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STUDIES IN THE BIOCHEMISTRY OF MICROORGANISMS

Part XIII.—The Biosynthesis of Yasimin, a Metabolic Product of Aspergillus unguis

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The biosynthesis of yasimin was established from head-to-tail condensation of one acetate and three malonate units for ring A.

In the previous communications,[1] we have described the isolation and structures of yasimin and other metabolites of *Aspergillus unguis*.

In the present communication we are describing the biosynthesis of yasimin. Yasimin is a very attractive depsidone for biosynthetic studies, because of its origin from three different precursors using acetate–malonate, mevalonic acid and methionine. We have undertaken a preliminary study of the biosynthesis of ring A of yasimin, which could be derived from one acetate and three malonate units, due to the condensation and cyclisation of head-to-tail polyketide chain by aldol condensation, forming orsellinic acid moiety of yasimin. The origin of ring B could be attributed to two isoprene units, which will be tested by feeding (2-C^{14})-mevalonic acid. The C-methyl of the ring B was derived from methionine due to transmethylation at this position. It was already shown few years ago that ring B of nidulin was derived from isoleucine and the methyl group in the same ring was speculated to be derived from formaldehyde.[2] The role of mevalonic acid for C-5 unit had been demonstrated in a wide variety of natural products such as terpenes, alkaloids and xanthones and as such it could be quite safe to expect that mevalonic acid will be incorporated in a high degree in the ring B of yasimin in comparison to isoleucine. Biosynthetic studies on the ring B are underway and will be reported later.

For biogenetic studies of ring A, a solution of sodium (1-C^{14})- acetate (0.6 m.c.) was fed to *Aspergillus unguis* after 3 days growth, on a modified Czapek–Dox medium enriched with carrot extract. After 22 days the mycelium was separated and worked up to yield pure yasimin, which was recrystallised to constant radioactivity (incorporation 0.9%, 1.9%; r.m.a. 2.6×10^5 and 5.3×10^5, respectively).

When yasimin (r.m.a. 2.6×10^5) was hydrolysed and decarboxylated to decarboxyyasimin, the evolved carbon dioxide was assayed as barium carbonate (r.m.a. 0.34×10^5). The ether linkage of decarboxyyasimin was cleaved with a mixture of hydroiodic and nitric acids yielding pure orcinol (r.m.a. 1.2×10^5, $3\times$C*). Orcinol was oxidised by Kuhn–Roth method and Schmidt reaction to obtain carbon dioxide, which was trapped as barium carbonate (r.m.a. 0.42×10^5, $1\times$C*) which is slightly higher than the one-third activity (0.4×10^5) of orcinol. The remaining reaction mixture was reacted with 2,4-dinitrochlorobenzene, to get *N*-methyl-2,4-dinitroaniline which was inactive, indicating that the whole activity was in the carboxyl group.

These results were further verified by hydrolysing yasimin (incorporation 1.9%, r.m.a. 5.3×10^5) esterifying and breaking the ether linkage with nitric acid to get methyl orsellinate (r.m.a. 2.24×10^5, $4\times$C*). Kuhn–Roth oxidation of methyl orsellinate gave sodium acetate. This was further degraded by Schmidt reaction and the carbon dioxide gas evolved was trapped as barium carbonate (r.m.a. 0.6×10^5, $1\times$C*) which was again slightly higher than the exact one-fourth activity (r.m.a. 0.56×10^5). As in the previous case, the remaining methylamine hydrochloride was once again found to be inactive. These results are summarised in Chart 1.

The above results were supported by the degradation of yasimin derived from sodium (2-C^{14})-acetate (incorporation 1.2%, r.m.a. 4.5×10^5). On alkaline hydrolysis and decarboxylation with copper–bronze in quinoline, followed by ether cleavage with a mixture of hydroiodic and nitric acids, yasimin yielded pure orcinol (r.m.a. 2.35×10^5, $4\times$C*). Kuhn–Roth oxidation and Schmidt degradation of the resulting sodium acetate gave barium carbonate which was found to be inactive but the *N*-methyl-2,4-dinitroaniline derivative of methylamine hydrochloride had again quite high activity (r.m.a. 0.77×10^5, $1\times$C*) against the exact one-fourth expected activity (r.m.a. 0.58×10^5, $1\times$C*).

