158. *The Reaction of Ninhydrin with Cyclic α-Imino-acids.*

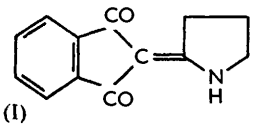
By A. W. Johnson and D. J. McCaldin.

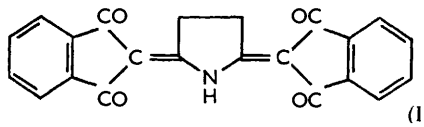
Structures are proposed for the yellow and the purple condensation products from ninhydrin and the cyclic α-imino-carboxylic acids. Ring size is shown to have a marked influence on the course of the reaction. The purple condensation products are also obtained from ninhydrin and the cyclic bases and the mechanism of these condensations, which resembles that of the oxidative decarboxylation of α-amino-acids, is discussed.

It has been recognised for many years that, whereas most of the common α-amino-acids give purple colours with ninhydrin, proline and hydroxyproline give yellow colours.[1] When this reaction is used for identifications after chromatography on paper, it is sometimes observed that the yellow proline spot has a purple fringe, especially if the colour has been developed at temperatures rather higher than usual; and indeed, after prolonged heating, the yellow colour may be completely transformed into purple. The chemistry of these coloured proline condensation products was studied originally by Grassmann and Arnim [2] who suggested that their formation might be used as the basis for estimation of proline and hydroxyproline. Moore and Stein [3] realised that the cyclic α-imino-acids were anomalous in the method they developed for the photometric determination of α-amino-acids with ninhydrin. However, a modified procedure for the estimation of proline by means of ninhydrin was worked out in detail later by Chinard [4] who also showed that ornithine, lysine, and hydroxylysine gave comparable results, probably because of initial cyclisations to α-imino-acids. Chinard's method was again modified by later workers [5] with the result that details for the estimation of all the naturally occurring cyclic α-imino-acids, on the basis of the absorption spectra of the colours given with ninhydrin, are now available. In the case of proline, for example, a yellow colour ($\lambda_{max.}$ 350 mμ) is formed with ninhydrin in acetic acid at room temperature whereas a reddish-purple colour ($\lambda_{max.}$ 515 mμ) is formed with the same reagent at 100° in 35 minutes. If the latter reaction is carried out in neutral solution the resulting compound shows maximum absorption at 550 mμ, and it was this coloured product which was investigated chemically by Grassmann and Arnim.[2] They advanced structures (I) and (II) respectively for the yellow and the purple product and showed that the former was intermediate in the formation of the latter. These formulæ, which have been accepted by later workers,[5] were derived largely by analogy with the structures then proposed for compounds of the isatin-blue series, which are derived from pipecolic acid, proline, and related compounds by condensation with two equivalents of isatin.

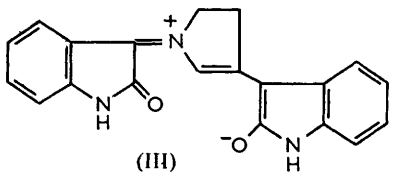
[1] Abderhalden and Schmidt, *Z. physiol. Chem.*, 1911, **72**, 37.
[2] Grassmann and Arnim, *Annalen*, 1934, **509**, 288; 1935, **519**, 192.
[3] Moore and Stein, *J. Biol. Chem.*, 1948, **176**, 367.
[4] Chinard, *ibid.*, 1952, **199**, 91; see also Weisiger, *ibid.*, 1950, **186**, 591; Stein and Moore, *ibid.*, 1951, **190**, 103; Touster, *J. Amer. Chem. Soc.*, 1951, **73**, 491.
[5] Troll *et al.*, *J. Biol. Chem.*, 1953, **200**, 804; 1955, **215**, 655; Schweet, *ibid.*, 1954, **208**, 603; Piez, Irreverre, and Wolff, *ibid.*, 1956, **223**, 687.

