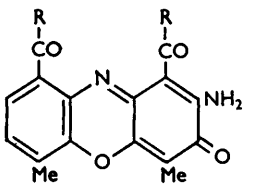
## 307. *Actinomycin. Part IV.* An Oxidative Degradation of Actinomycin B.*

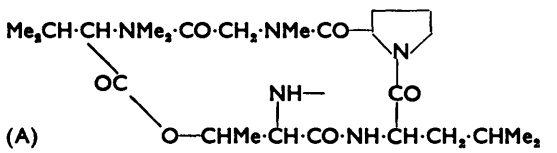
By E. Bullock and A. W. Johnson.

Oxidation of actinomycin B with alkaline hydrogen peroxide gives an acidic peptide of 7-methylbenzoxazolone-4-carboxylic acid. The lactone of the peptide group of actinomycin is hydrolysed during the oxidation but all of the original amino-acids are retained. Acid hydrolysis of the oxidation product gives 7-methylbenzoxazolone-4-carboxylic acid, the structure of which is confirmed by synthesis.

As a result of an extensive and elegant series of degradative experiments, Brockmann and his colleagues [1] have deduced structure (I) for actinomycin $C_3$, where R = L-threonyl-D-*alloiso*leucyl-L-prolyl-sarcosyl-L-*N*-methylvaline with the terminal carboxyl group esterified by the hydroxyl group of the threonyl fragment, as in (A). The chemistry of the actinomycins [2] as well as the rather confused nomenclature of the group [3] have been reviewed recently. The chemical differences between the various actinomycins involve the nature and order of the amino-acids in the peptide chains; the phenoxazin-3-one nucleus seems



(I)



(A)

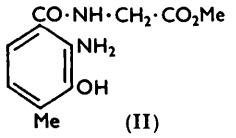
to be common to all the actinomycins so far examined.[4] It is of particular interest that the actinomycins can arise from the oxidative condensation of peptides derived from 3-hydroxy-4-methylanthranilic acid and that this reaction has been achieved *in vitro* by the aerial oxidation of a solution of *N*-(3-hydroxy-4-methylanthraniloyl)glycine methyl ester (II) in aqueous ammonium carbonate,[4] to give (I; R = $NH{\cdot}CH_2{\cdot}CO_2Me$), a reaction which recalls the formation of the insect pigment xanthommatin (III) by a similar oxidation of 3-hydroxykynurenine.[5]

Our own work on actinomycins B [6] and D [7] gives further support to formulæ of the type (I). Thus, from the action of alkaline hydrogen peroxide on actinomycin B we have obtained a colourless crystalline acid, $C_{32}H_{44}O_{10}N_6,2H_2O$ which has been purified by counter-current distribution between ethyl acetate and a phosphate buffer at pH 5·9. This acid still contained all five of the constituent amino-acids, L-*N*-methylvaline, sarcosine, L-proline, D-valine, and L-threonine, of the original actinomycin B peptide chains.

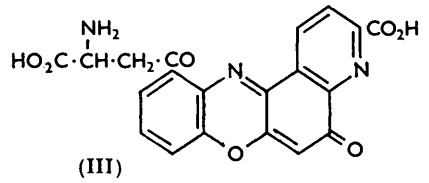
* Part III, preceding paper.

[1] Brockmann, Bohnsack, Franck, Gröne, Muxfeldt, and Süling, *Angew. Chem.*, 1956, **68**, 70.
[2] Johnson, Chem. Soc. Special Publ., No. 5, 1956, 82.
[3] Roussos and Vining, *J.*, 1956, 2469.
[4] Brockmann and Muxfeldt, *Angew. Chem.*, 1956, **68**, 69.
[5] Butenandt, Schiedt, Biekert, and Cromartie, *Annalen*, 1954, **588**, 106; 1955, **590**, 75.
[6] Dalgliesh, Johnson, Todd, and Vining, *J.*, 1950, 2946.
[7] Vining and Waksman, *Science*, 1954, **120**, 389.

