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Laser Temperature Jump Study of Solvent Effects on Proflavin Stacking

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Abstract: The Raman laser temperature jump technique has been used to determine rate constants for proflavin dimerization in aqueous solutions of methanol, ethanol, 1-propanol, glycerol, and urea. Forward rates for solutions in aqueous ethanol are quantitatively analyzed using a specific solvation model. The reverse rates are characterized by the molecularity of ethanol attack on the dye dimer. Thionine requires approximately three ethanols to disrupt a dimer, whereas proflavin requires only one. Poor correlations are found between the reverse rates and bulk solvent properties. The results suggest that solvent effects on dye stacking are determined by specific dye-solvent interactions. Comparison with previous results for thionine stacking indicates the detailed electronic structure of the dye determines these interactions, and that solvent-solvent contributions are relatively unimportant.

Stacking interactions play a fundamental role in nucleic acid chemistry and in the solution chemistry of dyes. Despite an ever increasing amount of thermodynamic data on stacking systems, these interactions are still poorly understood. While quantum chemists have emphasized electronic interactions,[1-3] experimentalists have demonstrated the importance of the aqueous environment.[4,5] Initially, the requirement of water for stacking was thought to implicate traditional hydrophobic interactions.[6,7] However, this hypothesis is contradicted by thermodynamic investigations that have shown that the driving force has a large favorable enthalpy and a significant unfavorable entropy.[8,9] Sinanoglu suggested a model in which the reduction of surface area in the solvent cavity upon stacking resulted in a favorable enthalpy contribution in liquids of very high surface tension.[10] Unfortunately, there has been little experimental work on solvent effects on stacking. Thus, the relative contributions of these various interactions remain unknown.

A more detailed picture of stacking can be obtained by studying the kinetics of the reaction as a function of solvent. In this paper, we present the kinetic results on the association of a cationic dye, proflavin, in various mixed aqueous solvents (see Figure 1). These results are compared with our previous study on thionine.[11] The comparison of these two dyes is of interest, since they both have essentially the same size and shape, while their equilibrium constants for stacking differ by almost an order of magnitude.

Experimental Section

Materials. Proflavin, 3,6-diaminoacridine, was obtained from Aldrich, and recrystallized from water as described previously.[12] Gravimetric analysis showed 1 mol of water per mol of proflavin. Concentrations were made by weight using a molecular weight of 227.26. Water was doubly distilled and absolute ethanol was used. Methanol was spectral grade from Mallinckrodt, 1-propanol "distilled in glass" from Burdick and Jackson, and urea Ultra Pure grade from Schwarz/Mann.

Kinetics. The association of proflavin has a relaxation time of less than 1 μs, thus requiring the use of the Raman laser temperature jump method described previously.[13,14] The probe beam was filtered with a Corning CS5-57 filter and monitored at 440 nm, close to the absorption maximum for the monomer. For studies involving small equilibrium constants, the probe beam was intensified by pulsing the Xe arc lamp to increase the light intensity a factor of ten.[15] As a check for photochemical effects, a 3.96×10^{-3} M solution in D_2O was tested. The temperature jump in D_2O is over 100 times smaller than in H_2O, and no signal was observed. Relaxation data were photographed on 35-mm film, projected onto a Tektronix 4662 plotter, and digitized and analyzed with a Tektronix 4051 terminal. The data were fit to a single exponential by a nonlinear least-squares procedure. Each relaxation time represents an average of at least 12 shots. The estimated error in the rate constants is ±15%. All solutions contained 0.01 M KH_2PO_4.

Results

The stacking of planar dye molecules has been demonstrated for acridine orange and thionine by the characteristic upfield shift of the NMR peaks of the ring protons.[11,16] Although no NMR measurements have been made on proflavin, it is expected to behave similarly. Proflavin does exhibit deviations in absorbance from Beer's law at high concentrations, which suggests that stacking does occur.[17-23]

Solvent effects were studied with aqueous mixtures, since dye stacking has not been observed in nonaqueous solvents.[24] Attempts were made to observe relaxations for both proflavin

