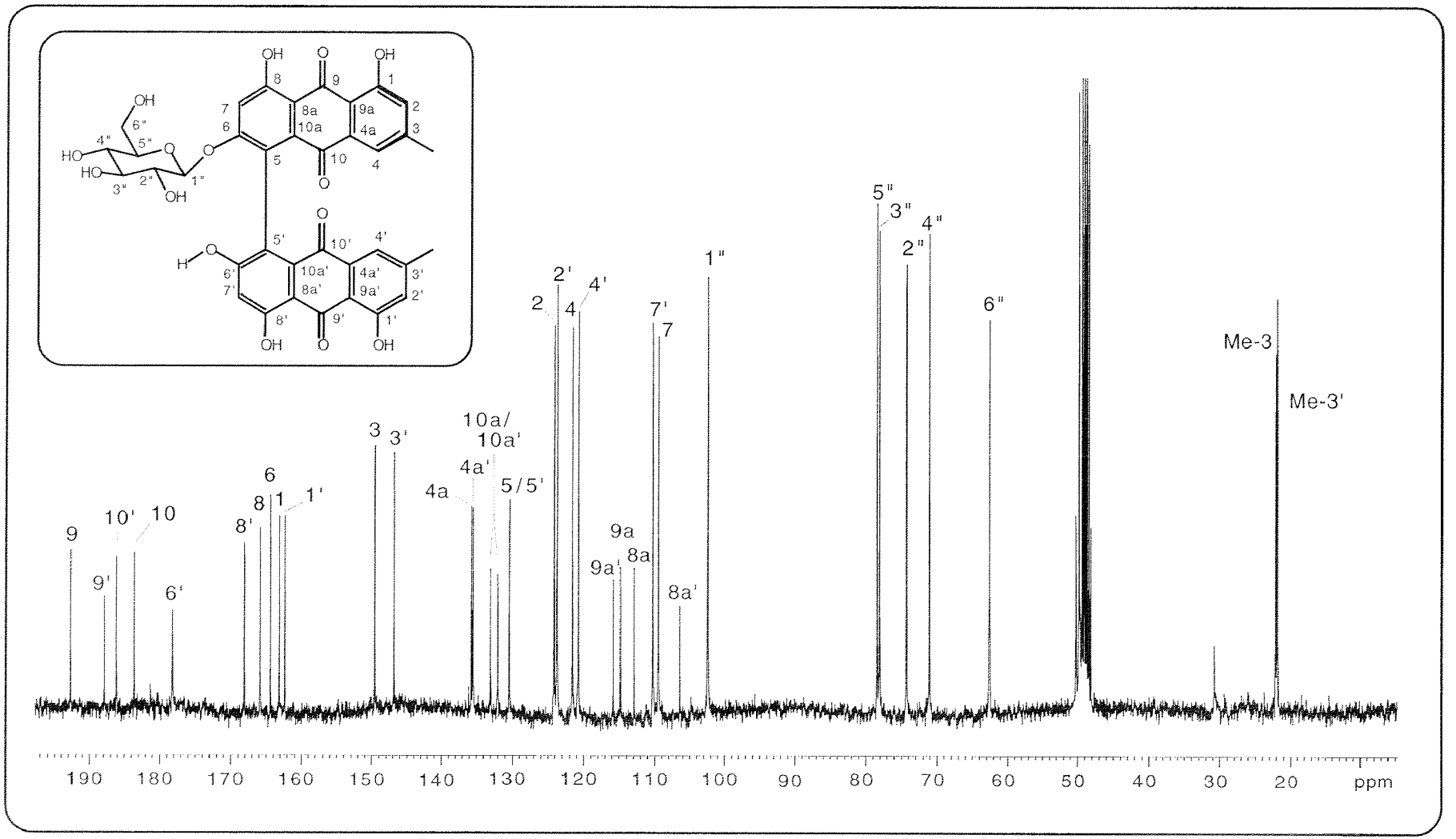


Figure 6.15 ^{13}C-NMR spectrum of **2** (75.47 MHz, methanol-d_4)

