The ΔH and ΔS values for the *cis,cis* $\rightleftarrows$ *trans,cis* equilibrium are consistent with a twist-boat $\rightleftarrows$ chair interconversion. The possibility that the *cis,cis* isomer might exist in a chair $\rightleftarrows$ twist-boat equilibrium was investigated by variable-temperature infrared techniques (3% solution in carbon disulfide). No changes in relative intensities were noted, indicating that the *cis,cis* isomer must exist almost exclusively in a single conformation. As the *cis,cis* isomer exists essentially only in the twist-boat conformation, one can readily calculate ΔG_{298}, ΔH, and ΔS for the 1,4-di-*t*-butylcyclohexane twist-boat $\rightleftarrows$ chair interconversion by removing the effects of the hydroxyl. Subtracting the appropriate values of ΔG_{298}, ΔH, and ΔS for $OH_{ax} \rightleftarrows OH_{eq}$ interconversion[11] in the cyclohexane system from the data given in Scheme I gives $\Delta G_{298} = -5.7$ kcal/mol, $\Delta H = -7.7 \pm 0.29$ kcal/mol, and $\Delta S = -8.0 \pm 0.7$ eu.

The thermodynamic parameters for the various equilibria involving the *trans,trans* isomer, as well as the lack of linearity of the log K *vs.* $1/T$ plots at higher temperatures, indicate that the *trans,trans* isomer is not conformationally homogeneous. The infrared spectra of the *trans,trans* isomer (3% solution in carbon disulfide) showed significant changes in band intensities with changes in temperature. Using the variable-temperature infrared data,[12] values for ΔH and ΔS for the $\text{chair}_{t,t} \rightleftarrows \text{twist-boat}_{t,t}$ equilibrium were calculated to be -0.42 kcal/mol and -1.30 eu, respectively, with $\Delta G_{298} \approx -0.05$ kcal/mol. The unexpectedly low value for ΔS for this chair $\rightleftarrows$ twist-boat equilibrium can be rationalized on the basis that the expected dipseudoequatorial *t*-Bu–OH interaction in the twist-boat conformation restricts the twist-boat to a small part of its ordinary pseudorotational circuit resulting in a reduction in entropy. A more detailed discussion of these results and those of related systems will appear at a later time.

Acknowledgment. The authors wish to thank Professor Ernest L. Eliel for helpful discussions concerning this work.

(11) E. L. Eliel, D. G. Nielson, and E. C. Gilbert, *Chem. Commun.*, 360 (1968).

(12) The temperature range covered was -90 to $+65°$ in carbon disulfide solution. Although several sets of peaks changed in relative intensity, two reasonably well-resolved, medium–weak peaks at 1032 and 1016 cm^{-1} were used in the determination of the thermodynamic parameters. The extinction coefficients of the two peaks were assumed to be equal.

(13) Alfred P. Sloan Research Fellow, 1967–1969.

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The Chemistry of the Modification of Tryptophan with 2-Hydroxy-5-nitrobenzyl Bromide

Sir:

The increasing use of the reagent 2-hydroxy-5-nitrobenzyl bromide for the modification and quantitative estimation of tryptophan in proteins[1,2] dictates the necessity of determining the structures of the products of the modification reaction. Recently, Spande, Wilchek, and Witkop[3] and Schellenberg, Chan, and McLean[4] made significant contributions to this end by elucidating the structures of some products of the reactions of model indole compounds with 2-hydroxy-5-nitrobenzyl bromide. In this work we wish to report the products formed during modification of tryptophan ethyl ester to produce 1:1 adducts. These and previous studies allow us to define quite completely the possible modes of reaction of 2-hydroxy-5-nitrobenzyl bromide with tryptophan in proteins to produce the monosubstitution products which are usually observed.

