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STUDIES IN THE BIOCHEMISTRY OF MICROORGANISMS

Part XIV.—Biosynthesis of Amudol, a Metabolic Product of Penicillium martinsii Biourge

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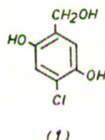
The biogenesis of amudol has been examined by feeding sodium (1-C^{14})- and (2-C^{14})-acetates. It has been shown that *amudol* is formed by head-to-tail aldol condensation of one acetate and three malonate units.

In the earlier communications[1,2] we have described the isolation and structures of amudol, amudane, amudene and amujane—last three identified as griseofulvin, dehydrogriseofulvin and dihydrogriseofulvin—obtained as metabolites of the mold *Penicillium martinsii.*

The structure of amudol (I) brought out some very interesting features. The starting unit is oxidised to a primary alcoholic group, one of the



(I)

hydroxyl group, which should have been located in position 3 on the polyketide chain is located at position 2 due to the phenomenon of hydroxylation-induced intramolecular migration,[3] 'NIH shift' and lastly a chlorine atom is substituted in position 4. With these features available in the molecule of amudol it was considered desirable to study its biogenesis as a start leading to our projected studies of biogenesis in the cell-free systems.

Amudol is built up as a very simple molecule formed due to the head-to-tail condensation of one acetate and three malonate units. The present communication describes the biogenesis of *amudol* employing sodium (1-C^{14}), and also (2-C^{14})-acetate as precursors. *Penicillium martinsii* was grown on modified Czapek–Dox medium and pure amudol was obtained derived from (1-C^{14})-acetate and also from sodium (2-C^{14})-acetate separately. The incorporation was 1.45% and 2.8% respectively.

Amudol derived from sodium (1-C^{14})-acetate (r.m.a. 3.9×10^5) was oxidised and decarboxylated to give carbon dioxide, assayed as barium carbonate, which was almost inactive (r.m.a. 0.031×10^5). This small activity could be due to randomisation.

When amudol was nitrated[4] and subjected to hypobromite degradation,[5] it gave bromopicrin, which was reduced and the resulting methylamine hydrochloride was assayed as its *N*-methyl-2,4-dinitroaniline derivative (r.m.a. 1.18×10^5 for 1C* against 1.30×10^5). This indicates that the lower activity was due to the benzene ring carbons, which were formed due to decarboxylation of malonate unit.

Dechlorination,[4] reduction and Kuhn–Roth oxidation and Schmidt reaction of the resulting 2,5-dihydroxytoluene gave barium carbonate (r.m.a. 1.34×10^5 for 1C* against 1.30×10^5). This high activity was indicative of the starting unit of the molecule. The methylamine hydrochloride the second product in the Schmidt reaction, was assayed as *N*-methyl-2,4-dinitroaniline and was found to be inactive. All these results are outlined in Chart 1. Amudol derived from sodium (2-C^{14})-acetate (r.m.a. 6.27×10^5) when oxidised and decarboxylated to give carbon dioxide (assayed as barium carbonate) was found to be, as expected, active (r.m.a. 1.6×10^5 against 1.5×10^5). This higher activity can be safely attributed to the starting unit of the molecule.

Similarly, amudol on nitration[4] and hypobromite degradation,[5] yielded bromopcrin which was reduced and assayed as *N*-methyl-2,4-dinitroaniline, having slightly lower activity (r.m.a. 1.54×10^5; 1C*; against 1.56×10^5).

Similarly dechlorination,[4] reduction and Kuhn–Roth oxidation followed by Schmidt reaction of the resulting 2,5-dihydroxytoluene gave barium carbonate (r.m.a. 0.00). The methylamine hydrochloride, the second product in the Schmidt reaction, was assayed as *N*-methyl-2, 4-dinitroaniline (r.m.a. 1.59×10^5 for 1C* against

1.50×10^5). The slightly higher activity was indicative of the starting unit of the molecule. These results, summarised in Chart 2, clearly supported and confirmed the previous results.

When the mold was fed with generally labeled C^{14}-glucose, the isolated amudol (3.1% incorporation) had the r.m.a. 3.07×10^5. It was interestingly observed that the distribution of the activity was randomised all over the molecule of amudol, as was evident from the r.m.a. values of the successive products (as shown in Chart 3), obtained through identical degradtive steps, earlier carried out on sodium (1-C^{14})- and (2-C^{14})-acetates-derived amudol. This randomisation could be due to the uptake of the C^{14}-carbon dioxide by the mold, evolved during the metabolism of glucose by a number of enzymes.