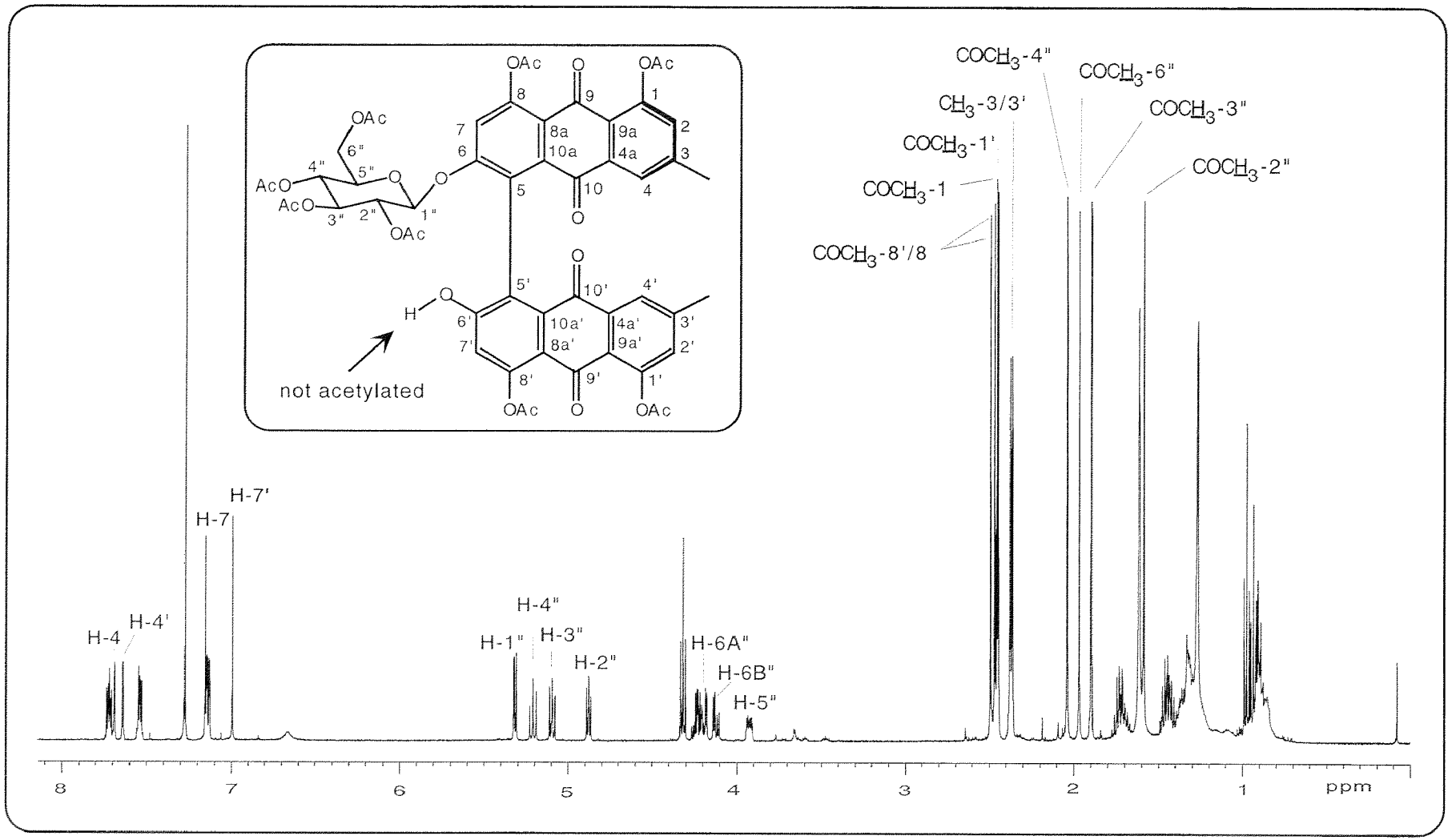


Figure 6.16 ^{1}H-NMR spectrum of **2a** (500.13 MHz, chloroform-d_1)