Potentiometric titration of the acid revealed the presence of only one carboxyl group and the above molecular formula was supported by determinations of the equivalent. The ultraviolet absorption spectrum suggested the presence of a benzenoid ring and the

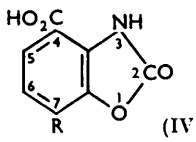


(II)



(III)

resemblance of the spectrum to those of 3-hydroxyanthranilic acid and its derivatives [8] was especially striking. The infrared spectrum of the product as a mull in Nujol showed a band in the carbonyl region at 1786 cm.$^{-1}$ which is generally associated with the presence of a $\beta\gamma$-unsaturated five-membered lactone,[9] and, combining all of the foregoing evidence, it seemed that the degradation product might well be a peptide derived from benzoxazolone-4-carboxylic acid (IV; R = H).



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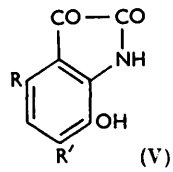


(V)

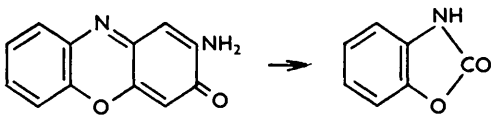
This was confirmed by acid hydrolysis of the peptide, the amino-acids being removed and another crystalline carboxylic acid, $C_9H_7O_4N$, obtained, the ultraviolet absorption of which closely resembled that of the parent peptide. This product contained no free hydroxyl or amino-group, but the presence of a $\beta\gamma$-unsaturated five-membered lactone was again suggested by the band at 1799 cm.$^{-1}$ in the infrared spectrum of a chloroform solution. Bands at 1145, 1087, 1038, 971, and 810 cm.$^{-1}$ were consistent with the presence of two adjacent hydrogen atoms in the benzene nucleus; bands between 900 and 860 cm.$^{-1}$ usually associated with isolated hydrogen atoms on a benzene ring were absent.[10] The acid was therefore formulated as either 7- (IV; R = Me) or 5-methylbenzoxazolone-4-carboxylic acid, and a decision in favour of the 7-methyl isomer was reached after each isomer had been synthesised. Application of the Sandmeyer synthesis [11] to 2-(and 6-)methoxy-*m*-toluidine ($NH_2$ = 1) gave 7-methoxy-6-(and 4-[12])methylisatin which was hydrolysed to the corresponding 7-hydroxy-6-(and 4-)methylisatin (V; R = H, R′ = Me, and *vice versa*, respectively). Oxidation of the 7-hydroxyisatins with alkaline hydrogen peroxide gave the 7- and 5-methylbenzoxazolone-4-carboxylic acids, presumably with intermediate formation of a labile carbamic acid.[13]

The formation of a benzoxazolone on oxidation of a phenoxazin-3-one was rather unexpected and for a time led us to believe that actinomycin was a derivative of isatin (see Part III, preceding paper). However, the reaction appears to be fairly general, as shown by the isolation of benzoxazolone itself from a similar oxidation of 2-aminophenoxazin-3-one (VI). Very recently, Butenandt, Biekert, and Neubert [14] reported the formation of 4-acetylbenzoxazolone on oxidation of 1 : 8-diacetyl-2-aminophenoxazin-3-one with chromic acid.

Besides the benzoxazolone peptide, the other major product from the reaction of

[8] Nyc and Mitchell, *J. Amer. Chem. Soc.*, 1948, **70**, 1847.
[9] Grove and Willis, *J.*, 1951, 881.
[10] Bellamy, " The Infra-red Spectra of Complex Molecules," Methuen, London, 1954, p. 67.
[11] Marvel and Hiess, *Org. Synth.*, Coll. Vol. I, 1944, 327.
[12] I. G. Farbenind., B.P., 308,740; *Chem. Zentr.*, 1930, II, 2185.
[13] Cf. Mitchell and Trautner, *J.*, 1947, 1330.
[14] Butenandt, Biekert, and Neubert, *Angew. Chem.*, 1956, **68**, 379.

actinomycin with alkaline hydrogen peroxide is a peptide, possibly attached to a small molecule. Its investigation is still in progress but it is obvious from the yields that the two peptide-containing products cannot be derived from the same peptide chain in the actinomycin molecule. This oxidative fission of actinomycin therefore provides a method for distinguishing the two peptide groups and hence should prove to be of value in elucidating




(VI)

the precise structure of the various members of the actinomycin group. The techniques used by Brockmann and his colleagues for elucidation of the order of the amino-acids in the peptides were applied to the whole molecule, and such methods are much less valuable when the peptide chains are not identical.

