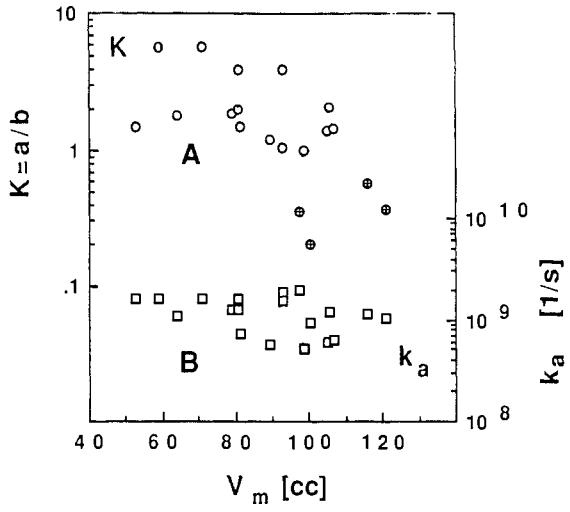

Figure 7. (A) The logarithm of the equilibrium constant for the conformers of $ZnPQ(Ac)_4$ at 300 K (O) is plotted versus the molar volume of the solvent. The points on the left edge are molecules close to spherical (e.g., benzene), whereas those on the right edge are more elliptical (e.g., diethyl ether). The symbol ⊕ indicates hexafluorobenzene, CH_3CCl_3, $CHCl_2CCl_3$, and CCl_4. (B) The logarithm of the rate constant k_a (□) is plotted versus the molar volume of the solvent. See Table I for data.

size (Figure 7A), albeit with some scatter. A plot of log k_a in the same manner shows no such correlation (Figure 7B). The molecules on the left edge of the scatter zone of the log K plot are close to spherical (CH_3CN, PhH, CCl_4). If the minimum axis of the more ellipsoidal molecules are used as a volume measure, the points on the right edge of the zone shift horizontally to smaller volumes, decreasing the scatter. It is striking that the solvents with $K < 1$ (crossed circles) are hexafluorobenzene or contain the $XCCl_3$ group with X = Cl, $CHCl_2$, or CH_3. Molecular models indicate that such molecules are too large to fit in the cavity of PQ_a. Assuming the molar volume at $K = 1$ (100 cc in Figure 7A) defines the size of the cavity, the radius of the equivalent sphere is 3.4 Å. This is just the spacing estimated for the cavity in PQ_a.[13]

Conclusion

The weak solvent dependence and the temperature independence of the electron-transfer reactions of $ZnPQ(Ac)_4$ are best interpreted by nonadiabatic electron tunneling. The somewhat larger dependencies of the triplet state reactions are explained by the closeness of the energy levels of the triplet state and that of $ZnP^+Q^-(Ac)_4$: 1.6 eV and 1.4 eV in polar solvents. Thus over 90% of the energy of this state is converted to free energy of the products.

Although the complexity of this macropolycyclic porphyrin–quinone cage molecule causes difficulties in the kinetic analysis of its electron-transfer reactions, this complexity is also a source of valuable information on the parameters which control the rate of these reactions. The polarizable spacer groups holding the porphyrin–quinone molecule together both isolate the reactants from the environment and favor the reaction, as in the photosynthetic reaction center.

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Determination of a Precise Interatomic Distance in a Helical Peptide by REDOR NMR

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Abstract: A new spectroscopic technique, rotational-echo double-resonance (REDOR) NMR, for solids utilizes magic-angle spinning and measures directly the dipolar coupling between stable-isotope-labeled nuclei and, thus, interatomic distances. REDOR has been used to measure the ^{13}C–^{15}N interatomic distance in a nine-residue fragment, Ac-Phe-MeA(1-^{13}C)-MeA(d_6)-MeA-Val-Gly(^{15}N)-Leu-MeA-MeA-OBzl (MeA = α-methylalanine or aminoisobutyric acid (Aib)), of the peptide antibiotic emerimicin. The crystal structure of the peptide emerimicin 1–9 benzyl ester was determined previously, and the measurement by REDOR of a known interatomic distance allows both validation and a practical demonstration of the precision of REDOR. The ability to map precisely intermolecular distances suggests applications of REDOR in the solid, or aggregated state, for determinations of the conformations of ligand molecules in drug–receptor, inhibitor–enzyme, and antigen–antibody complexes.