A complex mixture resulted when 500 mg of L-tryptophan ethyl ester hydrochloride (**1**) was allowed to react with 500 mg of 2-hydroxy-5-nitrobenzyl bromide (**1**) in aqueous acetone at pH 4.7. Unreacted **1**, 2-hydroxy-5-nitrobenzyl alcohol, and its methyl ether were identified by conventional methods. Two components, **3** and **4**, in a ratio of 57:43, were identified as monosubstitution products, and the two remaining components, **5** and **6**, were shown to be disubstitution products derived from **3** and **4**, respectively, by further reaction with 1 equiv of 2-hydroxy-5-nitrobenzyl bromide.

Compounds **3** (mp 189–190°) and **4** (mp 198–199°) had identical elemental analyses, identical infrared spectra, and identical electronic spectra: λ_{max} (95% ethanol) 240 mμ (ε 11,800), 310 mμ (8900); λ_{max} (2 *N* NaOH) 422 mμ (19,800).[5] When **3** and **4** were refluxed in 3:1 (v/v) ethanol–concentrated HCl, both yielded the same compound, **7**, which upon acetylation gave an N,O-diacetyl derivative (**7**-Ac_2) possessing characteristic indole uv absorption. The similarity of the two nonidentical compounds **3** and **4** can be explained by the formation of a new asymmetric center when the hydroxynitrobenzyl group adds at the 3 position of the indole ring;[6–8] this addition would yield diastereomeric adducts, because the asymmetric α carbon of the tryptophan ethyl ester is present as the L enantiomer. The transformation of **3** and **4** to **7** is evidently an example of an indolenine rearrangement.[7,8]

The 220-MHz nmr spectra confirmed these ideas, except that there was no signal in the aromatic region attributable to the 2-proton of an indolenine ring. This problem was resolved when it became clear that the initial indolenine products, **3′** and **4′**, had undergone nucleophilic attack at the 2 position of the ring by either the side-chain amino group of tryptophan or the *o*-hydroxyl group of the hydroxynitrobenzyl moiety to create a third asymmetric center.[3,7,8] The singlet resonance at δ 5.2 in the spectra of both compounds supports these alternatives.[3,8–10] The presence of a

(1) (a) D. E. Koshland, Jr., Y. D. Kharkhanis, and H. G. Latham, *J. Amer. Chem. Soc.*, **86**, 1448 (1964); (b) H. R. Horton and D. E. Koshland, Jr., *ibid.*, **87**, 1126 (1965).

(2) T. E. Barman and D. E. Koshland, Jr., *J. Biol. Chem.*, **242**, 5771 (1967).

(3) T. F. Spande, M. Wilchek, and B. Witkop, *J. Amer. Chem. Soc.*, 90, 3256 (1968).

(4) (a) K. A. Schellenberg, T. Chan, and G. W. McLean, *Federation Proc.*, 27, 453 (1968); T. Chan and K. A. Schellenberg, *J. Biol. Chem.*, **243**, 6284 (1968).

(5) Because of the importance of the value of the extinction coefficient of these compounds in the assay and modification procedures, this number was determined in strong base from a Beer's law plot to be ϵ_{410} 18,450, essentially identical with the value for the free alcohol (*cf.* ref 2).

(6) E. H. Rodd, Ed., "The Chemistry of Carbon Compounds," Vol. IV, Elsevier Publishing Co., New York, N. Y., 1951, p 41 ff.

(7) A. H. Jackson and A. E. Smith, *Tetrahedron*, **21**, 989 (1965).

(8) P. L. Julian, E. W. Meyer, and H. C. Printy in "Heterocyclic Compounds," Vol. III, R. C. Elderfield, Ed., John Wiley & Sons, Inc., New York, N. Y., 1952, p 103 ff.

