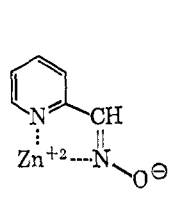
Table II. Second-Order Rate Constants[a] for Nucleophilic Attack

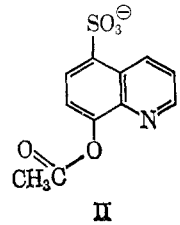
Nucleophile	pK_a	k_2, M^{-1} sec.$^{-1}$ for II	k_2, M^{-1} sec.$^{-1}$ for III	Rate ratio III/II
OH^-	15.7[b]	2.01 ± 0.03	14.8[b]	7.4 ± 0.1
PCA anion	10.04	6.98 ± 0.85	77.2 ± 0.9	11 ± 1
H_2O	−1.7[b]	(7.3 ± 0.9) × 10^{-7}	1.0 × 10^{-8}[b]	0.014 ± 0.002
I	6.5	10 ± 2[c]	10 ± 2[c]	1.0 ± 0.1[d]

[a] At 25°. The pseudo-first-order rate constants for anion liberation from II and III were determined spectrophotometrically in solutions 0.0010 M in $ZnSO_4$, 0.0015 M in PCA, and 0.010 M in 2,6-lutidine buffer, at pH values from 5.89 to 7.53. At any pH, all measurements were made on aliquots of the same reaction mixture, and the substrate concentration was 1.4 × 10^{-4} M or less. [b] W. P. Jencks and J. Carriulo, *J. Am. Chem. Soc.*, **82**, 1778 (1960). [c] Determined over a range of 5.89 to 7.0. Above this pH, nucleophilic attack by ZnPCA(OH) becomes appreciable. [d] The ratio is more accurate than the rate constants, since the latter contain uncertainties in equilibrium constants.

that in a mixed complex of I and II the oxygen atom of I is in a position to attack the acetyl of II.



I



II

Spectroscopic and titrimetric methods establish the equilibrium constants listed in Table I for complexes between zinc and pyridinecarboxaldoxime (PCA). Using these constants and the pseudo-first-order rates of phenoxide formation, we have derived the second-order rate constants listed in Table II for attack on II and on *p*-nitrophenyl acetate (III).[3] These are rates for the spectrophotometric appearance of zinc–oxyquinolinesulfonate complex (λ_{max} 365 mμ) and *p*-nitrophenoxide ion. Under our conditions, subsequent hydrolysis of acetylated I is about ten times slower than acetyl transfer from II. It is still fast enough that the over-all titrimetric rate of hydrolysis of II with 0.01 M Zn^{2+} and 0.015 M PCA is increased by a factor of 10 over the uncatalyzed rate (which has k_1 = 4.6 × 10^{-3} min.$^{-1}$) at neutrality, and a factor of 2 over the rate in 0.01 M Zn^{2+} and 0.015 M glycine. It is apparent that I is an extraordinary[4] nucleophile toward both substrates; it is comparable in reactivity to hydroxide ion, although its pK_a is only 6.5.

The data in Table II show that *p*-nitrophenyl acetate (III) is more reactive than is II toward OH^- and PCA anion; this is as expected since the *p*-nitrophenoxide ion (pK_a = 7.0) is a better leaving group than is the oxyquinolinesulfonate ion (pK_a = 8.4). The very high reactivity of II toward water probably reflects intramolecular general base catalysis by the quinoline nitrogen. We suggest that the increased reactivity of II toward I (shown by the III/II reactivity ratio) reflects an improved rate because of I–II complex formation. Control runs have established that the acetyl transfer from II to I has the correct apparent kinetic order in zinc (calculated from the equilibria in Table I) and completely exclude a mechanism with 2 zinc atoms in the transition state. Furthermore, by quenching with EDTA and isolation, PCA acetate[5] has been identified (infrared, n.m.r., thin layer chromatography) as an intermediate in the hydrolyses, found in 84 ± 4% of the theoretical maximum yield.

