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Carbon-13 and Proton Nuclear Magnetic Resonance Spectroscopic Evidence for a Molecular Complex of Actinomycin D and 10,11-Dihydro-3*H*-naphth[1,2-*g*]indazol-7-ol

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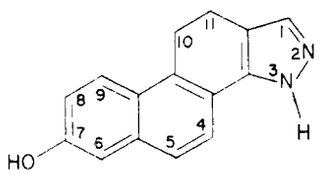
The role of molecular complexation in the activity of actinomycin D in the presence of heterosteroid-type substances has been recently investigated[1-3] by a variety of spectrometric methods, including fluorescence and proton magnetic resonance spectroscopy. While ^{1}H NMR studies have proven to be useful in the study of molecular complexes,[4,5] the complexity of the spectra of large biomolecular complex systems is a limiting factor except in those cases in which a well-resolved signal is available or the system is a highly symmetrical molecule.[6]

It has been recognized for many years that the application of ^{13}C NMR analysis to large, unsymmetrical molecular complexes could be instructive in that the chemical shifts of most of the carbon atoms of the acceptor and donor may be examined simultaneously. With the advent of pulse Fourier transform techniques, examination of molecular complexation of the charge transfer type has become feasible, and recent work on some known charge transfer systems[7,8] has provided advances in this area.

It has been reported that a combination of actinomycin D, a clinical anticancer agent, and 10,11-dihydro-3*H*-naphth[1,2-*g*]indazol-7-ol (**1**) readily inhibited growth of selected microorganisms and the L–M cell line to a greater extent than the individual compounds alone.[1,2] The later report[1] gave evidence for the hypothesis that this potentiation in activity could be due to the formation of a molecular complex between the two compounds in solution. A tentative structure for the complex was also postulated on the basis of ^{1}H NMR spectral analysis.[1] However, the data did not permit unequivocally the elimination of alternative structures and did not have the benefit of ^{13}C NMR analysis.



This paper describes additional evidence for the formation of a molecular complex between actinomycin D and **1** as well as a postulated orientation of the complex under the stipulated conditions. All spectrometric determinations were done with D_3COD as the solvent to attain the concentrations necessary for ^{13}C NMR. Although the previous complexation studies for this system[1] were done in D_2O, unfortunately, low solubility of **1** in D_2O prevented a practical ^{13}C spectral analysis in this solvent, and also some decompostion of the actinomycin D occurred during the extremely long acquisition times. Thus, the concentrations used in the D_2O study were less than those used in the D_3COD study by a factor of 10 (1.3 mM vs. 12.7 mM).

Results and Discussion

The ^{13}C NMR spectrum of actinomycin D had been previously analyzed in $DCCl_3$.[9,10] Assignments in D_3COD were made by direct comparison with this published work. However, not every carbon atom could be identified due to the low solubility of the actinomycin D as well as the low intensity of some of the slower relaxing carbons.[10] No assignments were made of the carbon signals in the polypeptide chains, as several resonances were partially obscured by a large ^{13}C signal from the solvent and the high resonance density of carbon signals. Partial obscuring of ^{13}C signals by a large ^{13}C solvent signal was avoided in the previous study[10] by using $D^{12}CCl_3$. Unfortunately, isotopically pure $D_3{}^{12}COD$ is not available.

In the case of **1**, chemical shifts were identified for appropriate carbons on the basis of model compounds, as well as by use of an off-resonance, broad-band, proton-decoupled spectral analysis. The assignments for the ^{13}C shifts for the individual solutions of actinomycin D, **1**, and the complex are given in Table I.

The striking feature of the ^{13}C NMR spectrum of the complex is that *all* identifiable signals for carbon atoms in the phenoxazine ring of actinomycin D displayed a *negative* (*upfield*) shift in the spectrum relative to the corresponding signals in the ^{13}C spectrum of pure actinomycin D in D_3COD. In contrast, the ^{13}C chemical shifts in the complexed indazole **1** displayed *both* upfield and downfield shifts. A more graphic representation of these results can be seen in Figures 1 and 2, where the numerical differences between the ^{13}C chemical shifts of the free and complexed actinomycin D (Figure 1) and the free and complexed indazole **1** (Figure 2) are given.

