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Author(s):
Lends, Alons; Ravotti, Francesco; Zandomeneghi, Giorgia; Böckmann, Anja; Ernst, Matthias; Meier, Beat H.

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Direct amide $^{15}$N to $^{13}$C transfers for solid-state assignment experiments in deuterated proteins.

Alons Lends$^{1#}$, Francesco Ravotti$^{1#}$, Giorgia Zandomeneghi$^1$, Anja Böckmann$^2$, Matthias Ernst$^{1*}$, Beat H. Meier$^{1*}$

$^1$Physical Chemistry, ETH Zürich, Vladimir-Prelog-Weg 2, 8093 Zürich, Switzerland

$^2$Molecular Microbiology and Structural Biochemistry, Labex Ecofect, UMR 5086 CNRS/Université de Lyon 69367 Lyon, France

#Both authors share an equal contribution to this work

*Corresponding authors: maer@ethz.ch, beme@ethz.ch

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Abstract

The assignment of protein backbone and side-chain NMR chemical shifts is the first step towards the characterization of protein structure. The recent introduction of proton detection in combination with fast MAS has opened up novel opportunities for assignment experiments. However, typical 3D sequential-assignment experiments using proton detection under fast MAS lead to signal intensities much smaller than the theoretically expected ones due to the low transfer efficiency of some of the steps.

Here, we present a selective 3D experiment for deuterated and (amide) proton back-exchanged proteins where polarization is directly transferred from backbone nitrogen to selected backbone or sidechain carbons. The proposed pulse sequence uses only $^1$H-
**Introduction**

Protein backbone and side-chain NMR chemical-shift assignments are the first step towards the characterization of protein structure. The analysis of backbone chemical shifts can provide information about the secondary structure of the protein and guide 3D-structure calculations (Schütz et al. 2015; Wälti et al. 2016). The development of faster magic-angle spinning (MAS) probes (Samoson 2007; Nishiyama 2016) has enabled the detection of proton spins in solids with increasing resolution, due to an improved averaging of the homonuclear dipolar interactions (Knight et al. 2011; Agarwal et al. 2014; Barbet-Massin et al. 2014; Böckmann et al. 2015).

Proton detection (for recent reviews see (Struppe et al. 2017; Schubeis et al. 2018; Vasa et al. 2018)), the standard detection method in solution-state NMR, has in principle many advantages over $^{13}$C detection, which is traditionally used in solid-state NMR. First of all, the higher gyromagnetic ratio of the $^1$H nucleus compared to $^{13}$C provides a gain of eight in the integrated signal intensity. However, this gain can only be realized if the spinning is fast enough to efficiently average dipolar interactions to decrease the proton line widths and increase $T_2^*$ relaxation times (Penzel et al. 2015), both for deuterated and protonated samples (Lewandowski et al. 2011; Penzel et al. 2015). The need for faster spinning requires smaller-diameter rotors with less sample volume (Böckmann et al. 2015), which in turn leads to a signal decrease. Still, a considerable gain in sensitivity can be achieved (Agarwal et al. 2014). A proton spectral dimension for chemical-shift connectivity can be introduced for spinning frequencies above approximately 50 kHHz for perdeuterated and 100% back-protonated proteins. Above about 100 kHHz, also fully-protonated proteins can be investigated at reasonably high
resolution with a proton linewidth approaching 100 Hz for well-ordered preparations (Stanek et al. 2016; Andreas et al. 2016). In this article, we focus on deuterated proteins. Pulse sequences for sequential backbone assignment based on proton-detected spectroscopy under fast MAS typically employ cross polarization (CP) (Hartmann and Hahn 1962; Schaefer and Stejskal 1976) and/or INEPT (Morris and Freeman 1979) type of transfers to realize HNCA, HN(CO)CA, HN(CO)CACB experiments (Zhou et al. 2012; Barbet-Massin et al. 2013; Penzel et al. 2015; Xiang et al. 2016) using selective pulses for INEPT or selective low-power CP transfer to/from Cα, CO and Cβ (Baldus et al. 1998; Barbet-Massin et al. 2014; Penzel et al. 2015). The relative merit of CP and INEPT depends on the MAS frequency and is discussed in detail in Ref. (Penzel et al. 2015). In the CP version, the HNCA is typically realized as a (H)CANH experiment. In this notation, the letters in brackets stand for dimensions that are not sampled. In the (H)CANH experiment, (H) refers to the amide protons, possibly also of neighboring residues. The (H)CA transfer is usually the least efficient polarization-transfer step (~31% in rigid model systems, compared to 58% and 39% for HN and NC, respectively (Penzel et al. 2015)). Backbone assignment experiments contain also Cα-N and/or CO-N CP steps which require relatively long contact times typically ranging from 5 to 15 ms (Baldus et al. 1996), or “out-and-back” INEPT-type transfers for backbone walks (Linser et al. 2008). $T_{1π}$ relaxation times are longer than $T_2$ and CP efficiency should be less affected by long contact times. The efficiencies of these transfers were shown to be in the range 23 to 57% (Penzel et al. 2015). Incorporation of multiple CP and INEPT-type transfers into 3D experiments leads to a final signal intensity of only 2 to 24% of the theoretically possible intensity expected for proton-detected experiments (Chevelkov et al. 2014; Penzel et al. 2015).