Methyl orsellinate (r.m.a. 4.56×10^5, $4\times C^*$) was again isolated from yasimin (incorporation 3·3%, r.m.a. 12.4×10^5) through a series of reactions, viz. hydrolysis, esterification and ether cleavage. This methyl orsellinate was subjected to Kuhn–Roth oxidation and Schmidt degradation which gave inactive barium carbonate and the methylamine-HCl, characterised as *N*-methyl-2,4-dinitroaniline, showed slightly higher activity (r.m.a. 1.2×10^5) than the expected one-fourth of the total activity (r.m.a. 1.1×10^5). This clearly demonstrated that the methyl group of orsellinic acid moiety was the starting unit of this molecule. All these results are outlined in Chart 2.

The above findings were strongly supported by the degradation of yasimin derived from sodium (1-C^{14})-malonate prepared according to the method of Calvin[3] (incorporation 1.8%, r.m.a. 30.2×10^5) into methyl orsellinate (r.m.a. 10.4×10^5; $4\times C^*$) by the same reaction sequence, viz. hydrolysis, esterification and cleavage of ether linkage. This methyl orsellinate, when subjected to Kuhn–Roth oxidation, gave sodium acetate which was boiled with *p*-bromophenacyl bromide in ethanol to give *p*-bromophenacyl ester. It showed slightly lower activity (r.m.a. 2.10×10^5) than the exact one-fourth activity (r.m.a. 2.59×10^5, $1\times C^*$) of the molecule. All these reactions are graphically represented in Chart 3. These results indicated that malonate was not a starting unit but it was a building unit of the molecule. Thus all these results clearly verify the acetate malonate hypothesis.

The loss of half the activity of yasimin on degradation to orcinol and methyl orsellinate clearly showed that acetate is also used up in building up the isoprene units and as such the residual activity should be located in ring B. This was verified by the ozonolysis of yasmin derived from both sodium (1-C^{14})- and (2-C^{14})-acetate which gave acetaldehyde, assayed as its 2,4-dinitrophenylhydrazone derivative and this was found to be active.

Further work to establish the presence of other intermediate precursors like orcinol, salicylic acid and methyl orsellinic acid are being tested by feeding deuterium-labeled orcinol, salicylic acid and orsellinic acid.

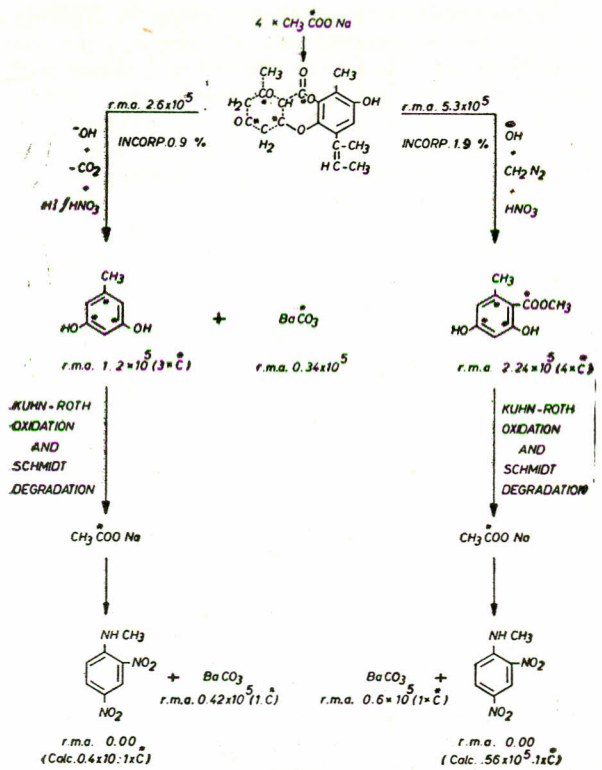

Chart 1.—Yasimin labeled from sodium [1-C^{14}]-acetate.

