The $^{13}C={}^{18}O/{}^{15}N$ Isotope Dependence of the Amide-I/II 2D IR Cross Peaks for the Fully-Extended Peptides

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Abstract

We have used a combination of 2D IR spectroscopy with $^{13}C=^{18}O$ labeled amide-I and ^{15}N labeled amide-II modes to reveal how vibrational coupling between labeled peptide units depends on secondary structure. Linear and 2D IR measurements and simulations of $C^{\alpha,\alpha}$-diethylglycine homo-tetrapeptide show that this compound adopts the fully-extended $(2.0_5$-helical) conformation in $CDCl_3$, consistent with previous work on the Ac-capped peptide. The amide-I/II cross peaks of isotopomers exhibit only a marginal isotope frequency shift between labeled modes that are separated by two peptide units, indicating a very weak coupling. This result is in sharp contrast with a large cross-peak shift observed in 3_{10}-helical peptides, in which the labeled amide-I and II modes are connected through an inter-residue $C=O\cdots H-N$ hydrogen bond. The discovered 3D-structural dependence indicates that the $^{13}C=^{18}O/^{15}N$ labeled amide I/II cross peaks can distinguish the formation of a single 3_{10}-helical turn from the fully-extended polypeptide chain, and increases the versatility of 2D IR spectroscopy as a conformational analysis tool of biomolecules.

I. Introduction

Isotope editing empowers vibrational spectroscopic methods by not only facilitating the assignment of vibrational modes but also making it possible to probe specific local structure and dynamics even in relatively large systems.[1-6] For example, labeling amide units in peptides and proteins with ^{13}C, ^{18}O, ^{15}N or D isotopes can significantly modify the vibrational properties of the labeled amide modes. The amide-I frequency decreases by about 65 cm^{-1} with the ^{13}C and ^{18}O labeling,[2,6] which is large enough to isolate the labeled residue from the rest. The hydrogen and deuterium exchange leads to a redshift of the amide-II frequency by 60–90 cm^{-1},[7,8] which enables researchers to trace water dynamics, solvent accessibility, and structural evolution.[7,8]

Combining the site specificity of isotope labeling with the conformational sensitivity of two-dimensional infrared (2D IR) spectroscopy is a powerful approach to reveal more detailed structural information.[9-20] Our previous experimental studies on unlabeled and isotope labeled Aib (α-aminoisobutyric acid or C$^{\alpha,\alpha}$-dimethylglycine)-rich 3_{10}-helical model hexapeptides discovered that the cross peaks between the amide-I and amide-II modes in the 3_{10}-helical ^{13}C=^{18}O···H–^{15}N hydrogen-bonded peptide units can be detected and manifested as an isotope shift in nonrephasing 2D IR spectra.[17,18,21] Conversely, if the peptide backbone extends and the ith and (i+2)th residues no longer interact with each other through the formation of an inter-residual hydrogen bond, the amide-I/II cross peaks will not exhibit isotope shift, based on our theoretical calculation for a peptide in the semiextended structure [(ϕ, ψ) = (−78°, 146°)].[18] Therefore, we expect that the isotope dependence of the amide-I/II cross peaks can serve as a sensitive probe to monitor the formation of a single helical turn from an extended structure.

The above theoretical prediction needs to be further consolidated by experimental confirmation on model peptides that are in the semiextended or fully-extended conformations under the same condition. To this end, the fully-extended conformation, or the so-called 2.0_5-helix, with the backbone dihedral angles (ϕ, ψ) centered at (±180°, ±180°),[22] is an excellent choice. Our recent 2D IR study of Ac-(Deg)$_n$-OtBu (Ac = acetyl; Deg =C$^{\alpha,\alpha}$-diethylglycine; OtBu = $tert$-butoxy, n = 2–5), showed that the solution conformation of this series of Deg homo-peptides in deuterated chloroform is well characterized as fully extended and the onset of 2D cross-peak spectral patterns occurs at n = 3.[23] Although one can distinguish between the fully-extended structure and the 3_{10}-helix based on their distinctly different linear IR spectra and 2D IR spectra, the spectral signatures originate from the exciton couplings of the amide modes over the entire peptide chain and do not provide site-specific information. Isotope labeling is still required to reveal whether the C=O group of the ith peptide unit couples with the N–H group in the (i+2)th peptide unit in the fully-extended structure.

In this study, we measured 2D IR spectra of the unlabeled and isotope labeled Deg homo-tetrapeptide, Tfa-(Deg)$_4$-OtBu (Tfa = trifluoroacetyl) to investigate the isotope effects on the cross-peak pattern between the amide-I and amide-II modes. The backbone conformation of this peptide is fully extended in the crystalline state.[24] The FT IR spectrum of unlabeled tetrapeptide (TD4UL, Figure 1 top)

in CDCl$_3$ exhibits a very similar spectral signature to that of the Ac-capped peptides.[23] It is conceivable that the Tfa-capped peptide also forms the fully-extended conformation in solution, as suggested by ^{1}H-NMR studies,[25,26] but this requires confirmation from 2D IR studies. Isotope labeling on the second peptide unit with ^{13}C and ^{18}O (TD4SL) can isolate the amide-I mode of this unit from the other unlabeled amide-I modes, and the 2D IR amide-I/amide-II cross-peak pattern will tell us the coupling between the labeled amide-I and the unlabeled amide-II modes. However, the experimental studies on *N*-methylacetamide indicated that the amide-I and amide-II modes in the same peptide unit are strongly coupled.[27,28] Another isotope label on the amide-II mode of interest is required to separate the inter-residue and intra-residue amide-I/amide-II couplings. To this end, we prepared bis-labeled tetrapeptide (TD4DL) which has the ^{13}C=^{18}O group in the second peptide unit and ^{15}N in the fourth peptide unit. The labeled groups form a C=O···H−N inter-residue hydrogen bond if the backbone adopts the 3$_{10}$-helical conformation [(ϕ, ψ) = (−57°, −30°)],[29] but they are distant from each other if the backbone adopts the fully extended conformation (Figure 1 bottom). If the amide-I/II cross-peak patterns are the same for TD4SL and TD4DL, it will provide an important experimental evidence that the vibrational coupling strength between the labeled amide-I and amide-II modes is much smaller in the fully-extended conformation than in the 3$_{10}$-helix and therefore the labeled amide-I/II 2D IR cross peaks can be utilized as a unique probe to get insight into the helix nucleation process.

This paper is organized as follows. The next section describes the synthesis and characterization of Tfa-capped tetrapeptides as well as FT IR and 2D IR measurements. Experimental spectra are presented in section III. In Section IV, vibrational property calculations using the ONIOM method and model calculations of the linear and 2D IR spectra are described. Experimental and simulation results are compared. In section V, we discuss the isotope effects on the amide-I/II cross-peak patterns of the fully-extended structure, then point out the differences from those of the 3$_{10}$-helical conformation we studied previously.[17,18] Concluding remarks are given in the last section.

