Yeast sRNA (100 mg, Calbiochem) was dissolved in 2 ml of water and heated to 85°. The denatured sRNA was added with rapid stirring to 10 ml of a 5% solution of tetradecyltrimethylammonium bromide. The resulting tetradecyltrimethylammonium salt of sRNA formed a thick precipitate which was removed by centrifugation, washed several times, and dried thoroughly *in vacuo* over P_2O_5. Attachment of the sRNA to cellulose (100 mg of Cellex N-1, Calbiochem) was accomplished by adding 100 mg of dicyclohexylcarbodiimide (Aldrich) to a suspension of the cellulose in 10 ml of an anhydrous pyridine solution of the tetradecyltrimethylammonium salt of sRNA (formation of this salt of sRNA renders it soluble in a number of anhydrous organic solvents) containing catalytic amounts of pyridinium hydrochloride and stirring the mixture for 10 days. After completion of the reaction the cellulose was removed by filtration, washed with 200 ml of pyridine, and treated with 50 ml of a 10% solution of sodium dodecyl sulfate to remove the tetradecyltrimethylammonium ions from the attached sRNA.[3] Further washings with water were carried out until no material absorbing at 260 mμ was observed in the washings. The resulting cellulose containing sRNA molecules linked by way of 5′-terminal phosphodiester bonds was dried, weighed (130 mg), and stored at −20°.

The sequential cleavage of the 3′-terminal bases from the cellulose-supported sRNA was carried out on 20-mg samples by repetition of the following procedure: (1) heat for 2 hr at 75° in the presence of 0.6 ml of a 1 *M* cyclohexylamine solution and 0.6 ml of a 0.1 *M* sodium periodate solution; (2) separate the cellulose-supported sRNA from the reaction solution and the cleaved terminal base by filtration; (3) exhaustively wash the cellulose-supported sRNA with water; (4) incubate the washed cellulose-supported sRNA in 1 ml of a 0.1 *M* NH_4HCO_3 solution containing 25 units of alkaline phosphatase (Calbiochem) at 37° for 1 hr; (5) exhaustively wash the cellulose-supported sRNA with 0.1 *M* NH_4HCO_3 solution to remove all traces of phosphomonesterase; (6) analyze the solution resulting from step 2 using thin-layer chromatography on cellulose plates developed with distilled water (adenine, R_f 0.61; cytidine, R_f 0.80) and paper chromatography developed with 2-propanol–acetic acid–water[4] (85:5:10, v/v).

Yeast sRNA carried through the above procedure three times yielded the sequence ···CpCpA. Each step appeared to be quantative since no observable adenine spot was found on the thin layer or paper chromatographic analysis of the solution resulting from step 2 in the second or third cycle through the procedure. When a fourth step was carried out, paper chromatographic analysis showed that all four bases were present as products of the cleavage reaction. Although Whitfeld[4] and Heppel[5] observe breakdown of RNA at high temperatures in the periodate–amine step, we were unable to detect the release of any nucleotide material from the cellulose-supported sRNA upon heating the material from step 3 with additional periodate–amine solution at 75° for 24 hr. As well as facilitating the recovery of reacted RNA, attachment to cellulose apparently also serves to stabilize the RNA to the high temperatures required for complete reaction in the periodate–amine step. Thus, the use of a modified Whitfeld procedure on a cellulose-supported polyribonucleotide seems to provide a fast, efficient method for the sequential analysis of RNA molecules which should lend itself to automation with little difficulty.

(3) R. L. Letsinger and T. E. Wagner, *J. Am. Chem. Soc.*, **88**, 2026 (1966).
(4) P. R. Whitfeld, *Biochim. Biophys. Acta*, **108**, 202 (1965).
(5) H. C. Neu and L. A. Heppel, *J. Biol. Chem.*, **239**, 2927 (1964).