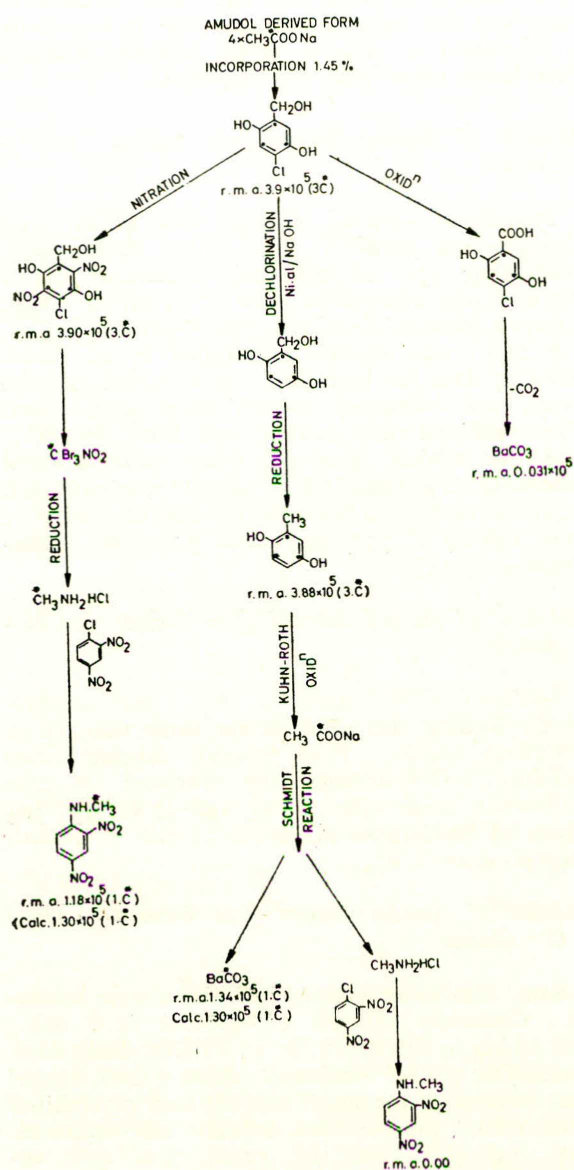


Chart 1

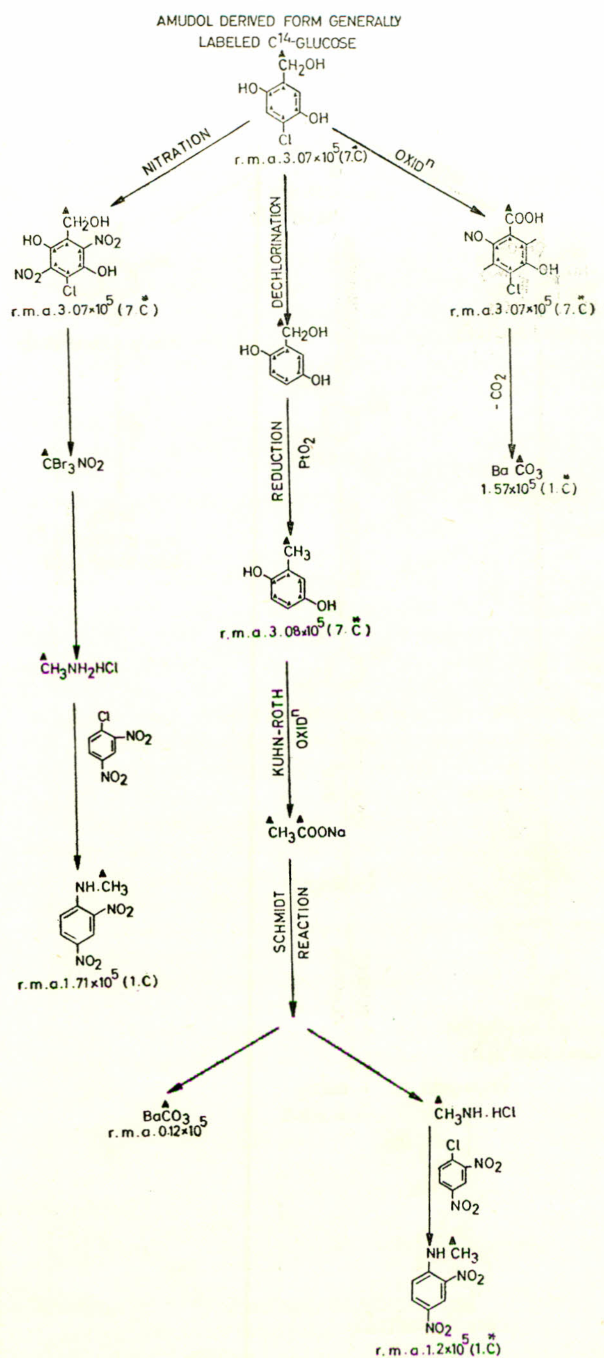


Chart 2

The studies clearly prove that $—CH_2OH$ in amudol was originally a methyl group of the starting unit, which was later on oxidised to $—CH_2OH$. This view is being confirmed through our feeding the mold with (2,4,6-$^2H_3$)-orcinol, (3,4,6-$^2H_3$)-toluquinol and (1,2,3,4-$^2H_4$)-salicylic acid which are most probable earlier precursors of amudol. The work is in progress and will be communicated later.