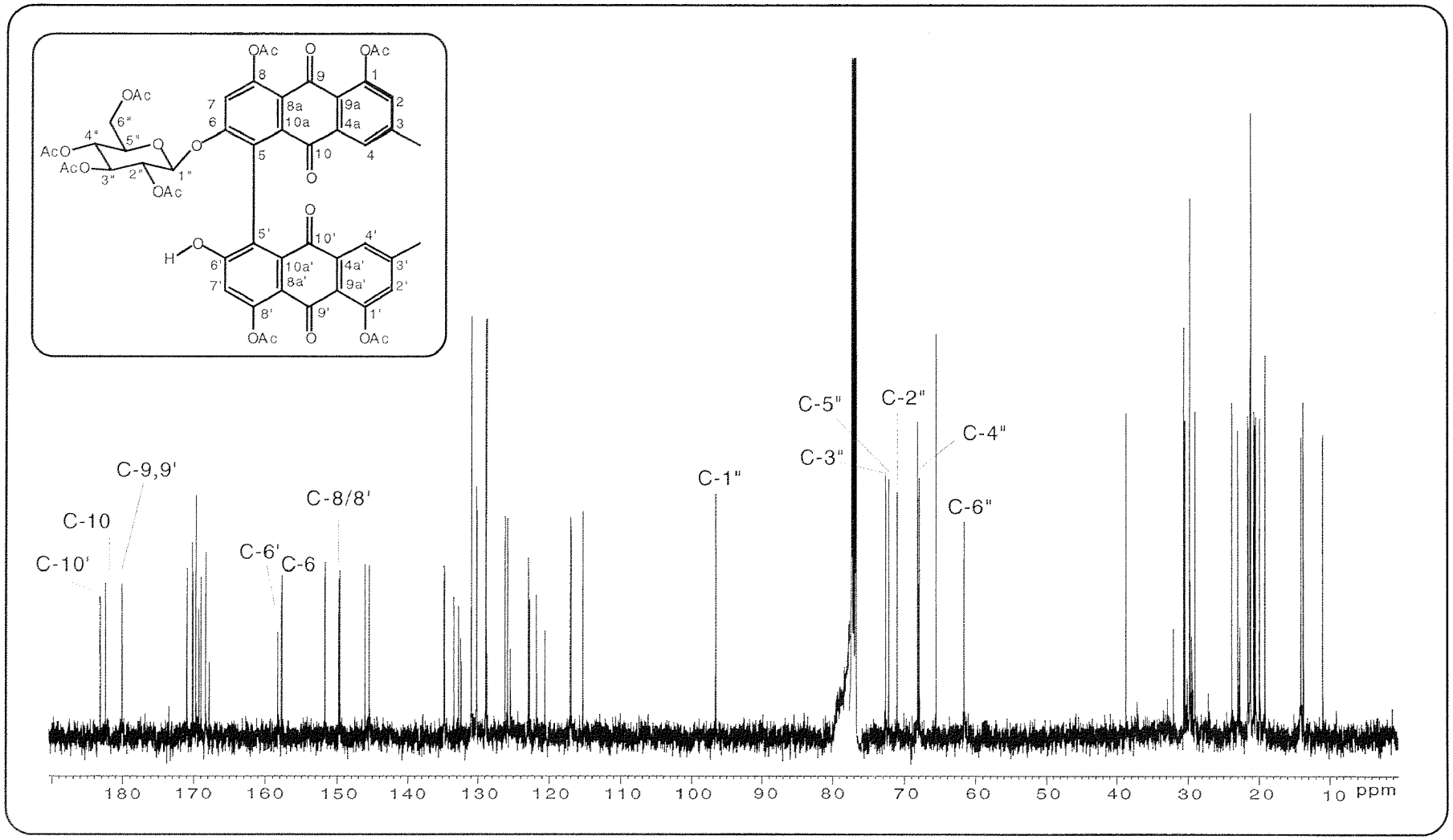


Figure 6.17 ^{13}C-NMR spectrum of **2a** (125.77 MHz, choroform-d_1)