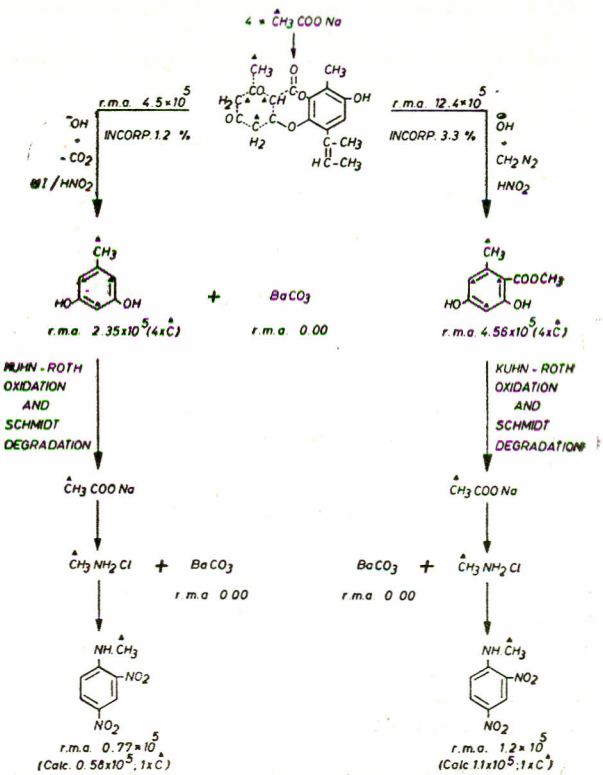

Chart 2.—Yasimin labeled from sodium [2-C^{14}]-acetate.

Experimental

Melting points were taken on a Kofler block and are uncorrected. Radioactivity was assayed on infinitely thick samples to an error of $\pm 3\%$ by a thin window Geiger system coupled to an EKCO N-530 F Scalar. Radioactivity is described in relative molar activity (r.m.a.). All the samples were crystallised and purified to constant radioactivity. Petroleum ether used had b.p. 65–85°C.

Isolation of Sodium (1-C^{14})-*Acetate-labeled Yasimin*

Aspergillus unguis Emile-Weil and Gaudin (C.M.I. catalogue No. IMI. 138767) was grown on standard Czapek–Dox medium enriched with carrot extract at 24°C in 15 flasks containing 350 ml in each. After 3 days a solution of sodium (1-C^{14})-acetate (0.6 m.c., 10 ml) was equally distributed in the above flasks. The mycelium was separated from the broth after 22 days and was dried at 60°C in the oven. The dried mycelium was powdered and extracted with petroleum ether in a Soxhlet apparatus (72 hr) followed by ether (120 hr). Ether was removed under reduced pressure and the residue was boiled with hexane which yielded (1-C^{14})-acetate labeled pure yasimin, m.p. 201°C, 66 mg (incorporation 0.9%, r.m.a. 2.6×10^5). In a similar experiment, 1 m.c. sodium (1-C^{14})-acetate was used under conditions described above. Sodium (1-C^{14})-acetate-labeled yasimin obtained had incorporation 1.9%, r.m.a. 5.3×10^5.

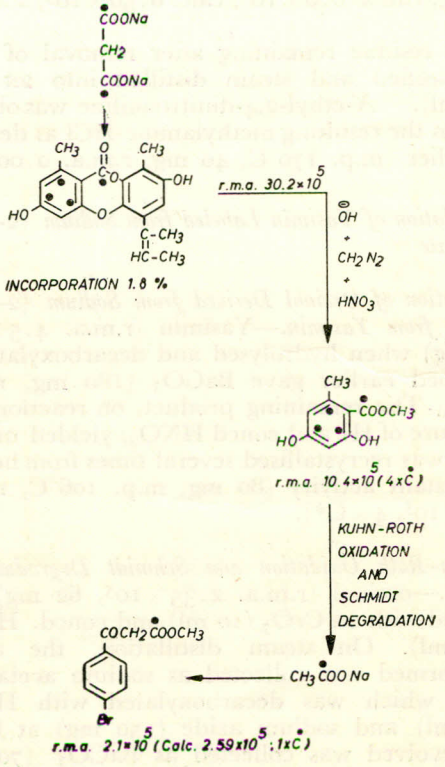

Chart 3.—Yasimin labeled from sodium [1-C^{14}]-malonate.

Isolation of Sodium (2-C^{14})-*Acetate-labeled Yasimin*

The mold grown under the conditions described above when fed with sodium (2-C^{14})-acetate similarly yielded (2-C^{14})-acetate-labeled pure yasimin, m.p. 201°C, 860 mg (incorporation 1.2%, r.m.a. 4.5×10^5). In a similar experiment 1 m.c. sodium (2-C^{14})-acetate used under conditions described above gave yasimin (incorporation 3.3%, r.m.a. 12.4×10^5).