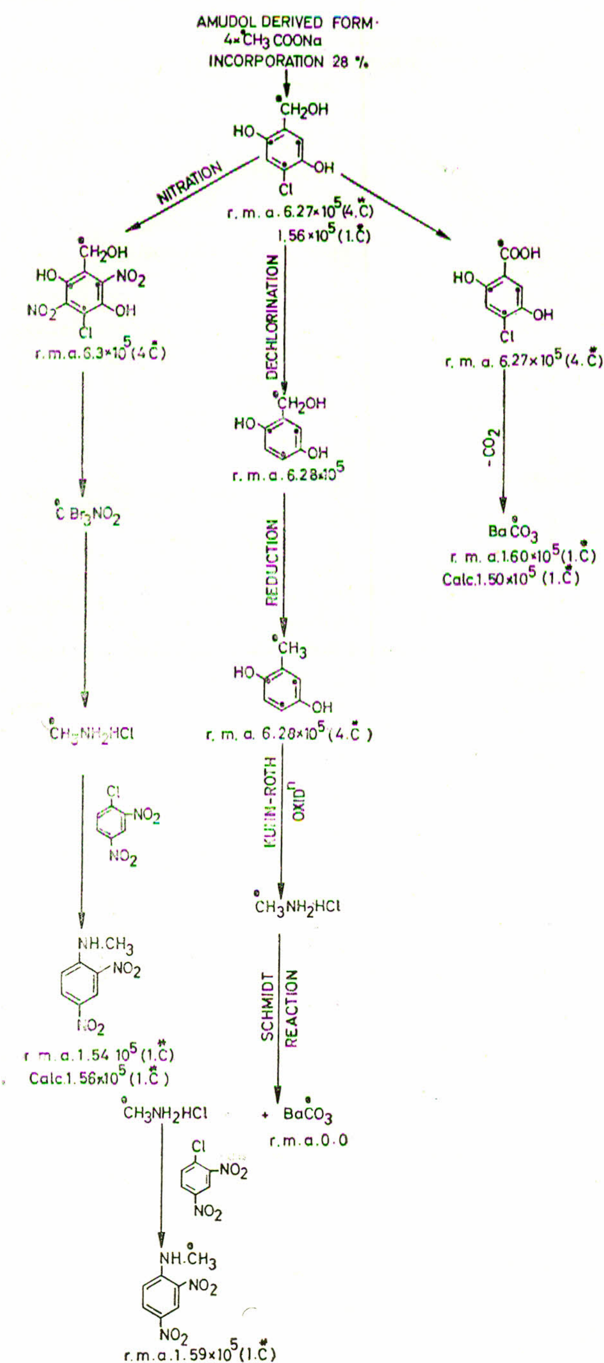


Chart 3

Experimental

Melting points were taken on Kofler block. Radioactive samples were assayed at infinite thickness in 0.3 cm^2 planchettes to an error of $\pm 3\%$, by a thin window Geiger system coupled to an EKCO N-530-F scaler. All compounds were crystallised to constant activity. The counts were corrected for background and the radioactivity is described in relative molar activity (r.m.a.). Petroleum ether had b.p. 65–85°C.