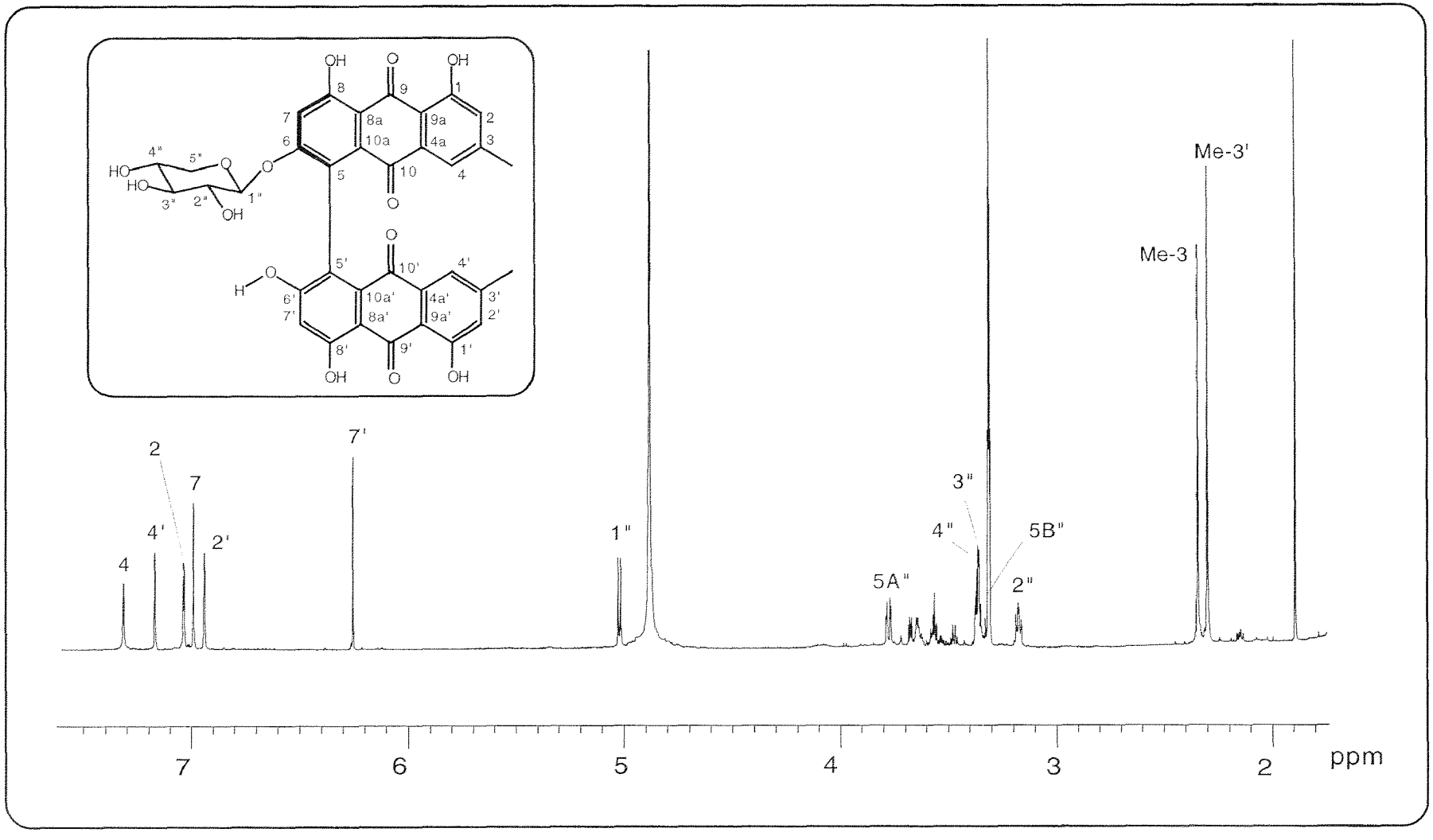


Figure 6.18 ^{1}H-NMR spectrum of **3** (600.13 MHz, methanol-d_4)

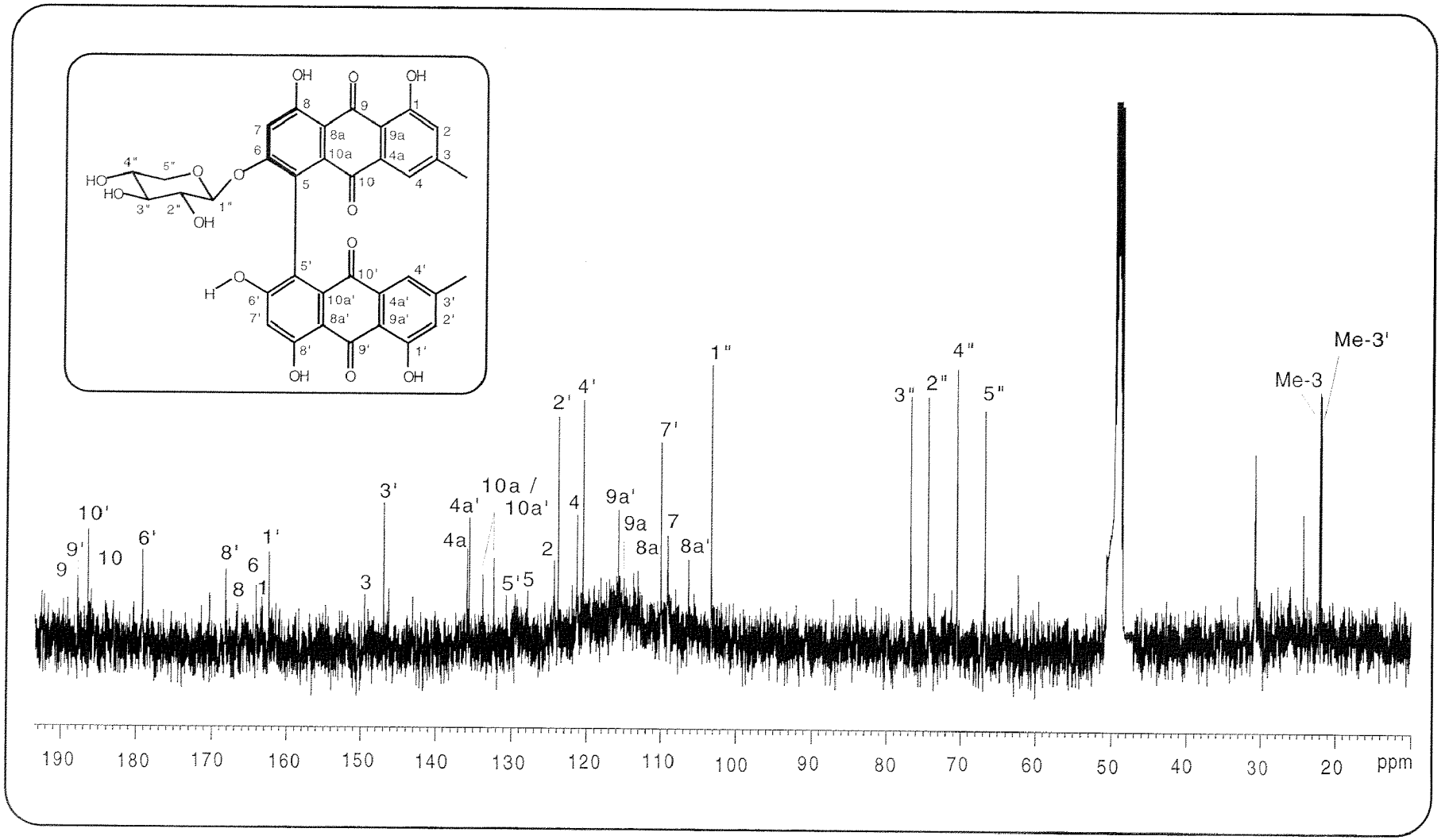


Figure 6.19 ^{13}C-NMR spectrum of **3** (125.77 MHz, methanol-d_4)

