Total Assignment of the ^{13}C NMR Spectrum of the Cembranoid Diterpene Eunicin through the Use of Two-Dimensional Proton–Carbon Chemical Shift Correlation[1]

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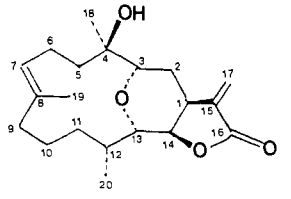
Abstract: Total assignment of the ^{13}C NMR spectrum of the oxa-bridged cembranoid diterpene eunicin was accomplished through the combined use of methylene carbon chemical shift regularity behavior associated with (*E*)-isoprene-derived structural fragments, lanthanide induced shifts, and two-dimensional proton–carbon chemical shift correlation NMR spectroscopy.

Cembranoid diterpenes present a substantial challenge to the structural chemist since the elucidation of the structure of each new member of the series, in essence, requires the total establishment of the carbon skeleton.[4] Generally, structures of new cembranoid diterpenes have been established through the use of either degradative techniques or single-crystal X-ray diffraction methods. There has, to the best of our knowledge, been no successful attempt at establishing a cembranoid structure using NMR spectroscopic techniques alone. However, with the growing body of papers reporting partial and total assignments of the ^{13}C NMR spectra of members of this interesting class of compounds,[5–10] accompanied by the development of two-dimensional NMR spectroscopy, it is perhaps only a matter of time until the results of such a study appear. In the interim, the reporting of additional total ^{13}C NMR assignments in the cembrane series will help to establish a data base of sufficient size to be useful in efforts directed at the $^{13}C/^{1}H$ NMR based structure elucidation of new cembranoids of unknown structure. Thus, we now wish to report the total assignment of the ^{13}C NMR spectrum of the oxa-bridged cembranoid diterpene eunicin (**1**),[11] which has been accomplished



on the basis of methylene carbon chemical shift regularities of (*E*)-isoprenoid structural subunits, lanthanide induced shift (LIS) studies, and two-dimensional proton–carbon chemical shift correlation NMR spectroscopy.

Results and Discussion

The ^{13}C NMR spectrum of eunicin (**1**), on initial inspection, appears to contain only 19 rather than the expected 20 carbon resonances. The remaining resonance was, however, subsequently shown to be a quaternary carbon resonance having an accidentally degenerate chemical shift with the methine carbon resonating at $\delta = 73.22$. Resonance multiplicities were established via the acquisition of decoupled INEPT spectra[12] (see Table I). Identical resonance multiplicities were reflected in the measured spin–lattice (T_1) relaxation times of the methine and methylene carbons[13–15] although the distribution of methine carbon relaxation times was nonuniform, in contrast to previous studies in the cembrane series.[6,7,9,10] The expanded range of observed methine–carbon re-

Table I. ^{13}C NMR Chemical Shift Assignments, Multiplicities, and Spin–Lattice (T_1) Relaxation Times for Eunicin (1) in Deuteriochloroform

resonance	$\delta\,^{13}C^a$	multiplicity[b]	T_1, s[c]
C16	170.26	s	
C15	136.65	s	
C8	130.11	s	
C7	128.34	d	0.74
C17	121.33	t	0.49
C13	77.20	d	1.46
C3	73.50	d	1.66
C14	73.22	d	*d*
C4	73.22	s	
C5	40.87	t	0.48
C1	38.14	d	1.35
C9	37.52	t	0.55
C12	33.24	d	0.74
C10	28.66	t	0.46
C2	24.30	t	0.51
C18	23.52	q	
C11	22.70	t	0.48
C6	20.32	t	0.49
C19	16.80	q	
C20	13.99	q	

[a] Chemical shifts are reported in ppm downfield from Me_4Si at 100.6 MHz. [b] Resonance multiplicities were determined using the INEPT experiment and are denoted as s, d, t and q for singlet, doublet, triplet and quartet, respectively. [c] Spin–lattice (T_1) relaxation times were measured at 25.2 MHz by using the inversion–recovery pulse sequence. The data were processed by using the three-parameter fitting program described by: Kowalewski, J. G.; Levy, G. C.; Johnson, L. F.; Palmer, L. *J. Magn. Reson.* 1979, *26*, 553. No effort was made to accurately measure the quarternary carbon or methyl-carbon relaxation times. [d] The relaxation time of the C14 resonance could not be measured because of overlap with the C4 quaternary resonance even at 100.6 MHz.

