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The Dimerization of Δ^1-Piperidine-2-carboxylic Acid

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The L-amino acid oxidase of *Mytilus edulis* has been used to oxidize L-lysine on a large scale in the presence of catalase. The α-oxo acid derived from lysine cyclizes to a Schiff base, which readily dimerizes. The dimer undergoes spontaneous dehydration and decarboxylation to form 1,2,3,4,5,6,7,8-octahydropyrido[3,2-*a*]-indolizin-10(4b*H*)-one. This structure was established by a study of its molecular weight and infrared, nuclear-magnetic-resonance and mass spectra.

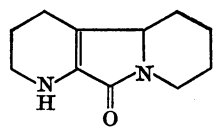
ε-Amino-α-oxohexanoic acid, the α-oxo acid derived from lysine, has been isolated as the hydrobromide after oxidation of ε-*N*-carbonylbenzyloxy-L-lysine by snake-venom L-amino acid oxidase (for which L-lysine itself is a poor substrate) and subsequent removal of the protecting group (Meister, 1954). The α-oxo acid appeared to be in equilibrium with the cyclic anhydro form, Δ^1-piperidine-2-carboxylic acid, in neutral aqueous solution. This intramolecular condensation was confirmed by Schweet, Holden & Lowy (1955), who also reported that the compound readily polymerized, although the nature of the product(s) was not established.

In a recent study of the enzymic oxidation of lysine and of oxalysine by the L-amino acid oxidase of *Mytilus edulis*, L-lysine was oxidized to 2-piperidone in the absence of catalase (Hope & Horncastle, 1967). In the presence of catalase the expected product was Δ^1-piperidine-2-carboxylic acid; however, little of this substance could be detected after complete oxidation of the substrate L-lysine. We now report the isolation of a substance of molecular weight 192 from a large-scale experiment. The structure (I) shown in Fig. 1 is consistent with its physical and chemical properties. This substance is formed when Δ^1-piperidine-2-carboxylic acid dimerizes with subsequent loss of the elements of 1 molecule of water and 1 molecule of carbon dioxide.

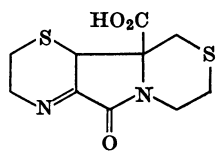
MATERIALS AND METHODS

Preparation of enzyme. Common mussels, *Mytilus edulis*, were obtained from the Marine Biological Laboratory in Plymouth. On arrival the digestive glands, including a portion of the intestine, were dissected. The fresh tissue (217g.) was homogenized in 750ml. of 67mM-sodium phosphate buffer, pH7·15, in a Waring Blendor. The suspension was centrifuged at 1500*g* for 2hr. at 0°. The sediment was resuspended in 750ml. of the same buffer and dialysed against a total of 10l. of buffer with four changes. The crude enzyme suspension was adjusted to pH3·0 with 5N-HCl (10·5ml.). After being stirred for 24hr. at 4°, the suspension was centrifuged at 2150*g* for 70min. The sediment was resuspended in 750ml. of water and dialysed against 20l. of water with four changes and centrifuged at 2500*g* for 40min. The sediment was resuspended in 390ml. of water.

Manometric experiments. The enzymic activity of the preparation was determined manometrically. A 1ml. sample of enzyme suspension was used in the main compartment of a Warburg flask together with 0·6ml. of either water or the phosphate buffer. The inner tube contained a filter paper and 0·3ml. of N-KOH, and the side bulb contained 0·4ml. of either water or a solution containing 10μmoles of L-amino acid (lysine and arginine for comparison). The initial substrate concentration was 5mM and this gave maximum rates with both substrates: there was little inhibition of enzymic activity at high substrate concentrations (up to 0·1M). L-Lysine monohydrochloride was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Crystalline bovine catalase was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.



(I)



(II)

Fig. 1. (I) 1,2,3,4,5,6,7,8-Octahydropyrido[3,2-*a*]indolizin-10(4b*H*)-one, the compound isolated in the present work. (II) The compound isolated by Hermann (1961).

Spectroscopic measurements. (*a*) Ultraviolet spectra. The u.v. spectra were obtained with a Bausch and Lomb Spectronic 505 spectrophotometer. Solutions of compounds of suitable strength were prepared in spectroscopically pure ethanol, water, 0·01 N-HCl and 0·01 N-NaOH.

(*b*) Infrared spectra. The i.r. spectra of solids were measured by using dispersions in KCl disks with a Perkin–Elmer Infracord spectrophotometer and a Perkin–Elmer 125 grating spectrophotometer.

(*c*) Mass spectrum. An A.E.I. MS 9 mass spectrometer was used.