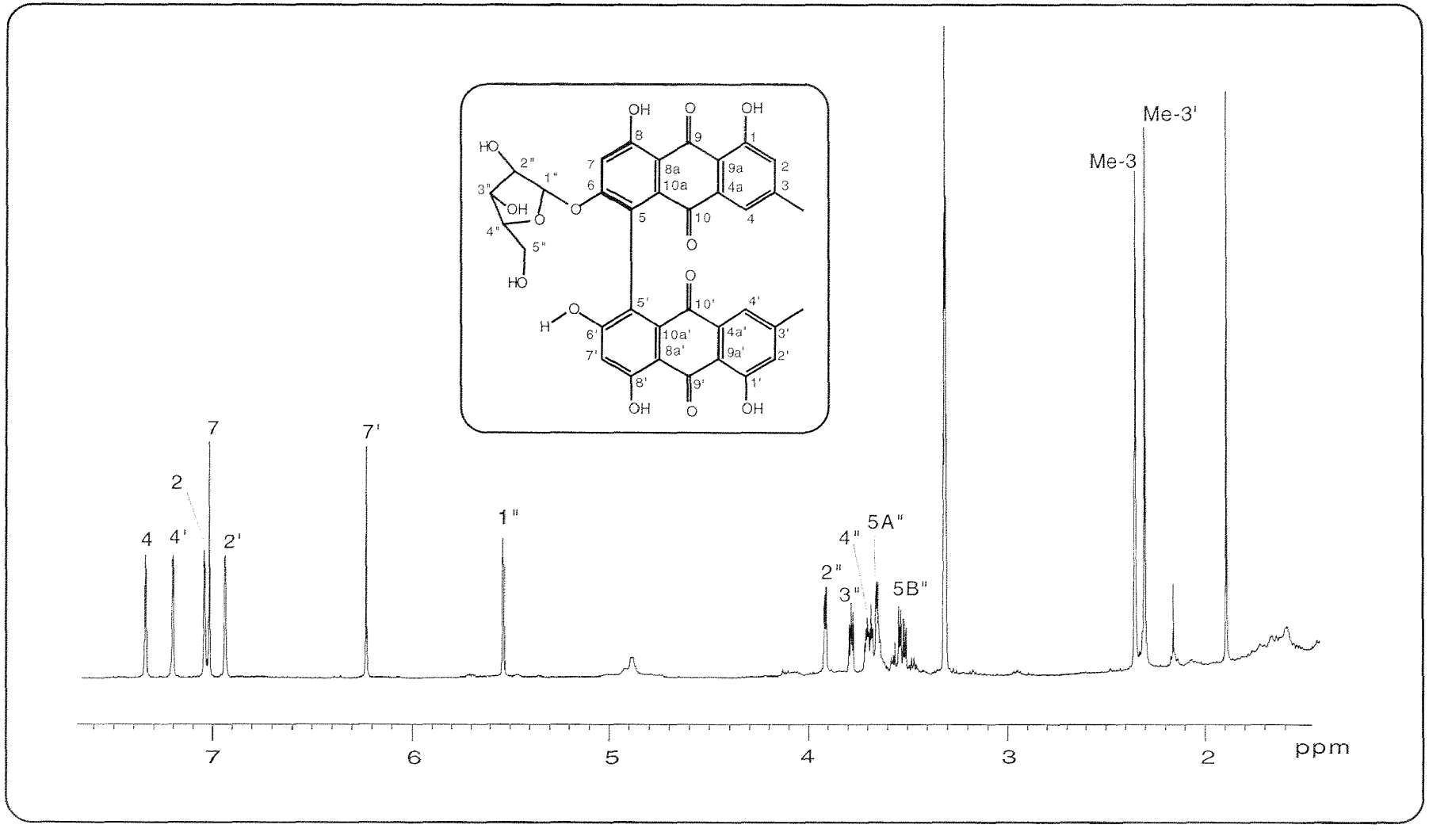


Figure 6.20 ^{1}H-NMR spectrum of **4** (500.13 MHz, methanol-d_4)