Side-chain assignment experiments are even less sensitive with respect to signal intensity. Pulse sequences previously used to assign the aliphatic side chains in deuterated protein use rotor-synchronized RFDR (Bennett et al. 1992), DARR (Tang et al. 2010) or DONER (Akbey et al. 2012) and TOBSY (Baldus and Meier 1997; Hardy et al. 2003; Linser 2011) and MOCCA (Kulminskaya et al. 2016) isotropic mixing schemes for homonuclear $^{13}$C polarization transfer. In these experiments, the in-phase magnetization is transferred from the Cα carbon to the side-chains carbons through $J$ couplings. After encoding $^{13}$C chemical shifts, the magnetization is transferred back to backbone amides for $^1$H detection. In these types of transfer schemes, magnetization
gets distributed over many $^{13}$C nuclei, irrespectively of their chemical shift, leading to a reduced intensity for the interesting signal. In solution NMR, side-chain connectivity is achieved by homonuclear CC-TOCSY transfer (Sattler et al. 1999). In particular, assignment of aromatic side chains is used for both structure determination (Williams et al. 2015) and investigation of protein-ligand interactions (Creemers et al. 2002). In solid-state NMR the assignment of aromatic side-chains is also important for structure determination (Wälti et al. 2016). Previously, carbon-detected experiments at low MAS frequency have been shown to be able to provide only 1-2% of polarization transfer to nuclei in aromatic side-chains using RFDR (Bennett et al. 1992) or DONER (Akbey et al. 2012). These experiments were performed on deuterated, 100% back-exchanged ubiquitin at 14 kHz MAS using 4 mm rotors.

Here, we are interested in efficient transfer from $^{15}$N into the side-chains atoms. We describe a pulse sequence that starts with $^1$H-$^{15}$N CP transfers and then employs a dipolar version of the INEPT experiment (3D TEDOR) for N-C transfer. While TEDOR has most often been used in the context of measuring $^{13}$C-$^{15}$N internuclear distances (Nieuwkoop et al. 2009; Nieuwkoop and Rienstra 2010), we employ it to obtain efficient polarization transfer into the sidechains to establish connectivities. Inefficient H$^N$-C and C-C transfers as encountered in alternative scheme (vide supra) are avoided. It will be demonstrated that this leads to significantly more efficient experiments for backbone (e.g. HNCA) and, particularly, for side-chain assignment.

**Pulse sequence**

The TEDOR experiment follows the pulse scheme of Fig. 1. It is a selective version of the REDOR 3D experiment introduced by Michal and Jelinski (Michal and Jelinski 1997) and later modified by Jaroniec et al. (3D TEDOR) (Jaroniec et al. 2002). We note that the naming is somewhat inconsistent but we stick to the previously used TEDOR naming convention even though no transferred echo is observed in this implementation. The main difference between our pulse sequence and the one by Jaroniec is the choice of the REDOR pulses which are in our sequence applied on the carbon channel. Moreover, the pulse sequence shown here is designed with an additional selective Gaussian $\pi$ pulse, followed by a non-selective $\pi$ pulse, both pulses being rotor
synchronized. In that way, only selected parts of the spectrum are inverted and polarization is selectively transferred to these $^{13}$C spins (Bayro et al. 2009).