## Experimental

95% Ethanol was used as the solvent in determinations of ultraviolet absorption spectra except where otherwise stated.

*Oxidation of Actinomycin B with Alkaline Hydrogen Peroxide.*—Actinomycin B (recrystallised; 1·01 g.) was dissolved in the minimum amount (*ca.* 80 c.c.) of methanol, and an excess of alkaline hydrogen peroxide (100 c.c. of a 3% solution in 0·75N-sodium hydroxide) was added with stirring. After 1 hr. at room temperature the pH was adjusted to 3—4 by dilute hydrochloric acid, and the solution was diluted with water (250 c.c.). The product was extracted with butan-1-ol (3 × 100 c.c.), the combined butanol extracts were washed, and the solvent was removed under reduced pressure, to leave a pale yellow solid resin (0·86 g.). Examination of this product by chromatography on Whatman No. 1 paper, with propan-2-ol–aqueous ammonia (*d* 0·88)–water (7 : 1 : 2) as solvent, revealed the presence of four spots fluorescent in ultraviolet light, $R_F$ 1·0 (*a*), 0·75 (*b*), 0·45 (*c*), and 0·40 (*d*) respectively. Of these, spot (*b*) was intensely fluorescent and the others were only faintly fluorescent; material (*a*) was non-acidic and the other three were acidic. A rough separation of the mixture was achieved by dissolving the crude product in acetone–ethyl acetate (1 : 9; 70 c.c.), separating any undissolved solid, and extracting the solution with saturated sodium hydrogen carbonate solution (3 × 40 c.c.). The aqueous layer was then acidified with hydrochloric acid and extracted with chloroform (3 × 50 c.c.) and then with butan-1-ol (2 × 50 c.c.). Examination of the various solutions by paper chromatography as before showed that the residual yellow ethyl acetate solution contained substance (*a*) of $R_F$ 1·0, and evaporation of the solution gave it as a bright yellow solid (125 mg.) which was not examined further. The mixed chloroform extracts contained most of substance (*b*) of $R_F$ 0·75 and evaporation gave a pale yellow solid (260 mg.). The butan-1-ol extracts contained substances (*c*) and (*d*), of $R_F$ 0·45 and 0·40 respectively, and a little of substance (*b*). Evaporation of the solvent gave a pale yellow solid (304 mg.) which showed only very weak absorption at 250 mμ and was therefore largely composed of peptides. The examination of this product will be described in a later paper.

Fraction (*b*) was purified by counter-current extraction between ethyl acetate and 0·1M-phosphate buffer (pH 5·9). The solution was subjected to 24 transfers and the distribution of the product followed by measurement of the intensity of light absorption at 300 mμ; it was found that most of substance (*b*) was located in tubes 2—13. The contents of these tubes were mixed and acidified and the organic layer was separated and washed. After removal of the solvent, the *product* was obtained as a pale buff solid (90 mg.) which crystallised from ethyl acetate–light petroleum (b. p. 60—80°) as very small prisms, m. p. 233° (Found : C, 53·9; H, 7·2; N, 11·3. $C_{32}H_{44}O_{10}N_6,2H_2O$ requires C, 54·2; H, 6·8; N, 11·85%). The product was acidic, having $pK_a$ 3·7 in glycol monomethyl ether–water (1 : 9), equiv. (titration), 632 ($C_{32}H_{44}O_{10}N_6$ requires 673), and light-absorption max. at 246 and 296—297 mμ (log ε 4·05 and

3·63 respectively). The principal bands in the infrared spectrum (Nujol mull) were at 3300, 1786, 1740, and 1666—1625 (broad) cm.$^{-1}$. The product gave a brownish-red ferric reaction.