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Table I. Rate Constants for Dye Stacking at 22 °C and Physical Constants of Aqueous Solvent Systems at 25 °C[a]

solvent	thionine		proflavin		surface tension, dyn/cm	viscosity, cP	dielectric constant	Y value[c]
	$10^{-9}k_1$, M^{-1} s^{-1}	$10^{-6}k_{-1}$, s^{-1}	$10^{-9}k_1$, M^{-1} s^{-1}	$10^{-6}k_{-1}$, s^{-1}				
H_2O (0.01 M KH_2PO_4)	2.4	0.9	1.1	3.0	72.0	0.89	78.5	3.493
H_2O (0.2 M KCl-0.05 M KH_2PO_4)			0.96	1.8				
5% MeOH			0.94	2.8	57.32	1.07	74.9	3.263
10% MeOH	1.6	1.4	0.92	3.9	50.0	1.24	71.5	3.016
1% EtOH	0.49	0.8	1.1	4.5	63.2	1.00	77.2	3.432
5% EtOH	0.20	1.4	0.63	6.2	46.4	1.40	72.0	3.201
7.5% EtOH	0.20	3.0	0.56	8.0	40.4	1.65	68.8	3.028
10% EtOH	0.18	5.1	0.48	9.5	36.6	1.90	65.5	2.852
1% PrOH	0.36	1.0	0.16	5.8	48.8	1.05	76.4	
10% PrOH		(18.0)			26.4	2.15	59.7	
1% urea			0.35	6.4		0.91[b]	80.2	
5% urea				(14.0)		1.00[b]	85.5	
10% urea	0.61	1.7				1.15[b]	90.7	
10% glycerol	0.4	0.7	1.0	2.9	68.1	2.74	69.8	
15% glycerol				(4.2)	67.5	4.46	66.6	

[a] Obtained by linear interpolation of tables in J. Timmerman, "Physico-Chemical Constants of Binary Systems", Vol. 4, Interscience, New York, 1960. [b] K. Kawahara and C. Tanford, *J. Biol. Chem.*, **241**, 3228 (1966). [c] A. H. Fainberg and S. Winstein, *J. Am. Chem. Soc.*, **78**, 2770 (1956).

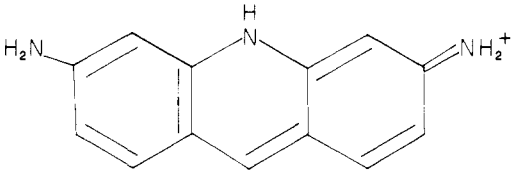

Figure 1. Structure of proflavin.

and thionine (4×10^{-3} M, 22 °C) in pure glycerol and ethylene glycol, but were unsuccessful. Thionine in 15 mol % 1-propanol also showed no relaxation. The relaxations observed in aqueous mixtures are due to the monomer–dimer equilibrium:

$$P + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P_2 \qquad (1)$$

Rate constants are determined from the concentration dependence of the relaxation time according to eq 2:

$$\tau^{-2} = k_{-1}^2 + 8k_1k_{-1}[P]_T \qquad (2)$$

where $[P]_T$ is the total concentration of proflavin. Figure 2 shows plots of τ^{-2} vs. $[P]_T$. Additional kinetic plots are available in the microfilm edition (see paragraph at end of paper). Each solvent mixture exhibits the expected linear behavior. The rate constants obtained from these plots are listed in Table I. Additional results for thionine are also included. The results in 0.2 M KCl–0.05 M KH_2PO_4 are identical with those reported previously,[25] except in this work the proflavin concentrations have been corrected for the contribution of one water of hydration to the molecular weight. Aqueous solutions do not show a large salt dependence, and equilibrium constants obtained from the kinetic data are 370 (0.01 M KH_2PO_4) and 530 M^{-1} (0.2 M KCl–0.05 M KH_2PO_4). These are in agreement with the equilibrium constants from calorimetric measurements but are considerably different from the spectrophotometric numbers.[17,19]

It was not possible to accurately determine the rate constants in 5 mol % urea. This is because the low equilibrium constant in this solution results in weak signals. Relaxation times were measured at three high concentrations and were found to be independent of concentration. This suggests the k_{-1}^2 term dominates the relaxation and the k_1 for the reaction is small. This is consistent with the large decrease in k_1 already observed at 1 mol % urea. The relaxation times were used to determine k_{-1} alone.