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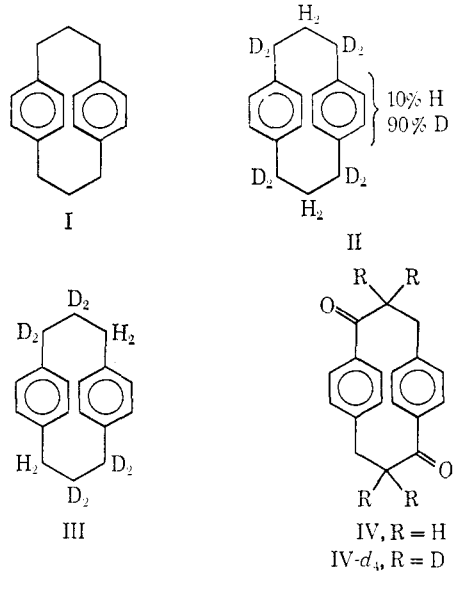
Nuclear Magnetic Resonance Studies of Conformation and Ring Inversion in the [3.3]Paracyclophane System

Sir:

In the crystalline state [3.3]paracyclophane (I) has been shown to exist in the chair conformation[1] (Figure 1). We now report[2] variable-temperature nmr data on I and partially deuterated I. The results show that, in solution, I exists as a mixture of boat and chair conformations in the ratio of about 2:1, and that the free-energy barrier for the boat–chair conversion is about 11.7 kcal/mol.

At room temperature, the aromatic protons of I (4% solution in 1:1 $CDCl_3$–$CDCl_2F$) appeared as a singlet (τ 3.33). At −88°, the aromatic region showed four partially resolved broad bands (Figure 1). This complex and unsymmetrical spectrum could not have arisen from a single A_2B_2 system and suggested the presence of both chair and boat conformations. To



(1) P. K. Gantzel and K. N. Trueblood, *Acta Cryst.*, **18**, 958 (1965).
(2) Variable-temperature nmr studies have been done on: [3.2]-metacyclophane (R. W. Griffin, Jr., and R. A. Coburn, *Tetrahedron Letters*, 2571 (1964)); [8]paracyclophane (G. M. Whitesides, B. A. Pawson, and A. C. Cope, *J. Am. Chem. Soc.*, **90**, 639 (1968)); various heterophanes (I. Gault, B. J. Price, and I. O. Sutherland, *Chem. Commun.*, 540 (1967); H. Nozaki, T. Koyama, T. Mori, and R. Noyori, *Tetrahedron Letters*, 2181 (1968); F. Vögtle, *ibid.*, 3623 (1968)); [2.3]-, [3.4]-, and [4.4]paracyclophanes (F. A. L. Anet, unpublished work); [2.4]-paracyclophane derivatives (D. J. Cram and H. Reich, unpublished work).

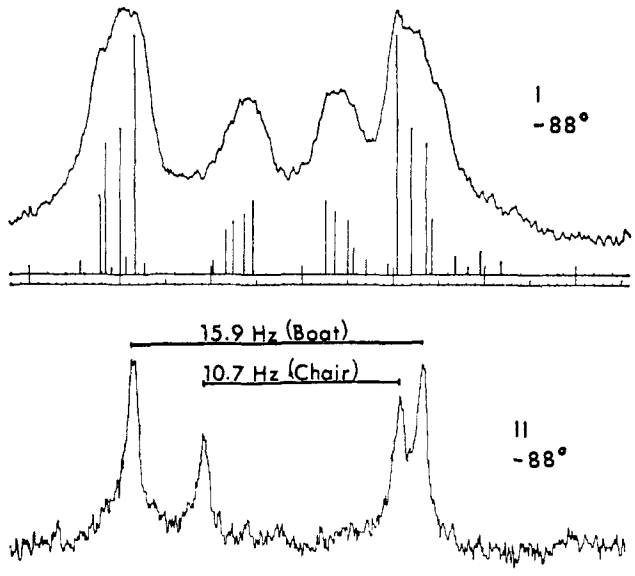


Figure 1. Spectra (100 MHz) of the aromatic regions of undeuterated [3.3]paracyclophane (I) and the ring-deuterated compound II. The composite calculated spectrum of a 2:1 mixture of boat and chair forms is shown below the spectrum of I. The spectrum of II was obtained by summing ten sweeps on a computer.

