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Hyperpolarization

Peptide Hydrogenation and Labeling with Parahydrogen**

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The assignment of specific fragments of peptides or proteins in NMR spectra can be challenging for complex structures with overlap of signals possessing similar chemical shifts. One attractive choice to address this issue is the use of isotopically labeled molecules. The labeling process may be difficult, however, for naturally occurring biomolecules. Recently, the use of hyperpolarized molecules has attracted much attention. Hyperpolarized ^{129}Xe gas has been used to examine protein interactions and binding dynamics.[1] Dynamic nuclear polarization (DNP) methods, including chemically and photochemically induced polarization (CIDNP) can be used to obtain information, including structural features and folding dynamics of proteins.[2] Herein, we demonstrate hyperpolarization of the naturally occurring peptide antibiotic, thiostrepton, selectively at terminal dehydroalanine residues using parahydrogen-induced polarization (PHIP).[3] This approach revealed NMR spectral features for the hydrogenated residues among heavily overlapped peaks, demonstrating site-specific spin polarization labeling of a biomolecule. Surprisingly, polarization transfer to a remote alanine residue was also observed. The diastereoselectivity of the hydrogenation reaction was determined using PHIP in conjunction with other data. These results show that parahydrogen can be used to enhance signals and elucidate the hydrogenation process of dehydropeptide units in complex biomolecules.

PHIP is an efficient and economical strategy to gain a substantial signal boost.[3] In this method, polarization enhancement can be achieved either by direct hydrogenation, or by reversible polarization exchange in intermediate processes. In the signal amplification by reversible exchange (SABRE) approach,[4] the polarization is exchanged from parahydrogen (p-H_2) to the target molecule via an organometallic intermediate complex. SABRE achieves polarization transfer without permanently altering the chemical structure of the substrate, but not all molecules can be polarized, and only particular examples of relatively small molecules have been polarized effectively to date.[4a,c] The other method, involving hydrogenation with p-H_2, is relatively simple to implement and, in principle, can be performed on any sample possessing an asymmetric multiple bond. In this case also, only relatively simple molecules have been examined by this method to date.[5] As an example of applying PHIP methods to molecules typical of those found in biology, we describe herein the monitoring of a homogeneous hydrogenation reaction with Rh^I complexes, taking advantage of the "only parahydrogen spectroscopy" (OPSY) technique[6] for the analysis of reaction stereoselectivity and conformational dynamics of peptide units.

Although in prior work the hydrogenation of a dehydroamino acid derivative was demonstrated,[7] scaling up the procedure to larger molecules is nontrivial. Herein, we sought to examine a peptide by PHIP. Thiostrepton (Scheme 1) is a naturally occurring antibiotic belonging to a family of thiopeptides including micrococcin P1 and nosiheptide, and is active against Gram-positive pathogens. It has also shown antimalarial, chemotherapeutic, and other biological activ-

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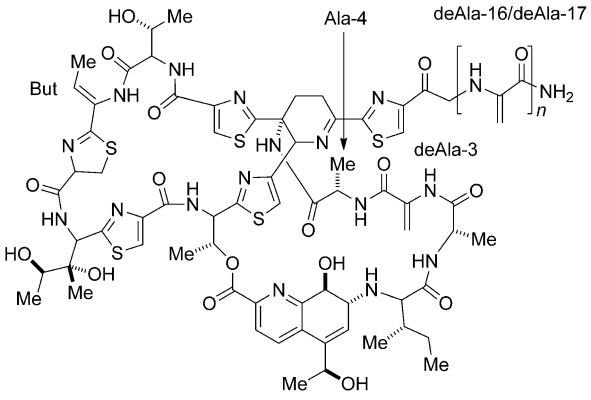