The suggestion that the relatively high reactivity of II toward I is a reflection of reaction within a mixed complex is supported by kinetic runs in the presence of 1 mole of *o*-phenanthroline/mole of Zn^{2+}. The *o*-phenanthroline complex of I is actually four times more nucleophilic toward *p*-nitrophenyl acetate, apparently because of increased basicity, but the rate ratio for *p*-nitrophenyl acetate/II is again up to 5.6 (pH 7.51) to 6.1 (pH 7.22). *o*-Phenanthroline is a competitive inhibitor which at least partially blocks binding of II.

The effects seen here are relatively modest since II is a poor ligand with high intrinsic reactivity. We are currently extending these studies to more firmly bound substrates, with I and other chelate catalysts, to examine the scope of this approach to enzyme models.

(3) All kinetic runs were performed with the substrate at less than one-fifth the concentration of zinc to avoid inhibition by product.

(4) Although *p*-nitrophenyl acetate is used as an example of an uncomplexing substrate, the high reactivity toward I probably indicates that here too there is stabilizing interaction between zinc and the leaving group during acetyl transfer.

(5) S. Ginsburg and I. B. Wilson, *J. Am. Chem. Soc.*, 79, 481 (1957).

(6) Predoctoral Fellow of the National Science Foundation. Partial support of this work by the National Institutes of Health is also gratefully acknowledged.

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The Use of the Tyrocidines for the Study of Conformation and Aggregation Behavior

Sir:

Protein studies undertaken from many viewpoints have focused attention on the importance of interacting forces other than those involved in covalent linkages. These forces largely determine the specific physical and biological properties of a given protein. The terms hydrogen bonds, hydrophobic bonds, hydrophilic bonds, π bonds, and ionic bonds in recent years have been widely used in the attempt to describe more precisely the nature of the interactions which determine the stability, conformation, solubility, and state of aggregation of such large polymeric molecules. Obviously a number of parameters, some perhaps not yet even defined, are involved in the concerted interactions to be considered.

This communication is written to point out the value of the tyrocidines and the related peptide gramicidin S-A in this connection and the reason they can serve as particularly valuable models. The tyrocidines are a unique class of naturally occurring cyclic decapeptides. Thus far the individual members can be isolated in preparative amounts only by countercurrent distribution probably because of their very strong degree of aggre-

gation in aqueous solution. The amino acid sequences of the first three members of the tyrocidine series[1-3] and of the peptide gramicidin S-A[4] are known.

Thin-film dialysis studies[5] supported by ultracentrifugal measurements have shown that gramicidin S-A has much less tendency to aggregate than the tyrocidine peptides do. It therefore seemed of interest to investigate the structural requirements for the aggregation phenomenon.

It was found that splitting the peptide ring of tyrocidine B at the Phe-Pro linkage[6] eliminated the tendency to aggregate as well as the antibiotic property. This result indicated that the particular conformation dictated by the ring is required for aggregation, but this alone is not the basis for the association phenomenon, since gramicidin S-A with the intact polypeptide ring as well as half of the amino acid sequence common to the tyrocidines does not show the strong association tendency. Therefore the proper spatial alignment of a particular sequence of groups in the cyclic peptide is necessary in order for aggregation to take place.

An important result was obtained with studies on tyrocidine A in which all of the aromatic groups were fully hydrogenated with platinum oxide catalyst. It was surprising to find that this hydrogenated peptide still showed the aggregation phenomenon even to an enhanced degree. This result demonstrated that the so-called π bonding through the aromatic residues was not responsible for the intermolecular association of the tyrocidine peptides. The hydrogenation of aromatic side chains in polypeptides may thus be a useful technique for detecting π bonding.

Table I. Rotatory Dispersion Constants[a]

Peptide	λ_0, mμ	b_0, deg.
Tyrocidine B	246 ± 10	−321 ± 60
Gramicidin S-A	241 ± 6	−720 ± 90
Linear tyrocidine B	233 ± 12	−49 ± 8

[a] The measurements were made on a Rudolf spectropolarimeter, using a 1-dm. polarimeter tube with concentrations of the order of 2 mg./ml. of peptide in water at 25°.