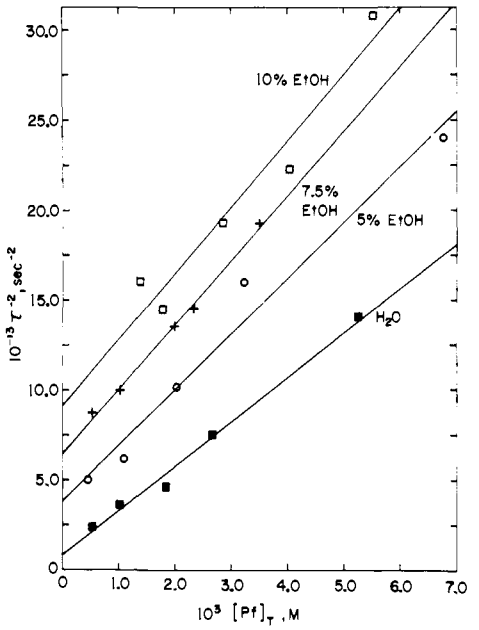

Figure 2. Plots of square of reciprocal relaxation time vs. total proflavin concentration for ethanol–water mixtures at 22 °C: (■) 100% water; (○) 5 mol % ethanol; (+) 7.5 mol %; (□) 10 mol %. All solutions contained 0.01 M KH_2PO_4.

Discussion

The goal of this research is to understand how solvent affects stacking reactions. The results presented here make it possible to compare solvent effects on proflavin stacking with those previously determined for thionine.[11] Both reactions share the general mechanism:

$$P + P \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} P,P \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} P_2 \qquad (3)$$

where P,P is an encounter complex consisting of monomers separated by one or more solvent molecules. The first step is the diffusion-controlled formation of the encounter group, and the second step is a rearrangement to yield the dimer. Applying the steady-state assumption to the encounter group gives the following expressions for the observed rates:

$$k_1 = \frac{k_{12}k_{23}}{k_{21} + k_{23}} \qquad (4)$$

$$k_{-1} = \frac{k_{21}k_{32}}{k_{21} + k_{23}} \quad (5)$$

Provided there are no long-range interactions, the diffusion-controlled rates, k_{12} and k_{21}, can be estimated from the Smoluchowski equation.[26] Since proflavin and thionine have similar sizes, they should have similar diffusion-controlled rates. These have been previously calculated as 4.1×10^9 M^{-1} s^{-1} and 2.6×10^9 s^{-1} for k_{12} and k_{21}, respectively.[11] For proflavin, the measured forward rate, 1.1×10^9 M^{-1} s^{-1}, is almost four times slower than the calculated rate. This might suggest that electrostatic repulsion between the cations should be considered. However, the forward rate changes by only 10% when the ionic strength is varied from 0.01 to 0.25, indicating that proflavin's charge is sufficiently delocalized over the ring system to make electrostatic effects negligible. This is consistent with CNDO/2 calculations of partial charges.[27] An alternative explanation is that k_{23} is partially rate determining. In this case, consideration of eq 4 gives k_{23} values of 9×10^8 and 4×10^9 s^{-1} for proflavin and thionine, respectively. This could correspond to rearrangement of dye monomers, or to the rate of solvent ejection from the encounter complex. The rate of orientational relaxation of these dyes in water is expected to be 10^{10} s^{-1} or higher and should be the same for proflavin and thionine, since they have identical volumes.[28] Thus, k_{23} appears to be a measure of the solvent exchange rate, suggesting that water solvates proflavin more strongly than thionine.