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laxation times suggests that **1** undergoes some form of anisotropic reorientation in solution rather than the isotropic tumbling previously observed.[6,7,9,10]

Prior to undertaking the specific assignment of any of the ^{13}C resonances of **1**, it is useful to consider the consequences of the patterned methylene carbon chemical shift behavior of (*E*)-trisubstituted double bonds[16] and the utility of this behavior as an adjunct in the assignment of isoprenoid methylenes in the ^{13}C NMR spectrum of **1**. On the basis of the compliance of the relevant carbons in other members of the cembrane series, the C5 and C9 methylene carbons of **1** were expected to resonate in the range $\delta = 38.55 \pm 2.60$ while their counterpart C2[17] and C6 methylene carbons, were expected to resonate in the range $\delta = 25.71 \pm 2.37$. As expected, the upfield region of the spectrum contains two methylene carbons which resonate downfield at 40.87 and 37.52 ppm. Although no assignments can be made within this pair of resonances at this point, they may safely be attributed to C5 and C9. Within the normal range of chemical shifts for the C2 and C6 methylene carbons, three methylene resonances were observed as well as a fourth resonance observed slightly upfield of this range. While the patterned chemical shift behavior does not provide a basis for making specific assignments, it does provide a logical means of subgrouping resonances prior to beginning a total assignment. A knowledge of such subgroupings was extremely useful in the completion of the assignments in the present study.

Beyond the subgrouping of the resonances on the basis of the patterned chemical shift behavior, several resonances in the ^{13}C NMR spectrum of **1** may be assigned solely from chemical shift and multiplicity considerations. Thus, the C7 resonance may be assigned to the vinyl signal observed at $\delta = 128.34$, the C16 resonance to the carbonyl signal at $\delta = 170.26$, the C17 resonance to the vinyl signal at $\delta = 121.33$, and the C18 methyl resonance tentatively to the signal observed at $\delta = 23.52$.[18] Arguments could certainly be developed that would also justify the assignment of additional resonances on the basis of chemical shift considerations. However, without any evidence with which to corroborate such assignments, they would remain speculative and should be undertaken only with caution.

Examination of Dreiding models of eunicin (**1**), in conjunction with the already established crystal structure of the molecule[19] suggested that the only site in the molecule that would be sterically accessible for binding with the lanthanide complex would be the hydroxyl group at the 4-position.[20] The quaternary carbon resonance observed at $\delta = 73.22$, which experienced the largest LIS, was assigned to C4. By assuming a monotonic decline in the observed LIS, the establishment of two additional subgroups of resonances was also possible. The first consisted of the C3, C5, and C18 resonances which, on the basis of chemical shift considerations and resonance multiplicities, were assigned to the signals observed at 73.50, 40.87 and 23.52 ppm, respectively. One of these resonances (40.87 ppm) was one of two methylenes designated previously as C5 or C9 by the (*E*)-isoprenoid shift regularity,[16] thus permitting the assignment of the remaining downfield methylene carbon at 37.52 ppm to C9. Within the upfield group of methylene resonances, the C2 and C6 resonances would be expected to exhibit considerably larger LIS than the C10 and C11 resonances. Thus, a second group of resonances comprised of C2 and C6 could be established, representing the resonances at 24.30 and 20.32 ppm. The remaining upfield resonances at 28.66 and 22.70 ppm may then be attributed to the C10 and C11 resonances. Specific assignments could not be made within either of the groups of methylene carbons solely on the basis of the LIS data at this point. Other than the aforementioned atoms, the remaining carbons contained in the ^{13}C NMR spectrum of eunicin (**1**) uniformly exhibited only small induced shifts, thus precluding the use of these data for assignment purposes without first establishing the precise geometry of the eunicin–lanthanide complex, which, although possible, was not the specific intent of this investigation.[21]

As described above, partial assignment of the ^{13}C NMR spectrum of eunicin (**1**) can be made with a reasonable degree of confidence on the basis of conventional chemical shift arguments when supplemented by a knowledge of INEPT-based spin multiplicities and lanthanide induced shifts. Total assignment of the spectrum, in contrast, cannot be made without resorting to additional and more sophisticated experiments. Total assignment of the ^{13}C NMR spectrum of eunicin (**1**) was accomplished through the execution of a two-dimensional proton–carbon