(*d*) Nuclear-magnetic-resonance spectrum. A Perkin–Elmer R10 60 Mcyc./sec. nuclear-magnetic-resonance spectrometer was used.

Melting points. A Kofler block with a Reichert microscope and crossed Nicol prisms was used. The melting points are recorded uncorrected.

Molecular weights. These were determined by using a Mechrolab vapour pressure osmometer on solutions of compounds in either benzene or chloroform. Approx. 12 mg. of the substance was dissolved in 2 ml. of solvent; determinations were made on the solutions again after suitable intervals.

Thin-layer chromatography. The homogeneity of the products was investigated by chromatography on 250 μ-thick layers of u.v.-fluorescent silica gel (Shandon; dried at 110° for 30 min.) on glass plates (5 cm. × 20 cm.). The solvents used were (*a*) methanol and (*b*) chloroform–methanol (9:1, v/v). The solvent front moved 15 cm. from the origin. Substances were detected either by their ability to quench the fluorescence of the dry gel or by the chlorine/tolidine–KI reagent (Pataki, 1963).

Elementary analyses. Microanalyses were by Dr H. Bieler, Organisch-Chemisches Institüt der Universität, Vienna, Austria.

RESULTS

Enzymic oxidation of L-*lysine.* L-Lysine hydrochloride (10 g.) was incubated at 25° in an atmosphere of oxygen with 130 ml. of the L-amino acid oxidase preparation together with 30 000 units of bovine catalase in a total volume of 200 ml. without added buffer. The mixture was shaken in a Dubnoff shaker throughout the incubation. The rate of oxidation was followed in a pilot manometric experiment in which 1 ml. of the reaction mixture was maintained under the same conditions. After 30 hr. there was an abrupt fall in the rate of oxygen consumption and the incubation period was terminated. The mixture was divided into six equal portions and each was dialysed against 1 l. of distilled water for 2 hr. with shaking. The combined dialysates were passed through a column (30 cm. × 2 cm.) of Dowex AG-1 (X4; formate form; 200–400 mesh) to remove Cl^- ions. The column also abstracted some coloured material. A flash evaporator, which permitted the removal of water below 20°, was used to reduce the volume to 50 ml. Further concentration was effected by freeze-drying, which removed not only water but also ammonium formate. The dry residue was taken up in 15 ml. of water and after a few minutes colourless crystals began to separate. The solid was collected, washed with ice-cold water and dried over calcium chloride *in vacuo*. A further crop of crystals was obtained when the mother liquors were again freeze-dried and the powder was taken up in 5 ml. of water. The product (0·9 g.) had m.p. 101–103° (Found: C, 68·6; H, 8·3; N, 14·4. $C_{11}H_{16}N_2O$ requires C, 68·7; H, 8·4; N, 14·6%). It was soluble in water and gave a neutral solution. It was also soluble in methanol, ethanol, chloroform and benzene but not in light petroleum.

The elementary analysis suggested that the compound had been formed by cyclization of the α-oxo acid followed by dimerization and loss of carbon dioxide and water. This was confirmed when the molecular weight was measured in chloroform. A solution containing 12·9 mg. dissolved in 2 ml. of chloroform was 34 mM. This corresponded to a molecular weight of 190·5; the expected value was 192·3. The choice of solvent proved to be important, because an experiment in benzene solution (12·66 mg. in 2 ml.) indicated that a rapid further polymerization occurred in this solvent. The final stable reading (17·6 mM) obtained after 3 days corresponded to a molecular weight of 360.

Infrared spectrum. The i.r. spectrum in a potassium chloride disk showed strong absorptions at 3305 $cm.^{-1}$ (NH), 2927 and 2835 $cm.^{-1}$ doublet (CH), 1670 $cm.^{-1}$ (C=C—C=O), 1495 $cm.^{-1}$ and 1427 $cm.^{-1}$.

Ultraviolet spectrum. The u.v. spectrum of an aqueous solution was strongly absorbing and had $\lambda_{max.}$ 222 and 262 mμ with extinction coefficients $9{\cdot}6 \times 10^3$ and $2{\cdot}9 \times 10^3$ l. $mole^{-1}$ $cm.^{-1}$ respectively. A similar spectrum was obtained in ethanol ($\lambda_{max.}$ 224 and 275 mμ), and in 0·01 N-sodium hydroxide the band maxima were identical with those obtained in water. In 0·01 N-hydrochloric acid, however, there was a shift of both bands to shorter wavelengths: these had $\lambda_{max.}$ 208 and 243 mμ with extinction coefficients $8{\cdot}2 \times 10^3$ and $3{\cdot}5 \times 10^3$ l. $mole^{-1}$ $cm.^{-1}$ respectively.