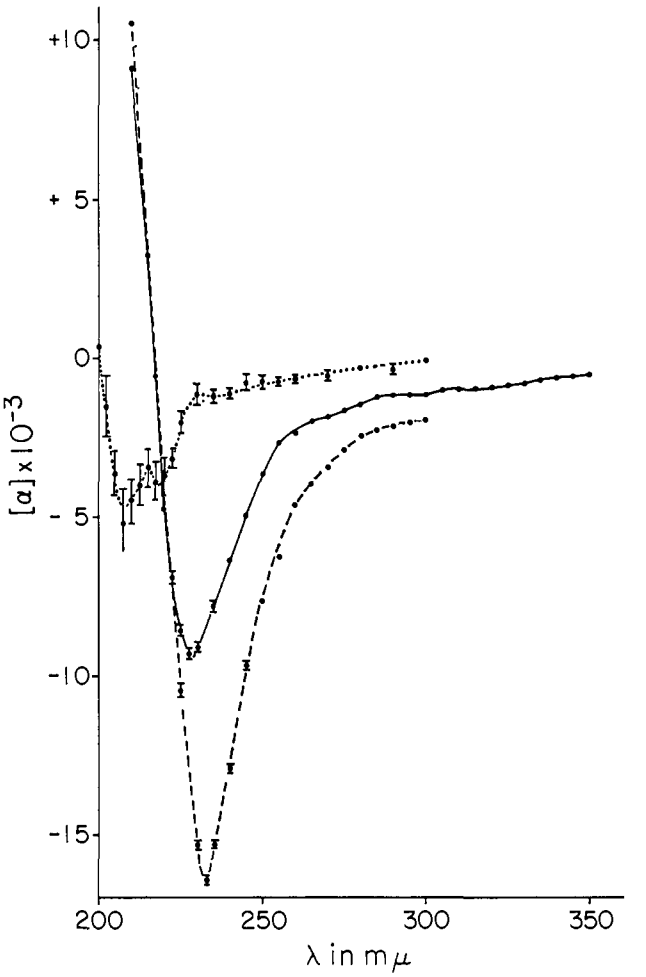

Figure 1. Rotatory dispersion curves. Solid curve, tyrocidine B; dashed curve, gramicidin S-A; dotted curve, linear tyrocidine B. Measurements were made on a Cary recording spectropolarimeter using a 1 mm. cuvette at ambient temperature with concentrations of 0.2–0.4 mg./ml. in water.

Selective alkylation and acylation of the δ-amino group on the ornithine residue and the phenolic group of tyrosine in tyrocidine have indicated that these charged groups are not necessary for the aggregation. It would therefore seem most likely that the strong association tendency of the tyrocidine peptides is the result of hydrophobic bonding allowed by a specific conformation. The hydrophobic association may not be unlike the micelle behavior of detergents such as sodium dodecyl sulfate. In fact, the properties of aggregation, detergent behavior, and antibiotic activity have so far appeared to be inseparable in the case of the tyrocidines.

Rotatory dispersion studies of the tyrocidines are of special interest. The dispersion was essentially monotonic in the 350–700 mμ region but a distinct Cotton effect was evident in the 200–350 mμ region. When the dispersion data of the longer wave length region for the peptides studied were treated by the method of Moffitt and Yang,[7] the values given in Table I were obtained.

In view of the accepted use of the b_0 value and of the trough at 233 mμ as measures of helicity in proteins,[8,9] the results shown in Table I and in Figure 1 are puzzling since a cyclic decapeptide cannot have a helical structure. Either the large aggregate has helicity (which is unlikely, since the dispersion data for the tyrocidine B and the gramicidin S-A are similar while their aggregation behavior is vastly different) or these types of rotatory dispersion data are not unique for helical structures.