II. Experimental Section

A. Materials. The Tfa-capped tetrapeptide and its isotope analogues were synthesized by reference to previous works,[30,31] and identified with mass spectrometry and ^{1}H-NMR measurements. The only way to prepare the Deg homo-peptides in a reasonable yield is to use their N^α-trifluoroacetylated peptides and to activate them as 5-(4*H*)oxazolones. However, this procedure does not allow us to obtain ^{18}O labeled peptides at high purity. We found that the isotope labels of ^{13}C and ^{15}N were perfectly (100%) introduced into the tetrapeptides whereas the ^{18}O content was about 33% based on the analysis of the measured mass spectra. Hence, the synthesized monolabeled peptide was a mixture of ^{13}C=^{16}O/^{14}N (TD4SL*) and ^{13}C=^{18}O/^{14}N (TD4SL) with a ratio of 2:1. In the same manner, the bis-labeled peptide contained ^{13}C=^{16}O/^{15}N (TD4DL*) and ^{13}C=^{18}O/^{15}N (TD4DL) with the same ratio. Deuterated chloroform (99.96%D, Cambridge Isotope Laboratory) was used without further purification and desiccation. Peptide solutions

with 10 mM concentration were prepared and held in a 250-μm-thick sample cell. Previous FT IR measurements confirmed that spectral features of Tfa-capped Deg homo-peptides remained unchanged in the concentration range of 0.5-50 mM in CDCl$_3$.[26]

B. Linear and 2D IR measurements. Linear IR spectra of the peptide solutions and neat CDCl$_3$ were measured using a FT IR spectrometer (Nicolet 860, Thermo Scientific) with a 4-cm^{-1} resolution and averaged over 64 scans under dry air purging.

Details of our 2D IR spectrometer, data acquisition and processing have been described previously.[18,32-35] We will only briefly describe the 2D IR experimental conditions for the peptides in this study. Three 100-fs IR pulses with wavevectors k_a, k_b and k_c were focused onto the sample solutions. An induced third-order nonlinear signal was measured using a spectral interferometric method with a combination of monochromator and a 64-element MCT array detector that gives a spectral resolution of ~4 cm^{-1}/pixel. Polarization directions of the three IR pulses (a, b, and c for the k_a, k_b, and k_c pulse) and the signal (d) are denoted as $\langle a, b, c, d \rangle$. Rephasing (R, a-b-c) and nonrephasing (NR, b-a-c) pulse sequences were generated by adjusting the delay time τ between the first and second pulses, and the delay time T between the second and third pulses. In both R and NR measurements, τ was scanned from 0 to ~3 ps with a 9-fs time step. The waiting time T was set to 0 for R under the double-crossed $\langle \pi/4, -\pi/4, Y, Z \rangle$ polarization configuration and NR under the $\langle \pi/4, Y, -\pi/4, Z \rangle$ polarization configuration, and set to 250 fs for both R and NR under the perpendicular $\langle Y, Y, Z, Z \rangle$ polarization configuration to minimize the nonresonant solvent response. The local oscillator pulse preceded the signal by 800 fs. The frequency axis ω_τ in the 2D IR spectra was obtained by Fourier transform along τ, and ω_t was experimentally determined at each element of the array detector. The central frequency of IR pulse was tuned to ~1666 cm^{-1} to measure the amide-I cross-peak patterns. In measurement of the amide-I/II 2D IR spectra, it was set to ~1580 cm^{-1} so that both of the amide-I and amide-II bands were excited with almost the same IR intensity. All spectra were collected at ambient temperature (20 ± 1 °C).

III. Experimental Results

A. Isotope and capping group effects on FT IR spectra. Figure 2 shows measured FT IR spectra of TD4UL, the mixture of monolabeled tetrapeptide (TD4SL/TD4SL*), and the mixture of bis-labeled peptide (TD4DL/TD4DL*) dissolved in CDCl$_3$. The FT IR spectrum of Ac-(Deg)$_4$-OtBu (AD4UL) was also measured for comparison. All the spectra were normalized by the peak absorbance of the ester C=O stretching band at 1721 cm^{-1} after subtracting the solvent background spectrum.

Characteristic IR active bands of TD4UL shown in Figure 2a are assignable to the amide-I modes (1620–1700 cm^{-1}), the amide-II modes (1460–1540 cm^{-1}) and the methyl deformations (~1460 cm^{-1}), according to the results of normal mode analysis (see Section IV A). The absorbance at 1721 cm^{-1} is much stronger than that observed in the spectrum of the Ac capped peptide (Figure 2b). This is because the carbonyl frequency of the Tfa group is higher than that of the Ac group and the band overlaps with the

ester C=O band in the same frequency range,[26] whereas the carbonyl of the Ac group overlaps with the amide-I band. The frequency difference between the Ac and Tfa groups also affects spectral shape of the amide-I mode, despite the same number of Deg residues. The peak separation of the amide-I doublet is 22 and 27 cm^{-1} for TD4UL and AD4UL, respectively. The capping group also affects the amide-II profile as well. Comparing to the AD4UL, the higher amide-II frequency due to the Tfa group leads to a 4 cm^{-1} blue shift of the strongest amide-II exciton band, appearing at 1492 cm^{-1}. It also gives rise to a peak that appears as a shoulder at ~1520 cm^{-1}. The integrated area of the amide-II band is larger than that of the amide-I, and the ratio is 2.0 for TD4UL. The appearance that this is larger than the ratio for AD4UL, 1.3, is because the area of the Tfa carbonyl band is not included. The linear IR features in the amide-I and II regions of TD4UL are not significantly different from those observed for AD4UL, although the capping groups are different.

The introduced isotope labels changed the amide-I and II spectra, while the capping modes remained intact. In the spectrum of the monolabeled peptide mixture (Figure 2c), two additional peaks appeared at 1623 and 1601 cm^{-1} due to ^{13}C=^{16}O and ^{13}C=^{18}O, respectively. The original amide-I doublet pattern is not as clear as that of TD4UL. The amide-II exciton band is also affected by the presence of ^{13}C, because this mode is mostly an out-of-phase combination of N–H bending and C–N stretching.[7] The peak frequency of the largest amide-II band is 1487 cm^{-1}, which is lower than that of TD4UL by 5 cm^{-1}. The mixture of bis-labeled peptide (Figure 2d) shows a similar amide-I spectral pattern to that of the monolabeled one because the mode is mainly the C=O stretching and hence is not affected by the ^{15}N label. The effects of ^{15}N can be discerned as the redshift of the largest amide-II peak by 2 cm^{-1} from that of the monolabeled mixture.