In a recent paper,[6] we showed that the structure of the condensation product from isatin and proline was (III), and it seemed to us *a priori* that the purple ninhydrin products would be of a similar type. Proline reacts readily with ninhydrin in alcoholic solution, to


(I)


(II)

give a crystalline golden-yellow condensation product which we consider to be the enol betaine (IV) and indeed its colour, spectra, and other physical and chemical properties closely resemble those of the enol betaines (*e.g.*, V) described by Stafford.[7] The related compound from hydroxyproline and ninhydrin has also been prepared. The infrared spectrum of compound (IV) shows no band corresponding to a free imino-group, and thus structures such as (I) can be discounted. On the basis of structure (IV) for the yellow intermediate and (III) for the isatin–proline condensation product, we now formulate the purple-red condensation product from ninhydrin and proline as (VI), and the properties of this compound, especially the ultraviolet and visible absorption spectrum, are similar to those of the compounds of the isatin-blue series.[6] Hydroxyproline with excess of ninhydrin also gives a purple-red colour which can be formulated as due to the hydroxy-derivative of (VI). The purple ninhydrin pigments (VI) differ from the corresponding isatin compounds (III) in that they do not form the characteristic red salts with mineral acids. This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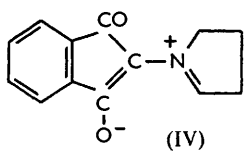
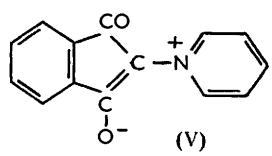
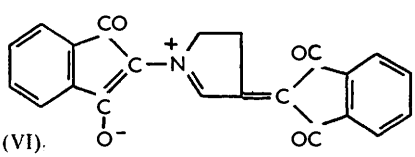
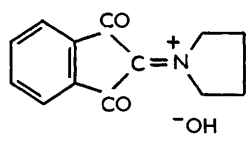

(IV)


(V)

property is associated with the oxindolylidene cyclic nitrogen atoms which are modified sufficiently in the resonant chromophore as to acquire basic character. It was shown [6] that the pyrolysis of isatin blue gave oxindole; a similar degradation of compound (VI) has given indane-1 : 3-dione, thus confirming that it is the 2-keto-group of ninhydrin which is involved in the condensation with proline.

Pipecolic acid and its derivatives are characterised by purple rather than yellow spots in paper chromatographic analysis by the ninhydrin method.[8] The yellow intermediate corresponding to (IV) is unstable, although it can often be observed as a transient colour, and the product obtained is the purple-red compound corresponding to (VI). This


(VI)


(VII)

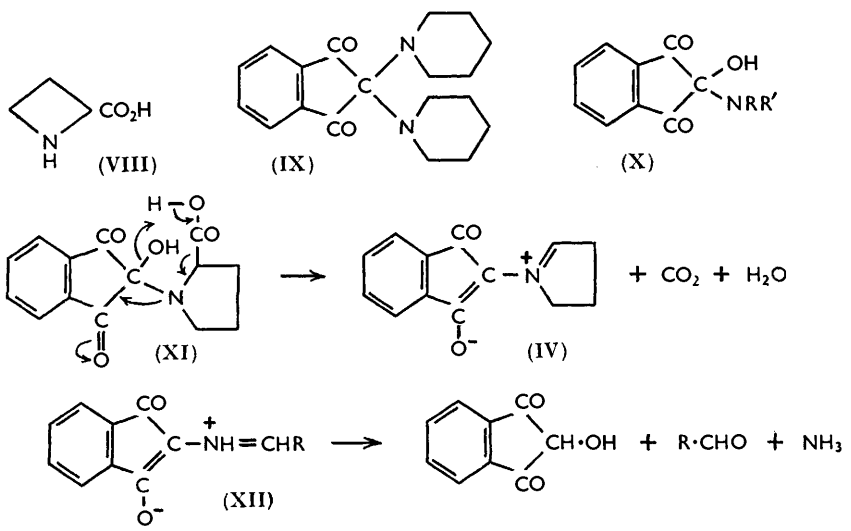
striking difference in the behaviour of the five- and six-membered cyclic α-imino-acids is obviously governed by steric factors and possibly depends upon the equilibrium between (IV) and (VII), enol betaines being known [7] to combine readily with water and alcohols. It has been postulated [9] that, whereas an *exo*-double bond stabilises a five-membered ring, the reverse obtains for six-membered rings. On this basis, the proportion of (VII) in