(1) A. Paladini and L. C. Craig, *J. Am. Chem. Soc.*, 74, 676 (1952).
(2) T. P. King and L. C. Craig, *ibid.*, 77, 6627 (1955).
(3) M. A. Ruttenberg, T. P. King, and L. C. Craig, *Biochemistry*, 4, 11 (1965).
(4) A. R. Battersby and L. C. Craig, *J. Am. Chem. Soc.*, 73, 1887 (1951). The term "Gramicidin" S is an unfortunate misnomer, since this peptide is really a homolog in the tyrocidine series. It is a monocyclic decapeptide which is a dimer of the pentapeptide sequence, Val-Orn-Leu-Phe-Pro. This pentapeptide sequence is also present in the cyclic decapeptides tyrocidines A, B, C, and D; however, in this series the other pentapeptide half of the molecule is different, and it is in this latter half that the stereospecific stepwise amino acid replacements occur to produce the variants.
(5) L. C. Craig, *Science*, 144, 1093 (1964).
(6) M. A. Ruttenberg, T. P. King, and L. C. Craig, *Biochemistry*, 3, 758 (1964).
(7) W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci. U. S.*, 42, 596 (1956).
(8) N. S. Simmons and E. R. Blout, *Biophys. J.*, 1, 55 (1960).
(9) J. A. Schellman and C. Schellman in "The Proteins," Vol. II, H. Neurath, Ed., Academic Press Inc., New York, N. Y., 1964, pp. 82–90.

Acknowledgment. We wish to thank Dr. D. Yphantis for the ultracentrifugal measurements and Mr. A. W. Kronk of Applied Physics laboratory for the measurements given in Figure 1. This work was supported in part by U. S. Public Health Grant No. AM 02493.

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Ceanothine-B, a Naturally Occurring Oxazacyclononadiene

Sir:

The root bark of *Ceanothus americanus* (rhamnaceae) has a long medical history and the presence therein of alkaloids, reported to have hypotensive properties,[1] has been realized for almost a century.[2] Although there have been sporadic attempts to resolve the complex mixture of bases, up to the present these efforts have been unsuccessful.[3,4]

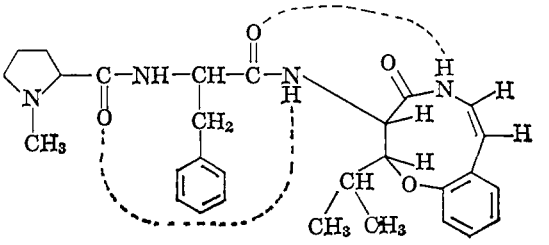
We wish to report evidence which establishes structure I for ceanothine-B, the major alkaloid of *C. americanus*. This structure includes the unusual feature of an oxazacyclononadiene ring common to all the ceanothus alkaloids we have examined.

Ceanothine-B, $C_{29}H_{36}O_4N_4$, m.p. 238.5–240.5°, $[\alpha]_D$ −293° (chloroform), is monobasic.[4] Quantitative comparison of the integrated carbonyl infrared absorption of the alkaloid, its derivatives, and model compounds disclosed the presence of three amide groups, accounting for the function of all four nitrogen atoms and three of the oxygen atoms. By elimination the remaining oxygen atom is ethereal since no hydroxyl or other carbonyl group could be detected. There are three amide NH peaks visible in the n.m.r. spectrum. Hydrogenation (Pd–C) saturated one *cis* double bond (–HC=CH–, δCD_3COOD = 6.01 and 6.82 p.p.m., J = 8 c.p.s.), whose vinyl protons are coupled only to each other in perdeuterioacetic acid, and produced dihydroceanothine-B, $C_{29}H_{38}O_4N_4$,[5] m.p. 272–278°, $[\alpha]_D$ −87° (methanol).

From the extremely intense base peak at *m/e* 84 in the mass spectra of the alkaloid and its dihydro derivative and the fact that the basic tertiary nitrogen atom bears one N-methyl group (n.m.r.), the N_{basic}-terminal group is N-methylproline.[4] The mass spectrum also contained peaks at *m/e* 91, 103, 120, and 131 characteristic of β-phenylalanine.[6] The presence of both these amino acids was verified by 6 *N* hydrochloric acid hydrolysis of dihydroceanothine-B and thin layer chromatographic comparison with authentic specimens. From the hydrolysate was isolated a compound, $C_{23}N_{29}O_3N_3$, m.p. 248–254°, $[\alpha]_D$ +63° (ethanol), dihydroceanothine-B minus the N-methylprolyl group.