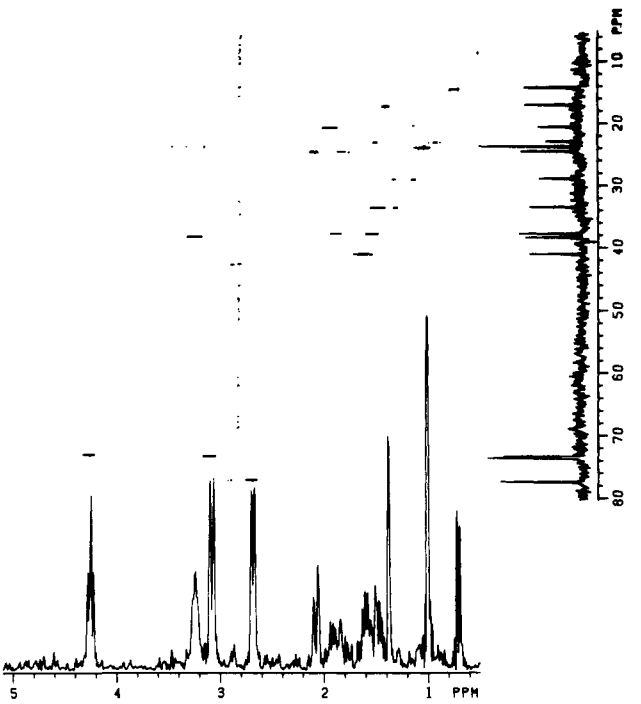

Figure 1. Four-level contour plot of the $S(F_2,F_1)$ data matrix from the two-dimensional proton–carbon chemical shift correlation experiment performed on eunicin (**1**) in deuteriochloroform with a carbon observation frequency of 90.793 MHz and a proton observation frequency of 361.062 MHz.

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(18) Assignment of the C18 methyl is based on the lack of a steric interaction, resulting in a chemical shift analogous to that observed for a methyl of a *Z*-trisubstituted double bond; cf. refs 7 and 11.
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(21) Individuals specifically interested in the LIS data may obtain this information upon request from the authors.

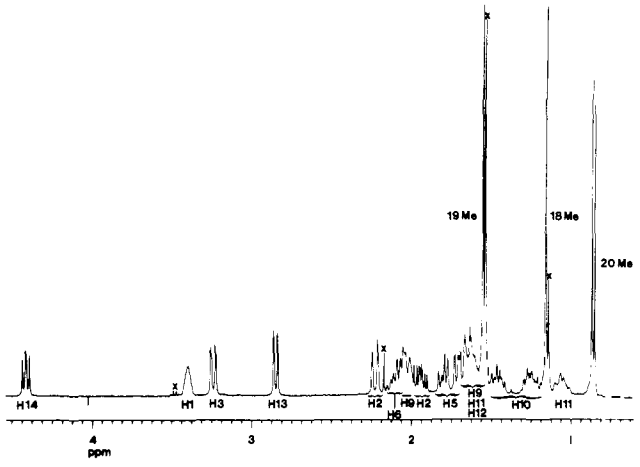

Figure 2. Conventional proton spectrum of the high-field region of eunicin (**1**) in deuteriochloroform at 400.13 MHz.

chemical shift correlation experiment.

Two-dimensional NMR spectroscopy was originally proposed by Jeener in 1971.[22] Intensity responses in the two-dimensional NMR experiment are a function of two frequencies, F_1 and F_2, which are obtained by the performance of a double-Fourier transformation[23,24] on a suitably constituted data set. Although many such experiments are possible by the selection of the spin Hamiltonians that operate during the various periods of the experiment,[25] the specific experiment germane to the problem at hand is the proton–carbon chemical shift correlation experiment first described by Freeman and co-workers[26-34] and subsequently applied to the study of steroids and carbohydrates. The two-dimensional NMR spectrum of eunicin (**1**) obtained by this technique is shown in Figure 1. The proton spectrum (projected sum) is shown across the bottom of the figure and corresponds to F_1 while the carbon spectrum (projection) is shown vertically and corresponds to F_2. Associated proton and carbon resonance frequencies appear as points of intensity in the contour plot bounded by the two projections. Assignments within the contour plot may be made straightforwardly, given a knowledge of either the ^1H or ^{13}C chemical shift of a given atom in the molecule. In practice, it is often most convenient to establish the proton connectivities through either decoupling or the use of autocorrelated proton two-dimensional NMR spectra[22,23,35] although the converse may be useful if ^{13}C-resonance assignments can be made on the basis of alternative techniques.