Scheme 1. Molecular structures of thiostrepton (**1**: $n=2$ with deAla-16 (proximal) and deAla-17 (distal) at the tail) and derivatives (**2**: $n=1$ with deAla-16 at the tail, **3**: $n=0$). Compound **4** is the product of hydrogenation of both deAla-16 and deAla-17 in thiostrepton, while **5a** is the product of hydrogenation of only deAla-16, and **5b** is the product of hydrogenation of only deAla-17. Compound **6** is the product of hydrogenation of deAla-16 in compound **2**.

ity.[8] Recently, thiostrepton was reported to exhibit activity against breast cancer cells.[9]

The hydrogenation reaction of thiostrepton with a heterogeneous Pd/C catalyst was reported, but required harsh conditions and long reaction times.[10] We found that homogeneous catalysis with $[Rh(cod)(diop)]BF_4$ (cod = 1,5-cyclooctadiene, diop = 2,2-dimethyl-1,3-dioxolane-4,5-diylbis(methylene)bis(diphenylphosphine)) for one hour at room temperature in a 4:1 mixture of $CHCl_3$/MeOH gave a 90% yield of hydrogenated product **4** (Scheme 1). Thiostrepton possesses several unsaturated groups in the structure, including three dehydroalanines (deAla) and a dehydrobutyrine (But). Electrospray ionization mass spectrometry (ESI-MS, negative mode) of the product under hydrogenation for one hour showed a peak at m/z 1665.9 $[M-H^+]$, an increase of mass by four units, compared with thiostrepton (m/z = 1661.8), indicating a selective reduction of only two of the double bonds (Supporting Information, Figure S1). Further addition of hydrogen at longer reaction times was not observed. The ^{1}H NMR spectra of the product showed depletion of the peaks at δ = 5.56/6.46 ppm and δ = 5.65/6.64 ppm, corresponding to the CH_2 protons of deAla-16 and deAla-17 (Scheme 1, numbering according to linear peptide sequence; Supporting Information, Figures S2 and S3).[11] The signals for deAla-3 (δ = 5.77/5.30 ppm) and But (δ = 6.17/1.55 ppm) were unaffected.

PHIP was used to monitor the reaction in combination with OPSY, which suppresses thermal peaks and isolates only hyperpolarized peaks. The ^{1}H OPSY spectra observed by hydrogenation of thiostrepton are shown in Figure 1. Resonances at δ = 4.5–4.6 ppm and δ = 1.4–1.5 ppm correspond to hydrogenated Ala-16 and 17 in **4**. At δ = 4.33–4.40 ppm and δ = 1.33–1.41 ppm, these resonances correspond to products with addition of one equivalent of H_2 (that is, product **5a** with Ala-16/deAla-17 or **5b** with deAla-16/Ala-17), the formation of which has been confirmed by high-resolution ESI-MS-TOF spectrometry and with ^{1}H–^{13}C HSQC NMR experiments with samples examined at comparable states of hydrogenation (Supporting Information, Figures S4 and S5). Surprisingly, additional hyperpolarized peaks were observed at δ = 4.69 and 1.37 ppm, which correspond to Ala-4 protons according to assignments by 1D and 2D NMR spectra and values found in the literature (Supporting Information, Figures S5–S7; Ref. [11b]). The OPSY spectrum was simulated using chemical shifts and J-couplings from both literature values[11b] and 2D data, and it agrees well with the experimental results (Figure 2 A). Interestingly, however, the Ala-4 group does not possess any unsaturated bonds and hence cannot give a hyperpolarized signal in the OPSY spectrum from simple PHIP, and the signal must therefore derive from polarization transfer.