Selective versions of the REDOR sequence and the 3D TEDOR experiment of Jaroniec et al. (Jaroniec et al. 2002) have already been proposed before. For example, a REDOR experiment with selective irradiation of $^{13}$C-$^{15}$N spin pairs to determine selected $^{15}$N-$^{13}$C dipole couplings was introduced (Jaroniec et al. 2001). The sequence was applied to small molecules (single amino acids, dipeptides and tripeptides), where $^{13}$C and $^{15}$N chemical shift are well resolved, thus enabling the accurate measurement of several $^{15}$N-$^{13}$C distances. However, this approach would be much more difficult for larger systems, like proteins, where the $^{15}$N chemical-shift overlap is significantly higher. More recently, Bajaj et al. (Bajaj et al. 2010) showed also the possibility of using a frequency-selective TEDOR experiment with $^{15}$N selective pulses in combination with DNP to measure a 2D correlation spectrum of only arginine $^{13}$C-$^{15}$N pairs in $^{13}$C, $^{15}$N]-labeled bacteriorhodopsin. Such an approach could be an alternative to, e.g., specific isotope labelling to obtain distance information from specific, well resolved $^{15}$N sites, as $^{15}$Nε in arginine.

![Fig. 1. Selective 3D TEDOR pulse sequence. Narrow empty and filled rectangles represent $\pi/2$ and $\pi$ pulses, respectively. Bell shapes represent selective inversion $\pi$ pulses, grey rectangles stand for $z$-filter periods. The MISSISSIPPI sequence was used for water suppression (WS) and the XY-8 phase cycling scheme was applied with following phase values: $\varphi_1=y,-y$; $\varphi_2=x,-x,-x,-x$, $\varphi_3=y,y,y,-y,-y,-y,-y$, $\varphi_{rec}=x,-x,-x,x,-x,-x$, $\varphi_1$ and $\varphi_2$ correspond to a multiple of ($2\tau_R$), where $\tau_R$ is the rotor period.](image)

The 3D TEDOR experiment starts with an adiabatic CP transfer from protons to I spins ($^{15}$N), creating Iε magnetization followed by $t_1$-evolution (Fig. 1). The subsequent REDOR period contains a train of rotor-synchronous $\pi$ pulses on the S spins ($^{13}$C) and a central refocusing-pulse pair on I and S spins. While this pulse is non-selective on the
I spins, it is frequency selective for the S spins and consists of a Gaussian pulse (Li et al. 2006) that inverts only the spins being polarized followed by a non-selective inversion pulse. The net rotation on the S spins is 0° for the spins in the bandwidth of the selected pulse and 180° for the others. The net effect of the period $t_{\text{mix}1}$ is to produce $I_S$ antiphase magnetization for the inverted S spins, and no magnetization on other S spins. After the $t_2$ evolution, the antiphase magnetization is then refocused into observable magnetization on I using a second REDOR period. Jaroniec et al. (Jaroniec et al. 2002) incorporated z-filter periods $\Delta$ in the pulse sequences in order to suppress unwanted coherences arising from $^{13}$C-$^{13}$C $J$ couplings. Subsequently, two $\pi/2$ pulses are applied on $^{15}$N channel for MISSISSIPPI water suppression (WS). The last step is the CP transfer from $^{15}$N to $^1$H spins for detection during $t_3$.

During the $t_1$, $t_2$ and REDOR periods, low power SW rf-TPPM proton decoupling (Thakur et al. 2006) is applied. The XY-8 phase cycling scheme (Gullion and Schaefer 1989) is used in order to minimize the effects of finite pulses and phase transients (Hellwagner et al. 2018). In order to fit two $\pi$ pulses in one rotor period during the REDOR recoupling, the nutation frequency of the $^{13}$C $\pi$ pulse (we used 100 kHz) must exceed the MAS frequency, which was in our case 55.5 kHz. In contrast to previous implementations (Michal and Jelinski 1997), the 3D TEDOR sequence applies the REDOR pulses to the $^{13}$C channel. Therefore, we investigated if the rotor-synchronized $\pi$ pulses on the $^{13}$C channel can cause unwanted homonuclear recoupling (see Fig. S1, Supplementary Information). We found, however, such effects to be negligible (Fig. S1).