As can be seen in Figure 1, the largest ^{13}C shift differences in the spectrum of complexed actinomycin D occur with carbons in the quinoid portion of the phenoxazine ring. Such differences are reduced for the carbon atoms furthest removed from this part of the ring system. This strongly implies that the primary interaction between the compounds in the complex occurs at the quinoid end of the phenoxazine ring behaving as the acceptor.

The observation of solvent-induced shifts for substituted aromatic systems for carbons several atoms removed from the site of substitution has been shown to correlate in a nearly quantitative manner with electronic perturbations in the ring.[11] Similar electronic perturbations can be postulated as the cause of the "tapering off" effect observed in the phenoxazine ring of actinomycin D, although it is impossible to judge the degree of perturbation for any one carbon atom

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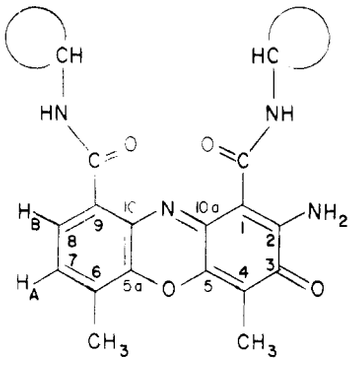
Table I. Chemical Shifts for Actinomycin D, Indazole 1, and the Complex in D_3COD



actinomycin D, δ[a]			indazole 1, δ[b]		
carbon	free	complexed	carbon	free	complexed
C(9) C=O	167.91	167.80	C(7)	155.84	155.80
C(1) C=O	169.69	169.60	C(5a)	136.27	136.18
C(2)	148.87	148.54	C(9b)	133.06	132.89
C(10a)	147.03	146.65	C(9a)	127.78	127.78
C(4a)	146.36	145.98	C(4)	126.17	*b*
C(5a)	141.69	141.40	C(5)	126.17	*b*
C(9a)	133.59	133.50	C(11a)	115.62	115.56
C(9)	130.52	130.35	C(6)	110.92	110.98
C(6)	129.03	128.75	C(10)	25.52	25.57
C(4)	114.27	113.89	C(11)	19.79	19.87
C(6) CH_3	15.11	14.99			
C(4) CH_3	7.76	7.58			

[a] In parts per million from the Me_4Si external standard (corrected). [b] Obscured in the spectrum of the complex.

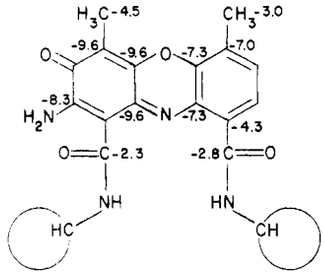


Figure 1. Shift differences in hertz for actinomycin D in the actinomycin D–indazole 1 complex (negative values correspond to upfield shifts).

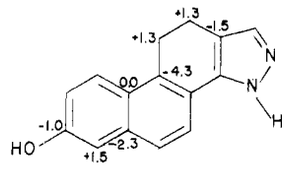


Figure 2. Shift differences in hertz for indazole 1 in the actinomycin D–indazole 1 complex.

without extensive molecular orbital calculations. Such calculations would involve a prohibitive amount of computer time and storage for such large multinuclear systems.