In the solvent mixtures, there is an additional equilibrium due to interaction of the dye with cosolvent, A:

$$P + A \rightleftharpoons PA \qquad K_A \quad (6)$$

Here K_A is the equilibrium constant for this association. If both P and PA can form dimers, then it is necessary to consider the following parallel reactions:

$$P + P \underset{k_{21}^{(0)}}{\overset{k_{12}^{(0)}}{\rightleftharpoons}} P,P \underset{k_{32}^{(0)}}{\overset{k_{23}^{(0)}}{\rightleftharpoons}} P_2 \quad (7a)$$

$$k_A \downarrow\uparrow$$

$$PA + P \underset{k_{21}^{(1)}}{\overset{k_{12}^{(1)}}{\rightleftharpoons}} PAP \underset{k_{32}^{(1)}}{\overset{k_{23}^{(1)}}{\rightleftharpoons}} P_2 + A \quad (7b)$$

$$\downarrow\uparrow$$

$$PA + PA \underset{k_{21}^{(2)}}{\overset{k_{12}^{(2)}}{\rightleftharpoons}} PAP + A \underset{k_{32}^{(2)}}{\overset{k_{23}^{(2)}}{\rightleftharpoons}} P_2 + 2A \quad (7c)$$

The assumption that PA and P are in equilibrium during the observed rate processes leads to the following expression for the observed forward rate:

$$k_1 = \frac{k_1^{(0)} + k_1^{(1)}K_A[A] + k_1^{(2)}K_A^2[A]^2}{(1 + K_A[A])^2} \quad (8)$$

where $k_1^{(0)}$, $k_1^{(1)}$, and $k_1^{(2)}$ are overall rates defined by analogy with eq 4, and [A] is the molar concentration of cosolvent. The rate $k_1^{(0)}$ is essentially that measured in pure water. The other constants can be estimated by fitting the observed dependence of k_1 on cosolvent concentration.

For thionine in water–ethanol mixtures, $k_1^{(0)}$ is 2.4×10^9 M^{-1} s^{-1} and $k_1^{(2)}$ is 0.2×10^9 M^{-1} s^{-1}. This latter value is derived from the limiting rate observed at high ethanol concentration (see Figure 3). The only constants left to be determined are $k_1^{(1)}$ and K_A. A nonlinear least-squares fit gives excellent agreement with the data when $k_1^{(1)}$ is 5.4×10^8 M^{-1} s^{-1} and K_A is 5.8 M^{-1}. The predicted curve is shown in Figure 3. The derived K_A agrees well with the value of 3.9 M^{-1} determined previously from equilibrium spectrophotometric measurements.[29]

For proflavin, the forward rate never levels off at high ethanol concentration, so $k_1^{(2)}$ cannot be measured directly. Therefore, three constants were determined by a nonlinear least-squares fit. Assuming a $k_1^{(0)}$ of 1.1×10^9 M^{-1} s^{-1}, the fitted values are $K_A = 1$ M^{-1}, $k_1^{(1)} = 2.6 \times 10^9$ M^{-1} s^{-1}, and $k_1^{(2)} = 2.0 \times 10^8$ M^{-1} s^{-1}. In comparing $k_1^{(0)}$ and $k_1^{(1)}$ it must be remembered that $k_1^{(0)}$ is a rate for a self-association, whereas $k_1^{(1)}$ refers to a reaction of two different species. With this in mind, it appears that a collision of a PA complex with P is just as likely to result in reaction as a collision of two P's. On the other hand, a collision of two PA complexes is only one-fifth as likely to result in dimer formation. Equation 4 indicates this may reflect changes in either k_{23} or k_{21} for this last case, a point we will return to later. Comparison of both K_A and $k_1^{(1)}$ for thionine and proflavin suggests the ethanol–dye complex is much stronger for thionine than for proflavin. Of course, these fitted numbers can only be considered order of magnitude estimates due to the error in the experimental rate constants.

The effect of cosolvents on the dimerization of dyes is not confined to changes in the forward rates, but is also seen in the reverse rates. The increase in k_{-1} may be due to cosolvent attack on the dimer being more effective than water attack. The molecularity of such an attack can be estimated by plotting k_{-1} as a function of cosolvent molarity. Plots for proflavin and thionine in aqueous ethanol solutions are shown in Figure 4. Within experimental error, this plot is linear for the proflavin data, whereas the thionine data are better fitted by the cube of the cosolvent concentration. Apparently, the proflavin dimer is broken up by a single alcohol, while the thionine dimer requires approximately three. This is consistent with the greater stability of the thionine dimer in water as reflected by its lower dissociation rate (0.9×10^6 vs. 3.0×10^6 s^{-1}).