[6] Johnson and McCaldin, *J.*, 1957, 3470.
[7] Stafford, *J.*, 1952, 580.
[8] Grobbelaar, Pollard, and Steward, *Nature*, 1955, **175**, 703; Witkop and Foltz, *J. Amer. Chem. Soc.*, 1957, **79**, 192.
[9] Brown, Brewster, and Shechter, *ibid.*, 1954, **76**, 467.

the product would be greater in the proline condensate than in the pipecolic acid product, and hence the activation of the β-position of the ring, a prerequisite for further condensation to the purple compounds of type (VI), is more pronounced with the six-membered cyclic α-imino-acids.

In the same connection, it is of interest to consider the behaviour of azetidine-2-carboxylic acid [10] (VIII) with ninhydrin. This α-imino-acid normally gives a brown colour on paper after treatment with ninhydrin, but if the condensation product [presumably the analogue of (IV)] is treated with excess of ninhydrin and warmed, the colour of the spot changes to blue. The main absorption of the new product in the visible region is at 575 mμ which differs from that for (VI) and corresponds to the colour obtained from a primary amino-acid with ninhydrin.[3] Azetidine-2-carboxylic acid is known [10] to undergo ring fission to homoserine under comparatively mild conditions and it is probable therefore that the blue condensation product is the same as that derived from ninhydrin and homoserine. The effect of ring size on the reaction of the cyclic α-imino-acids with ninhydrin thus causes the formation of a different type of stable condensation product from each of the four-, five-, and six-membered cyclic acids.

As in the condensations with isatin, the corresponding free base may be substituted for the α-imino-acid in the formation of the coloured products of type (VI), if the condensation with ninhydrin is carried out in the presence of hot acetic acid. Pyrrolidine, piperidine, 2-methylpiperidine, and morpholine have been treated with ninhydrin in this manner, and it should be noted that the formation of a coloured compound from 2-methylpiperidine and ninhydrin would not be possible on the Grassmann and Arnim formulation (II) of these compounds. Intermediate compounds were isolated in the reactions of both piperidine and morpholine with ninhydrin. The former has been formulated as (IX) and the latter, a colourless compound, as (X; RR′ = $[CH_2]_2{\cdot}O{\cdot}[CH_2]_2$). Whereas the proline adduct (IV) crystallised from hot acetic anhydride, the compound (IX) was immediately converted in this solvent into the purple-red condensation product of type (VI); this provides another example of the difference in reactivity between five- and six-membered rings. Proline, piperidine, and morpholine thus yield the three different intermediates (IV), (IX), and (X) which are theoretically possible on the way to the purple pigments.

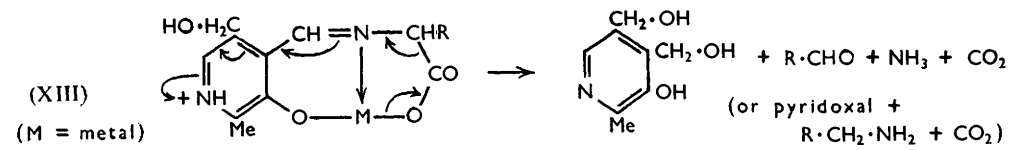


The ease of reaction of ninhydrin with the α-imino-acids (phosphate buffer at pH 7 and 60°), with concomitant loss of carbon dioxide, contrasts with the conditions required for condensation of the corresponding free bases and suggests that the carboxyl group is

[10] Fowden, *Biochem. J.*, 1956, **64**, 323.

involved in the process. It is suggested that the amines or α-imino-acids first form the adducts (X) by a reversible process; but, in the case of the α-imino-acid adducts (XI), operation of the electronic shifts shown, which involves afavourable transition state, results in ready elimination of water and carbon dioxide.