B. The amide-I 2D cross-peak patterns. Figure 3a shows the absolute magnitude 2D IR amide-I spectrum of TD4UL measured with the R pulse sequence and $\langle \pi/4, -\pi/4, Y, Z \rangle$ polarization configuration. This polarization is capable of suppressing diagonal peaks and therefore cross peaks appear more prominent in the 2D spectrum.[36] There are two clear peaks in the off-diagonal region, one at $(\omega_\tau, \omega_t) = (-1678, 1653)$ cm^{-1} and the other at $(-1656, 1678)$ cm^{-1}. This 2D IR spectral pattern looks very similar to that observed for AD4UL in the previous study.[23]

The amide-I NR cross-peak patterns measured with $\langle \pi/4, Y, -\pi/4, Z \rangle$ are plotted in Figure 3c. Two dominant cross peaks appear in the off-diagonal regions because cross peaks in the diagonal region are suppressed in this polarization.[23] This pattern is quite different from the previous NR cross-peak patterns obtained for other peptides using the double-crossed $\langle \pi/4, -\pi/4, Y, Z \rangle$ configuration where cross peaks in the off-diagonal regions are suppressed but those in the diagonal region remain.[32,37] Using the $\langle \pi/4, Y, -\pi/4, Z \rangle$ polarization to acquire NR spectra provides the advantage that cross peaks can in principle be more clearly observed without overlapping with residual diagonal peaks that are not completely suppressed. The observed R and NR 2D IR spectra in Figures 3a and 3c are quite distinct because the two pulse sequences have different molecular response functions and the ways that vibrational dynamics

manifest in the spectra are different. Besides strong cross peaks coming from amide-I couplings, weak cross peaks resulting from couplings between the amide-I modes and the C=O modes of the ester and/or Tfa capping groups are also observed.

C. The amide-I/II 2D IR spectra. The NR spectra of the amide-I and II modes collected with the perpendicular polarization configuration are presented in the left column of Figure 4. We chose to use the NR pulse sequence because our previous studies have shown that diagonal peaks are better resolved and cross peaks are more distinct in the NR spectra than in the R spectra.[18,23] Also, cross peaks are usually more pronounced using the perpendicular polarization than the parallel polarization. All the spectra were normalized by the largest amplitude of the amide-II diagonal peak.

Compared to the cross-peak spectral patterns in Figure 3, two diagonal peaks $[(\omega_\tau, \omega_t) = (1654, 1648)$ and $(1677, 1677)$ cm$^{-1}]$ are observed in the amide-I frequency region of TD4UL, along with the two cross peaks between them (Figure 4 top-left panel). The amide-II diagonal peak is elongated upward along the ω_τ axis, and to the right along the ω_t axis. The elongation along the frequency axes can be a result of the tails of the diagonal peak and/or cross peaks between the main peak and the shoulder. Several weak cross peaks between the amide-I and II modes appear at $(\omega_\tau, \omega_t) = (1650, 1490)$ and $(1480, 1650)$ cm^{-1}.

The mixture of monolabeled peptides exhibited additional amide-I/II cross peaks in the 2D NR spectrum (Figure 4 middle-left panel). The cross peaks at $(1623, 1492)$ and $(1601, 1492)$ cm^{-1} originate from the couplings between the ^{13}C=^{16}O amide-I and amide-II modes, and between the ^{13}C=^{18}O amide-I and amide-II modes, respectively. How the peak frequency of the latter cross peak changes upon adding a ^{15}N isotope label will provide us with insight into whether the labeled peptide units are coupled or not. In Figure 5a, we plot slices of the absolute magnitude 2D NR spectra along the horizontal dashed lines at ω_τ = 1601 cm^{-1} (Figure 4) to show how the spectral profiles depend on the isotope labeling. The slices mainly contain the information of couplings between the ^{13}C=^{18}O amide-I and amide-II modes with very little contamination due to the undesirable remnant of ^{13}C=^{16}O. Although TD4UL does not have a ^{13}C=^{18}O labeled amide-I mode, we still see a lobe in this frequency range due to the strong amide-II diagonal peak spreading along the vertical axis. For the monolabeled peptide, the increased intensity is due to the cross peaks from couplings between the ^{13}C=^{18}O amide-I mode on the second peptide unit and the amide II modes. The 2D spectral pattern of the bis-labeled peptide is shown in Figure 4 (bottom-left panel). The slice (Figure 5a) has almost the same cross-peak intensity as that of the monolabeled one. The peak maximum is at $\omega_t = 1489$ cm^{-1}, and hence introducing a ^{15}N label on the fourth peptide unit causes a frequency shift of about 3 cm^{-1} for the cross peak. The very weak shoulder at ~ 1500 cm^{-1} might be related to an additional isotope effect induced by ^{15}N.

IV. Computations

A. Vibrational properties from ONIOM calculations. The amide-I/II linear and 2D IR spectra were calculated based on vibrational exciton model.[38] The success of model calculation depends on proper construction of the exciton Hamiltonian, namely, the local mode energies and vibrational couplings of the system. In our previous study of a series of Ac-capped Deg homo-peptides, we already obtained a parameter set that reasonably well reproduced the experimental results of their linear and 2D IR spectra. Therefore, we first checked the effects of the Tfa-capping group on the parameters, and amended them as needed.

Quantum mechanical calculations were carried out using the Gaussian 03 package.[39] Structural optimization and normal mode analysis of TD4UL were performed with all the dihedral angles fixed at 180° using the ONIOM method.[40] Because the peptide has large side chains, treating it fully quantum mechanically would be quite time consuming. Instead, the AM1 semi-empirical method was applied to the atoms in the Deg ethyl side chains and the three methyl groups of the C-capping *tert*-butyl group. All other atoms were treated with the DFT method at the B3LYP/6-31+G(d) level. We chose this basis set because it provides a good balance between computational time and accuracy. It has been shown that using a large basis set such as 6-311++G(d,p) does not improve accuracy substantially over the smaller 6-31G(d) and 6-31+G(d) basis sets.[41] To obtain local mode frequencies, each peptide unit was isolated from the others by isotope labeling ^{12}C, ^{16}O and H atoms of the peptide units with ^{13}C, ^{18}O and D. The local mode frequencies, and the magnitude and orientation of transition dipole derivatives are summarized in Table 1. The frequencies of the amide-I modes and the Tfa and ester C=O stretchings were scaled by a factor of 0.9774, and those of the amide-II modes by 0.9665.[41]

Comparisons of the calculated results between the Ac- and Tfa-capped peptides reveal the effects of the capping group. The most significant difference is the local amide-I mode frequency of the first peptide unit to which the Tfa group directly connects. The frequency increased by 48 cm^{-1} from that of the Ac capping, and becomes closer to the ester C=O stretching frequency. This behavior is consistent with what is observed in the FT IR spectra (Figures 2a and b). The amide-I frequency of the second unit also slightly increased. In contrast to the amide-I modes, Tfa capping only marginally affected the amide-II local mode frequency of the first unit. Changing the capping group from Ac to Tfa affects the amide-I and -II transition dipole strength by less than 7%.