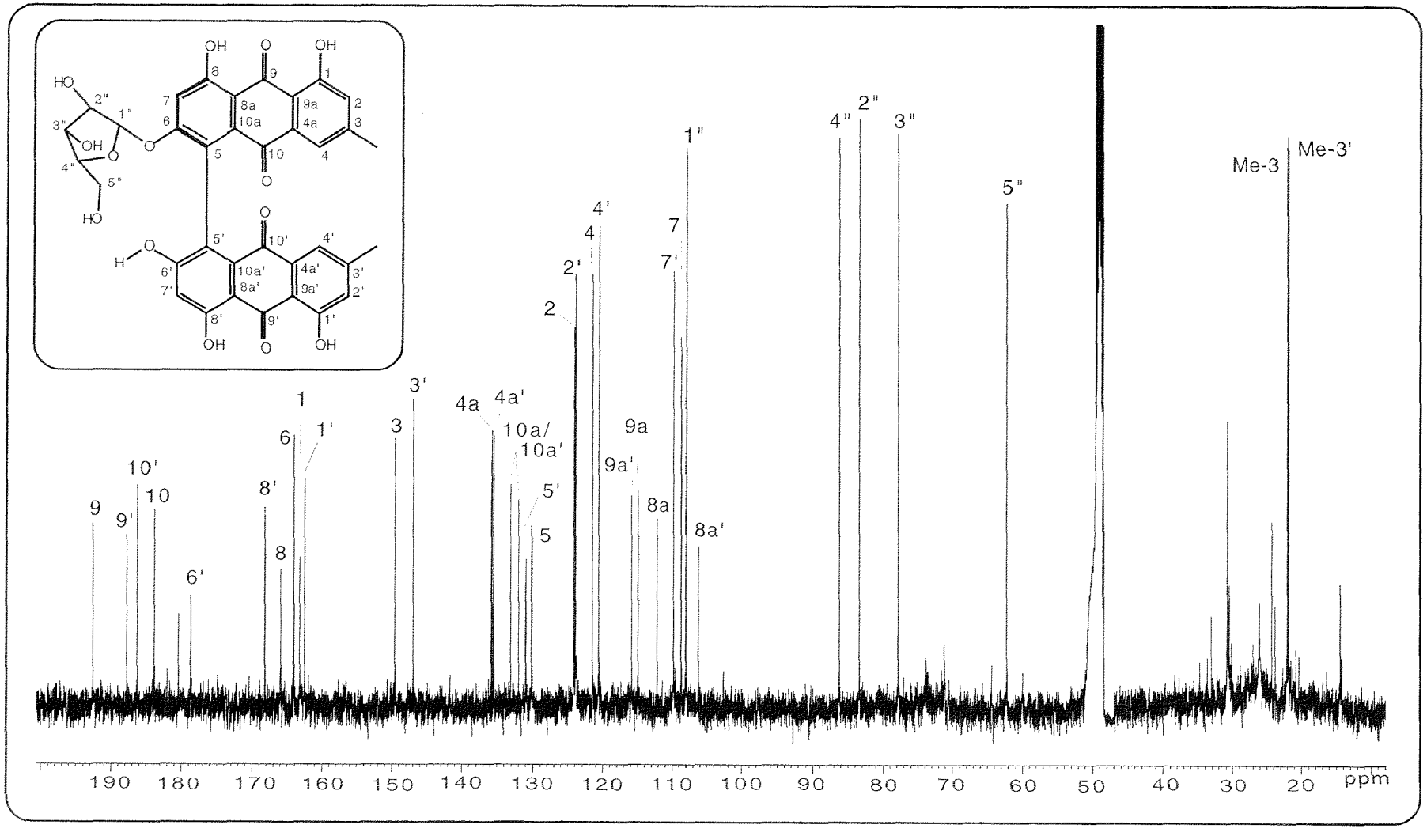


Figure 6.21 ^{13}C-NMR spectrum of **4** (125.77 MHz, methanol-d_4)

6.5 References

Antonowitz, A., Gill, M., Morgan, P. M. and Yu, J.: Coupled Anthraquinones from the Toadstool *Dermocybe icterinoides*. *Phytochemistry* **37**: 1679-1683 (1994)

D'Auria, M. V., Fontana, A., Minale, L. and Riccio, R.: Starfish Saponins. Part XLII. Isolation of Twelve Steroidal Glycosides from the Pacific Ocean Starfish *Henricia laeviuscola*. *Gazz. Chim. Ital.* **120**: 155-163 (1990)

Banks, H. J., Cameron, D. W. and Crossley, M. J.: Chemistry of the Coccoidea. IV Polyhydroxyanthraquinones and their Glucosides from *Eriococcus coriaceus* (Hemiptera: Insecta). *Aust. J. Chem.* **29**: 2231-2245 (1976)

Baureithel, K. H., Berger-Büter, K., Engesser, A., Burkard, W. and Schaffner, W.: Inhibition of Benzodiazepine Binding in Vitro by Amentoflavone, a Constituent of Various Species of *Hypericum*. *Pharm. Acta Helv.* **72**: 153-157 (1997)

Berghöfer, R.: Analytik und Isolierung phenolischer Inhaltsstoffe von *Hypericum perforatum* L. aus Anbau und Wildvorkommen. Thesis. *Dissertationes Botanicae* **106**. Cramer, J. Berlin, Stuttgart, 1987

Billen, G., Karl, U., Scholl, T., Stroech, K. D. and Steglich, W.: Stereochemical Studies on Pre-Anthraquinones and Dimeric Anthraquinone Pigments. In: *Natural Products Chemistry III*. Atta-ur-Rahman and Le Quesne, P. W. (Ed.); Springer: Berlin, Heidelberg, 1988 pp 305-315

Butterweck, V.: Beitrag zur Pharmakologie und Wirkstoff-Findung von *Hypericum perforatum* L. Thesis. Mathematisch-Naturwissenschaftliche Fakultät. Westfälische Wilhelms-Universität Münster, 1997

Cameron, D.W., Edmonds J.S. and Raverty, W.D.: Oxidation of Emodin Anthrone and Stereochemistry of Emodin Bianthrone. *Aust. J. Chem.* **29**: 1535-1548 (1976)