Such a mechanism could apply also to the oxidative decarboxylation [11] of α-amino-acids, $NH_2 \cdot CHR \cdot CO_2H$, to aldehydes, R·CHO, a reaction which can be brought about by ninhydrin, isatins,[12] and a variety of other carbonyl compounds containing electron-withdrawing groups adjacent to the carbonyl group. The reaction is also facilitated by an increase in the electron-attracting powers of the substituent R.[13] The adduct from ninhydrin and the α-amino-acid is visualised as undergoing a concerted electronic change similar to that shown in (XI) to give a Schiff's base (XII) which is



hydrolysed to the aldehyde, R·CHO, ammonia, and 2-hydroxyindane-1 : 3-dione, as observed. This hydrolysis is more difficult in the case of the product from proline where the quaternary nitrogen atom is fully substituted and prototropic rearrangement cannot occur. Somewhat related mechanisms have been discussed by Hine,[14] by Sweeley and Horning,[15] and also in the particular case of the transaminations and decarboxylations of α-amino-acids catalysed by vitamin B_6 in the presence of metal ions.[16] The intermediate (XIII) involved in these reactions is clearly related to (XI), and the analogy is strengthened by the observation [17] that substitution on the primary amino-group of the amino-acid, as in proline, prevents the decarboxylation–transamination reaction with pyridoxal. Alternative mechanisms, not involving decarboxylation as an intrinsic step, for the oxidation–decarboxylation of α-amino-acids have been advanced by Schönberg, Moubasher, and their collaborators [18] and by Baddar *et al.*,[19] and it has also been suggested [20] that the effective reagent might be OH^+ in certain cases.

Work on the structures of the blue pigments derived from the acyclic *N*-methylamino-acids and ninhydrin is in progress.

Experimental

Absorption spectra were determined in 95% EtOH except where otherwise stated.

Reaction of Proline with Ninhydrin. The Yellow Condensation Product (IV).—This was prepared by the method of Grassmann and Arnim [2] from ninhydrin (900 mg.) and L-proline (540 mg.). The product (875 mg., 82%) crystallised from warm methanol and the yellow crystals, m. p. 190—195° (decomp.), were separated and washed with ether (Found: C, 72·8; H, 5·45. Calc. for $C_{13}H_{11}O_2N$: C, 73·2; H, 5·2). Light absorption max. were at 231, 303, and 354 mμ (log ε 4·35, 4·04, and 4·26 respectively). The infrared spectrum (Nujol) showed bands at 1661, 1622, 1579, 1446, 1410, 1365, and 1360 $cm.^{-1}$. The compound is readily soluble in polar solvents and sparingly soluble in hydrocarbons.

Oxidation of the Yellow Proline–Ninhydrin Condensation Product.—The pigment (400 mg.)

[11] Schönberg and Moubasher, *Chem. Rev.*, 1952, **50**, 261.
[12] Langenbeck *et al.*, *Ber.*, 1927, **60**, 930; 1928, **61**, 942; 1937, **70**, 367, 672, 1039.
[13] Bergel and Bolz, *Z. physiol. Chem.*, 1933, **215**, 25; **220**, 20; 1934, **223**, 66; Herbst and Clarke, *J. Biol. Chem.*, 1934, **104**, 769; Baddar and Sherif, *J.*, 1956, 4292.
[14] Hine, " Physical Organic Chemistry," McGraw-Hill Book Co., New York, 1956, p. 288.
[15] Sweeley and Horning, *J. Amer. Chem. Soc.*, 1957, **79**, 2622.
[16] Metzler, Ikawa, and Snell, *ibid.*, 1954, **76**, 648; Rothberg and Steinberg, *ibid.*, 1957, **79**, 3274.
[17] Herbst, *Adv. Enzymol.*, 1944, **4**, 75; Snell, *J. Amer. Chem. Soc.*, 1945, **67**, 194.
[18] Moubasher *et al.*, *J.*, 1948, 176; 1949, 1137; 1951, 231, 1928; *J. Amer. Chem. Soc.*, 1950, **72**, 2666.
[19] Baddar *et al.*, *J.*, 1949, S 163; 1950, 136; 1954, 209; 1956, 4292; see also Hammick, Roe, Weston, and Whiting, *J.*, 1953, 3825.
[20] Spenser, Crawhall, and Smyth, *Chem. and Ind.*, 1956, 796.

was suspended in an alkaline solution of hydrogen peroxide (0·4 c.c. of 100-vol. hydrogen peroxide in 8 c.c. of 0·5N-aqueous sodium hydroxide) and shaken until all the solid had dissolved. The solution was then acidified with the minimum quantity of concentrated hydrochloric acid and kept overnight; then the white crystalline material (168 mg.) was separated, washed, and dried. It crystallised from benzene–methanol as small prisms, m. p. 185—186° (sealed tube), and was shown to be identical with phthalic acid by colour reactions and conversion into the anhydride.