Figure 6 shows the scaled normal mode frequencies and infrared intensities of TD4UL, TD4SL, and TD4DL as well as TD4SL* and TD4DL* in stick spectra. The spectrum of AD4UL[23] is also shown at the top for comparison. The ester C=O normal mode frequency for all of the Tfa-capped Deg peptides is 1727 cm^{-1}, shifted by 3 cm^{-1} from the local mode frequency. The ONIOM calculation reproduced the spectral features of the amide-I and II modes observed in the FT IR spectra of TD4UL, as it did for AD4UL.[23] TD4UL exhibits two large peaks in the amide-I frequency region, resembling the observed doublet, and a very strong single amide-II peak with three weak peaks at the higher frequency side that appears as the observed shoulder. In the monolabeled peptides, the two amide-I peaks become weaker and slightly

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shifted from the unlabeled peptide by 3 cm^{-1}. The isotope shifted amide-I peak shows up at 1614 cm^{-1} for TD4SL and 1639 cm^{-1} for TD4SL*. The strong amide-II peak is red shifted from that of the unlabeled peptide by 5 cm^{-1} with a lower intensity, and a stronger shoulder appears at 1490 cm^{-1}. Comparing the spectra of TD4SL and TD4DL (also TD4SL* and TD4DL*), ^{15}N labeling in the bis-labeled peptides causes the main amide-II normal mode peak to red shift by 2 cm^{-1} with a stronger intensity and reduces the higher frequency shoulder. It also results in an 1 cm^{-1} red shift in the unlabeled amide-I doublets, but does not affect the isotope labeled amide-I peak.

The nearest-neighbor couplings between the amide-I/I ($\beta_{\text{I-I}}$), I/II ($\beta_{\text{I-II}}$), II/I ($\beta_{\text{II-I}}$), and II/II ($\beta_{\text{II-II}}$) modes can be calculated by the finite energy difference method after obtaining the local mode coordinates in each peptide unit.[18] Here the first and second letters in the subscript of β specify the amide mode in the ith and (i+1)th peptide units, respectively. The values previously computed for the Ac-capped Deg homo-peptide were $\beta_{\text{I-I}}$ = 7.0 cm^{-1}, $\beta_{\text{I-II}}$ = −3.8 cm^{-1}, $\beta_{\text{II-I}}$ = −24 cm^{-1} and $\beta_{\text{II-II}}$ = −3.0 cm^{-1}.[23] The calculation for the Tfa-capped peptide indicated that these nearest neighbor couplings are not affected by the capping group. The amide-II/I coupling strength is quite large compared to the other three couplings. This is likely due to the fact that the N–H and C=O groups of these two modes form a C$_5$ intramolecular hydrogen bond and thus they are more strongly coupled. All of the non-nearest-neighbor couplings were modeled by the transition charge couplings (TCC) to include multipole interactions, as have been previously calculated.[23,42-44] Since these TCC couplings are all less than 1 cm^{-1} in the ideal fully-extended structure, the nearest-neighbor couplings affect the linear and 2D IR spectra to a much greater degree.

The TCC parameters (partial charges and charge derivatives) of the capping ester C=O group were taken from the previous model calculation on methyl acetate (CH$_3$CO-OCH$_3$).[18] The nearest-neighbor couplings between the ester C=O and the adjacent amide modes were taken from the previous model calculation on CH$_3$CO-Aib-OtBu.[18]

To model the isotope effects for the labeled peptides, we scaled the amide-I and II local mode frequency and transition dipole strength based on how they vary in the isotopomers of N-t-butyl-2,2-dimethyl-propionamide, (CH$_3$)$_3$CCONHC(CH$_3$)$_3$. This amide was employed as a model to mimic local peptide units in C$^{\alpha,\alpha}$-dialkylated peptides.[18] Previously, we have calculated the vibrational properties of the unlabeled model amide and its ^{13}C=^{18}O labeled and ^{15}N labeled isotopomers at the B3LYP/6-311++G(d,p) level using the Gaussian package.[18] In this work we performed additional calculations for the ^{13}C=^{16}O labeled isotopomers. The results are summarized in Table 2.

B. Model calculation of linear and 2D IR spectra. A tetrapeptide taking the ideal fully-extended conformation [(ϕ, ψ) = (±180°, ±180°)] was built based on the average conformational parameters of a Deg residue.[24] In the calculation, we started by building the one- and two-quantum exciton Hamiltonians using the vibrational properties described in the previous Section and then slightly adjusted some of the parameters in order to reproduce the measured linear and 2D IR spectra. The best set of local mode frequencies and transition dipole strengths extracted from this model calculation are summarized in Table

1. The local transition dipole of the ester C=O mode was orientated 8° away from the C=O bond and that of the amide-II was set to 87° away from the N–H based on the results from the ONIOM calculation.[23] The angle between the amide-I dipole and the C=O bond was set to 0°, about 10° smaller than the value determined by DFT calculation (Table 2). This slight adjustment improved the overall agreement between the measured and simulated spectra. The amide-I and II coupling in the same peptide unit was set to –27 cm^{-1}, the experimentally determined value for NMA,[27] and the nearest-neighbor couplings were set to the best values obtained previously: β_{I-I} = 4.3 cm^{-1}, β_{I-II} = –3.8 cm^{-1}, β_{II-I} = –24 cm^{-1} and β_{II-II} = –3.5 cm^{-1}.[23] The two-exciton Hamiltonian was built from the one-exciton Hamiltonian with the harmonic approximation and bilinear couplings. The diagonal anharmonicities of the amide-I and II modes were set to 16 cm^{-1} [38] and 10 cm^{-1},[28] respectively. The diagonal anharmonicity of the ester C=O stretching mode was set to the same value as the amide-I mode. The Hamiltonians were diagonalized to obtain the transition frequencies and dipole strengths of the exciton states. The formulas to compute linear and 2D IR spectra for $\langle Y, Y, Z, Z \rangle$ and $\langle \pi/4, -\pi/4, Y, Z \rangle$ in the R pulse sequence, and $\langle \pi/4, Y, -\pi/4, Z \rangle$ in the NR pulse sequence have been described previously.[23,33,34,45] The homogeneous line width of the fundamental transitions was set to 12 cm^{-1} (FWHM). To take into account the inhomogeneous frequency distribution, mainly due to the fluctuations in intramolecular C_5 hydrogen bonds, 7500 different Hamiltonians were constructed with varying amide-I and II local mode frequencies having a standard deviation of 7.5 cm^{-1} around the mean values. To compare with the experimental linear and 2D IR spectra, we calculated the spectra of the $^{13}C=^{16}O$ and $^{13}C=^{18}O$ labeled peptides separately and took a weighted sum based on the population ratio determined by the mass spectrometry measurements.

