favored, leading directly to the prominent fragments of *m/e* 178 (IIIa) or 180 (Va) and 96.

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A Procedure for the Addition of Amino Acid Residues to the Amino Groups of Insulin. Trimethionyl-insulin

Sir:

Amino acid residues have been added to the amino groups of insulin through the reaction of carbamino anhydrides of amino acids (Leuchs' anhydrides) with aqueous solutions of insulin.[1,2] The resulting products retained a considerable fraction of the biological activity of insulin. Although the anhydrides reacted to some extent with both of the α-amino groups at the N terminus of the two chains of insulin as well as with the ϵ-amino group of lysine, in no case did all of the amino groups completely react. This was true even when large excesses of reagent were used, resulting in incorporation of up to 7.3 amino acid residues per mole of insulin.[1] Such products must be considered as mixtures of molecules in which some of the original amino groups are unsubstituted while others may be substituted by single or poly amino acid residues. The significance of biological activity in such mixtures is understandably subject to some ambiguities in interpretation.

We wish to report a procedure by which new amino acid residues can be attached to all of the amino groups in insulin in such a fashion as to put only one amino acid residue on each amino group and thereby give essentially one product. The procedure, which is based on recent developments in peptide chemistry,[3-5] involves the reaction of *t*-butyloxycarbonyl (*t*-BOC) amino acid *p*-nitrophenyl esters with insulin in dimethylformamide, followed by removal of the *t*-BOC groups in anhydrous trifluoroacetic acid. The success of this method rests in part on the marked stability of insulin in acidic media.

Insulin hydrochloride[6] (60 mg, 10 μmoles) was dissolved in 5.0 ml of dimethylformamide and then triethylamine (10.0 mg, 100 μmoles) was added along with the *p*-nitrophenyl ester of *t*-BOC-methionine[7] (110 mg, 300 μmoles). The reaction was allowed to proceed for 18 hr at room temperature and the insulin derivative was precipitated and washed with ether. The resulting product, which was ninhydrin negative, was dried thoroughly over P_2O_5 under high vacuum and then dissolved in 2.0 ml of anhydrous trifluoroacetic acid. The solution was kept for 1 hr at room temperature by which time the ninhydrin color had reached a maximum value. The insulin derivative was precipitated, washed with ether, and dried. The residue was subjected to two isoelectric precipitations by dissolving it in 7 ml of 0.5 *M* acetic acid (containing 1 mg of zinc acetate) and precipitating at pH 5.9 with ammonium hydroxide. The final precipitate was collected by centrifugation, washed with water, acetone, and ether, and dried under vacuum; yield 65 mg, 93%.

Acid hydrolysis and amino acid analysis[8] revealed the incorporation of 3.0 methionine residues/mole of insulin. The trimethionyl-insulin was further characterized by a modified Edman degradation.[9] The ethylene chloride soluble thiazolinone was hydrolyzed in alkali.[10] Amino acid analysis of the hydrolysate showed the presence of three residues of methionine/mole of derivative and no other amino acids. Acid hydrolysis of the ethylene chloride insoluble residue, followed by amino acid analysis, afforded an amino acid composition identical with that of insulin. In another characterization, the trimethionyl-insulin derivative was treated with TPCK-trypsin.[11] No alanine was formed and a nonapeptide with the composition $Gly_{1.02}Phe_{1.93}Tyr_{0.98}Thr_{0.94}Pro_{0.99}Lys_{0.90}Met_{1.05}Ala_{1.00}$ was isolated. The action of trypsin on native insulin releases a heptapeptide, Gly-Phe-Phe-Tyr-Thr-Pro-Lys, and alanine.[12] The fact that no alanine was liberated on treating trimethionyl-insulin with trypsin,[13] along with the isolation of a methionine-containing nonapeptide, constitutes good evidence for locating one methionine residue attached to the ϵ-amino group of lysine at position 29 of the B chain. The remaining two methionine residues which were liberated by the Edman degradation must be attached to the N terminals of the A and B chains. These results indicate that the reagent was quite specific in that it reacted only with the free amino groups of insulin.

In the mouse convulsion assay the trimethionyl-insulin possessed a biological activity of 12.3 $\pm$ 1.7 units/mg. A control sample of insulin which had been put through all of the manipulations involved in this synthesis with the exception of deletion of the acylating reagent was recovered in crystalline form with essentially no loss in biological activity, 20.4 $\pm$ 4.5 units/mg.

The method described here should be applicable to the addition of a variety of amino acid residues to insulin and may be generally applicable to other proteins as a specific procedure for amino group modification. Experiments are in progress involving a correlation of biological activity and conformational changes with the addition of neutral, basic, and acidic amino acid residues on the insulin molecule.