The effects of ethanol on reverse rates suggest a possible explanation for the different behavior of $k_1^{(1)}$ and $k_1^{(2)}$ between proflavin and thionine (see eq 7). If the encounter complex P,P is not strictly diffusion controlled, but has some inherent stability, then the lifetime of this complex should be sensitive to the number of ethanol molecules present. The dependence of thionine reverse rate on alcohol suggests that more than one alcohol would be necessary to destabilize this complex. Thus, k_{21} should be essentially constant for the reactions in eq 7, and $k_1^{(1)}$ and $k_1^{(2)}$ are both determined largely by the rate of ethanol exchange on thionine. In contrast, a proflavin encounter complex will be destabilized by a single additional alcohol. If this complex consists of a single ethanol and two monomers, then the encounter group formed in reaction 7c will have a shorter lifetime than that formed in 7a or 7b. This increase in k_{21} results in the low value calculated for $k_1^{(2)}$. In this model, the rate of ethanol exchange on proflavin is too fast to slow down the observed rates. This is consistent with the high value of $k_1^{(1)}$, the low value deduced for K_A, and the relatively slow rate of water exchange on proflavin. There is an alternative explanation for the difference of $k_1^{(1)}$ and $k_1^{(2)}$ derived for proflavin. Solvent exchange could simply be slower when two ethanol molecules are present. While it is surprising this is not observed for thionine, the possibility cannot be strictly ruled out.

Attempts have also been made to correlate the measured rates with the solvent parameters listed in Table I. The forward rate could depend on viscosity if reaction occurred immediately on every encounter.[30] However, such a correlation is not observed. Thus, a number of collisions must occur between two monomers trapped in a solvent cage before the dimer forms. This is consistent with the proposed mechanism. Since both dyes have a positive charge, it might be expected that solvent polarity would influence the forward rates. However, no correlation is observed for $\log k_1$ with inverse dielectric constant or with solvent Y values, a measure of microscopic solvent polarity.[30-32] For example, the forward rates measured for proflavin in 1 mol % 1-propanol and 1 mol % urea are much

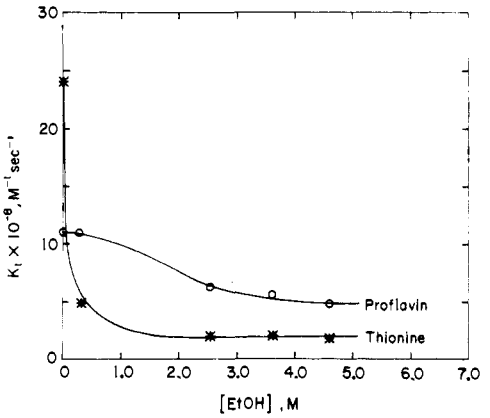

Figure 3. Plot of k_1 vs. molarity ethanol for thionine and proflavin. Solid lines are fitted curves.

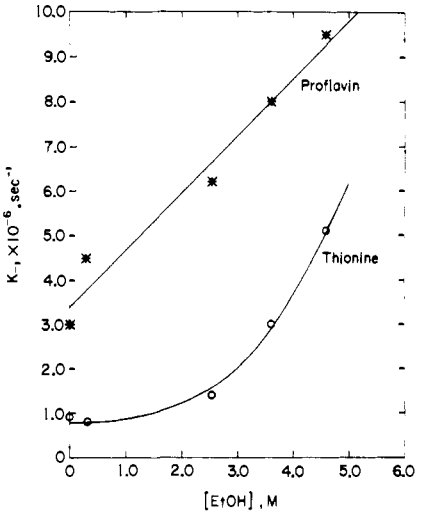

Figure 4. Plot of k_{-1} vs. molarity ethanol for thionine and proflavin. Solid line is fit to linear least squares for proflavin and cubic least squares for thionine.