The simulated linear spectra are superimposed on the measurement results in Figure 2. The calculation results agree with the observed spectra to within a few cm^{-1} and the important features of the amide-I and II modes were reproduced reasonably.

Figures 3b and 3d show the simulated 2D IR cross-peak patterns of the amide-I modes. Overall, we reach a quite good agreement between the measured and calculated spectra for both the R and NR cross-peak patterns. On the basis of the agreement of linear and 2D IR spectra, we concluded that Tfa-capped Deg homo-tetrapeptide in CDCl$_3$ took the molecular structure very close to the fully-extended (C_5) conformation.

The calculated amide-I/II 2D NR spectra are shown in Figure 4 (right column). Although lower intensity of some diagonal and cross peaks implies room for refinement of parameters used in the simulation, the agreement with the experimental spectral patterns is acceptable. Figure 5b shows the slices of the calculated 2D IR spectra at ω_τ = 1601 cm^{-1} to reveal the profiles of the cross peak between the $^{13}C=^{18}O$ labeled amide-I and the amide-II modes. As observed in the experimental results, the intensity of the unlabeled tetrapeptide in this cross-peak region originates from the tail of the amide-II diagonal peak. The mixed monolabeled and bis-labeled peptides show a peak at ω_t = 1485 cm^{-1} and 1484 cm^{-1}, respectively. The cross-peak shift due to the ^{15}N label is 1 cm^{-1}. The tiny shift is close to the 3 cm^{-1} shift

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extracted from the measured 2D IR spectra. As a test to check whether the elongation of the amide-II diagonal peaks along the ω_τ-axis affects the isotope shift, we turned off the contributions from the diagonal peaks in the simulation. The result showed that diagonal-peak elongation has no effect on the calculated isotope shift within the parameters used here.

V. Discussion

A. Effects of the Tfa-capping group. Before discussing the amide-I and II couplings between specific peptide residues, we need to confirm that the Tfa-capped tetrapeptides used in this study form the fully-extended conformation. We previously established the IR signatures of the amide-I and amide-II modes for the fully-extended peptide conformation of Deg homo-peptides.[23] However, those peptides were N-terminated with the Ac group, and it is known that the capping group can affect the structure of short peptides.[1,46] Based on our results, we ascertain that the Tfa group does not induce a significant structural change of the fully-extended structure.[47] The linear and 2D IR spectral signatures obtained for TD4UL clearly indicate that the peptide backbone is fully extended in CDCl$_3$, and essentially retains the crystal structure determined by the X-ray diffraction analysis.[24]

The Tfa-capping group mainly affects the local amide mode frequencies, especially of the first peptide unit. The local amide-I mode frequency of the Ac group in AD4UL in the previous model spectral calculation was set to 1660 cm^{-1},[23] whereas that of the first peptide unit in TD4UL was set to 1718 cm^{-1} in this work. The frequency shift of 58 cm^{-1} is huge, and hence the first amide-I mode in TD4UL is very much isolated from the excitons created by the other three local amide-I modes. However, as discovered in the main-chain length dependence of the 2D IR spectral pattern for the Ac-capped Deg peptides,[23] three peptide units are sufficient to create the characteristic IR signatures for the fully-extended conformation. This is the reason that the observed linear and 2D IR spectra for TD4UL are still similar to those for AD4UL.

The local amide-II frequency of the first peptide unit is also affected by the Tfa group. The shift is not as large as that of the amide-I mode, and hence the local mode is not completely isolated from the other amide-II modes. The local mode frequency used in the simulation is 1528 cm^{-1} for TD4UL and 1510 cm^{-1} for AD4UL. The blueshift of 18 cm^{-1} due to the Tfa group leads to a more prominent shoulder at the higher frequency side of the amide-II exciton band. However, the most fascinating feature of the amide-II band remains: its integrated area is larger than that of the amide-I band. This behavior also endorses that the peptide chain forms the fully-extended structure with a successive chain of intra-residue C$_5$ N–H···O=C hydrogen bonds. A recent theoretical study revealed the mechanism for the enhanced transition dipole strength of the amide-II mode in the C$_5$ conformation.[48] Hence, for the Deg tetrapeptide, the differences between the Ac and Tfa capping groups cause negligible effects on the peptide structure and the amide vibrational excitons.

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B. The amide-I/II cross-peaks of the isotope labeled tetrapeptides in the fully-extended structure. We focus on how the cross peaks between the amide-I and amide-II modes depend on the isotope labels. The synthesized monolabeled peptides contain both of the $^{13}C=^{16}O$ and $^{13}C=^{18}O$ labels on the second peptide unit with the ratio of 2:1. However, these labeled amide-I modes are well separated in their frequency (~30 cm^{-1}) and therefore we could observe their cross peaks with the amide-II modes separately. The presence of amide-I/II cross peaks in monolabeled peptides indicates that the amide-I mode of the second unit couples with the amide-II modes of the peptide backbone. It most strongly couples with the amide II modes of the same unit and the first unit, based on the vibrational coupling strengths (−27 and −24 cm^{-1}, respectively). The calculated transition charge coupling of the labeled amide-I mode and the amide-II mode of the fourth unit is −0.7 cm^{-1}. This small coupling implies that the cross-peak position would not be affected so much when the amide-II mode of the fourth unit is labeled with ^{15}N. This was indeed experimentally observed and the shift of the cross peak is only 3 cm^{-1}. The peak frequency of the largest amide-II band was 1492 cm^{-1} for the unlabeled peptide, 1487 cm^{-1} for the monolabeled peptide, and 1485 cm^{-1} for the bis-labeled peptide in the FT IR spectra. The values of redshift are consistent with the ONIOM calculation results: −5 cm^{-1} between TD4UL and TD4SL (also TD4SL*) and −7 cm^{-1} between TD4UL and TD4DL (also TD4DL*).

C. Different isotope effects in the 3_{10}-helix and fully-extended structures. This study and our previous work on the isotope labeled 3_{10}-helical peptides[17,18] enable us to discuss how the cross peaks between the labeled amide-I and amide-II modes depend on the peptide backbone conformation. As demonstrated here, the amide-I of the second peptide unit and the amide-II mode of the fourth unit are very weakly coupled in the fully-extended conformation. It is useful to compare the coupling of these modes in other secondary structures, such as a 3_{10}-helical system where the three isotope labels, ^{13}C, ^{18}O, and ^{15}N are involved in a C=O···H−N inter-residue hydrogen bond. A recent work reported that PyrAc-(Deg)$_4$-OtBu (PyrAc = 1-pyrenylacetyl) forms a 3_{10}-helix in acetonitrile.[49] It may therefore serve as a reference system for comparison. However, comparing 2D IR spectra of different secondary structures in different solvents is not straightforward. The frequencies of the amide modes greatly depend on the property of the solvent,[50] and consequently their 2D IR spectral patterns can exhibit strong solvent dependence that makes it difficult to definitively argue the structural effects on the labeled cross-peak profiles.