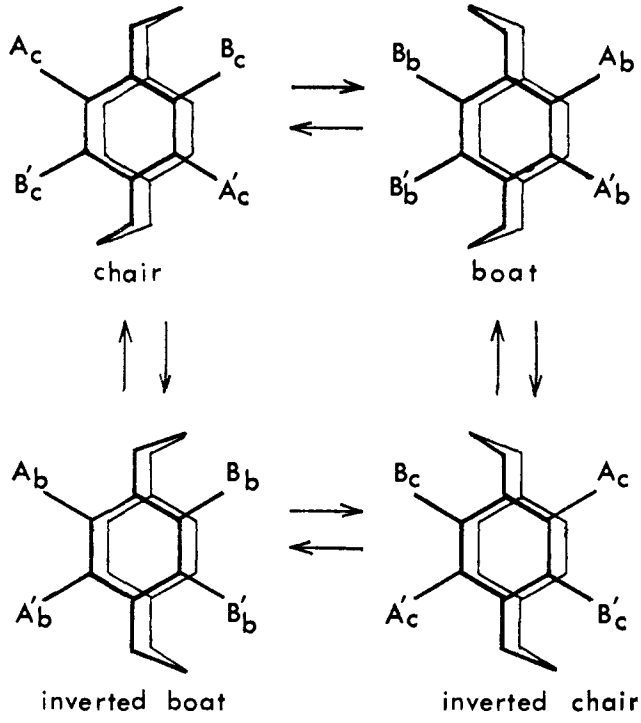
simplify the spectrum, I was massively deuterated[3] by exchange with deuterium oxide in the presence of platinum at 180° for 48 hr. The product (II), separated by vpc from ring-opened products, contained about 90% deuterium in the aromatic moiety and virtually complete deuteration in the benzylic region, as shown by nmr. With the assumption of statistical distribution, the isotopic composition of II per aromatic ring was calculated to be 66% D_4, 29% D_3H, and 5% D_2H_2, and thus the H–H couplings within the aromatic rings of II can be neglected. At −88°, the aromatic protons (deuterium decoupled) of II appeared as four lines (Figure 1). The two strong lines of equal intensities separated by 15.9 Hz are assigned to the absorption of protons in the A and B sites of one conformation. The two weak lines of equal intensities separated by 10.7 Hz represent the A and B protons of a second conformation, which is present in approximately one-half the concentration of the first. The center of the A and B resonances of the minor form is 1.3 Hz upfield from that of the major form.

From the spectrum of [3.3]paracyclophane and a knowledge of the chemical shifts and relative concentrations of the two conformations obtained from II, together with expected coupling constants, it is possible to assign the resonances of the chair and the boat forms. The aromatic spectrum of the chair form of I will have considerable intensity toward the center of the A_2B_2 system, due to the strong coupling between A and B protons (Scheme I); in the boat form, the A and B protons are weakly coupled (Scheme I) and will appear as two approximate triplets separated by $\Delta\nu_{AB}$, with no intensity toward the center of the spectrum.[4] With these considerations, a comparison of the spectra of I and II (Figure 1) shows that the major form must be the boat. Low-temperature spectra of the chair and

(3) J. L. Garnett and W. A. Sollich, *Australian J. Chem.*, 14, 441 (1961).

(4) K. W. Wiberg and B. J. Nist, "Interpretation of NMR Spectra," W. A. Benjamin, Inc., New York, N. Y., 1962.

Scheme I. Interconversion of Chair and Boat Forms of [3.3]Paracyclophane[a]



[a] The placements of the labels "inverted" are arbitrary.

boat forms were calculated,[5] using $\Delta\nu_{AB}$'s from II given above and J_{ortho} = 7.5, J_{meta} = 1.6, and J_{para} = 0.0 Hz. The composite calculated spectrum, assuming twice as much boat as chair form, is shown in Figure 1, and is in good agreement with the experimental spectrum.