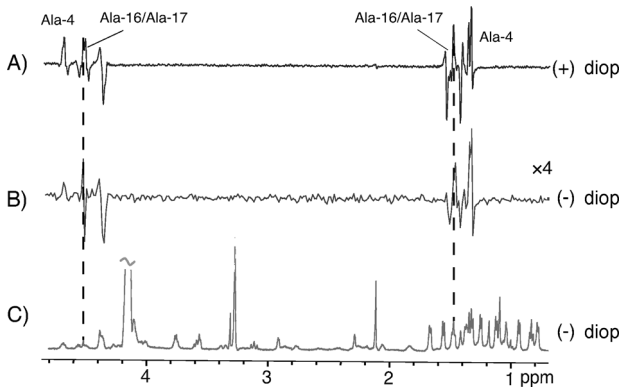

Figure 1. ^{1}H NMR spectra of hydrogenated thiostrepton: OPSY spectra of A) product hydrogenated with $[Rh(cod)(+diop)]BF_4$; B) $[Rh(cod)(-diop)]BF_4$; C) thermal spectrum with $[Rh(cod)(-diop)]BF_4$.

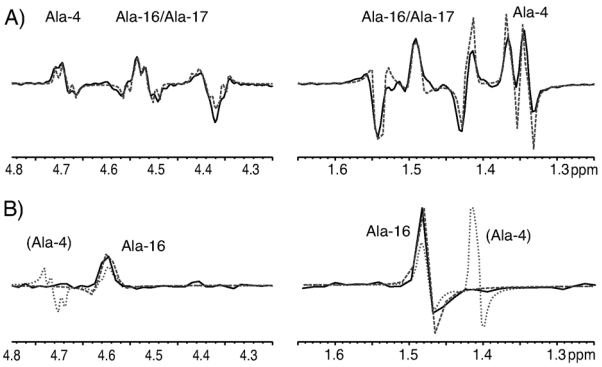

Figure 2. Expanded spectra in the regions of δ = 4.1–4.8 ppm and δ = 1.2–1.7 ppm. A) Hydrogenation of **1** (forming **4**) using (+)diop; B) hydrogenation of **2** (forming **6**) using (+)diop. —— experimental OPSY spectra, ----- simulated OPSY spectra, ••••• simulated OPSY spectra assuming there is hyperpolarization transfer to Ala-4. The disagreement between experiment and simulation in (B) indicates that there is no transfer to Ala-4 in compound **6**.

In an effort to determine the role of the newly formed alanine residues in providing polarization to the Ala-4 residue, compound **3**, which does not possess any deAla residues in the tail, was synthesized. The PHIP reaction with this compound did not show any signal in the OPSY spectrum. From this it can be concluded that the polarization transfer to Ala-4 could arise either from an intermediate transfer during the hydrogenation of deAla-16 and deAla-17 or from a through-space transfer after the hydrogenation. This conclusion is reinforced by the fact that attempting the PHIP experiment with **4** under the same conditions did not produce any polarization enhancement. Therefore, a plain SABRE-type nonhydrogenation mechanism for Ala-4 can be ruled out.

Anti-phased signals at the Ala-4 frequency are continuously observed in sequential OPSY experiments, indicating that the hydrogenation reaction continues at high magnetic field, and therefore the PASADENA mechanism applies here. At high field, the isotropic mixing effect, which is required for an efficient through-bond transfer, is extremely unlikely. Therefore, an NOE-type through-space transfer appears to be the more plausible explanation.

Both mechanisms require a relatively short distance between Ala-4 and the hyperpolarized spins in the tail region. ^{1}H NMR results suggest that the dehydroalanine tail is oriented away from the main body of the molecule,

although it is also proposed that the tail is very flexible.[11b] Indeed, examination of molecular models indicates that rotations about the C–C and C–N sigma bonds within the deAla-16 and deAla-17 residues bring the deAla-17 and Ala-4 residues within approximately 3 Å of each other (Supporting Information, Figure S8), which may allow such a polarization transfer to occur.

To test which newly formed Ala residue (Ala-16 or Ala-17) is responsible for the polarization transfer, compound **2** was synthesized. Possessing only deAla-16, models indicate that the shorter tail of compound **2** does not have enough flexibility to achieve proximity to Ala-4. Figure 2B shows the OPSY spectrum from the hydrogenation reaction of **2** (forming **6**) together with a simulated spectrum, assuming hyperpolarization of both Ala-16 and Ala-4. The experimental spectrum, however, shows only the newly formed Ala-16 signals. As is clearly seen, no transfer has occurred in compound **6**, which leaves the deAla-17 derived alanine as responsible for the polarization transfer in the case of thiostrepton.