Recent advances in NMR technology have allowed the determination in solution of the three-dimensional structure of small proteins at low resolution.[1,2] These techniques are based primarily on measurements of proton homonuclear nuclear Overhauser effects (NOE's). NOE-based methods for distance determination suffer a number of shortcomings, among them the need to approximate a specific correlation time in the context of a model of microscopic motion. While the range for determining interproton distances by NOE may extend to 5 Å, the error range is also large. Only by iteratively fitting the calculated structures to the experimental data can this range be reduced to ±10%. The

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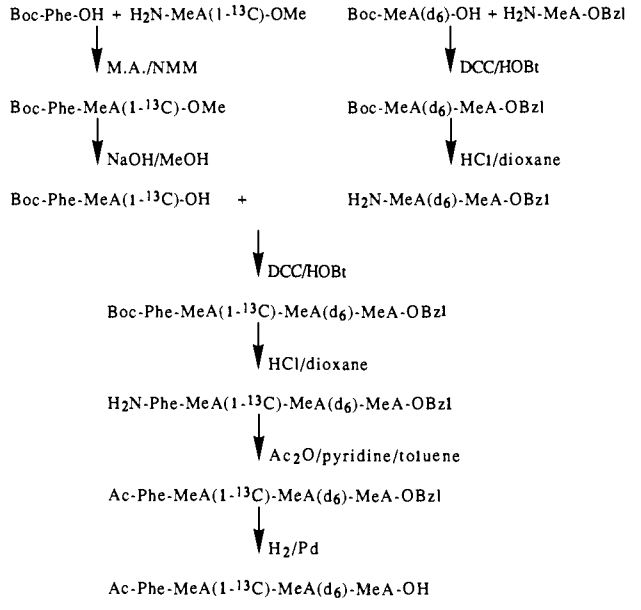
Scheme I



need to make geometric approximations due to the inability to assign uniquely to individual protons the cross-peaks seen with methyl and methylene groups also significantly compromises the precision of the NOE method.

A new spectroscopic technique, rotational-echo double-resonance (REDOR) NMR, has been described recently by Gullion and Schaefer.[3,4] This technique for solids utilizes magic-angle spinning and measures directly the heteronuclear dipolar coupling between isolated pairs of labeled nuclei, which allows interatomic distances to be measured without the need to invoke simplifying assumptions. In a solid with ^{13}C–^{15}N dipolar coupling, the ^{13}C rotational spin echoes that form each rotor period following a ^{1}H–^{13}C cross-polarization transfer can be prevented from reaching full intensity by inserting two ^{15}N π-pulses per rotor period. One of the π-pulses is synchronized with the completion of the rotor period and the other with a time less than or equal to half the rotor period. The difference between a ^{13}C NMR spectrum obtained under these conditions and one obtained with no ^{15}N π-pulses measures the ^{13}C–^{15}N coupling. The method offers both precision and accuracy due to its simplicity of mechanism: the dephasing of magnetization of one spin in the presence of the local dipolar field of the other spin. We have applied this technique to measure the interatomic distance in a nine-residue fragment of the peptide antibiotic emerimicin, in which we have incorporated one ^{13}C- and one ^{15}N-labeled amino acid. The crystal structure of the peptide emerimicin 1–9 has been determined[5] previously, and the measurement by REDOR of the interatomic distance established previously validates the NMR technique.