Nuclear-magnetic-resonance spectrum. The n.m.r. spectrum of a $CDCl_3$ solution of the compound is in good agreement with the proposed structure (I) as it shows a complete absence of absorption bands below 5·5 τ.

The addition of D_2O to the $CDCl_3$ solution showed the presence of one exchangeable hydrogen atom by the appearance of a one-proton-intensity band (HOD) at 5·35 τ.

Mass spectrum. The mass spectrum (Fig. 2) confirms the molecular weight (M^+, 192) and the high-resolution data (Table 1) confirm the empirical formula.

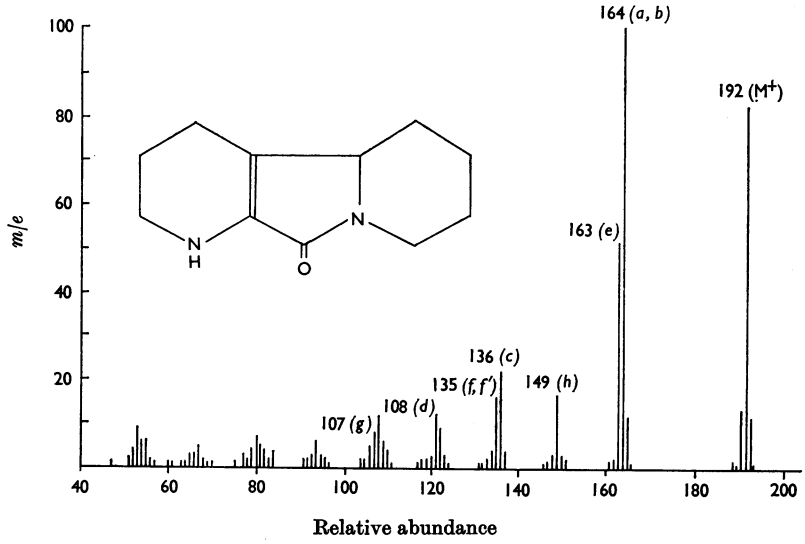


Fig. 2. Mass spectrum of the dimer of Δ¹-piperidine-2-carboxylic acid. An A.E.I. MS9 mass spectrometer was used. The source temperature was 130°,

Table 1. *Compositions of the non-isotopic ions with mass greater than m/e* 100 *in the mass spectrum of the compound* (I) (*Fig.* 1)

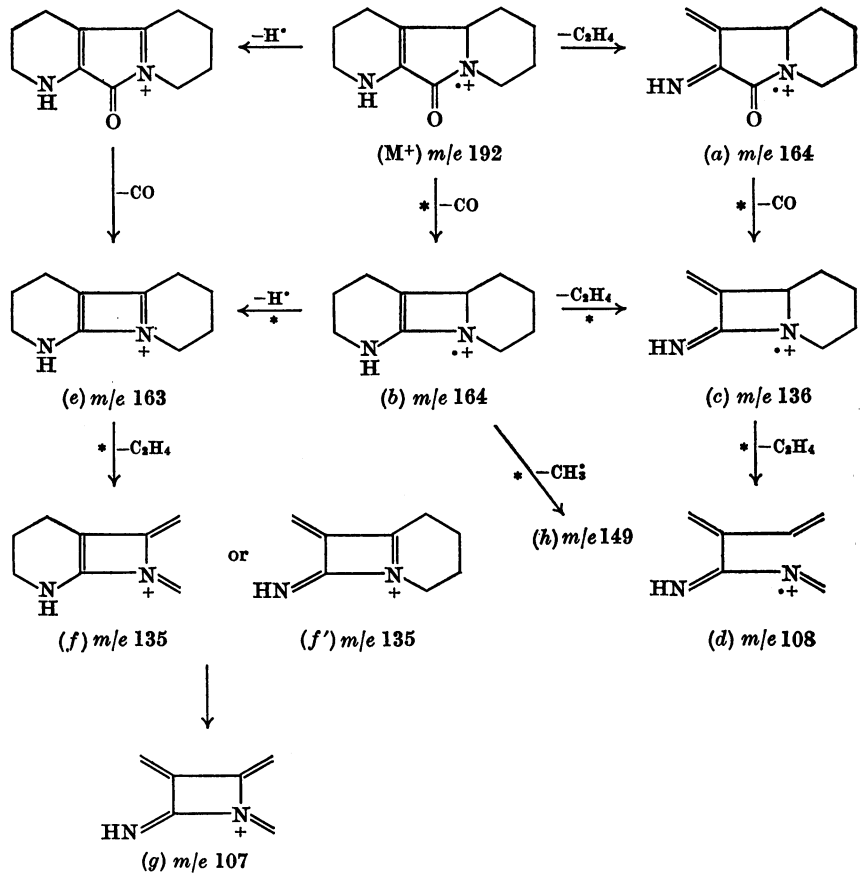
	Composition of fragments			
m/e	N	N_2	N_2O	Ratio
192			$C_{11}H_{16}N_2O$	
191			$C_{11}H_{15}N_2O$	
164		$C_{10}H_{16}N_2$	$C_9H_{12}N_2O$	3:2
163		$C_{10}H_{15}N_2$	$C_9H_{11}N_2O$	9:1
149		$C_9H_{13}N_2$		
136		$C_8H_{12}N_2$	$C_7H_8N_2O$	3:1
135		$C_8H_{11}N_2$		
122	$C_8H_{12}N$	$C_7H_{10}N_2$		1:1
121	$C_8H_{11}N$	$C_7H_9N_2$		6:1
108	$C_7H_{10}N$	$C_6H_8N_2$		1:1
107	C_7H_9N	$C_6H_7N_2$		1:1