Isolation of Amudol Derived from Sodium (1-C^{14})-*Acetate*

Penicillium martinsii Biourge was grown on Czapek–Dox medium (15×1-litre flasks, each containing 350 ml medium) containing carrot extract[6] and allowed to grow at 24–26°C. After 3 days a solution of sodium (1-C^{14})-acetate (15 ml, 0.6 m.c.) was equally distributed in all flasks. After 17 days the mycelium was removed and the broth was extracted several times with ether. The combined ether extract were dried (Na_2SO_4) and the solvent removed, when pure amudol labeled from sodium (1-C^{14})-acetate was obtained. It was recrystallised from ether to constant activity, m.p. 146–47°C, 750 mg, r.m.a. 3.9×10^5, incorporation 1.45%.

Isolation of Amudol Derived from Sodium (2-C^{14})-*Acetate*

Sodium (2-C^{14})-acetate (0.5 m.c.) was employed for feeding the mold in the same manner as described above. Pure amudol labeled from sodium (2-C^{14})-acetate was obtained on crystallisation from ether, m.p. 146–47°C, 550 mg (from 15 flasks, 4500 ml broth); r.m.a. 6.2×10^5, incorporation 2.8%.

Isolation of Amudol Derived from Generally Labeled C^{14}-*Glucose*.

Same conditions as described above were followed. Generally labeled C^{14}-glucose (0.6 m.c.) was added to the broth (in 15×1-litre flasks each containing 350 ml medium). After 3 days, *amudol* was isolated in the usual manner and crystallised from ether till constant activity was obtained, m.p. 146–47°C, 600 mg, r.m.a. 3.0×10^5), incorporation 3.10%.

Degradation of Amudol Derived from Sodium (1-C^{14})-*Acetate.*

(i) *Oxidation of Hot Amudol.*—Amudol (100 mg) was dissolved in water (2 ml), cooled in ice, and concd H_2SO_4 (0.5 ml) was added to the mixture, $K_2Cr_2O_7$ solution (1 g in 3 ml water) was then added gradually under stirring. After 2 hr the mixture was extracted with ethyl acetate. The extract on removal of the solvent gave 4-chloro-2,5-dihydroxybenzoic acid, m.p. 120 °C; 85 mg.

(ii) *Decarboxylation of* 4-*Chloro*-2,5-*dihydroxybenzoic Acid.*—4-Chloro-2,5-dihydroxybenzoic acid (85mg) was dissolved in quinoline (2 ml) and Cu–bronze (200 mg) was added. The mixture was heated (180–225°C) in a current of N_2 gas. The evolved CO_2 was trapped as $BaCO_3$ which was collected, washed with freshly boiled distilled water, ethanol and finally with ether. The residue was dried at 85°C, r.m.a. of $BaCO_3$ 0.03×10^5.

(iii) *Dechlorination of Amudol.*—To a refluxing solution of amudol (174 mg) in aqueous NaOH (10%, 5 ml), nickel–aluminium alloy (50% Ni, 50% Al; 200 mg) was added in small portions. After the reaction was over (2 hr) the mixture was acidified (2N HCl) and the resulting 2, 5-dihydroxybenzyl alcohol was isolated from ethyl acetate, m.p. 100°C, 102 mg, r.m.a. 3.9×10^5.

(iv) *Reduction of* 2,5-*Dihydroxybenzyl Alcohol.*—2, 5-Dihydroxybenzyl alcohol (80 mg) was dissolved in ethanol (15 ml) containing platinum oxide catalyst (20 mg) and shaken under H_2 atmosphere overnight. The reaction mixture was filtered. The filtrate on removal of the solvent gave 2,5-dihydroxytoluene. It was recrystallised from benzene; m.p. 121–22°C; 50 mg; r.m.a. 3.88×10^5.

(v) *Kuhn–Roth Oxidation of* 2,5-*Dihydroxytoluene.*—2,5-Dihydroxytoluene (48 mg) was dissolved in aqueous Cr_2O_3 (2N, 10 ml) containing concd H_2SO_4 (0.5 ml) and refluxed (3 hr). During this period O_2 gas was bubbled through the reaction mixture (1 bubble/sec). Excess of the oxidant was decomposed at 0°C with hydrazine hydrate (aqueous 50% solution). The mixture was basified with powdered pellets of NaOH, acidified with orthophosphoric acid and steam-distilled into a solution of 2N NaOH till 150 ml distillate was collected.

(vi) *Schmidt Degradation of Sodium Acetate.*—The above obtained distillate containing sodium acetate was concentrated down to 5 ml, transferred to a 10-ml round bottoned flask and evaporated to complete dryness under N_2 at 120°C. The residue was taken up in concd H_2SO_4 (0.5 ml) and sodium azide (100 mg) was added under cooling. The mixture was heated (N_2 atmosphere) at 60–70°C (2 hr). The evolved CO_2 was trapped as $BaCO_3$ which was collected and purified as described earlier, 50 mg, r.m.a. 1.34×10^5 ($1 \times C^*$, against 1.30×10^5).