*Acid Hydrolysis of the Hydrogen Peroxide Oxidation Product of Actinomycin.*—The crude oxidation product (1 g.) was dissolved in 6N-hydrochloric acid (25 c.c.) and heated overnight at 80° under oxygen-free nitrogen. The brown solution was cooled, adjusted to pH 4 by sodium hydrogen carbonate, and extracted with butan-1-ol (3 × 50 c.c.). The butanol extract was washed and the solvent removed under reduced pressure, to leave a brown gum (80—100 mg.), which was sublimed at 140°/0·5 mm., to give a sticky yellow solid (40—60 mg.). This was crystallised from water, and the pale yellow solid so obtained further purified by sublimation as before. The colourless solid (5 mg.) had no definite m. p. but darkened above 235°. After recrystallisation from methanol *7-methylbenzoxazolone-4-carboxylic acid* formed needles [Found : C, 56·5; H, 4·0, 3·9; N, 7·9, 7·3%; *M* (isopiestic method),[15] 187 ± 7. $C_9H_7O_4N$ requires C, 56·0; H, 3·6; N, 7·3%; *M*, 193]. Light absorption : (i) max. at 300—303, 244—245, and 212—213 mμ (log ε 3·68, 4·02, and 4·48 respectively), unchanged on acidification with dilute hydrochloric acid; (ii) in 95% ethanol–0·1N-aqueous sodium hydroxide (1 : 1) : max. at 303—304, 250, and 218—219 mμ (log ε 3·72, 4·03, and 4·32 respectively). The infrared spectrum (KBr) showed bands at 3175, 3077, 2915, 2740—2500 (diffuse), 1764 (s), 1695, 1653, 1623, 1605, 1508, 1422 (s), 1389, 1333 (s), 1290, 1258 (s), 1205, 1171, 1145, 1117, 1087, 1038, 1003, 971, 961, 833, 810, 775 (s), 758 (s), 739 (s), and 680 cm.$^{-1}$. A similar determination on a chloroform solution showed bands at 1799 (s), 1695, and 1647 cm.$^{-1}$ in the carbonyl region.

The product was acidic and could be extracted from aqueous solutions of pH <5 into organic solvents, *e.g.*, ether, ethyl acetate, and butan-1-ol. It coupled with diazotised sulphanilic acid to give a red product but could not be diazotised itself. Tests for a primary amide group were negative and the compound gave no ferric reaction. With Ehrlich's reagent it gave a yellow colour similar to that obtained from 3-hydroxyanthranilic acid.

*Oxidation of 2-Aminophenoxazin-3-one.*—2-Aminophenoxazin-3-one[16] (100 mg.) was dissolved in methanol (10 c.c.) and treated with an alkaline solution of hydrogen peroxide (1·5 c.c. of 30%; 3 c.c. of 10% aqueous sodium hydroxide; and 10 c.c. of water). No visible reaction occurred after 30 min. at room temperature but at 50° the solution was rapidly decolorised. After cooling and acidification, the mixture was extracted with chloroform (3 × 20 c.c.) and the combined extracts, which showed a strong green fluorescence in ultraviolet light, were washed, dried, and evaporated. The dark brown resinous product (15 mg.) was sublimed at 140°/0·1 mm. and gave two products. The less volatile (2 mg.) had m. p. 140° alone and mixed with sublimed benzoxazolone.

*2-Methoxy-m-toluidine Hydrochloride.*—Raney nickel (10 g. as sludge in ethanol) was added to a solution of 2-methoxy-3-nitrotoluene[17] (35 g.) in dry ethanol (50 c.c.) and hydrogenated in an autoclave at room temperature and 4 atm. of hydrogen. The reduction was complete in 20 min., then the catalyst was removed and the filtrate diluted with ether (150 c.c.). The solution was saturated with hydrogen chloride, and the precipitated hydrochloride of 2-methoxy-*m*-toluidine (35 g.) was separated and washed with ether. It had m. p. 112° (sealed capillary) after crystallisation from methanol–ether.

*7-Methoxy-6-methylisatin.*—A solution of 2-methoxy-*m*-toluidine hydrochloride (10 g.) in 3N-hydrochloric acid (50 c.c.) was added to an aqueous solution (120 c.c.) of chloral hydrate (9 g.) and anhydrous sodium sulphate (130 g.). A solution of hydroxylamine hydrochloride (11 g.) in water was then added and the whole brought to 90° during 1 hr., then boiled for 10 min., cooled, and kept overnight. Crystalline hydroxyiminoacetyl-2-methoxy-*m*-toluidine (9 g.; air-dried) separated in a form pure enough for the subsequent cyclisation.