**Results and discussion**

**Backbone assignment**

We first tested the 3D TEDOR experiment of Fig. 1 to detect HNC$\alpha$ intra-residue connectivity. In the (H)NCAH experiment the carrier frequency of the selective $^{13}$C pulse and the mixing time $t_{\text{mix}}$ need to be optimized for the transfer to $C\alpha$. The protocol we used to set up the selective pulse is described in the Supplementary Information (see Fig. S3 and S5). The carbon carrier frequency was set to the $C\alpha$ region. Using a 1.2 ms mixing time, the magnetization was transferred predominantly between directly bonded $^{13}$C-$^{15}$N pairs (Jaroniec et al. 2002), i.e., between $N^H$ and $C\alpha$ of the same residue. Using
a mixing time of 7.2 ms, the magnetization was transferred also to Cα of neighboring residues, without detectable transfer to the spectrally detuned side chains (Fig. S5).

We applied the 3D TEDOR experiment optimized for HNCα connectivities ($\tau_{\text{mix}} = 1.2$ ms and setting the carrier frequency of the $^{13}$C selective pulse to 50 ppm) on 100% back-exchanged $[^2\text{H}, ^{13}\text{C}, ^{15}\text{N}]$-labeled ubiquitin. On the same sample we also performed the 3D (H)NCAH experiment based only on CP transfers (see Supplementary Information, Fig. S2), which has been previously described (Barbet-Massin et al. 2014; Penzel et al. 2015). The 1D spectra obtained from the first FID of the two 3D experiments are compared in Fig. 2. The TEDOR experiment yielded about 50% higher intensity, due to the higher frequency selectivity and efficiency of the transfer. A quantitative comparison of the intensities with respect to a (H)NH experiments under the same conditions is found in Table 2. We note that the CP efficiency depends on the $^{13}$C and $^{15}$N $T_{1\rho}$ which are typically long (>20 ms) for our samples but also for fibrils (Smith et al. 2016) or the NS4B membrane protein (Fogeron et al. 2016) (unpublished data). The TEDOR efficiency, in contrast, depends on $T_2^*$ which was determined to be 30 ms for $^{15}$N on deuterated MLF and 40 ms for deuterated ubiquitin. The corresponding values for membrane proteins seem comparable (18 ms bulk $T_2^*$ for proteorhodopsine at 60 kHz (Lalli et al. 2017)). The $^{13}$C values are comparable but typically slightly shorter.

![Fig. 2](image_url)  

**Fig. 2.** A comparison of the first FID from two 3D (H)NCAH experiments applied to 100%-HN-
[\textsuperscript{2}H,\textsuperscript{13}C,\textsuperscript{15}N]-labeled ubiquitin recorded at 850 MHz. We compare TEDOR with a mixing time of 1.2 ms (blue trace) and only CP transfers (Penzel et al. 2015) (red trace). Both spectra were recorded with the same acquisition parameters and processed in the same way (Exp. 1 and 2, Table 1). Integrated intensities are compared in Table 2.

**Side-chain assignment: Transfer to aliphatic side chains**

Using a selective pulse with a suitable carrier frequency and optimized \textsuperscript{15}N-\textsuperscript{13}C mixing time in the pulse sequence of Fig. 1, it was possible to transfer polarization directly to side-chain carbons. Thus, we could apply the TEDOR experiment to detect connectivities between the HN pair and side-chain resonances within one residue for side-chain assignment (Fig. S6 in Supplementary Information). In order to transfer polarization to side-chain aliphatic carbons, the carrier frequency was set to the middle of the region of interest and the rf-power of the selective pulse for the inversion of the desired aliphatic part of the \textsuperscript{13}C spectrum was fine-tuned, as described in Supplementary Information.