The most readily assigned group of signals contained in Figure 1 are those due to the methyl resonances. Beginning with the 18-methyl protons, which appear as a singlet at about 1.1 ppm[36] (see Figure 2), we observe a point of intensity in the contour plot that correlates with the carbon resonance at 23.52 ppm, confirming the assignment made from the LIS experiments. The remaining methyl signals, the 19-vinyl methyl singlet and the 20-methyl doublet at about 1.6 and 0.9 ppm, respectively, were observed to correlate with the carbon resonances at 16.80 and 13.99 ppm, respectively, thus providing an unequivocal assignment of the methyl resonances of the molecule. Returning to the problem associated with the C2 and C6 methylene resonances, selective decouplings in the 400-MHz ^1H NMR spectrum served to establish the locations of the corresponding H2 and H6 protons. Having established these proton chemical shifts (see Figure 2), the assignment of the resonances at 24.30 and 20.32 ppm to C2 and C6, respectively, was a relatively simple undertaking. In a similar fashion, the assignment of the two aliphatic methine carbons in this molecule, C1 and C12, was also accomplished. Thus, the H1 resonance at about 3.4 ppm may be seen to correlate with the resonance at 38.14 ppm, thus permitting the assignment of the remaining methine carbon at 33.24 ppm to C12. By utilization of the assigned resonance for C12, the location of the H12 resonance is readily established from the two-dimensional spectrum (also confirmed by decoupling the H20 methyl doublet). Given the location of the H12 resonance, specific decoupling of the H12 resonance unequivocally established the location of one of the H11 protons at about 1.05 ppm. When the identity of the H11 resonance was known, the C11 carbon resonance was assigned to the signal at 22.70 ppm. The total assignment was completed by attribution of the remaining methylene resonance at 28.66 ppm to C10.

Conclusions

Through the use of patterned methylene carbon chemical shift behavior coupled with lanthanide induced shifts and two-dimensional proton–carbon chemical shift correlation spectroscopy, the total and unequivocal assignment of the ^{13}C NMR spectrum of eunicin (**1**) has been achieved. Although the structure and stereochemistry of this molecule are known in detail, the value, even necessity, of the two-dimensional spectrum in the successful completion of the assignment is quite clear. In conclusion, it is the opinion of these authors that two-dimensional NMR spectroscopy, particularly double quantum coherence two-dimensional NMR experiments,[37-39] will play a role in the eventual establishment of cembrane structures, thus obviating the need to destroy samples of these interesting and often scarce compounds using more classical structure elucidation methods.

Experimental Section

The sample of eunicin (**1**) employed in this study was isolated and purified as previously described[11] and exhibited physical characteristics consistent with those previously reported. Lanthanide induced shift experiments were performed by using $Yb(dpm)_3$[20] on a Varian XL-100 spectrometer equipped with a Nicolet 1180 computer interfaced through a Model 293A′ pulse programmer and operating at a frequency of 25.158 MHz for ^{13}C observation. Spectra were obtained by using a routine sweep width of 5000 Hz digitized with 8K data points. Proton spectra and specific proton decouplings were performed on a Bruker WM-400 spectrometer equipped with an ASPECT 2000 computer and operating at a frequency of 400.130 MHz for ^1H observation. Spectra were obtained with a sweep width of 4000 Hz digitized with 64K data points. The two-dimensional proton–carbon chemical shift correlation experiment was performed on a Nicolet WB-360 spectrometer equipped with

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a model 1280 computer and operated at frequencies of 361.062 MHz for proton and 90.793 MHz for ^{13}C observation. The pulse sequence employed for the experiment was essentially that of Freeman[27] modified to deliver a composite 180° pulse.[40] Pulse widths were 30 μs for carbon and 32 μs for proton via the decoupler coils. Fixed delays around the acquisition pulse (Δ_1 and Δ_2) were set to 3.0 and 2.5 ms (respectively) with a spectral width of ±833 Hz for proton and ±3425 Hz for carbon, with phase cycling to provide the equivalent of quadrature data in both dimensions. The initial $S(t_1,t_2)$ data matrix was generated using 256 × 2K blocks of data followed by processing in the usual fashion. The experiment was performed on a sample prepared by dissolving 200 mg of **1** in approximately 2 mL of deuteriochloroform. Accumulation of the initial $S(t_1,t_2)$ data matrix required approximately 3 h. The contour plot (Figure 1) was prepared by using four contour levels. The proton reference spectrum was the projected sum of the data matrix through the F_1 dimension[36] and the carbon reference spectrum was the projection through the F_2 dimension.