(1) J. T. Groot, *J. Pharmacol. Exptl. Therap.*, 30, 275 (1927); H. Wastl, *Federation Proc.*, 7, 131 (1948); A. A. Manian, Ph.D. Thesis, Purdue University, 1954.
(2) J. H. M. Clinch, *Am. J. Pharm.*, 56, 131 (1884).
(3) A possible exception is the ceanothine of A. Bertho and W. S. Liang, *Arch. Pharm.*, 271, 273 (1933), which may be identical with the ceanothine-B described in this work.
(4) See E. W. Warnhoff, S. K. Pradhan, and J. C. N. Ma, *Can. J. Chem.*, 43, 2594 (1965), for details of the isolation and purification.
(5) Satisfactory analytical data have been obtained for all new compounds reported.
(6) K. Heyns and H.-F. Grützmacher, *Ann.*, 669, 191 (1963).

Subtraction of the N-methylprolyl and phenylalanyl residues from the molecular formula of ceanothine-B leaves $C_{14}H_{18}O_2N_2$. A C-methyl determination on the alkaloid or its dihydro derivative gave, as one of the volatile acids, isobutyric acid which could only have come from the C_{14} unit. In the n.m.r. spectrum of ceanothine-B the isobutyl *gem*-dimethyl group is the only C-methyl absorption present and appears as a pair of doublets at δ_{CDCl_3} 0.93 and 1.22 p.p.m. (J = 6.5 c.p.s.). The presence of an *ortho*-disubstituted benzene ring in addition to the phenyl group was shown by the n.m.r. (9 aromatic H) and infrared (strong peak at 757 cm.$^{-1}$) spectra. The ultraviolet chromophore, λ_{max} ~250 mμ (ε 4000), removed on hydrogenation is that of an enamide group (II). The C_{14} unit must therefore include isobutyl, *ortho*-substituted phenyl, and enamide fragments, further elaboration of which is provided by the following experiments.



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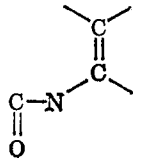

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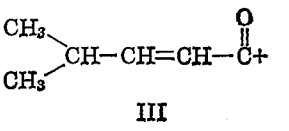

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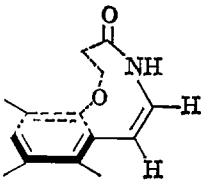

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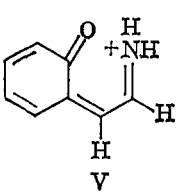

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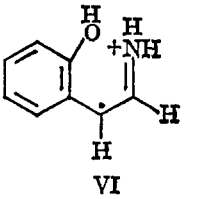

VI

Since lithium aluminum hydride reduction of all three amide groups to amino functions did not change the ultraviolet chromophore (λ_{max} 230 mμ (ε 9000) and 280 mμ (ε 1000)) of dihydroceanothine-B, whether the polyamine was in ethanol or ethanolic hydrochloric acid solution, the aromatic moiety in the C_{14} unit is present as an *o*-alkyl phenol ether. In the mass spectra of all five pure ceanothus alkaloids and of dihydroceanothine-B is an intense peak at *m/e* 97. This peak must arise from some common unit and not from the N-methylprolyl or phenylalanyl groups which are known to be absent from most of the alkaloids. Nor can the peak result from the enamide or *o*-alkyl phenol ether,[7] but must, by exclusion, involve the isobutyl group. The most reasonable formula for the *m/e* 97 ion is $C_6H_9O^+$ for which the structure III follows. In support of a substituted isocaproic acid

(7) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Interpretation of Mass Spectra of Organic Compounds," Holden-Day, Inc., San Francisco, Calif., 1964, pp. 174–183.