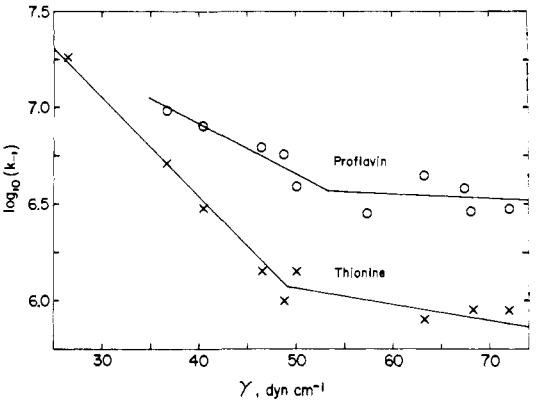

Figure 5. Plot of surface tension vs. common logarithm of k_{-1}.

slower than would be predicted from solvent polarity. For the reverse rate, there are slight correlations of log k_{-1} with viscosity, reciprocal of the dielectric constant, and surface tension. Both dyes correlate well with these parameters when only alcohol–water mixtures are considered, but the urea, glycerol, and formamide results are not consistent. The surface tension gives the best correlation and the plot is shown in Figure 5. This plot is unusual in that it shows two linear regions. The reverse rate seems to level off at large surface tension. If simple cavity considerations were important, this plot would be expected to be linear. It is also important to note that pure glycerol, ethylene glycol, and 15 mol % 1-propanol do not appear to correlate with this plot. These solvents have surface tensions at 25 °C of 62.5, 48.0, and 25.3, respectively, and would be expected to have a stable dimer. However, no relaxations could be detected for either proflavin or thionine in these solvents. The data, therefore, indicate that cavity terms do not dominate solvent effects on stacking. Of course, there is no guarantee that macroscopic surface tension adequately reflects the microscopic surface tension. One possible interpretation of the ethanol effects on reverse rates is that the alcohol interacts with the solvent cavity rather than directly with the dimer. However, the slope of the proflavin plot shown in Figure 4 yields a rate constant of 1.2×10^6 M^{-1} s^{-1} for dimer disruption by ethanol. Thus, only one in every 10^4 collisions results in dimer destruction. This efficiency seems too small for a mechanism involving cavity perturbation.

It is interesting to speculate on the intermolecular forces responsible for the effects reported here. The two dyes have essentially identical sizes, so they should be affected similarly if solvent–solvent interactions are dominant. This does not appear to be the case. On the other hand, the electronic structures of the dyes are significantly different. Proflavin has a monomer absorption maximum at 444 nm with $\epsilon = 3.3 \times 10^4$ and an oscillator strength of 0.43, whereas thionine has a maximum at 598 nm with $\epsilon = 5.6 \times 10^4$ and an oscillator strength of 0.52. Dispersion forces will surely be involved in the interaction of the dyes with cosolvents, and to a first approximation they will be stronger for thionine than for proflavin.[29,33,34] This could explain the stronger solvation of thionine by ethanol. However, the forward rate constants measured in 1 mol % propanol suggest that this solvent interacts more strongly with proflavin than with thionine. Thus, it seems that the detailed electronic structure must be considered to explain the kinetic data. Preliminary CNDO/2 calculations indicate that proflavin has a more polar structure than thionine.[27] This is consistent with the stronger interactions of water and urea with that dye. Further understanding of the solvation of these large molecules as reflected in the kinetics will require more extensive calculations.

The reverse rates measured in this work indicate that ethanol is more effective than water in disrupting proflavin and thionine dimers. If the results are extrapolated to high alcohol concentrations, this is the dominant effect that would prevent stacking. This is somewhat surprising for proflavin, since the forward rates indicate the interactions of ethanol and water with the monomer are of equal strength. This may suggest that the size of the attacking solvent molecule is important in determining k_{-1}. Recent theoretical work has highlighted the importance of this parameter on solvent interactions, and this may be another manifestation of its influence.[35,36]

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Supplementary Material Available: Kinetic plots (2 pages). Ordering information is given on any current masthead page.

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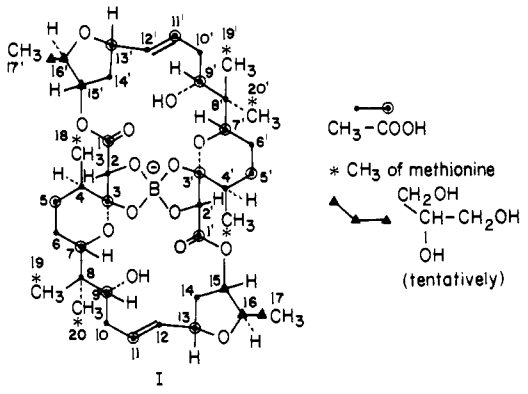
Communications to the Editor