The major fragmentation pathways, as shown by the appropriate metastable peaks and high-resolution data, provide very good support for the structure (I) depicted in Fig. 1.

The molecular ion undergoes two major cleavages, a retro Diels–Alder fragmentation (Budzikiewicz, Brauman & Djerassi, 1965) to give the ion (*a*) *m/e* 164, or the loss of CO to give the ion (*b*) *m/e* 164. Both of these ions then decompose by loss of either CO or C_2H_4 to give the ion (*c*) *m/e* 136, which undergoes a further retro Diels–Alder fragmentation to form the ion (*d*) *m/e* 108. The fragment ion (*b*) exhibits two other fragmentation pathways: either the loss of H· to give the ion (*e*) *m/e* 163, which then undergoes two successive retro Diels–Alder fragmentations to give the ions (*f* or *f'*) *m/e* 135 and the ion (*g*) *m/e* 107; or the loss of CH_3 to give the ion (*h*) *m/e* 149. The formation of these fragments can be rationalized as shown in Scheme 1.

Autoxidation in benzene. It was observed that the change of molecular weight in benzene solution was associated with the consumption of oxygen. The effect of solvent on this autoxidation was studied manometrically. The dry substance (2mg.) was placed in the side bulb and the solvent (water, ethanol, chloroform or benzene, 2·3ml.) was placed in the main compartment of the flask. After equilibration at 25° for 15min. the solvent was tipped into the side bulb. No oxygen consumption was detected in any of the flasks in the first 5min. The material in benzene began to absorb oxygen rapidly in a further period of 5min.; after a total of 20min. the reaction was complete and 176μl. of oxygen had been consumed. Oxygen was not consumed by the material in the other solvents in 24hr. All the solutions were then investigated by thin-layer chromatography.

The original compound (I) had R_F 0·59 in chloroform–methanol (9:1, v/v). The solutions in water and ethanol gave single blue spots with unchanged R_F values. The benzene solution gave two intense blue spots R_F 0·27 and 0·45; none of the original material could be detected.



Scheme 1. Major fragmentation pathways as revealed by the metastable peaks and high-resolution data obtained from the mass spectrum. * Metastable peak observed.

DISCUSSION

The value obtained for the molecular weight of the compound by mass spectrometry was 192. This value agrees well within the limits of experimental error with the result obtained by vapour-pressure measurements (190·5). The molecular weight indicated that the compound was formed by dimerization of the α-oxo acid corresponding to lysine. The precise molecular weight and the elementary analysis could be accounted for if the cyclic Schiff-base form of the α-oxo acid (Δ^1-piperidine-2-carboxylic acid) dimerized with loss of the elements of 1 molecule of water and 1 molecule of carbon dioxide. The formulation of the structure (I) (Fig. 1) was based on the behaviour of Δ^1-piperidine. This Schiff base is known to dimerize readily to form tetrahydroanabasine (Schoepf, Zomzak, Braun & Jacobi, 1948; Hasse & Maisack, 1955; Hasse & Berg, 1957).

A similar compound has been described by Hermann (1961). He prepared the compound (II) shown in Fig. 1 by condensation of β-bromopyruvic acid with β-mercaptoethylamine and decarboxylated it in aqueous solution at 100°.

The formation of the compound (I) reported in the present paper presumably accounts in part for the low yield of α-oxo acid when L-lysine was oxidized by the L-amino acid oxidase of *Mytilus edulis* or of *Neurospora* wild-type strain 4A (Schweet *et al.* 1955).

The compound was extremely sensitive to oxygen in benzene solution; after a short lag phase 1 mol.prop. of oxygen was absorbed within a few minutes in the absence of a catalyst. No evidence was found for the formation of a hydroperoxide and the products were not further investigated.

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