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Acknowledgment. This paper is dedicated to Professor William von Eggers Doering on the occasion of his 65th birthday. His excitement about the science of chemistry has been continuously transmitted to M.R.W. for the past 25 years. In some small way, this enthusiasm has also been transmitted to all of the other authors of this paper. Several of the authors, G.E.M., M.A., A.J.W., and M.R.W. wish to thank the Robert A. Welch Foundation for its generous support through Grants No. E-792, E-745, E-744, and E-183. The authors also acknowledge the support of the National Science Foundation in the form of Grants No. CHE-7506162 and CHE-7818723, which provided the funds for the acquisition of the XL-100 spectrometer system at the University of Houston and for the establishment and operation of the South Carolina Regional NMR Facility, respectively. Lastly, G.E.M. also acknowledges the partial support of this work by the University of Houston Limited Grant in Aid Program.

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^1H NMR Investigation of the Active Site of Cobalt(II)-Substituted Liver Alcohol Dehydrogenase

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Abstract: The pH dependence of the ^1H NMR spectra of active-site specifically substituted cobalt(II) horse liver alcohol dehydrogenase and its complexes with NAD^+ and NADH are reported. The ^1H NMR signals of the cysteine and the histidine ligands are well shifted from the diamagnetic position. The δ-NH of histidine 67 probably deprotonates with a pK_a of 9.0 ± 0.2; in the complex with NAD^+ the same group exhibits a pH-dependent shift without deprotonation with a pK_a of 8.3 ± 0.2. The complex with NADH is pH independent up to pH 9.3. Both the ^1H NMR and near-IR spectra indicate that no major change in the coordination sphere of the catalytic metal ion occurs upon binding coenzyme. The results suggest the participation in catalysis of groups not considered in previously proposed mechanisms.

Liver alcohol dehydrogenase (LADH, EC 1.1.1.1) is a dimeric zinc enzyme of molecular weight 80 000, which catalyzes the reversible oxidation of alcohols. Each subunit contains two zinc ions. One zinc is essential for the substrate binding and activation while the other presumably plays a structural role. X-ray crystallographic studies of the native enzyme[1] and the cobalt(II)- and cadmium(II)-substituted enzymes[2,3] have shown that the catalytic metal ion is bound to histidine 67, cysteine 46, and cysteine 174 in a distorted tetrahedral geometry, the fourth ligand being a solvent water molecule. At least four protonation steps with pK_a values of 6.4, 7.6, 9.2, and 11.2 have been shown to be important for the catalytic action of the enzyme by kinetic studies.[4,5] The assignments of these ionizations to functional groups of the enzyme given in the literature do not have a firm physical basis. The pK_a of 9.2 has been attributed to the deprotonation of the zinc-coordinated water in the free enzyme and the pK_a's of 7.6 and 11.2 to the deprotonation of the same moiety in the binary complexes with oxidized and reduced coenzymes, respectively.[5,6] The interaction of the positive charge of the nicotinamide ring of NAD^+ with the catalytic zinc ion was proposed to cause the decrease of the pK_a of metal-bound water.[7] It should be noted, however, that the pH dependence of the coenzyme dissociation processes for the enzyme depleted of the catalytic zinc ion is similar to that of the native enzyme.[8] The pK_a of 6.4 has been tentatively assigned to the deprotonation of the zinc-coordinated alcohol on the basis of an apparent Brønsted relationship between the pK_a's of the free and bound alcohols.[9]

Cobalt(II) can be selectively substituted for zinc(II) in the catalytic sites of LADH.[10] The resulting derivative (Co(c)$_2$Zn(n)$_2$-LADH) retains catalytic activity although differences in the rate constants of elementary steps along the catalytic pathway have ben observed.[11,12] The electronic spectra of Co-

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