Coskun, M., Satake, T., Hori, K., Saiki, Y. and Tanker, M.: Anthraquinone Glycosides from *Rhamnus libanoticus*. *Phytochemistry* **29**: 2018-2020 (1990)

Cott, J.M.: In Vitro Receptor Binding and Enzyme Inhibition by *Hypericum perforatum* Extract. *Pharmacopsychiat.* **30** (suppl. 2): 108-112 (1997)

Danielsen, K., Aksnes, D. W. and Francis, G. W.: NMR Study of Some Anthraquinones from Rhubarb. *Magnetic Resonance in Chemistry* **30**: 359-363 (1992)

Fuchino, H., Satoh, T. and Tanaka, N.: Chemical Evaluation of *Betula* Species in Japan. I. Constituents of *Betula ermanii*. *Chem. Pharm. Bull.* **43**: 1937-1942 (1995)

Gill, M. and Giménez, A.: Pigments of fungi. Part 40. Dermocanarins 1-3, Unique Naphthol- and Naphthoquinone-anthraquinone Dimers that Contain a Macrocyclic Lactone Ring from the Fungus *Dermocybe canaria* Horak. *J. Chem. Soc. Perkin Trans. I*: 645-651 (1995)

Gill, M. and Giménez, A.: Austrovenetin, the Principal Pigment of the Toadstool *Dermocybe austroveneta*. *Phytochemistry* **30**: 951-955 (1991)

Gill, M., Gimenez, A. and McKenzie, R. W.: Pigments of Fungi. Part 8. Bianthroquinones from *Dermocybe austroveneta*. *J. Nat. Prod.* **51**: 1251-1256 (1988)

Gill, M. and Steglich, W.: Pigments of Fungi. In: *Progress in the Chemistry of Organic Natural Products 51*. Herz, W., Griesbach, H. and Tamm, Ch. (Ed.); Springer: Wien, New York, 1987

Gottowik, J., Goetschy, V., Henriot, S., Kitas, E., Fluhman, B., Clerc, R. G., Moreau, J.-L., Monsma, F.J. and Kilpatrick, G.J.: Labelling of CRF_1 and CRF_2 Receptors Using the Novel Radioligand, [^{3}H]-urocortin. *Neuropharmacology* **36**: 1439-1446 (1997)

Hirota, H., Takayama, S., Miyashiro, S., Ozaki, Y. and Ikegami, S.: Structure of a Novel Steroidal Saponin, Pachastrelloside A, obtained from a Marine Sponge of the Genus *Pachastrella*. *Tetrahedron Lett.* **31**: 3321-3324 (1990)

Höfle, G.: ^{13}C-NMR-Spektroskopie chinoider Verbindungen-II. Substituierte 1,4-Naphthochinone und Anthrachinone. *Tetrahedron* **33**: 1963-1970 (1977)

Hölzl, J. and Ostrowski, E.: Johanniskraut (*Hypericum perforatum* L.). HPLC-Analyse der wichtigsten Inhaltsstoffe und deren Variabilität in einer Population. *Dtsch. Apoth. Ztg.* **127**: 1227-1230 (1987)

Howard, B. H. and Raistrick, H.: Studies in the Biochemistry of Micro-organisms. 91. The Colouring Matters of *Penicillium islandicum* Sopp. Part. 3. Skyrin and Flavoskyrin. *Biochem. J.* **56**: 56-65 (1954)

Huot, R. and Brassard, P.: Fungi. Hypocreaceae. Skyrin from *Hypomyces lactifluorum*. *Phytochemistry* **11**: 2879 (1972)

Iorizzi, M., De Marino, S., Minale, L., Zollo, F., Le Bert, V. and Roussakis, C.: Investigation of the Polar Steroids from an Antarctic Starfish of the Family *Echinasteridae*: Isolation of Twenty-Seven Polyhydroxysteroids and Steroidal Oligoglycosides, Structures and Biological Activities. *Tetrahedron* **52**: 10997-11012 (1996)

Jägers, E.: Untersuchungen über die Farbstoffe von Pilzen der Gattungen *Cortinarius anthracophyllum* und *boleopsis*. Thesis. Hohe Mathem.-Naturw. Fakultät. Rheinische Friedrich-Wilhelms-Universität Bonn, 1980

Karl, U.: Untersuchungen zur Bestimmung der absoluten Konfiguration axial-chiraler Pilzfarbstoffe. Thesis. Hohe Mathem.-Naturw. Fakultät. Rheinische Friedrich-Wilhelms-Universität Bonn, 1988

Kartnig, T. and Wegschaider, O.: Eine Möglichkeit zur Identifizierung von Zuckern aus kleinsten Mengen von Glykosiden oder aus Zuckergemischen, *J. Chromatogr.* **61**: 375-377 (1971)

Koyama, K., Aida, S. and Natori, S.: Supplemental Observations on Atropisomerism of Fungal Bis(naphtho-g-pyrone)s. *Chem. Pharm. Bull.* **38**: 2259-2261 (1990)

Kusano, A., Shibano, M. and Kusano, G.: Studies on the Constituents of *Cimicifuga* Species. XVII. Four New Glycosides from the Aerial Parts of *Cimicifuga simplex* Wormsk. *Chem. Pharm. Bull.* **43**: 1167-1170 (1995)