Reaction of Hydroxyproline with Ninhydrin.—Ninhydrin (400 mg.) was dissolved in warm alcohol (5 c.c.), cooled, and added to an aqueous solution (3 c.c.) of L-4-hydroxyproline (250 mg.). The solution became red and carbon dioxide was evolved. The product was concentrated (to *ca.* 3 c.c.) until crystals separated and, after cooling, the solid *product* was removed and washed with a little ethanol, being obtained as dark-brown needles (290 mg.), which after crystallisation from methanol had m. p. 132—133° (decomp.) (Found: C, 67·6; H, 5·0; N, 6·35. $C_{13}H_{11}O_3N$ requires C, 68·1; H, 4·84; N, 6·1%), $\lambda_{max.}$ 233, 303, and 354 mμ (log ε 4·35, 4·00, and 4·26 respectively).

Reaction of Proline with Ninhydrin. The Purple Condensation Product (VI).—(i) Ninhydrin (178 mg.) and L-proline (50 mg.) were dissolved in separate portions of phosphate buffer (pH 7; 2·5 c.c. each), mixed, and heated at 60° on a water-bath for 10 min. The purple-brown precipitate was separated (120 mg.), washed with hot water, and dried; it had m. p. 174—176° (decomp.). Purification was effected by chromatography of a chloroform solution of the product on a column (35 × 2 cm.) of deactivated alumina, most of the impurities being retained on the column. Concentration of the eluate under reduced pressure gave a solution from which the product crystallised as purple needles with a metallic sheen, m. p. 176° (decomp.) (Found: N, 4·1. Calc. for $C_{22}H_{13}O_4N$: N, 3·94%), $\lambda_{max.}$ (in $CHCl_3$) 250, 269, 285, 320, 340, and 545 mμ (log ε 4·42, 4·16, 4·03, 3·56, 3·59, and 4·96 respectively).

(ii) The yellow condensation product (IV) (200 mg.) from proline and ninhydrin was condensed with excess of ninhydrin (530 mg.) according to the method of Grassmann and Arnim.[2] The product (280 mg.) crystallised from chloroform–light petroleum (b. p. 60—80°; 1 : 2). Its ultraviolet and visible absorption spectrum was identical with that of the product of the previous experiment.

(iii) Pyrrolidine (125 mg.) was added to a solution of ninhydrin (500 mg.) in warm 2N-acetic acid (10 c.c.), and the mixture heated to boiling. After cooling, the solid product (280 mg.) was separated and crystallised as before from chloroform–light petroleum (b. p. 60—80°; 1 : 2). The product was identified by means of the visible and ultraviolet absorption spectrum.

Pyrolysis of the Purple Proline–Ninhydrin Condensation Product.—The pigment (200 mg.) was heated in a sublimation apparatus at 210—220°/0·1 mm. White crystals (*ca.* 3 mg.), m. p. 127—128°, together with a brown oil, were obtained as sublimate. The crystalline product was separated and gave a negative fluorescein reaction but a strong yellow colour with dilute aqueous sodium hydroxide. The m. p. was not depressed on admixture with authentic indane-1 : 3-dione, m. p. 129—131°. A similar yield of the same product was obtained from the pyrolysis of the purple morpholine–ninhydrin pigment (see below).