The hydroxyimino-compound was added gradually with stirring to concentrated sulphuric acid (56 g.; *d* 1·8), at 55° on the steam-bath. The temperature of the mixture rose rapidly to 80—90° and, after the addition of all of the solid, the mixture was kept at this temperature for a further 20 min. The cooled dark red solution was poured on ice (300 g.) and kept for 2 hr., then the deep red aqueous suspension was extracted with ether (5 × 50 c.c.). Removal of the solvent from the combined washed and dried ethereal extract yielded an orange solid (3 g.) which was crystallised from a small volume of chloroform and sublimed at 140°/0·5 mm., to give *7-methoxy-6-methylisatin* as orange-red needles, m. p. 169° (Found : C, 62·8; H, 4·8; N, 7·55.

[15] Morton, Campbell, and Ma, *Analyst*, 1953, **78**, 722.
[16] Zincke and Hebebrand, *Annalen*, 1884, **226**, 60.
[17] Robinson, *J.*, 1916, **109**, 1084.

$C_{10}H_9O_3N$ requires C, 62·8; H, 4·75; N, 7·3%). Light absorption: (i) max. at 420—424, 317—319, 246, and 224 mμ (log ε 2·97, 3·76, 4·22, and 3·97 respectively); (ii) in 95% ethanol–0·1N-aqueous sodium hydroxide (1 : 1): max. at 370, 272—273, and 231 mμ (log ε 3·68, 4·05, and 4·34 respectively). Infrared spectrum (Nujol mull): max. at 3140, 2945, 1740, 1620, 1593, 1490, 1430, 1370, 1317, 1280, 1240, 1195, 1150, 1062, 1013, 957, 875, 828, 787, 772, and 730 $cm.^{-1}$.

Contrary to the recommended procedure for the cyclisation of hydroxyiminoacetanilide [11] which was dried before addition to the sulphuric acid, it was found in this case that the cyclisation was more rapid if slightly damp samples of our 2-methoxy-compound were used. In one experiment with material which had been dried in a vacuum-desiccator, no isatin was obtained after the treatment with hot sulphuric acid.

*7-Hydroxy-6-methylisatin.*—7-Methoxy-6-methylisatin (0·89 g.) was mixed with freshly prepared pyridine hydrochloride (2·5 g.) and heated at 210° (oil-bath) for 1 hr.[18] The melt was cooled, then treated with water (25 c.c.), and the dark red insoluble material separated. The filtrate was extracted with ether, and the extract washed, dried, and evaporated. The residue was combined with the water-insoluble material and dissolved as far as possible in boiling ethanol (150 c.c.). After clarification with charcoal, most of the solvent was removed and water added until solid began to separate. This redissolved when the solution was heated and, after cooling, dark brownish-red needles (0·6 g.) were obtained which decomposed at 220° without melting. The *product* sublimed at 180°/0·5 mm. as long dark red needles (Found: C, 61·4; H, 4·1; N, 8·1. $C_9H_7O_3N$ requires C, 61·0; H, 4·0; N, 7·9%). Light absorption: (i) max. at 448—452, 430—446 (broad), 335—337, and 224—225 mμ (log ε 3·04, 3·04, 3·79, and 4·29 respectively); (ii) in 95% ethanol–0·1N-aqueous sodium hydroxide (1 : 1): max. at 406, 301, and 255 mμ (log ε 3·49, 4·09, and 4·28 respectively).

*7-Methylbenzoxazolone-4-carboxylic Acid.*—7-Hydroxy-6-methylisatin (0·15 g.) was dissolved in methanol (4 c.c.) and a solution of 30% hydrogen peroxide (1·5 c.c.) in aqueous 2·5% sodium hydroxide (13·5 c.c.) was added. The mixture was kept for 5 min. at room temperature, then acidified with 2N-hydrochloric acid to pH 2 and extracted with butan-1-ol (2 × 25 c.c.). The extract was washed and dried and the solvent removed, to leave a brownish solid which was purified by sublimation at 160°/0·1 mm. The main product (0·1 g.) was a colourless solid which was crystallised from hot water and resublimed. A minor product was not investigated further. The principal oxidation product, *7-methylbenzoxazolone-4-carboxylic acid*, decomposed without melting at 240° (Found: C, 55·7; H, 3·9; N, 7·5. $C_9H_7O_4N$ requires C, 56·0; H, 3·65; N, 7·25%). It gave no ferric reaction and on chromatography on paper with propan-2-ol–aqueous ammonia (*d* 0·88)–water (7 : 1 : 2) it had $R_F$ 0·6. The ultraviolet and infrared spectra were identical with those obtained for the product from actinomycin (see above).