Similar results were observed[8] in the known charge transfer acceptor *o*-chloranil with a variety of aromatic donors, including benzene, mesitylene, anisole, naphthalene, and anthracene. The upfield shift of *all* carbon signals for the acceptor was attributed to a *combination* of charge transfer and magnetic anisotropic effects. The shifts in the spectra of each

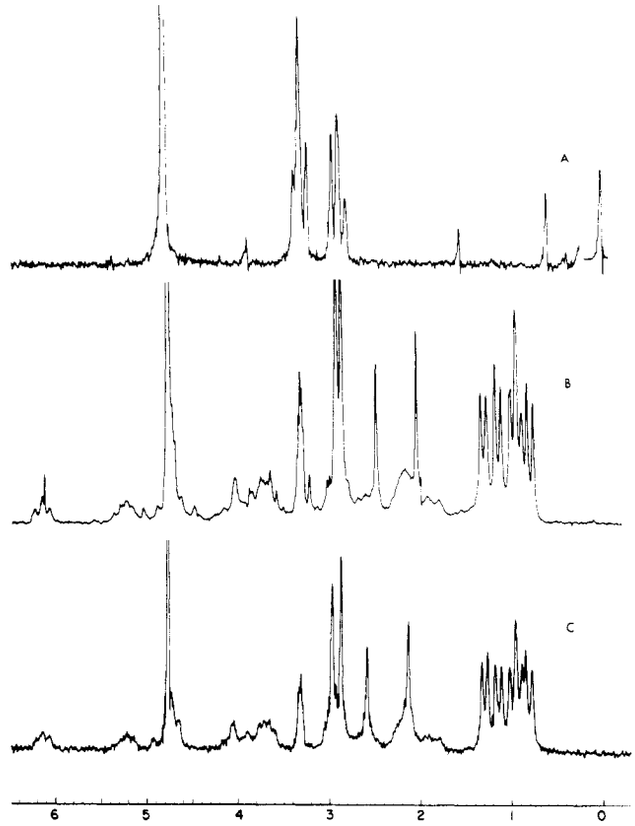


Figure 3. ^1H NMR spectra recorded at 37 °C in D_3COD for indazole 1 (A), the complex (B), and actinomycin D(C).

of the donors, however, showed both upfield and downfield changes in the complexes. This same phenomenon was observed in charge transfer complexes of 1,3,5-trinitrobenzene with the same set of donors.[7] In both papers, this behavior in the donors was attributed by the authors to charge transfer effects *competing* with magnetic anisotropic effects.

As mentioned previously, shifts in the acceptor show that the primary site of interaction apparently involves the quinoid portion of the phenoxazine ring. Indeed, quinoid-type molecules are known to be good charge transfer acceptors due to the availability of low-lying unoccupied electronic energy levels which will readily accept electrons from donors.[12] The site of primary donation in **1**, however, is not so easily defined. Donations from nonbonded electrons on nitrogen (n–π) as well as from aromatic π electrons (π–π) are possible. Earlier evidence[1] suggests that hydrogen bonding also plays an important role in stabilizing complexation under different conditions. Distinctions between each of these effects in contributing to the donor's ability to form a complex cannot be made with absolute certainty. Thus, while **1** must act as a donor in some fashion, as evidenced by the induced changes in the actinomycin D spectra when **1** is added, other evidence was necessary to establish the most likely orientation.

Portions of the ^1H NMR spectra of **1**, actinomycin D, and the complex recorded at 37 °C are shown in Figure 3. The methylene protons on C(10) and C(11) of **1** appear as two triplets as seen in curve A of Figure 3. The upfield triplet of the two is obscured in the spectrum of the complex (curve B) by overlapping signals from the polypeptide portion of actinomycin D. The downfield triplet, though partially obscured in the spectrum of the complex by a solvent peak, displays an upfield shift of −6 Hz in the complex compared to the spectrum of the free indazole **1**. A considerably larger shift (−20 Hz) was observed for these signals at lower concentrations in D_2O.[1] However, as can be seen by comparing curves B with C