Instead, we compare the results with the isotope effects on the amide-I/II cross peaks of a Aib-rich 3_{10}-helical hexapeptide, Z-Aib-L-Leu-(Aib)$_2$-Gly-Aib-OtBu and its $^{13}C=^{18}O$-Leu mono-labeled and $^{13}C=^{18}O$-Leu/^{15}N-Gly bis-labeled isotopomers in CDCl$_3$.[17,18] The Aib also belongs to the family of $C^{\alpha,\alpha}$-dialkylated amino acids, and the effects to 2D IR spectra caused by residue differences from Deg would be minimized. Many studies indicate that the Aib has a propensity to fold the peptide backbone into a 3_{10}-helical structure with the average dihedral angels of (ϕ, ψ) = (−57°, −30°).[29] The amide-I 2D IR cross peaks of the unlabeled hexapeptide also exhibited the doublet pattern characteristic of 3_{10}-helix.[33-35]

Our previous studies showed that ^{15}N labeling induces a frequency shift of 9 cm^{-1} for the cross peak between the labeled amide-I and II modes that are separated by two residues but form a single 3_{10}-helical turn (Figure 5c).[18] The transition charge coupling of the amide-I and amide-II modes connected through a 3_{10}-helical C=O···H–N hydrogen bond was estimated to be −11.6 cm^{-1}, more than two times larger than the nearest neighbor amide-I/I coupling.[18] Our previous simulations reproduced the experimental observed trend, but a smaller isotope shift of 5 cm^{-1} (Figure 5d) using two different levels of theory.[18,21] The isotope dependence of the cross peak in different conformations, that is, 3_{10}-helix and fully-extended conformations, leads us to conclude that the combination of 2D IR spectroscopy and [13]C=[18]O/^{15}N labeling is useful for detecting the formation of a single 3_{10}-helical turn in a specific part of a peptide chain when the solvent environment is unchanged. Thus, the information acquired from the labeled amide-I and II modes will complement what we are able to obtain about peptide conformations through the vibrational couplings of the labeled amide-I modes solely.[10,13,16,20]

The fully-extended conformation with $(\phi, \psi) = (\pm 180°, \pm 180°)$ taken by Deg residues is not a common structure in protein secondary structures, while its rigidness can be utilized as a molecular spacer as well as a template for artificial molecules.[22,51] The semiextended conformation with $(\phi, \psi) = (-78°, 146°)$, which is taken by poly-L-proline, is a more interesting extended conformation.[52] In this structure, all peptide units are exposed to solvent without forming intramolecular C=O···H–N hydrogen bonds. The local amide modes are not well aligned with one another in a plane and they do not form the kind of linear excitons as in the fully-extended structure. Although we have not experimentally measured the cross-peak shift of the labeled amide-I/II modes for the semiextended structure, we have shown through calculation that the ^{15}N label causes less than 0.1 cm^{-1} isotope shift of the cross peak.[18] This is because the amide-I/II transition charge coupling strength is only −0.1 cm^{-1} when the modes are separated by two amino acid residues in the semiextended polypeptide chain. The isotope dependence is similar to that experimentally and theoretically observed for the fully-extended structure. We expect that the strategy of utilizing [13]C=[18]O/^{15}N labels to distinguish secondary structures will be equally applicable to polypeptide chains composed of coded amino acid residues under physiological conditions.

VI. Conclusions

We applied 2D IR spectroscopy and studied the isotope dependence of the amide-I/II cross peaks of Tfa-capped Deg homo-tetrapeptides. The linear and 2D IR signatures of the unlabeled peptide indicated that its solution 3D-structure in CDCl$_3$ is fully extended (2.0_5-helix). Although the Tfa group affects vibrational properties of the local amide modes of the first peptide unit,[26] the overall structure and spectral features are very similar to those observed for Ac-(Deg)$_4$-OtBu previously.[23] In both of the measured and simulated 2D NR spectra, we found a marginal isotope shift in the cross peaks between the [13]C=[18]O amide-I on the 2nd peptide unit and the ^{15}N amide-II mode on the 4th peptide unit, in great contrast with the strong isotope shift observed in the 3_{10}-helix.[17,18] The difference in the isotope dependence for the two secondary structures is due to the difference in the vibrational coupling strength between the labeled

amide-I and II modes: the coupling is very weak (-0.7 cm^{-1}) for the fully-extended, but quite strong (-11.6 cm^{-1}) for the 3_{10}-helical structure. The positions of ^{13}C$=^{18}$O/^{15}N labels, therefore, can be strategically designed to detect the presence or absence of local couplings. The method experimentally demonstrated in our studies will be generally applicable to other secondary structures. For example, the formation of a single α-helical turn from an extended chain can be studied by changing the position of isotope labels. The nearest inter-strand hydrogen bond in small antiparallel β-sheet peptides may also be probed through the amide-I and amide-II couplings. The combination of 2D IR spectroscopy with amide-I and amide-II isotope editing can have useful and versatile applications in revealing the 3D-structure and dynamics of biomolecules.