*6-Methoxy-m-toluidine.*—Raney nickel (5 g. as sludge in ethanol) was added to a solution of 4-methoxy-3-nitrotoluene in ethanol (40 c.c.) and the mixture hydrogenated in an autoclave at room temperature and 4 atm. hydrogen. Reduction was complete in 4 hr., then the catalyst and solvent were removed. The residue (13·5 g.) formed colourless crystals, m. p. 45°, which, without purification, were used in the following experiment.

*7-Methoxy-4-methylisatin.*—A solution of 6-methoxy-*m*-toluidine (4·5 g.) in 3N-hydrochloric acid (25 c.c.) was added to an aqueous solution (60 c.c.) of chloral hydrate (4·5 g.) and anhydrous sodium sulphate (65 g.). A solution of hydroxylamine hydrochloride (6·5 g.) in water (25 c.c.) was added and the whole brought slowly to 90° (1 hr.) and then boiled for 10 min. After cooling, the colourless crystals (4 g.) of hydroxyiminoacetyl-6-methoxy-*m*-toluidine were separated and air-dried. This product (2 g.) was added gradually with stirring to sulphuric acid (15 g.; *d* 1·8) at 50°. The temperature of the mixture rose rapidly to 90—100° and, after the addition of all of the solid, was kept thereat for a further 20 min. The cooled dark red solution was poured on ice (100 g.), and the red precipitate separated. A further quantity was deposited from the filtrate on storage. The combined solid product (1·1 g.) was crystallised from ethyl acetate, forming dark red crystals, m. p. 234° (lit.,[12] 235—236°), which sublimed at 140°/0·5 mm. (Found: C, 63·05; H, 4·5; N, 7·65. Calc. for $C_{10}H_9O_3N$: C, 62·8; H, 4·75; N, 7·3%). Light absorption: max. at 459, 327—332, and 227—229 mμ (log ε 3·17, 3·53, and 4·27 respectively).

*7-Hydroxy-4-methylisatin.*—7-Methoxy-4-methylisatin (100 mg.) was heated with freshly prepared pyridine hydrochloride (200 mg.) at 200° (oil-bath) for 5 min. The melt was cooled and treated with water (5 c.c.), and the red insoluble material separated, washed, and dried. Fractional vacuum-sublimation at 140°/0·5 mm. gave the unchanged methoxy-compound but

[18] Cf. Prey, *Ber.*, 1941, **74**, 1219.

at 190°/0·5 mm. the hydroxy-compound was obtained as dark red needles (10 mg.). The sublimed *product*, when crystallised from aqueous ethanol and resublimed, had m. p. 269° (Found : C, 61·3; H, 4·0; N, 8·15. $C_9H_7O_3N$ requires C, 61·0; H, 4·0; N, 7·9%). Light absorption : max. at 464—469, 335—336, and 227 mμ (log ε 3·22, 3·61, and 4·26 respectively).

*5-Methylbenzoxazolone-4-carboxylic Acid.*—7-Hydroxy-4-methylisatin (50 mg.) was dissolved in methanol (2 c.c.) and a solution of 30% hydrogen peroxide (0·5 c.c.) in aqueous 2·5% sodium hydroxide (5·5 c.c.) was added. The mixture was kept at room temperature for 5 min., then acidified with dilute hydrochloric acid and extracted with butan-1-ol (2 × 10 c.c.). The butanol extract was washed and the solvent removed, leaving a pale brown solid (35 mg.) which was purified by sublimation at 185°/0·5 mm. The colourless *acid* crystallised from very dilute ethanol and was then resublimed (Found : C, 56·1; H, 3·9; N, 7·5. $C_9H_7O_4N$ requires C, 56·0; H, 3·65, N, 7·25%). The infrared spectrum (Nujol mull) showed bands at 3226, 1908, 1779 (s), 1745 (s), 1709 (s), 1626, 1610, 1312, 1292, 1258 (s), 1136, 1124, 1040, 1026, 962, 943, 855, 826, 809, 800, 793, 786, 752, and 727 cm.$^{-1}$.

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