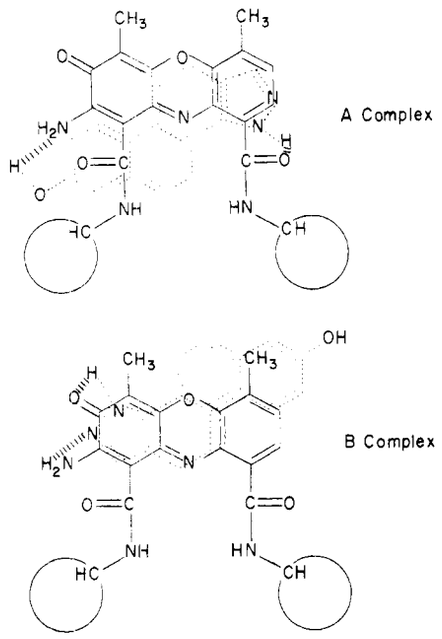


Figure 4. Proposed orientation of the actinomycin D–indazole 1 complex in D_2O[1] (A) and the newly proposed orientation in D_3COD (B).

in Figure 3, a shift of −10 Hz was obtained for protons on both methyl groups attached to C(6) and C(4) of actinomycin D. No such related proton shifts were observed in the ^1H NMR spectra recorded in D_2O in the earlier work.[1] Thus, while evidence in both solvent systems reveal the occurrence of complexation of a similar nature, identical orientations for the complexes in the different solvents with different concentrations *cannot* be assumed.

The orientation of the complex of actinomycin D and **1** in D_2O proposed earlier,[1] based in part on evidence from ^1H NMR data discussed above, is shown in complex A, Figure 4. The observed upfield ^1H shifts for the methyl protons at C(4) and C(6) of actinomycin D in the ^1H NMR spectrum of the complex in D_3COD at higher concentrations, coupled with the analysis of the ^{13}C NMR spectra stated above, support the arrangement shown in complex B of Figure 4 under these conditions. The upfield ^{13}C and ^1H shifts of the signals for the carbons and protons of the methyl groups attached to C(4) and C(6) of the actinomycin D must result from a combination of anisotropic and electronic charge transfer effects. Such a combination of effects could be operative in the proposed complex B (Figure 4) since the aromatic portion of **1** lies over the methyl groups at C(4) and C(6). The charge transfer effect could be readily transmitted through the π system of the phenoxazine ring. The carbon of the methyl group attached to C(4) shows a slightly larger ^{13}C shift difference compared to the difference for the carbon of the C(6) methyl group. This would be expected since the C(4) methyl carbon is closer to the primary site of acceptance. The protons on the methyl groups attached to C(4) and C(6), which are not as sensitive to the electronic perturbations, do not show any detectable difference in the shift changes between the methyl groups when recorded at 37 °C.

When the proton spectrum of the complex was recorded at ~60 °C, the signals of the protons on the C(6) and C(4) methyl groups displayed shifts to lower field relative to the ^1H spectrum of the complex recorded at 37 °C. These results can be seen in Figure 5. The shift is much larger for the signal of the methyl protons (+9 Hz) at C(4) than for the signal of the methyl protons at C(6) (+3 Hz). This suggests that the effects of an increase in molecular motion on the complex's orientation are more important at the C(4) methyl than at the C(6)

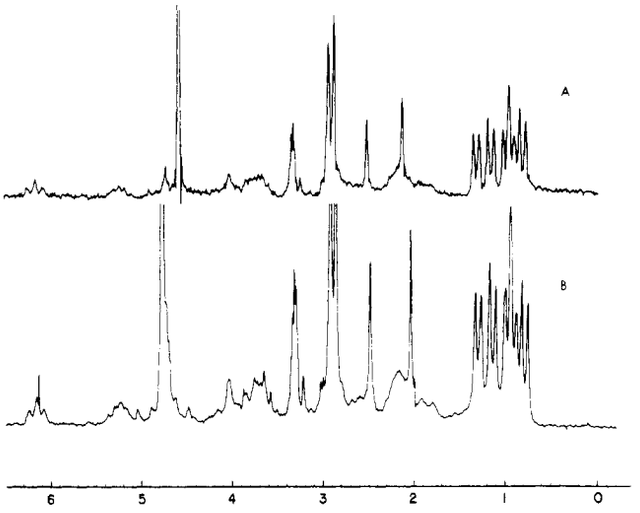


Figure 5. ^1H NMR spectra of the actinomycin D–indazole 1 complex recorded at 60 °C (A) and at 37 °C (B).