Experimental Section and Results

Synthetic Methods. α-Methylalanine-1-^{13}C, MeA(1-^{13}C), or aminoisobutyric acid -1-^{13}C, was prepared from K^{13}CN (98.5 atom % ^{13}C) obtained from MSD Isotopes; acetone and ammonium chloride were prepared by the Bucherer procedure.[6] The methyl ester was prepared with 2,2-dimethoxypropane and concentrated HCl. MeA-d_6 was prepared similarly from 99.5% perdeuterioacetone purchased from Sigma Chemical Co. [^{15}N]Glycine was purchased from MSD Isotopes, and the Boc derivatives of both MeA-d_6 and [^{15}N]Gly were prepared with Boc–ON reagent.[7] The emerimicin 1–9 benzyl ester was prepared by fragment condensation as shown in Schemes I and II. The sample was crystallized from ethyl acetate/hexane by normal synthetic procedures and used as microcrystalline material after drying.

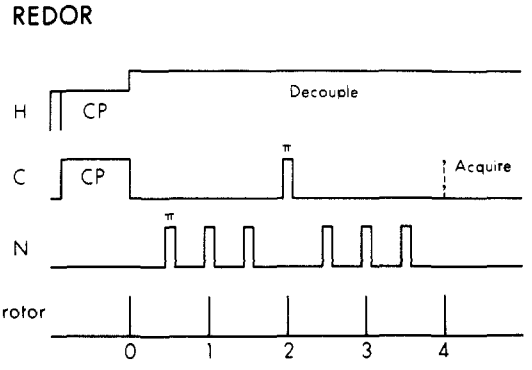
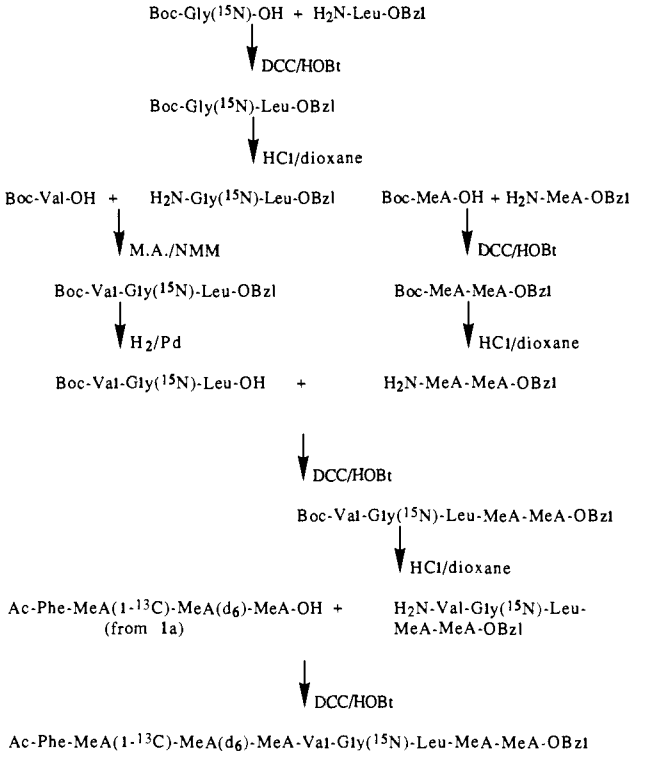


Figure 1. Pulse sequence for a version of rotational-echo, double-resonance (REDOR) ^{13}C NMR. Two equally spaced, ^{15}N 180° pulses per rotor period result in the dephasing of transverse carbon magnetization produced by a cross-polarization (CP) transfer from dipolar-coupled protons. Carbon–nitrogen dipolar coupling determines the extent of dephasing. A ^{13}C 180° pulse replaces the ^{15}N pulse in the middle of the dephasing period and refocuses isotropic ^{13}C chemical shift differences at the beginning of data acquisition. After the CP transfer, resonant decoupling removes the protons from the experiment. The illustration is for four rotor periods.