The spectrum of II at intermediate temperatures was not suitable for the determination of rate constants because of the low intensity of the proton signal resulting from the massive deuteration and also because of the limited amount of II that was available. A higher signal-to-noise ratio was obtained with compound III, which was prepared by base-catalyzed exchange of 1,10-diketo[3.3]paracyclophane (IV) with deuterium oxide to give IV-d_4, followed by a Wolff–Kishner reduction[6] with deuterated reagents. At room temperature at 60 MHz, the benzylic protons (deuterium decoupled) of III appeared as a singlet at τ 7.45. At low temperatures, an apparent AB quartet ($\Delta\nu_{AB}$ = 38.2 Hz, J_{AB} = 14.2 Hz) was observed, and this spectrum coalesced to a single broad line at −33°. In this case, the chemical shifts between boat and chair protons were not resolved. Unfortunately, the present system of exchanging AB quartets (*cf.* Scheme I) is different from those which have been treated so far.[7] If the spectrum is treated, as an approximation, as a simple

(5) The LAOCOON II program of S. Castellano and A. A. Bothner-By, *J. Chem. Phys.*, 41, 3863 (1964), was used; computing assistance was obtained from the Health Sciences Computing Facility, University of California, Los Angeles, Calif., sponsored by National Institutes of Health Grant FR-3.

(6) D. J. Cram and R. C. Helgeson, *J. Am. Chem. Soc.*, 88, 3515 (1966).

(7) Systems of two and three exchanging AB quartets have been treated (J. T. Gerig and J. D. Roberts, *ibid.*, 88, 2791 (1966); R. A. Newmark and C. H. Sederholm, *J. Chem. Phys.*, 43, 602 (1965)). If *direct* chair–chair and boat–boat interconversion paths are omitted (as in Scheme I), then the present system becomes one of four exchanging AB quartets, which can be analyzed by an extension of the procedure of Newmark and Sederholm. Work along these lines is in progress.

AB spectrum averaging to an A_2 spectrum,[8] then the free-energy barrier separating the boat and chair forms is calculated to be about 11.7 kcal/mol, with an estimated maximum error of ±0.5 kcal/mol.[7,9]

The diketone IV-d_4 also showed a temperature-dependent spectrum for the benzylic protons. At −110° at 60 MHz an apparent AB quartet ($\Delta\nu_{AB}$ = 24.4 Hz, J_{AB} = 12.6 Hz) was observed for the benzylic protons, and the coalescence temperature was −92°. Although the proportions of chair and boat forms were not determined, it is likely that both forms occur as with I. With the assumption previously mentioned for I, the free-energy barrier separating the conformations of the diketone is calculated to be about 8.8 kcal/mol. The lower barrier in the diketone compared to the hydrocarbon is understandable since the transition states should have expanded bond angles compared to the ground states, and the diketone has an sp^2-hybridized carbon atom on each bridge.

Acknowledgments. This work was supported by the U.S. Public Health Service. We thank Michael Sheehan for providing a sample of [3.3]paracyclophane and a mixture of paracyclophane diketones from which 1,10-diketo[3.3]paracyclophane was separated. We are grateful to Professor D. J. Cram for suggesting the problem and for his continued interest in this research.

(8) R. J. Kurland, M. B. Rubin, and W. B. Wise, *J. Chem. Phys.*, **40**, 2426 (1964).

(9) Because of the difference in populations and hence in the free energies of the boat and chair forms, the barrier for the chair–boat conversion is actually 0.25 kcal/mol less than for the boat–chair conversion.

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A Racemization Test in Peptide Synthesis[1]

Sir:

Several methods of measuring the extent of racemization in peptide synthesis have been reported in the literature.[2] We have been attempting to find a convenient racemization test with the application of an amino acid analyzer though Bodanszky and Conklin already reported the use of the analyzer in the system involving the coupling of acetyl-L-isoleucine and glycine ethyl ester.[3] We introduce here a simple and accurate procedure to detect the degree of racemization and report the results of the influence of several coupling reagents on the extent of racemization during peptide synthesis.

Our proposed sequence is shown in Scheme I.

Scheme I

$$\text{Z-Gly-L-A-OH} + \text{H-L-B-OBzl} \xrightarrow[\text{of A residue)}]{\text{(with partial racemization}} \text{Z-Gly-A-B-OBzl (L,L isomer plus D,L isomer)} \xrightarrow{H_2\text{–Pd}} \text{Gly-A-B (L,L isomer plus D,L isomer)}$$

(1) Presented at the 1st American Peptide Symposium, New Haven, Conn., Aug 14, 1968.