methyl. Such molecular motions were postulated as the source of similar temperature shifts in the ^1H NMR spectra of actinomycin D alone at concentrations (5 mM) sufficient to induce a dimer complex.[13] However, these investigators found that the proton signal of the C(6) methyl group was shifted to *higher* field with increasing temperature, in marked contrast to the shift to *lower* field found for the corresponding signal in the spectra of the actinomycin D–indazole **1** complex shown in Figure 5. In the newly proposed complex B (Figure 4), higher energy arrangements are more probable for the situation in which **1** is offset to one side or the other at temperatures above 37 °C. Such translations would be expected to lessen the aromatic anisotropic influence of **1** on the ^1H NMR signals of the actinomycin D protons. Since the protons of the methyl group attached to C(4) lie at the edge of this influence, these translations in the position of the indazole would give rise to greater ^1H NMR shift differences with temperature than would be the case for the protons on the methyl group attached to C(6). Although similar translational effects in the dimer complex of actinomycin D at 5 mM concentration in D_2O have been proposed,[13] the difference in the behavior for the proton signals of the methyl group at C(6) as stated above implies that a distinctly different interaction is occuring in the actinomycin D–indazole **1** complex.

The orientation in complex B (Figure 4) does bear a striking resemblance to the stacked structure proposed for the actinomycin D dimer in D_2O at concentrations above 5 mM.[13] Indeed, it is not unlikely that dimeric structures are also formed in solutions of D_3COD and that **1** must compete with the dimerization process for complexation. Such equilibria could be operating at rates much too fast to be seen on the NMR time scale, so that only a "time average" of signals for nuclei involved in the exchange would be observed. At higher concentrations of **1** relative to the actinomycin D concentration, ^1H shifts of as much as −30 Hz were observed for each of the signals for methyl groups at C(4) and C(6), relative to those observed in actinomycin D alone. This implies a shift in the possible competing equilibria toward the proposed complex of actinomycin D–indazole **1**.

Conclusions

All evidence to date strongly suggests that a molecular complex is formed between actinomycin D and indazole **1** in D_2O and D_3COD regardless of the concentrations and that for concentrations used for both the D_2O and D_3COD studies (1.3 and 12.7 mM, respectively), the preferred arrangement is stacked rather than side-by-side. Evidence for side-by-side

dimer orientations has been reported for D_2O solutions at concentrations from 10 to 100 μM.[14-16] However, we conclude that our evidence in D_3COD supports a 1:1 stacked complex based on (1) the ^{13}C shifts in the quinoid portion of the phenoxazine ring, (2) the upfield shift in the ^1H NMR spectrum of the C(6) and C(4) methyl protons in actinomycin D, and (3) the upfield shift in the ^1H NMR spectrum for the C(10) or C(11) methylene protons of the indazole **1**. This evidence is not inconsistent with the postulated orientation shown in complex B (Figure 4).[17]

Experimental Section

General. The ^{13}C NMR spectra were recorded (at 37 °C) on a Varian XL-100(15) NMR spectrometer, equipped with a TT-100 PFT accessory, operating at 25.2 MHz with tetramethylsilane (Me_4Si) as an external reference. Field stabilization was accomplished via an internal deuterium lock with broad-band proton decoupling. The pulse width was 15.5 μs (90° flip angle). The spectral width was 6024 Hz, using 16K data points for the real and imaginary portions of the spectra, and a total of 6000 pulses were acquired for each spectra. The ^1H NMR spectra were recorded with the same spectrometer in the field-sweep mode, with Me_4Si again as the external reference at 37 °C (ambient probe temperature). An internal deuterium lock provided field stabilization. The preparation of **1** was reported previously.[18] All samples were weighed on a microbalance to the nearest 0.1 mg.