Scheme II



Modeling. Dodecamers of MeA were modeled with use of the SYBYL software package.[8] Minimizations of the dodecamer, from both the α-helix and 3_{10}-helix starting conformations with the Kollman united atom parameters[9] and a dielectric of $4r$, were performed. The average interatomic distances derived from residues 2–11 were compiled for each atom of the residue at a position of each helix with respect to the following four residues of that helix. The distance matrix for the α-helix was then subtracted from that of the 3_{10}-helix to yield a differential distance matrix. The crystal lattice for the emerimicin 1–9 benzyl ester structure[5] was generated by the module CRYSIN, and the interatomic distances between ^{13}C and ^{15}N labels were measured by the GEOMETRY module.

NMR. Cross-polarization, magic-angle spinning ^{13}C NMR spectra were obtained at room temperature at 50.3 MHz. The single, 12-mm-diameter, radio-frequency coil was connected by a low-loss transmission line to a triple-resonance tuning circuit. ^{1}H, ^{13}C, and ^{15}N transmitters

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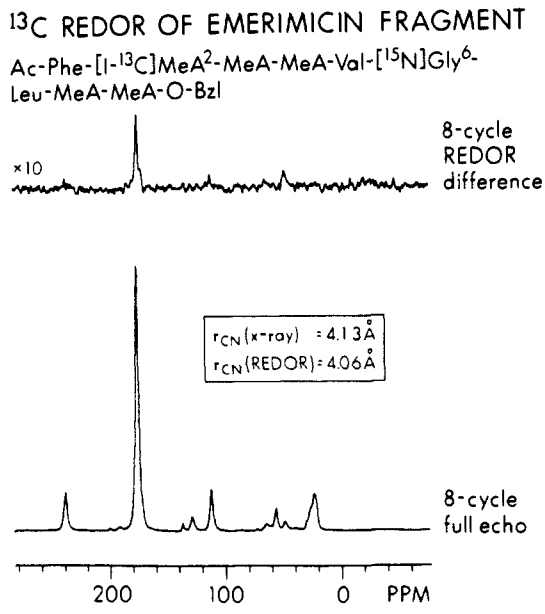

Figure 2. 50.3-MHz REDOR ^{13}C NMR spectra obtained from 120 mg of [*MeA*^{2}-1-^{13}C,Gly^6-^{15}N]emerimicin 1–9 benzyl ester. The pulse sequence of Figure 1 was used over eight rotor cycles with magic-angle spinning at 3205 Hz. The REDOR difference spectrum (top, 10×) is the difference between rotational-echo ^{13}C NMR with and without the ^{15}N 180° pulses. The difference peak at 178 ppm is due to carbonyl carbons and that at 45 ppm to α-carbons. The difference spectrum required a total of 162 000 scans collected in 2 days.

(1-kW tuned) produced maximum radio-frequency field amplitudes of 95, 80, and 40 kHz, respectively. Proton–carbon cross-polarization transfers are typically performed at 50 kHz, and proton decoupling is performed at 90 kHz. The rotor was made from a thin-wall ceramic (zirconia) barrel, fitted with plastic (Kel-F) end caps, and supported at both ends by air-pumped journal bearings. The rotor holds a 1-g sample.

A single ^{13}C 180° pulse in the center of the REDOR carbon-magnetization dephasing period refocuses all isotropic chemical shifts at the start of data acquisition (Figure 1). Weak REDOR difference signals (the difference between ^{13}C rotational-echo intensities with and without dephasing ^{15}N 180° pulses) are obtained reliably because the operating conditions of the observation channel do not change from scan to scan. The extent of REDOR dephasing was calculated by the methods of Griffin and co-workers.[10,11]