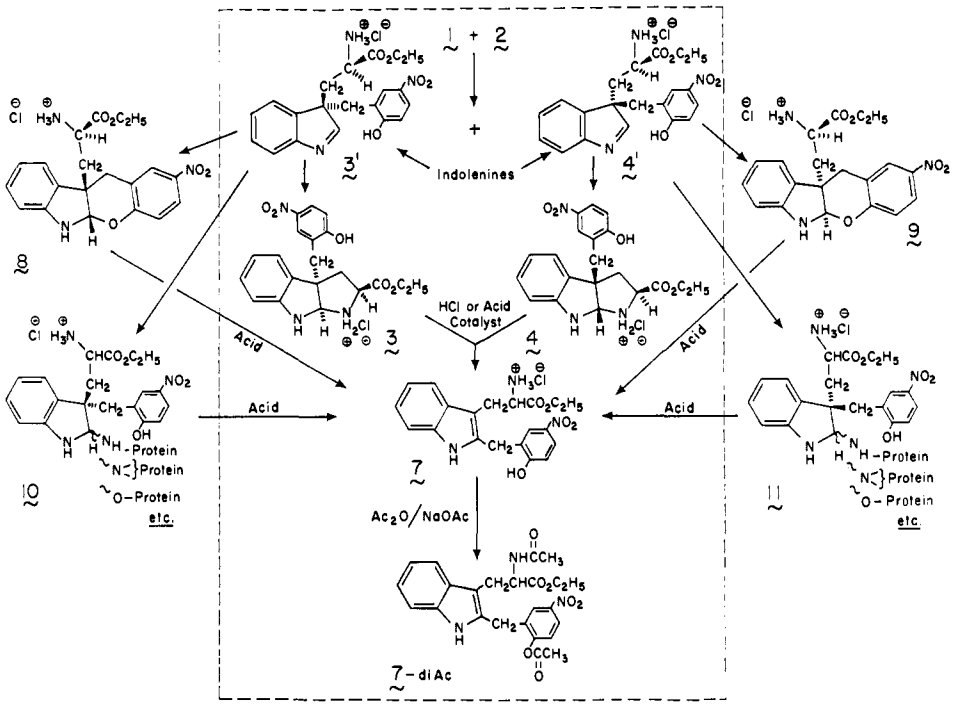

Figure 1. The possible modes of reaction of 2-hydroxy-5-nitrobenzyl bromide with L-tryptophan (indicated schematically as the ethyl ester) in proteins. The reaction sequences elucidated by this work are included within the blocked portion of the diagram.

fully ionized phenolic hydroxyl group of **3** and **4** in basic solution is readily demonstrated by the uv spectrum; yet, when the nmr spectra of **3** and **4** were each run successively in CD_3OD (un-ionized phenolic hydroxyl) and CD_3OD–NaOD (ionized phenolic hydroxyl), there was no shift in the position of the resonance of the ring-juncture proton. Thus, the geminal diamino alternative must be the preferred mode of ring closure in **3** and **4**. A *cis* ring juncture, which determines the configuration of the third asymmetric center, is postulated on thermodynamic grounds. Further support for these assignments is obtained from other features of the nmr spectra. For example, the 2-proton AB quartet (J_{AB} = 15 Hz) centered at δ 3.2 is characteristic of geminal nonequivalent methylene protons on a carbon bonded to a quaternary asymmetric center. In **7**-$(Ac)_2$, the quartet has collapsed to a singlet, a fact consistent with the rearomatization of the ring to which the carbon bearing these protons is bound; also, the δ 5.2 resonance has disappeared, and an indole ring–NH resonance has appeared at *ca.* δ 8.1. The proposed sequence of reactions and structures in this work is therefore shown in the blocked region of Figure 1.

The absolute configurations of **3** and **4** were deduced from the nmr spectra and the knowledge of the absolute configuration of the asymmetric carbon of the tryptophan side chain. An examination of models revealed that the time-average environment of the hydroxynitrobenzyl group includes a contribution from a rotamer in which the face of the phenyl ring is directly over the asymmetric carbon of the tryptophan side chain. In **3**, the methine proton at this asymmetric carbon is in the face of the ring and the carbethoxy group is relatively far removed from it, whereas, in **4**, the reverse is true. The nmr spectra show that, in isomer **3**, the carbethoxy methylene proton and the methine proton resonances are separated by 82 Hz. In isomer **4**, however, an upfield shift, relative to the position in isomer **3**, of the carbethoxy methylene proton and a downfield shift of the methine proton resonances have caused these signals to converge. These results are required by the ring-current effect due to the position of the phenyl ring of the hydroxynitrobenzyl group in the two isomers and define the absolute stereochemistry of the proposed structures, presented in Figure 1. The slight predominance of **3** and **4** obtained because of asymmetric induction is also predicted by this assignment, since the more bulky carbethoxy group will sterically hinder approach of 2-hydroxy-5-nitrobenzyl bromide from a "*syn*" direction (to give **4**), but cannot do so when approach is from the opposite direction (to give **3**).