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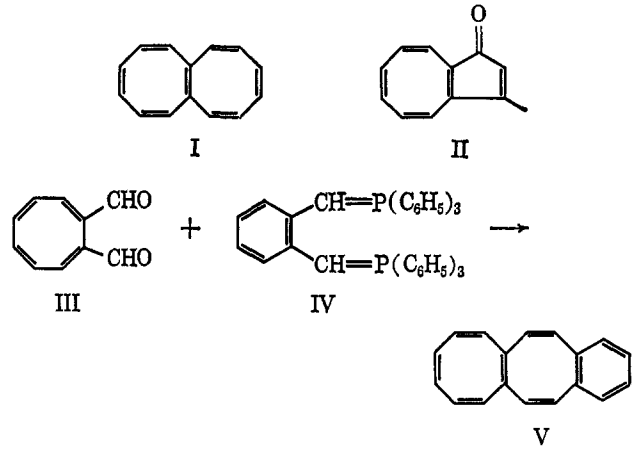
Octalene Derivatives

Sir:

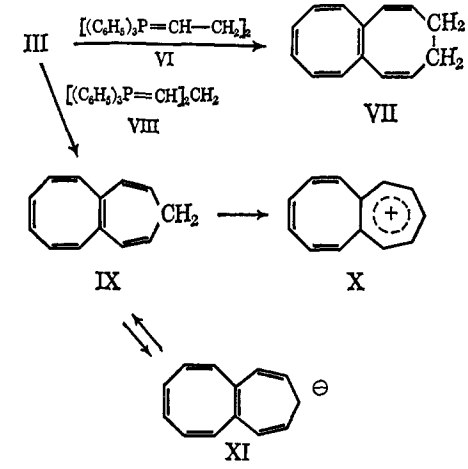
One of the most interesting problems remaining in the field of novel aromatic systems is whether there is a class of aromatic compounds in which the magic number of $4n + 2$ electrons is attained by fusion of two antiaromatic rings, with $4n$ electrons each.[1] A possible example of such a system is octalene (I), a molecule with 14 π electrons. The system has substantial resonance energy by a Hückel calculation (4.1β), albeit much less with a more sophisticated treatment,[2] and it fits Craig's rule[3] for aromaticity as well. Of course, in this case one must be concerned not only with the usual cavils about simple MO predictions but also with the question of whether the putative aromaticity in a planar octalene would be sufficient to induce flattening of two cyclooctatetraene rings, with consequent strain.[4] The only evidence to date on such matters is our ambiguous finding[1] that a cyclooctatetraenocyclopentadienone (II) is quite unstable, but that it is also very basic. The instability suggests nonaromaticity, while the high basicity suggests that the bicyclic ten-π-electron cation may be aromatic. We have now prepared some derivatives and relatives of octalene (I) and wish to report their properties.

1,8-Diformylcyclooctatetraene (III), mp 124–125°, can be prepared in 10–40% yield from 1,8-bis(hydroxymethyl)cyclooctatetraene[5] by oxidation with MnO_2 or, better, NiO_2; it is separated from the concomitant furan and γ-lactone by alumina chromatography. Reaction of III with the bisphosphorane IV affords a 1–2% yield of benzo[*c*]octalene (V), which is isolated by chromatography and reversible extraction into 70% aqueous silver nitrate. The mass and nmr spectra are unchanged after vacuum distillation or preparative vpc; the mass spectrum shows a parent peak at *m/e* 230 ($C_{18}H_{14}$) and the nmr has a peak at δ 7.06, a sharp peak at δ 6.34, and a multiplet at δ 5.70 in a ratio of approximately 4:4:6. For comparison, *sym*-dibenzocyclooctatetraene, prepared as above but from *o*-phthalaldehyde, has the aromatic protons at δ 6.95 and the vinyl protons at δ 6.61; it has similar behavior on thin layer chromatography, and on vapor phase chromatography under the same conditions it shows a retention time of 100 sec compared with 130 sec for V. Hydrogenation of V in benzene for 2 hr (1 atm) with 10% Pd–C gives a substance with parent *m/e* 240 (residual benzene ring and tetrasubstituted double bond), while a further 2 hr with 10% Pd–C in ethanol gives *m/e* 242 (residual benzene ring).



The ultraviolet spectrum of V shows only intense and absorption, with a shoulder at 245 mμ ($\epsilon \sim 17{,}000$), and it resembles that[6] of benzocyclooctatetraene (which has a nonplanar eight-membered ring). By contrast, we calculate (Pariser–Parr–Pople) λ_{max} 328 mμ with a reasonable intensity for planar aromatic benzooctalene (the longest wavelength absorption is predicted to be 1.6 μ).[7] Furthermore, the protons in the terminal cyclooctatetraene ring are unshifted (δ 5.70) from normal cyclooctatetraene (δ 5.69). Benzooctalene is not particularly stable, being destroyed on chromatography on activity II alumina, exposure to air for a length of time, or (in part) vapor phase chromatography at 160–210°. From all this we conclude that the eight-membered rings have ordinary tub conformations, and the benzooctalene is not aromatic.

Related evidence comes from the reaction of III with VI, producing 5–10% yield of dihydrooctalene (VII). After vpc purification VII has *m/e* 182, with expected fragments at *m/e* 167 and 154; hydrogenation at 25° for 2 hr in benzene over 10% Pd–C gives a small peak at *m/e* 194 (perhydrooctalene) and the main peak at *m/e* 192 (residual hindered olefin). In the nmr VII has four protons in a broad (room temperature) peak at δ 2.4 and ten vinyl protons in a complex pattern near



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