An examination of the ROESY spectrum of the hydrogenated thiostrepton finds no cross-peak between Ala-4 and Ala-16/Ala-17 (Supporting Information, Figure S7), indicating that the conformational change which brings the tail of thiostrepton close to Ala-4 must happen during the hydrogenation process in an intermediate state with the catalyst. It can therefore be concluded that the polarization transfer seen here is the result of a conformational change during hydrogenation, facilitating an efficient NOE-type transfer mechanism. Computational predictions and NMR structures show that the dehydroalanine tail is flexible, a factor thought to be important for proper binding to the target receptor.[11b]

Bargon et al.[12] determined the stereoselectivity of a mixture of unsaturated racemic alcohols with Rh^I metal complexes with parahydrogenation. The determination of enantioselectivity in peptides is very challenging; although differentiation between diastereomers of peptides by NMR spectroscopy has been performed for peptides possessing as many as 18 amino acid residues,[13] it is a nontrivial task. Hydrogenation was performed using the two enantiomers of the catalyst [Rh(cod)(±diop)]BF_4. The ^{1}H OPSY NMR sequence used for the analysis of the reaction showed that the peaks appearing from the newly formed alanines show 7–10 Hz difference in chemical shifts for both the CH and CH_3 peaks (Figure 1). The ROESY spectra also indicate that the two products have different structures in the tail region. Owing to the complexity of the spectrum of thiostrepton, the reaction was further examined with derivative **2**. ^{1}H NMR spectra of **6** using the two enantiomeric diop ligands are shown in Figure 3. The methyl peak at $\delta = 1.47$ ppm shows clearly that the spectra of the two diastereoisomers partially overlap. The difference in chemical shift of about 7 Hz between the two isomers suggested that the product from (+)diop-containing catalyst gave a downfield shifted resonance for the methyl substituent, with a diasteriomeric excess of approximately 59%. The (−)diop-containing catalyst favored the product with upfield shifted methyl group with a diastereomeric excess of approximately 65%. The methine groups in the $\delta = 4.58$ region overlap heavily. OPSY spectra confirmed these results (Supporting Information, Figure S9), showing two sets of methyl groups and only one set of resonances for CH.

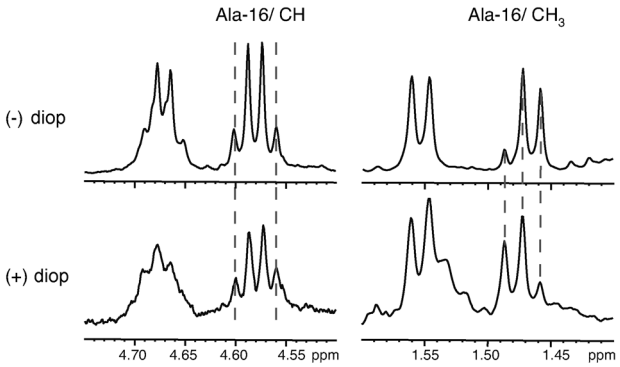

Figure 3. Methyl and methine regions of the ^{1}H NMR spectra of **6**.

In conclusion, we have examined a hydrogenated form of the thiostrepton antibiotic molecule with PHIP. Apart from observing enhanced (and labeled) polarization in the hydrogenated groups, we also identified and verified a polarization transfer mechanism to a remote amino acid residue, which is most likely due to a catalyst-mediated through-space transfer, in an intermediate state of the reaction. Furthermore, the diastereoselectivity of the hydrogenation reaction of this complex molecule was determined. It can be anticipated that similar studies should be possible with other peptides belonging to the important thiopeptide family to examine structural changes and specific features that would be otherwise undetectable with standard methods.

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