The experimental ratio of the carbonyl carbon REDOR difference signal to full echo, $\Delta S/S$, after eight rotor cycles with spinning at 3205 Hz is 0.0267 (Figure 2; δ(C) = 178 ppm). Approximately 7% of the full echo signal, S, arises from carbonyl carbons due to natural abundance, and correcting for this contribution increases $\Delta S/S$ to 0.0287. A part of the REDOR difference signal, ΔS, is due to dipolar coupling between the labeled nitrogen and carbon with natural-abundance carbons and nitrogens, respectively, which are one and two bonds distant. We evaluate these ΔS's using the experimental C–N dipolar coupling of 900 Hz in [2-^{13}C,^{15}N]alanine.[12] This coupling corresponds to a C–N internuclear distance of 1.49 Å. The peptide one- and two-bond, main-chain distances in emerimicin are 1.33 and 2.46 Å, respectively. On the basis of the known r^3 dependence of dipolar coupling, the one- and two-bond couplings in emerimicin are, therefore, 1265 and 200 Hz, respectively. We ignore indirect J couplings. The $\Delta S/S$ attributable to natural-abundance contributions to the REDOR difference signal is, therefore, 0.0158.[13] The remaining $\Delta S/S$, 0.0129, arises solely from coupling between the ^{13}C and ^{15}N labels. For this sort of weak coupling,[4] $\Delta S/S = K(N_c D/\nu_r)^2$, where N_c is the number of rotor cycles, D is the C–N dipolar coupling, ν_r is the spinning speed, and K is a dimensionless constant equal to 1.066. When the appropriate substitutions are made, $N_c = 8$, $\nu_r = 3205$ Hz, the ^{13}C–^{15}N dipolar coupling is 44.1 Hz, and the ^{13}C–^{15}N internuclear separation is 4.07 Å. The validity of the expression for $\Delta S/S$ was tested by varying both the spinning speed and the number of dephasing ^{15}N pulses. In a second experiment with $N_c = 4$ and $\nu_r = 2222$ Hz, the ^{13}C–^{15}N internuclear separation was determined to be 4.05 Å. The average of the two measurements is 4.06 Å, and we take this value as the REDOR-determined intramolecular C–N distance.

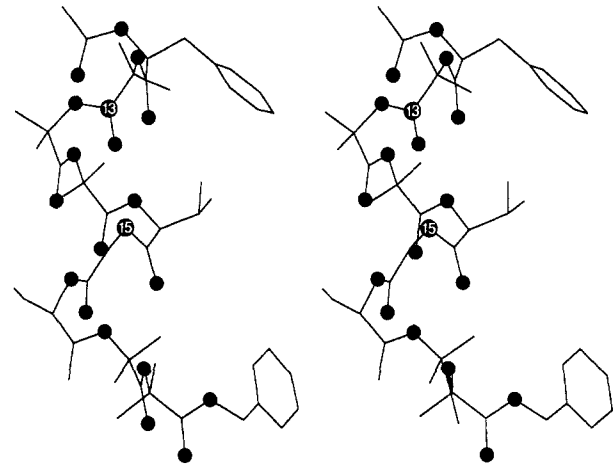

Figure 3. Stereoview of crystal structure of emerimicin 1–9 benzyl ester.[5] Heteroatoms are indicated by filled circles; hydrogens are deleted for clarity. Positions of ^{13}C and ^{15}N labels are indicated by numbers on filled circles.

Discussion

The choice of peptide to validate the REDOR method was based on the availability of the α-helical crystal structure of emerimicin 1–9 benzyl ester,[5] Ac-Phe-MeA-MeA-MeA-Val-Gly-Leu-MeA-MeA-OBzl, and interest in using this technique to investigate α-helix–3_{10}-helix transitions in peptides containing multiple α,α-dialkyl amino acids. The crystal structure of emerimicin 2–9, only one residue shorter than the peptide used in this study, shows a 3_{10}-conformation in the crystal,[14] emphasizing the ease of transition between these two conformations. In order to position our isotopes optimally to distinguish between these two conformations, interatomic distance matrices for the α-helix and the 3_{10}-helix were calculated. Subtraction of the two matrices clearly indicated that the location of the ^{13}C label in the carbonyl carbon of residue i and the ^{15}N label at the amino group of residue $i + 4$ was optimal in giving a large difference, 1.74 Å, between the two conformations while maintaining the absolute distances within the experimentally observable range. The estimated interatomic distance for the MeA α-helix is 4.13 Å, while the 3_{10}-helical distance is 5.87 Å. The ready availability of [^{15}N]glycine and the ease of synthesis of MeA(1-^{13}C) led to the decision to incorporate the ^{13}C label at position 2 and the ^{15}N label at position 6 of the nonapeptide.