These results therefore suggest that 2-hydroxy-5-nitrobenzyl bromide will react with tryptophan in proteins to produce first the 1:1 adduct at the 3 position of the indole ring (compounds **3′** and/or **4′**). Ring closure may then occur with any of a number of suitable nucleophiles. The free hydroxyl of the tryptophan reagent itself may be one such nucleophile.[3,4] If so, two isomers similar to **8** and **9** of Figure 1 would be expected as products resulting from the reaction with L-tryptophan in a peptide linkage. In addition, the adjacent nucleophiles such as free NH_3^+ groups or free OH groups could react as indicated schematically by **10** and **11**. Finally, adjacent acid groups in the protein might catalyze the rearrangement to derivatives such as **7**. The exact products will depend therefore on the nature of the groups in the neighborhood of the tryptophan being modified.

Thus, the 2-hydroxy-5-nitrobenzyl bromide reagent

(9) L. A. Cohen, J. W. Daly, H. Kny, and B. Witkop, *J. Amer. Chem. Soc.*, 82, 2184 (1960).

(10) N. S. Bhacca, D. P. Hollis, L. F. Johnson, and E. A. Pier, Compilers, "High Resolution NMR Spectra Catalog," Vol. II, Varian Associates, Palo Alto, Calif., 1963, Spectra No. 562–691.

provides an entire spectrum of possibilities for modes of reaction which should be immensely useful in probing the details of the active sites of enzymes.

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The Effect of Association on the Nuclear Magnetic Resonance Spectra of Tyrocidine B[1]

Sir:

The tyrocidines are cyclic antibiotic decapeptides of known amino acid sequence which are excellent models[2-8] for the study of many concepts which are currently much discussed in protein chemistry. These concepts are based on interpretations from physical measurements such as viscosity, diffusion, sedimentation, rotatory dispersion, circular dichroism, nuclear magnetic resonance, ultraviolet and infrared spectroscopy, etc. It seems possible to develop a more definitive understanding of the interacting forces which determine the shape (conformation), state of aggregation, solubility, etc., of polypeptides and proteins.

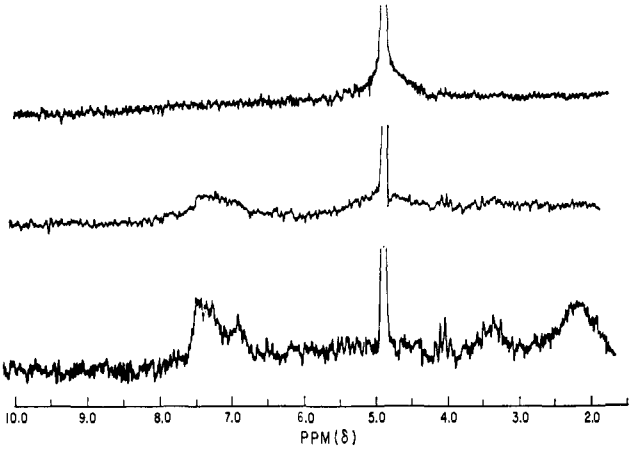

Figure 1. Effect of concentration on 100-MHz nmr spectrum of tyrocidine B in D_2O at 60°. Top pattern = 6%, middle pattern = 2%, bottom pattern = 1%.