Reaction of Pipecolic Acid with Ninhydrin.—A solution of ninhydrin (170 mg.) in phosphate buffer (pH 7; 2 c.c.) was mixed with a solution of L-pipecolic acid (85 mg.) in phosphate buffer (pH 7; 2 c.c.), and the reaction which commenced immediately was completed by warming at 60° on the water-bath for 10 min. The solid product (95 mg.) was separated, washed with hot water, and dried; it had m. p. 170° (decomp.) and was purified by adsorption of the impurities from a chloroform solution on a column of deactivated alumina. Concentration of the eluate (to 15 c.c.) and dilution with light petroleum (b. p. 60—80°; 30 c.c.) caused the product (40 mg.) to crystallise as purple needles with a metallic sheen, m. p. 238° (decomp.) (Found: N, 3·4. Calc. for $C_{23}H_{15}O_4N$: N, 3·8%), $\lambda_{max.}$ (in $CHCl_3$) 260, 350, and 571 mμ (log ε 4·42, 3·67, and 4·87 respectively).

Reaction of Piperidine with Ninhydrin.—(i) Piperidine (150 mg.) was added to a solution of ninhydrin (500 mg.) in warm 2N-acetic acid (10 c.c.), and the mixture heated to the b. p. After cooling, the solid product (330 mg.) was separated and crystallised from chloroform–light petroleum (b. p. 60—80°; 1 : 2) as purple needles, identical (spectra) with the product from the previous experiment.

(ii) 2 : 2-*Dipiperidinoindane*-1 : 3-*dione.*—Piperidine (190 mg.) was added to a solution of ninhydrin (178 mg.) in ethanol (2 c.c.). The mixture was heated on the water-bath for 20 min.

and, after cooling, the colourless crystals (190 mg.) were separated and washed with cold ethanol (Found: N, 8·6. Calc. for $C_{19}H_{24}O_2N_2$: N, 8·95%). Light absorption max. were at 228, 281, and 375 mμ (log ε 4·64, 3·31, and 2·59 respectively). The crystals were transformed into a coloured product at >100°.

Reaction of Morpholine with Ninhydrin.—(i) A solution of ninhydrin (500 mg.) and morpholine (500 mg.) in aqueous 10% acetic acid (10 c.c.) was boiled for 10 min., then concentrated to *ca.* 1 c.c. under reduced pressure. The precipitated purple product was separated, washed, dried (264 mg.), and purified, first by adsorption of the impurities from a solution in chloroform on deactivated alumina, and secondly by slow crystallisation from hot chloroform (extraction with hot solvent from a thimble) to yield the *product* as dark purple prisms, m. p. 258—260° (decomp.) (Found: C, 70·6; H, 3·75; N, 3·7. $C_{22}H_{13}O_5N$ requires C, 71·15; H, 3·55; N, 3·8%), $\lambda_{max.}$ (in $CHCl_3$) 250, 270, 345, and 554 mμ (log ε 4·43, 4·29, 3·67, and 4·80 respectively).

(ii) *2-Hydroxy-2-morpholinoindane-1 : 3-dione.*—Morpholine (500 mg.) was added to a solution of ninhydrin (500 mg.) in ethanol (5 c.c.). The mixture was warmed on the water-bath for 10 min., then cooled slowly. White crystals were obtained. These recrystallised from ether–light petroleum (b. p. 60—80°; 1 : 2) as needles (420 mg.) (Found: C, 63·1; H, 5·55; N, 5·65. $C_{13}H_{13}O_4N$ requires C, 63·15; H, 5·3; N, 5·65%), $\lambda_{max.}$ 228, 247, 279, and 351 mμ (log ε 4·42, 3·82, 3·00, and 2·15 respectively).

Reaction of 2-Methylpiperidine with Ninhydrin.—A solution of ninhydrin (500 mg.) and 2-methylpiperidine (500 mg.) in glacial acetic acid (15 c.c.) was heated on the steam-bath for 10 min. On addition of a few drops of water, the purple condensation *product* (128 mg.) was precipitated. It was separated, washed, dried, and crystallised from chloroform–carbon tetrachloride (1 : 8) as dark purple prisms, m. p. 195—197° (decomp.) (Found: C, 75·0; H, 4·4; N, 3·8. $C_{24}H_{17}O_4N$ requires C, 75·2; H, 4·45; N, 3·65%), $\lambda_{max.}$ (in $CHCl_3$) 248, 345, and 571 mμ (log ε 4·39, 3·69, and 4·11 respectively).

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