The actual intramolecular distance between the ^{13}C-labeled carbonyl carbon of MeA-2 and the amide nitrogen of Gly-6, determined by examination (Figure 3) of the crystal structure of emerimicin 1–9 benzyl ester,[5] was 4.128 Å. The average distance for the six pairs of carbonyl carbons and amide nitrogens involved in internal hydrogen bonding in the crystal was 4.153 ± 0.028 Å. This distance is not expected to coincide exactly with that of the calculated MeA oligomer (4.13 Å) since emerimicin contains several amino acids without α,α-dialkyl substituents. Hodgkin et al.[15] have shown that the optimal α-helical torsion angle values for the backbone of Ala oligomers and MeA oligomers differ. These differences result in different values for the interatomic distances of the carbonyl carbon and its hydrogen-bonded amide nitrogen, 4.07 Å for Ala and 4.13 Å for MeA. In the case of the 3_{10}-helix, the values are 5.82 Å for oligo-Ala and 5.87 Å for oligo-MeA.

With an experimental standard (the C–N distance in alanine[12] for which the coupling is known for a specific distance), the observed dipolar coupling of 44.1 Hz, and the r^3 distance de-

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pendence for dipolar coupling, a separation of 4.07 Å is calculated for the two emerimicin labels. Expected sources of error include differences between motional averaging of dipolar coupling for alanine and emerimicin, lack of consideration of the anisotropic J-coupling contribution to the directly bonded C–N coupling in alanine,[12] and the presence of intermolecular contributions to ^{13}C–^{15}N dipolar coupling in emerimicin.

The last source of error is the easiest to examine. Intermolecular distances as measured in the emerimicin crystal[5] between the ^{13}C label and ^{15}N labels in other molecules are all greater than 10.8 Å except for one distance of 8.27 Å. On the basis of the r^3 distance dependence of the dipolar interaction, the presence of ^{15}N labels on adjacent molecules introduces an underestimation of the distance determination of 2% (about 0.08 Å). Inclusion of a distance-independent J coupling for alanine decreases the intramolecular distance-dependent dipolar coupling extracted from an observed $\Delta S/S$ and therefore also leads to an underestimation of the C–N distance in emerimicin. However, we expect C–N J coupling to be small in alanine, no more than 5% of dipolar coupling.[4] Thus, ignoring J coupling will lead to an underestimate of the C–N distance of less than 1%.

Motional averaging is the most important source of error in REDOR. If motional averaging of dipolar coupling in emerimicin exceeds that in alanine (which seems likely), the experimental $\Delta S/S$ overestimates the C–N distance in emerimicin. A measure of the overestimate can be made by assuming molecular motion in the backbone of crystalline gramicidin A, a 15-residue peptide, matches that in the crystalline emerimicin nine-residue fragment. The experimental C–N dipolar coupling for the Gly^2–Ala^3 1.33-Å peptide bond in gramicidin A is 1220 Hz.[4] With this value as a reference, the $\Delta S/S$ of Figure 2 translates to a [1-^{13}C]-Gly^2–[^{15}N]Ala^6 C–N distance of 3.98 Å in emerimicin. Naturally, motion can vary from one location in a protein to another. A systematic strategy to minimize the effect of motion on REDOR distance measurements would involve separate experiments to determine the extent of motional averaging of ^{13}C chemical shift and ^1H–^{15}N dipolar tensors at the sites of interest. In any event, the probable accuracy in the present REDOR C–N distance determination is ±0.1 Å. This accuracy exceeds most, if not all, spectroscopic methods for determining interatomic distances in molecules.