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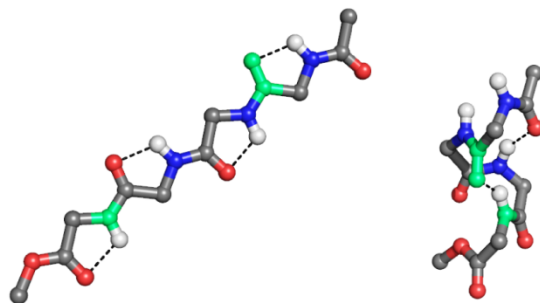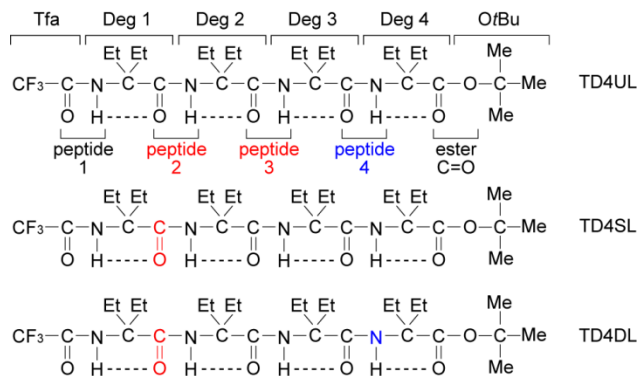
Figure 1. (Top) Structural formula of Tfa-(Deg)$_4$-OtBu. The peptide (amide) –CONH– units in chemically different environments are colored in black (between Tfa and Deg), red (between Deg and Deg), and blue (between Deg and OtBu). Dashed lines represent intramolecular C$_5$ hydrogen bonds. Three different isotopomers are studied: unlabeled (TD4UL), singly labeled with ^{13}C=^{18}O (TD4SL), and doubly labeled with ^{13}C=^{18}O and ^{15}N (TD4DL). The ^{13}C=^{18}O and ^{15}N labels are colored in red and blue, respectively. (Bottom) The ideal fully extended conformation with (ϕ, ψ) = (±180°, ±180°) (left) and 3$_{10}$-helical conformation with (ϕ, ψ) = (−57°, −30°) (right) of the tetrapeptide are shown in which the isotope labeled atoms are colored green. Dashed lines represent intramolecular hydrogen bonds.
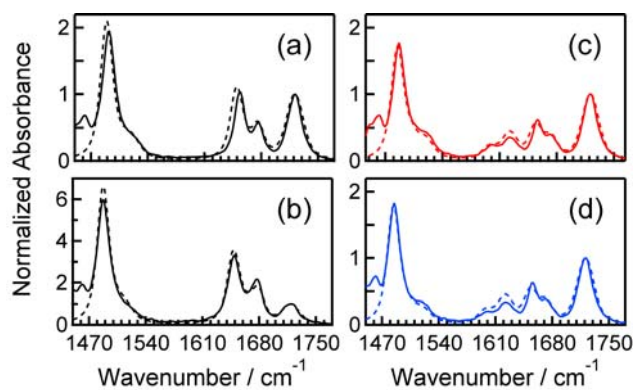
Figure 2. Measured FT IR spectra of the Deg homo-tetrapeptides in $CDCl_3$: (a) TD4UL; (b) AD4UL; (c) TD4SL/TD4SL*; (d) TD4DL/TD4DL*. After subtracting the solvent background spectrum, each spectrum was normalized by the peak absorbance of the ester C=O band at 1721 cm^{-1}. The dashed lines represent simulated linear IR spectra, normalized by the same procedure.
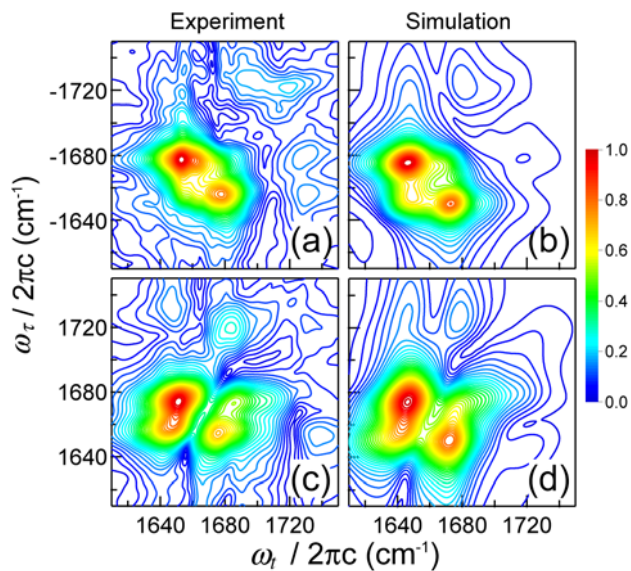
Figure 3. Absolute magnitude 2D IR amide-I spectra of TD4UL in CDCl₃ measured with the R pulse sequence and $\langle \pi/4, -\pi/4, Y, Z \rangle$ polarization configuration (a) and the NR pulse sequence and $\langle \pi/4, Y, -\pi/4, Z \rangle$ (c). Simulated 2D R and NR spectra are shown in the panels (b) and (d), respectively. Each spectrum is normalized by the maximum amplitude, and plotted with 40 equally-spaced contour lines from 0 to 1.
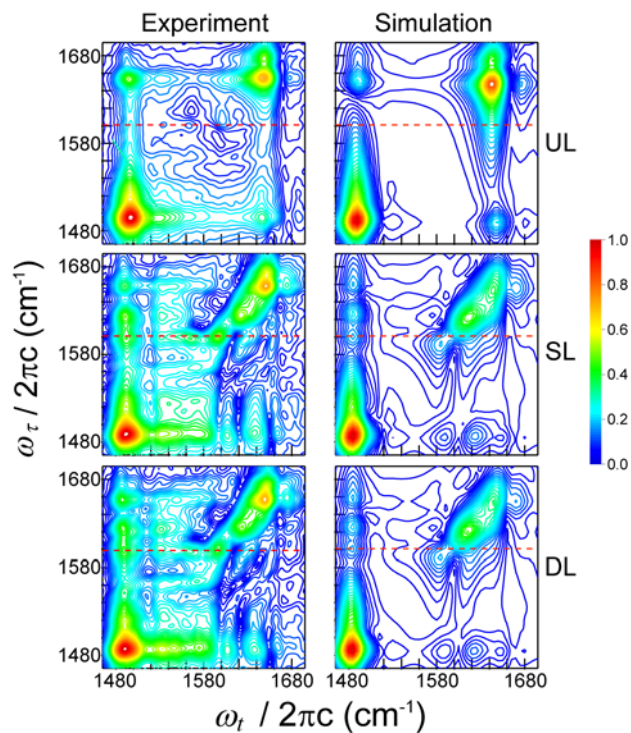
Figure 4. Absolute magnitude 2D IR amide-I/II spectra of TD4UL (top), TD4SL/TD4SL* (middle), and TD4DL/TD4DL* (bottom) in CDCl₃ with the NR pulse sequence and ⟨*Y, Y, Z, Z*⟩ polarization configuration (left column). Simulated spectra are shown on the right column. Each spectrum is normalized by the maximum amplitude of the diagonal amide-II band and plotted with 40 equally-spaced contour lines from 0 to 1.
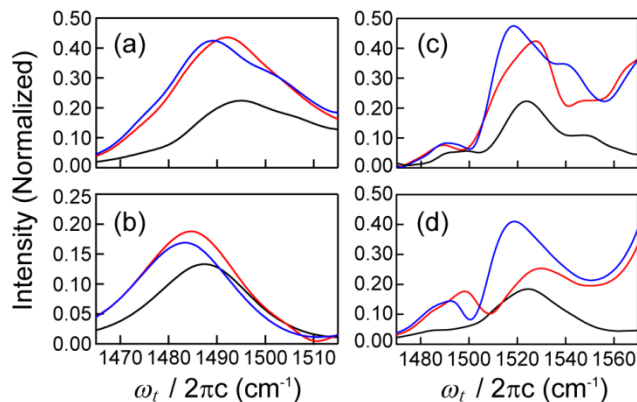
Figure 5. (a) Slices of the experimental absolute magnitude 2D NR spectra of TD4UL (black), TD4SL/TD4SL* (red), and TD4DL/TD4DL* (blue) along the horizontal dashed lines at ω_τ = 1601 cm^{-1} in Figure 4. (b) Slices of the simulated 2D NR spectra at ω_τ = 1601 cm^{-1} for the unlabeled (black), a mixture of monolabeled (red), and a mixture of bis-labeled (blue) tetrapeptides forming the ideal fully-extended structure with (ϕ, ψ) = ($\pm$180°, $\pm$180°). (c) Slices of the experimental absolute magnitude 2D NR spectra of unlabeled Z-Aib-L-Leu-(Aib)$_2$-Gly-Aib-OtBu (black), its ^{13}C=^{18}O-Leu mono-labeled (red), and ^{13}C=^{18}O-Leu/^{15}N-Gly bis-labeled (blue) isotopomers in CDCl$_3$.[18] (d) Slices of the simulated 2D NR spectra for the unlabeled (black), monolabeled (red), and bis-labeled (blue) hexapeptides forming the ideal 3$_{10}$-helix structure with the average dihedral angles (ϕ, ψ) = (−57°, −30°).[18] All 2D spectra were normalized by the strongest peak intensity of diagonal amide-II bands before slicing.
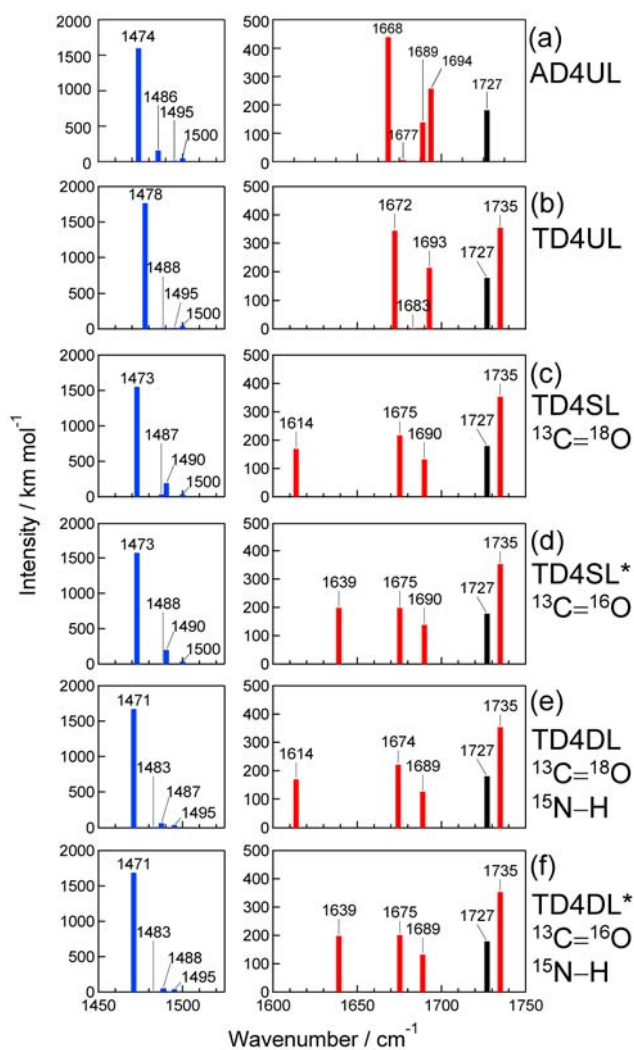
Figure 6. Scaled normal mode frequencies and infrared intensities of unlabeled and labeled Tfa-(Deg)$_4$-OtBu and unlabeled Ac-(Deg)$_4$-OtBu represented as stick spectra: (a) AD4UL; (b) TD4UL; (c) TD4SL; (d) TD4SL*; (e) TD4DL; (e) TD4DL*. Black, red and blue sticks correspond to the ester C=O mode, the amide-I modes, and the amide-II modes, respectively.