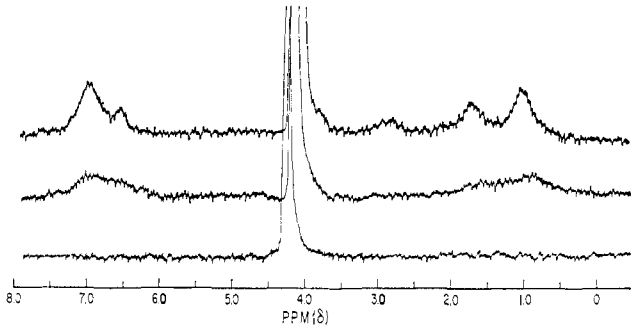

Figure 2. Effect of changing the temperature on the 60-MHz nmr spectrum of 8% tyrocidine B in D_2O. Top pattern = 100°, middle pattern = 80°, lower pattern = 45°.

Tyrocidines A, B, and C all show a strong tendency to aggregate.[3,9] In contrast, gramicidin S-A under similar conditions exhibits little tendency to associate. Nuclear magnetic resonance has been used to establish the conformation of gramicidin S-A in solution,[10] *viz.*, all the Ψ, ϕ, and ω angles were estimated; six of the amino acid residues were involved in an antiparallel β-pleated sheet with four intramolecular hydrogen bonds, and the molecule was unequivocally shown to have a C_2 axis of symmetry perpendicular to the molecular plane. This model agrees in many details with earlier formulations by other workers[11,12] and with proton exchange studies.[10,13]

Since nuclear magnetic resonance can provide much information about structure and interaction of molecules, it became of interest to apply this technique to a study of the self-association and structure of the tyrocidines. This communication describes a preliminary investigation by nmr of the phenomenon of aggregation of tyrocidine B.

When a 6 % (w/w) solution of tyrocidine B in heavy water was examined by high-resolution nmr at ambient temperature no signal was observed (Figure 1). A spectrum could be detected and resolution improved by lowering the concentration of tyrocidine B or by raising the temperature (Figures 1 and 2). The latter phenomenon was reversible. Addition of increasing proportions of methanol also gave nmr spectra of increasing resolution (Figure 3).

These phenomena are consistent with the known aggregation of the tyrocidines in water solution. The high molecular weight aggregates so formed have poorly resolved nmr spectra due to the well-known phenomenon of dipolar line broadening.[14,15] Dissociation of the aggregates by lowering the tyrocidine concentration, increasing the temperature, or adding so-called denaturing solvents gives smaller "polymers" and monomers in which freer rotation results in

(1) This research was supported in part by U. S. Public Health Grant No. A. M. 02493.
(2) M. A. Ruttenberg, T. P. King, and L. C. Craig, *J. Am. Chem. Soc.*, **87**, 4196 (1965).
(3) M. A. Ruttenberg, T. P. King, and L. C. Craig, *Biochemistry*, **5**, 2857 (1966).
(4) R. Williams, "The Non-covalent Association of Tyrocidine B," Ph.D. Thesis, The Rockefeller University, 1967.
(5) L. C. Craig, *Proc. Natl. Acad. Sci. U. S.*, **61**, 152 (1968).
(6) G. Vanderkooi, S. J. Leach, G. Némethy, R. A. Scott, and H. A. Scheraga, *Biochemistry*, **5**, 2991 (1966).
(7) A. M. Liquori and F. Conti, *Nature*, **217**, 635 (1968).
(8) R. Schwyzer and U. Ludescher, *Biochemistry*, **7**, 2519 (1968).
(9) M. A. Ruttenberg, T. P. King, and L. C. Craig, *ibid.*, **4**, 11 (1965).
(10) A. Stern, W. A. Gibbons, and L. C. Craig, *Proc. Natl. Acad. Sci. U. S.*, **61**, 734 (1968).
(11) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).
(12) D. C. Hodgkin and B. M. Oughton, *Biochem. J.*, **65**, 752 (1957).
(13) S. Laiken, M. Printz, and L. C. Craig, *Biochemistry*, **8**, 519 (1969).
(14) C. C. Macdonald and W. F. Phillips, *J. Am. Chem. Soc.*, **89**, 6332 (1967).
(15) J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," Vol. 2, Pergamon Press, Inc., New York, N. Y., 1965, p 829.