The optimal mixing time to transfer polarization to the \(C_\gamma1\) and \(C_\gamma2\) resonances of valine and leucine is about 10 ms, as established for protonated peptides (Jaroniec et al. 2002). We experimentally determined the optimal mixing time for the transfer to leucine \(C_\gamma2\) and methionine \(C_\varepsilon\) side chains to be between 7.3 ms and 8.7 ms (Fig. S7, Supplementary Information). Depending on the relaxation properties of the sample, such long times may become inefficient and a compromise using a shorter contact time must be found.

The advantage of the TEDOR sequence compared to, e.g. the TOBSY (Baldus and Meier 1997; Hardy et al. 2003; Linser 2011) experiment for the assignment of the aliphatic side chains, is the possibility to transfer the magnetization only to a predefined \textsuperscript{13}C spectral region, as shown by the comparison of the spectra in Fig. 3. In contrast to TOBSY, the transfer is through space and may also proceed to apically neighboring residues. The left panel in Fig. 3 shows the 2D H(N)-CB version of the experiment shown in Fig. 1 (where \(t_1\) in Fig. 1 is omitted) applied to 100% back-exchanged \([\textsuperscript{2}H, \textsuperscript{13}C, \textsuperscript{15}N]\)-labeled ubiquitin, with 7.3 ms mixing time and the \textsuperscript{13}C carrier frequency set to 10 ppm. Fig. 3 shows also the spectrum obtained on the same ubiquitin sample using a \textsuperscript{1}H detected TOBSY experiment optimized for side-chain detection, with the out-and-
back scheme and a 7.8 ms long $C9_{24}^1$ mixing period (Tan et al. 2014).

**Fig. 3.** Comparison of slices from 2D (H(N)-CB(N)H correlation spectra, with red color – selective 7.3 ms long TEDOR mixing period (Table 1, Exp. 4), with blue color – $C9_{24}^1$ 7.8 ms long TOBSY mixing period (Table 1, Exp. 4). Spectra were acquired with the same acquisition parameters on 100 %-HN-[²H,¹³C,¹⁵N]- labeled ubiquitin.

In the TEDOR experiment optimized for aliphatic residues, only the selected side-chain signals are visible and with significantly higher intensity than the corresponding signals in the TOBSY spectrum (Fig. 3). In the TEDOR experiment, polarization is transferred directly from $^{15}$N to Cβ, Cγ, Cδ (Fig. 4) and the signal intensity is concentrated on the selected side-chains carbons, and not spread also to Cα and CO as in the TOBSY spectrum. The consequence is a higher intensity for the selected signals.

**Fig. 4.** Schematic representation of polarization transfer pathways from amide protons to carbons for two different experiments which can be used for the side-chain assignments for deuterated proteins: A:
TEDOR, B: C9\textsubscript{14} TOBSY. For TEDOR also inter-residue transfer is possible.

We also applied the 3D TEDOR sequence of Fig. 1 with a mixing time of 7.3 ms and the carrier frequency of the selective $^{13}\text{C}$ pulse set to 10 ppm to 100% back-exchanged [$^2\text{H}, ^{13}\text{C}, ^{15}\text{N}$]-labeled ubiquitin. The strip plots from the 3D TEDOR spectrum are shown in Fig. 5 for some representative amino acids and show the correlation between backbone $^1\text{H}$ and $^{15}\text{N}$ chemical shifts and aliphatic side-chains $^{13}\text{C}$ resonances. From the connectivities detected in the spectrum and from the structure of ubiquitin (2L3Z (Huber et al. 2012)), we observe that in the TEDOR experiment optimized for aliphatic residues, it is possible to transfer polarization directly from $^{15}\text{N}$ to $^{13}\text{C}\beta$, $^{13}\text{C}\gamma$, $^{13}\text{C}\delta$ (Fig. 4) which are located within a sphere of 3-4 Å radius from the nitrogen atom. These are mostly carbons within the same residue. However, carbons from neighboring residues which are located within a similar distance can be also seen in the selected strips (see Fig. S8, Supplementary Information).
Fig. 5. Strip plots from 3D TEDOR spectrum of 100 %-HN-[²H,¹³C,¹⁵N]-labeled ubiquitin (Table 1, Exp. 5) showing the correlations between protein backbone HN and aliphatic side-chains carbons for some amino acids. The spectrum has been acquired with mixing time of 7.3 ms and Q3 selective pulse at 10 ppm in the ¹³C dimension.