TABLE 1: Scaled Local Mode Frequencies (cm⁻¹) and Transition Dipole Derivatives (DÅ⁻¹ amu⁻¹/²) of Fully Extended Ac-(Deg)₄-O*t*Bu and Tfa-(Deg)₄-O*t*Bu Calculated with the ONIOM (B3LYP/6-31+(d) and AM1) Method in Vacuo, and the Best Parameters Extracted from Model Calculation of Linear and 2D IR Spectra

unit[a]	ONIOM calculation[b]		model spectral calculation
	Ac [c]	Tfa	TD4UL
ester C=O	1725 (2.2)	1724 (2.2)	1717 (2.8)
amide-I			
1	1684 (2.7)	1732 (2.9)	1718 (4.0)
2	1670 (2.5)	1678 (2.6)	1656 (2.5)
3	1673 (2.6)	1672 (2.7)	1656 (2.5)
4	1678 (2.4)	1678 (2.3)	1656 (2.4)
amide-II			
1	1480 (3.0)	1486 (2.8)	1528 (2.9)
2	1486 (3.4)	1487 (3.5)	1510 (3.7)
3	1487 (3.5)	1487 (3.5)	1510 (3.7)
4	1495 (3.2)	1495 (3.2)	1515 (3.4)

[a] The peptide units are numbered from the N- to C-terminus. [b] A frequency scaling factor of 0.9774 was used for the ester C=O stretching and the amide-I modes, and 0.9665 for the amide-II modes, respectively.[41] Transition dipole derivatives are shown in parenthesis. [c] From Reference [23].

TABLE 2: Vibrational Frequency (cm^{-1}), Magnitude (DÅ^{-1} amu$^{-1/2}$) and Direction (degrees) of the Transition Dipole Derivatives of the Amide-I and Amide-II Modes in Unlabeled and Isotopically Labeled *N-t*-Butyl-2,2-dimethyl-propionamide, (CH₃)₃CCONHC(CH₃)₃, Calculated at the B3LYP/6-311++G(d,p) Level

	Frequency [a]				Transition dipole derivative [b]				ratio [c]
	amide-I		amide-II		amide-I		amide-II		
Unlabeled [d]	1669		1490		2.25	(11)	2.24	(87)	1.00
¹³C=¹⁶O	1626	(−43)	1483	(−7)	2.15	(8)	2.03	(84)	1.06
¹³C=¹⁸O [d]	1599	(−70)	1484	(−6)	2.06	(8)	2.05	(85)	1.00
¹⁵N [d]	1668	(−1)	1482	(−8)	2.25	(11)	1.95	(88)	1.15

[a] The normal mode frequency is scaled by a factor of 0.9679.[53,54] The number in parentheses indicates the frequency difference between the unlabeled and isotopically labeled species. [b] The number in parentheses is the angle between the transition dipole derivative and the C=O (N–H) bond axis for the amide-I (II) mode. [c] The ratio of magnitude of the amide-I transition dipole derivative to that of amide-II mode. [d] From Reference [18].

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2D IR spectroscopy of amide-I/II modes