Biosynthesis of the Boron-Containing Macrodiolide Antibiotic Aplasmomycin

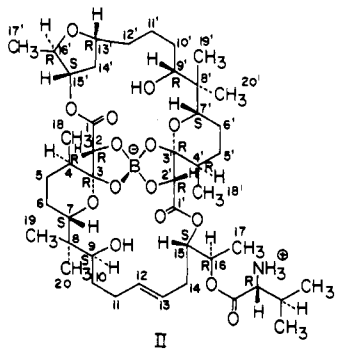
Sir:

Aplasmomycin (I) is a novel ionophoric macrodiolide antibiotic which was isolated from strain SS-20 of *Streptomyces griseus* obtained from a sample of sea mud.[1] Its structure has



I

been determined by a single-crystal X-ray analysis as a symmetric dimer built around a boron atom.[2] It is closely related to boromycin (II), the first boron-containing antibiotic found



II

in nature.[3,4] The two compounds have very similar conformations and identical configurations at all the asymmetric centers except C-9, but, in contrast to boromycin, aplasmomycin does not contain the D-valine moiety. In this communication, we present results on the biosynthesis of this unusual macrolide antibiotic.

Following preliminary studies with ^{14}C-labeled precursors, feeding experiments were conducted with 90% enriched sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C]acetate, and L-[*methyl*-^{13}C]methionine. The labeled precursors were added to shake cultures of *Streptomyces griseus* strain SS-20 at 48 h after innoculation, and the fermentation was continued for an additional 48 h.[5] The labeled antibiotic samples were then isolated in yields of ~10 mg/L by chloroform extraction of the broth followed by preparative TLC. The antibiotics thus obtained were analyzed by ^{13}C NMR spectroscopy.

The natural-abundance proton noise-decoupled ^{13}C NMR spectrum of aplasmomycin shows 20 signals corresponding to 40 carbon atoms of the symmetrical macrocyclic dilactone ring. Each signal represents two identical carbon atoms. An unequivocal assignment (Table I) of every signal in the spectrum was made using the characteristic chemical shifts, multiplicities, single-frequency decoupling, comparison with several derivatives and model compounds, specific deuteration experiments, and analysis of one-bond carbon–carbon couplings of pairs of carbon atoms.[7]

The ^{13}C NMR spectrum of [1-^{13}C]acetate-derived aplasmomycin showed seven enhanced carbon signals representing C-1, -1′, C-3, -3′, C-5, -5′, C-7, -7′, C-9, -9′, C-11, -11′, and C-13, -13′ of the macrolide ring. Conversely, [2-^{13}C]acetate increased the intensity of the seven carbon signals corresponding to C-2, -2′, C-4, -4′, C-6, -6′, C-8, -8′, C-10, -10′, C-12, -12′, and C-14, -14′. Incorporation of 14 intact acetate units was confirmed by analysis of the antibiotic enriched by sodium [1,2-^{13}C]acetate, which showed seven pairs of doublets due to carbon–carbon coupling as characteristic satellite signals on the natural-abundance peaks. The pattern of incorporation of acetate is consistent with the polyketide pathway in the sense that the polyketide chains extend from carbon atoms 14 and 14′ through the ring system to carbon atoms 1 and 1′ in the direction of decreasing numbers of the carbon atoms with the nonacetate derived carbons 17–15 and 17′–15′ as starter units. Table I lists the relative abundance values observed in this antibiotic after feeding various precursors and the respective $^1J_{C\text{-}C}$ values found.

Three of the four methyl groups of each chain, carbons 18, 19, and 20 are derived from methionine (Table I). This is unusual since the branching methyl groups of most macrolide antibiotics, with few exceptions, e.g., the lankacidins,[8] have been demonstrated to originate from propionate units.

No significant enrichment of carbons 15, 16, and 17 was observed by any of the ^{13}C-labeled precursors employed so far. Although [2-^{14}C]- and [3-^{14}C]propionate showed good specific incorporations, 75 and 80%, respectively, into aplasmomycin, surprisingly [1-^{14}C]- and [1-^{13}C]propionate did not give any

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