**Side-chain assignment: Transfer to aromatic side chains**

We also checked if the TEDOR pulse sequence can be used for transfer to the aromatic side-chain carbons. Setting the carrier frequency to 120 ppm, we could detect polarization transfer to all four aromatic residues present in the protein. The strip plots of the 3D TEDOR spectrum in Fig. 6 show correlations between backbone HN and aromatic side-chains carbons. For Phe and His residues, polarization was transferred until Cγ and Cδ side-chain carbons, but for Tyr only until Cγ.

Fig. 6. Strip plots from the aromatic part of 3D TEDOR spectrum of 100%-HN-[²H,¹³C,¹⁵N]-labeled ubiquitin (Table 1, Exp. 6). The experiment was acquired with a mixing time of 8.7 ms and Q3 selective pulse at 120 ppm.
Conclusions

We describe the application of frequency-selective TEDOR experiments for the correlation of HN and carbon spins selected according to their resonance frequency (e.g. either Ca, aliphatic or aromatic sidechains). We found that the polarization transfer is more efficient than for CP/INEPT alternatives. The experiments were recorded at 55 kHz MAS. Expansion to higher spinning frequencies would require stronger radio-frequency pulses not presently possible in, e.g., 110 kHz probes, but already demonstrated for small coil diameters (Brinkmann et al. 2010). Such experiments could be even more efficient at higher spinning frequencies, due to increased $T_2'$ relaxation times at higher MAS frequencies (Penzel et al. 2015).

Experimental

Sample preparation

100 %-HN-[2H,13C,15N] ubiquitin was expressed in *E. coli* and crystallized in protonated MPD with H2O to re-protonate the exchangeable sites, as previously described (Igumenova et al. 2004).

After crystallization, crystals were harvested and packed into a rotor with 1.3 mm diameter by using a home-built device (Böckmann et al. 2009). The temperature of the sample was set to 18 °C, as estimated from the resonance frequency of water.

NMR spectroscopy

Solid-state NMR spectra were acquired at 9.4, 11.7, and 20.0 T static magnetic field strengths, using a 1.3 mm Bruker MAS probe. The MAS frequency was set to 55.555 kHz, which resulted in $\tau_r = 18 \mu$s. We used $\pi/2$ pulses of 2.5 $\mu$s for $^1$H, $^{13}$C and $^{15}$N nuclei. The selective $\pi$ pulse was 0.3 or 1 ms long with Q3 shape (Li et al. 2006).

During $t_1$, $t_2$ and REDOR periods, a 5 kHz SW-$r$-TPPM decoupling was applied. More parameters for each experiment are summarized in the Table 1. All spectra were processed with the software Topspin (version 3.5, Bruker) and analyzed with the software CcpNmr (Vranken et al. 2005).

Resonances were internally referenced to the chemical shift of the CH in MPD at 4.1 ppm.
Table 1: Experimental parameters used for the solid-state NMR experiments

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<tr>
<th>Experiment</th>
<th>HNC (H)NCA H TEDOR (1)</th>
<th>HNC HNCA (2)</th>
<th>H(N)C TEDOR (3)</th>
<th>H(N)C TOBSY (4)</th>
<th>HNC aliphatic TEDOR (5)</th>
<th>HNC aromatic TEDOR (6)</th>
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<td>HC-CP</td>
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<td>8.9</td>
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**Table 2:** Transfer efficiencies of main- and side-chain TEDOR transfer compared to a \(^{1}\text{H}NH~2\text{D}\) spectrum with CP transfer. The quantitative data were obtained at 400 MHz and represent the overall intensity. While the TOBSY has a higher intensity than the HNCx TEDOR, the sidechains are more intense in the TEDOR (see Fig. 2).

<table>
<thead>
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<th>Experiment</th>
<th>Relative efficiency</th>
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<tr>
<td>((H)NH)</td>
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<tr>
<td>((H)NCA)H TEDOR 1.2 ms</td>
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<tr>
<td>((H)(N)Cx)H TOBSY 7.8 ms</td>
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<tr>
<td>((H)NC)x H TEDOR 8.7 ms (aromatic)</td>
<td>0.02</td>
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References