(2) For reviews, see: M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience Publishers, New York, N. Y., 1966, p 137; E. Schröder and K. Lübke, "The Peptides," Vol. I, Academic Press, New York, N. Y., 1965, p 319.

(3) M. Bodanszky and L. E. Conklin, *Chem. Commun.*, 773 (1967).

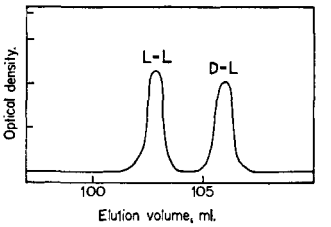

Figure 1. Chromatogram of Gly-Ala-Leu using an amino acid analyzer.

The crude benzyloxycarbonyl (Z) tripeptide benzyl ester (OBzl) is subjected to hydrogenolysis, and the hydrogenated material is submitted to the analyzer.

We first tried to discover a good system of glycyl tripeptide diastereomers for separation by the analyzer. We synthesized the pure L,L and D,L isomers of Gly-Lys-Glu, Gly-Lys-Asp, Gly-Orn-Glu, Gly-Orn-Asp, Gly-Glu-Lys, and Gly-Asp-Lys, with the surmise that a diastereomeric mixture of the polyfunctional neutral tripeptides might be efficiently separated under appropriate conditions. We observed, however, that all mixtures gave incomplete separation.[4] Therefore, we selected rather simple systems preparing several Gly-Ala-B tripeptides in which B could be Ala, Val, Leu, Pro, and Ser residues. We observed that a diastereomeric mixture of Gly-Ala-Val or Gly-Ala-Leu was separated completely with a Hitachi amino acid analyzer (Model KLA-3B) with spherical Dowex 50 resin in a 0.9 × 50 cm column under the conditions: flow rate 60 ml/hr, jacket temperature 55°, and 0.2 *M* standard citrate buffer at pH 4.25 as solvent. Gly-Ala-Leu (I) was our preferred system for the racemization test because it is not overlapped by either Leu or Gly-Ala; Leu was eluted at 58 ml of effluent volume, Gly-Ala at 73 ml, L,L-I at 129 ml, and D,L-I at 159 ml.

We prepared pure I (L,L and D,L) by the azide method. The azide in ethyl acetate derived from Z-Gly-L(or D)-Ala-$NHNH_2$[5] (mp 133°) was added to H-L-Leu-OBzl *p*-toluenesulfonate[6] (II) (mp 156°) in dimethylformamide (DMF) and triethylamine (TEA). The pure Z-Gly-Ala-Leu-OBzl (III) (L,L, 70%, mp 102°; D,L, 64%, mp 125°) obtained was hydrogenated to yield crystalline I (L,L with $0.25H_2O$, 92%, $[\alpha]^{25}D$ −84.5° (H_2O); D,L with $0.5H_2O$, 88%, $[\alpha]^{25}D$ +29.6° (H_2O)). Figure 1 shows the pattern of a mixture of 0.6 μmol each of I L,L and D,L by the analyzer. The limit of detection of D,L in L,L was studied with a synthetic mixture of both isomers. When a mixture of I (L,L) (6 μmol) and I (D,L) (0.06 μmol) was analyzed, a distinct peak of D,L isomer was observed. Even at 1000 parts of L,L (6 μmol) and 1 part of D,L, a small peak of D,L isomer could still be recognized. It will be evident that our method is more sensitive in detecting the slight occurrence of racemization than the Anderson test[2] which has been used widely nowadays. We applied this method in the detection of racemization in the azide procedure. Crude III (L,L) was directly hydrogenated, and a part of the filtrate was submitted to the analyzer. The material showed only single peak by I (L,L), using

(4) M. Muraoka, N. Yoshida, K. Noda, and N. Izumiya, *Bull. Chem. Soc. Japan*, 41, 2134 (1968).

(5) M. Bergmann and L. Zervas, *J. Biol. Chem.*, 113, 341 (1936).

(6) N. Izumiya and S. Makisumi, *J. Chem. Soc. Japan*, 78, 662, 1768 (1957).