Sample Preparation. The ^{13}C and ^1H NMR spectra of actinomycin D were recorded at 25.2 MHz on a 12.7 mM D_3COD (99.5% *d*) solution. After addition of 10.8 mg (0.046 mmol) of the indazole **1**, the ^{13}C and ^1H NMR spectra were again examined. Separate spectra of **1** at the same concentration (15.3 mM) were recorded.

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Registry No.—**1**, 31184-51-7; actinomycin D, 50-76-0; 1–actinomycin D complex, 55006-96-6.

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Practical Synthesis of Deuterium-Labeled Methyl Sulfide, Methyl Disulfide, Methanethiol, and Methanesulfenyl Compounds

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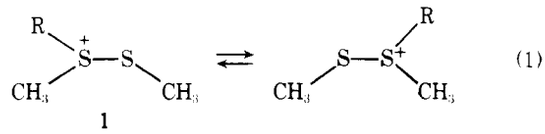
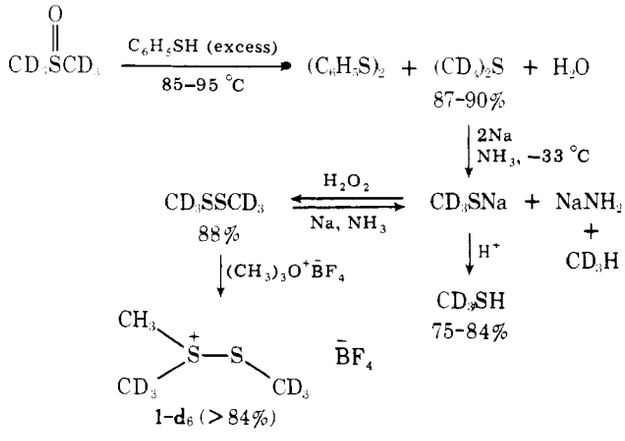
Previously we reported evidence that sulfenyl salts of the type shown in **1** can rearrange by migration of an alkyl group from positive sulfur to neutral sulfur (eq 1).[1,2] This rearrangement appears to be a reversible S_N1 dissociation when the R group can separate as a reasonably stable carbocation,[3] and a concerted 2,3-sigmatropic process when R is allylic.[4] The possibility of rearrangement occurring in the simplest analogue (R = CH_3) was considered unlikely but deserving of investigation. For this purpose we required a source of dimethyl-d_6 sulfide and disulfide. These labeled compounds are available commercially only by custom synthesis, and a recent price quotation for the synthesis of $(CD_3)_2S$ came in at $1500–2000 per 5 g. The high cost of this and related labeled methylthio compounds made it necessary to attempt the synthesis ourselves. The literature records the synthesis of methane-d_3-thiol by methylation of thiourea[5,6,7] and the synthesis of dimethyl-d_6 disulfide by the oxidation of methane-d_3-thiol. The yields are fair to good and the expense high because of the high cost of the methylating agents, CD_3I or $(CD_3)_2SO_4$.[8] We were forced to look for better procedures.



At the present time, the least expensive deuterium-labeled methylthio compound is dimethyl-d_6 sulfoxide,[8] and reduction of the sulfoxide to dimethyl-d_6 sulfide in modest yield has been reported.[9] We have found that reduction of $(CD_3)_2SO$ gives high yields of $(CD_3)_2S$ by the method of Yiannios and Karabinos[10] which involves heating the sulfoxide with excess benzenethiol. In turn, reductive cleavage of the labeled sulfide with sodium metal in liquid ammonia gives sodium methiolate-d_3 which can be converted to methane-d_3-thiol as desired by acidification. In the cleavage step, CD_3Na formed is subsequently lost as CD_3H. Sodium methiolate-d_3 with a molar equivalent of 30% hydrogen peroxide proved to be a convenient route to dimethyl-d_6 disulfide,[11] and methylation of the d_6 disulfide gave the labeled methanesulfenyl salt **1**-d_6. The disulfide can easily be reconverted to sodium methiolate-d_3 and hence to methane-d_3-thiol on treatment with sodium in liquid ammonia.



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