The remaining reaction mixture when basified (aqueous 2N NaOH) and steam distilled into 2N HCl gave methylamine hydrochloride in the distillate (50 ml) which was evaporated to dryness. The residue of methlamine hydrochloride was dissolved in ethanol containing K_2CO_3 (50 mg) (50 mg) and 2,4-dinitrochlorobenzene and refluxed (0.5 hr). Ethanol was removed (*in vauo*) and water (10 ml) was added to the mixture. The mixture was extracted with chloroform and the extract passed through a column of neutral aluminium oxide. The extract on removal of chloroform gave pure *N*-methyl-2, 4-dinitroaniline which was crystallised from ethanol or from benzene–ether mixture, m.p. 171°C, r.m.a. 0.00.

(vii) *Nitration of Amudol.*—Amudol (174 mg, 0.001 mole) was suspended in concd H_2SO_4 (0.6 ml) and to the ice-cooled mixture a mixture of concd H_2SO_4 (0.8 ml) and HNO_3 (0.4 ml) was added dropwise (5 min) under vigorous stirring. Additional quantity of HNO_3 (0.7 ml) was added and the mixture was allowed to stand (40 min). Addition of crushed ice to the mixture precipitated 3,6-dinitroamudol which was collected by suction and crystallised from methanol, orange-coloured needles, m.p. 220°C, 210 mg (yield 79.5%), r.m.a. 3.9×10^5 ($3 \times C^*$).

(viii) *Hypobromite Degradation of* 3,6-*Dinitroamudol.*—To a hot solution of 3,6-dinitroamudol (100 mg) in water (10 ml) sufficient $Ba(OH)_2$ was added to produce a clear solution. To the cooled solution maintained at 0°C a cooled solution of Br_2 (1.1 g) in water (100 ml) containing hydrated $Ba(OH)_2$ (1.2 g) was added. The mixture was stirred for 30 min and then rapidly heated to boiling. Bromopicrin formed was rapidly steam distilled and isolated from ether, 110 mg.

(ix) *Reduction of Bromopicrin.*—Bromopicrin (100 mg) was reduced with iron filings (50 mg) and 0.1N HCl (2 ml). After 3 hr the solution was basified and steam distilled into 2N HCl solution. Methylamine hydrochloride was isolated as *N*-methyl-2,4-dinitroaniline by usual method, m.p. 171°C, 55 mg, r.m.a. 1.18×10^5.

Degradation of Amudol Derived from Sodium (2-C^{14})-*Acetate*

(x) *Oxidation of Amudol.*—Amudol (174 mg, r.m.a. 6.27×10^5) was oxidised with $K_2Cr_2O_7$

(2g) in water (5 ml) and concd H_2SO_4 (0.5 ml). 4-Chloro-2,5-dihydroxybenzoic acid had m.p. 120°C, r.m.a. 6.26×10^5.

(xi) *Decarboxylation of* 4-*Chloro*-2,5-*dihydroxybenzoic Acid*.—4-Chloro-2,5-dihydroxy benzoic acid (50 mg) when decarboxylated in quinoline (1 ml) and Cu-bronze (100 mg) gave $BaCO_3$ r.m.a. 1.60×10^5 (1C*, against 1.56×10^5).

(xii) *Dechlorination and Reduction of Amudol*.—Amudol (174 mg), dechlorinated and reduced as described earlier, gave 2,5-dihydroxytoluene, m.p. 121°C, 110 mg, r.m.a. 1.55×10^5.

(xiii) *Kuhn–Roth Oxidation and Schmidt Degradation of* 2, 5-*Dihydroxytoluene*.—2,5-Dihydroxytoluene (50 mg) was oxidised with CrO_3 and on Schmidt reaction gave $BaCO_3$ (r.m.a. 0.0) and methylamine hydrochloride as *N*-methyl-2,4-dinitroaniline, m.p. 171°C, r.m.a. 1.59×10^5.

(xiv) *Nitration and Hypobromite Degradation of Amudol*.—Amudol (174 mg) was nitrated as described earlier. On hypobromite degradation, it gave bromopicrin which was reduced and isolated as *N*-methyl-2,4-dinitroaniline as derivative of methylamine hydrochloride, m.p. 171°C, r.m.a. 1.54×10^5.

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