The REDOR NMR approach offers precision of measurement and confidence in the distances being determined. In contrast with many of the other spectroscopic rulers that have been suggested, such as fluorescence energy transfer,[16] paramagnetic broadening,[17] lanthanide shift reagents,[18] and nuclear Overhauser effects,[1,2] REDOR depends on a simple physical mechanism. This simplicity eliminates many of the inherent assumptions that compromise the interpretations of the other procedures. The precision suggests that REDOR measurements can be used to refine the relative coordinates derived from difference Fourier maps, to distinguish between alternative interpretations on the basis of lower resolutions methods (such as the question of α- versus 3_{10}-helix), and to map precisely intermolecular distances such as those seen in drug–receptor, inhibitor–enzyme, or antigen–antibody complexes. An obvious attribute of REDOR NMR is the ability to examine systems in the aggregated, or solid state. Certainly, membrane-bound receptors and enzymes that resist crystallization may be approached more easily in this fashion. The primary disadvantage of using the ^{13}C–^{15}N dipolar coupling as a measure of interatomic distances is the relatively low sensitivity due to the low γ of ^{15}N. Other REDOR experiments based on isolated ^{19}F–^{13}C dipolar coupling, which are estimated to be approximately 90 times more sensitive due to the large γ of ^{19}F, are under way.

Acknowledgment. Support for this work was received from the NIH (Grants GM 24483 and GM 33918, G.R.M.; GM 40634, J.S.) and from NSF (DIR-8720089, J.S.). A.S.R., K.K., and M.T.L. acknowledge financial support for part of this work from the Polish Academy of Sciences (Grant CPBP 01.13.2.5). Mass spectra were obtained at the Washington University Mass Spectroscopy Resource (RR00945).

Registry No. BOC-Phe-OH, 13734-34-4; H_2N-MeA(1-^{13}C)-OMe, 124125-83-3; BOC-MeA(d_6)-OH, 124125-84-4; H_2N-MeA-OBzl, 55456-40-1; BOC-Phe-MeA(1-^{13}C)-OMe, 124125-85-5; BOC-MeA-(d_6)-MeA-OBzl, 124125-86-6; BOC-Phe-MeA(1-^{13}C)-MeA(d_6)-MeA-OBzl, 124125-87-7; BOC-Phe-MeA(1-^{13}C)-OH, 124153-29-3; H_2N-MeA(d_6)-MeA-OBzl, 124125-88-8; H_2N-Phe-MeA(1-^{13}C)-MeA(d_6)-MeA-OBzl, 124125-89-9; Ac-Phe-MeA(1-^{13}C)-MeA(d_6)-MeA-OBzl, 124125-90-2; Ac-Phe-MeA(1-^{13}C)-MeA(d_6)-MeA-OH, 124125-91-3; BOC-Gly(^{15}N)-OH, 106665-75-2; H_2N-Leu-OBzl, 1738-69-8; BOC-Gly(^{15}N)-Leu-OBzl, 124125-92-4; BOC-MeA-MeA-OBzl, 79118-36-8; BOC-Val-Gly(^{15}N)-Leu-MeA-MeA-OBzl, 124125-93-5; BOC-Val-OH, 13734-41-3; H_2N-Gly(^{15}N)-Leu-OBzl, 124125-94-6; BOC-MeA-OH, 30992-29-1; BOC-Val-Gly(^{15}N)-Leu-OBzl, 124125-95-7; BOC-Val-Gly(^{15}N)-Leu-OH, 124125-96-8; H_2N-MeA-MeA-OBzl, 124125-97-9; H_2N-Val-Gly(^{15}N)-Leu-MeA-MeA-OBzl, 124125-98-0; Ac-Phe-MeA-(1-^{13}C)-MeA(d_6)-MeA-Val-Gly(^{15}N)-Leu-MeA-MeA-OBzl, 124125-99-1; emerimicin, 71812-28-7.

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