Lin, C.-N., Chung, M.-I. and Lu, C.-M.: Anthraquinones from *Rhamnus formosana*. *Phytochemistry* **29**: 3903-3905 (1990)

Linde, K. and Mulrow, C. D.: St John's Wort for Depression (Cochrane Review). In: *The Cochrane Library* **3**: Oxford, 1999, Update Software. http://ovid.sams.ch/ovidweb/ovidweb.cgi

Linde, K., Ramirez, G., Mulrow, C. D., Pauls, A., Weidenhammer, W. and Melchart, D.: St John's wort for Depression - an Overview and Meta-analysis of Randomised Clinical Trials. *BMJ* **313**: 253-258 (1996)

Meier, B.: The Science Behind *Hypericum*. *Advances In Therapy* **16**: 135-147 (1999)

Rawat, M. S. M., Negi, D. S., Panwar, M. S., Pant, G., Shibata, S., Okada, Y., Oshima, Y. and Okuyama, T.: Anthraquinone Glycosides from *Rheum moorcroftianum* Royle. *Pharmazie* **44**: 509-510 (1989)

Reul, J. M. H., Stec, I., Söder, M. and Holsboer, F.: Chronic Treatment of Rats with the Antidepressant Amitriptyline Attenuates the Activity of the Hypothalamic-Pituitary-Adrenocortical System. *Endocrinology* **133**: 312-320 (1993)

Riccio, R., D'Auria, M. V. and Minale, L.: Two New Steroidal Glycoside Sulfates, Longicaudoside-A and -B, from the Mediterranean Ophiuroid *Ophioderma longicaudum*. *J. Org. Chem.* **51**: 533-536 (1986)

Sakamoto, I., Tanaka, T., Tanaka, O. and Tomimori, T.: Xanthone Glucosides of *Swertia japonica* Makino and a Related Plant: Structure of a New Glucoside, Isoswertianolin and Structure Revision of Swertianolin and Norswertianolin. *Chem. Pharm. Bull.* **30**: 4088-4091 (1982)

Sedmera, P., Podojil, M., Vokoun, J., Betina, V. and Nemec, P.: 2,2'-Dimethoxy-4a,4a'-dehydrorugulosin (Rugulin), a Minor Metabolite from *Penicillium rugulosum*. *Folia Microbiol.* **23**: 64-67 (1978)

Shibata, S., Tanaka, O. and Kitagawa, I.: Products of Fungi. Part V. The Structure of Skyrin. (1). *Chem. Pharm. Bull.* **3**: 278-290 (1955)

Simmen, U., Burkard, W., Berger, K., Schaffner, W. and Lundstrom, K.: Extracts and Constituents of *Hypericum perforatum* inhibit the Binding of Various Ligands to Recombinant Receptors expressed with the Semliki Forest Virus System. *J. of Receptor & Signal Transduction Research* **19**: 59-74 (1999)

Steglich, W. and Oertel, B.: Untersuchungen zur Konstitution und Verbreitung der Farbstoffe von *Cortinarius*, Untergattung *Phlegmacium* (Agaricales). *Sydowia* **37**: 284-295 (1984)

Takeda, N., Seo, S., Ogihara, Y., Sankawa, U., Iitaka, I., Kitagawa, I. and Shibata, S.: Studies on Fungal Metabolites-XXXI. Anthraquinoid Colouring Matters of *Penicillium islandicum* Sopp and some other Fungi (-)-Luteoskyrin, (-)-Rubroskyrin, (+)-Rugulosin and their Related Compounds. *Tetrahedron* **29**: 3703-3719 (1973)

Tanaka, O.: Metabolic Products of Fungi. Part XIV. The Structure of Skyrin. (3). On Pseudoskyrin. *Chem. Pharm. Bull.* **6**: 203-208 (1958)

R. H. Thomson: *Naturally Occurring Quinones*; 2nd edn. Academic Press: London, 1971

R. H. Thomson: *Naturally Occurring Quinones III: recent advances*; 3rd edn. Chapman and Hall: London, 1987

R. H. Thomson: *Naturally Occuring Quinones IV: recent advances*; 4th edn. Blackie Academic and Professional: London, 1997

Toma, F., Bouhet, J. C., Van Chuong, P. P., Fromageot, P., Haar, W., Rüterjans, H. and Maurer, W.: Carbon-13 NMR Spectroscopy of the Biological Pigments Luteoskyrin and Rugulosin and some Polyhydroxyanthraquinone Analogues. *Organic Magnetic Resonance* **7**: 496-503 (1975)

De Tommasi, N., Piacente, S., De Simone, F. and Pizza, C.: Characterization of Three New Triterpenoid Saponins from *Ardisia japonica*. *J. Nat. Prod.* **56**: 1669-1675 (1993)

Volz, H.-P.: Controlled Clinical Trials of Hypericum Extracts in Depressed Patients - an Overview. *Pharmacopsychiat.* **30** (suppl. 2): 72-76 (1997)

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