Isolation of Sodium (1-C^{14})-*Malonate labeled Yasimin.*

Sodium (1-C^{14})-malonate derived pure yasimin was obtained in the manner described above, m.p. 201°C, 800 mg (incorporation 1.8%, r.m.a. 30.2×10^5).

Degradation of Sodium (1-C^{14})-*Acetate-labeled Yasimin*

Hydrolysis.—Sodium (1-C^{14})-acetate-labeled yasimin (r.m.a. 2.6×10^5, 326 mg, 0.001 mole) was dissolved in dioxane (5 ml) and warmed. 2N NaOH aq (20 ml) was added slowly (N_2 atmosphere). After the addition was complete heating was continued (30 min). The resulting solution was chilled with ice, acidified with H_2SO_4 (concd) and extracted with ethyl acetate. Drying of ethyl acetate extract and removal of solvent gave carboxyyasimin (300 mg).

Decarboxylation and Cleavage of Ether Linkage of Carboxyyasimin.—Carboxyyasimin (300 mg) was dissolved in quinoline (3 ml) containing Cu–bronze (400 mg) and heated (1–2 hr) at 185–220°C (metal-bath), under N_2. The evolved CO_2 was collected as $BaCO_3$ which was purified by washing with freshly distilled water, ethanol and ether (three times with each solvent) and finally dried in an oven at 110°C (r.m.a. 0.34×10^5). The decarboxylated product mixed with Cu–bronze and quinoline was taken up in ethyl acetate, filtered and the solvent removed under reduced pressure. The residue containing quinoline was dissolved in acetic acid (3 ml) and a mixture of HI (6.5 ml) and HNO_3 (0.3 ml) was added and allowed to stand. The mixture was taken up in ethyl acetate and shaken up repeatedly with water, satd $NaHCO_3$ solution and then finally with 2N NaOH. The NaOH extract was acidified (2N HCl) and orcinol isolated from ethyl acetate, m.p. 106°C, 68 mg (r.m.a. 1.2×10^5, $3 \times C^*$).

Kuhn–Roth Oxidation of Orcinol Derived from Sodium (1-C^{14})-*Acetate.*—Orcinol (50 mg, r.m.a. $1.2 \times 10_5$) was dissolved in 2N CrO_3 solution (10 ml) containing concd H_2SO_4 (0.5 ml) and refluxed (3 hr) with O_2 bubbling through the solution. Hydrazine hydrate solution (50%) was added to decompose the excess oxidant. The reaction mixture was basified with 10% NaOH, acidified with orthophosphoric acid and the acetic acid formed was steam distilled into 2N NaOH solution containing a drop of phenolphthalein to obtain sodium acetate solution (distillate 150 ml).

Schmidt Degradation.—Water from the above sodium acetate solution was removed on sand bath and the last 5 ml transferred into a round-bottomed flask and dried under N_2 at 120°C (oil bath). Last traces of the moisture were removed by keeping the above flask in an oven at 100°C. The residue was dissolved in concd H_2SO_4 (0.6 ml) and then sodium azide (100 mg) was added, the apparatus was kept under N_2 and the flask slowly warmed to 65°C; the evolved carbon dioxide was collected as barium carbonate which was washed with water, ethanol and finally with ether and dried at 80°C (r.m.a. 0.42×10^5, 1 C*).

Formation of N-*Methyl-2,4-Dinitroaniline.*—The above residue was basified with NaOH solution and steam distilled into a flask containing 2N HCl. Distillation was stopped when 100 ml of the distillate had been collected. The water was removed on sand bath and the residual methylamine hydrochloride was dissolved in ethanol containing anhydrous K_2CO_3 and 2,4-dinitrochlorobenzene. The reaction mixture was refluxed on water-bath (2 hr), the solvent removed and the residue extracted with chloroform and washed with water. The extract was dried (Na_2SO_4) and passed over neutral alumina as absorbant, using chloroform, as eluant. On removal of chloroform, pure *N*-methyl-2,4-dinitroaniline was obtained, m.p. 170°C, r.m.a. 0.00.

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

Yasimin derived from sodium (1-C^{14})-acetate (r.m.a. 5.3×10^5, 326 mg) was dissolved in dioxane (5 ml) and 2N NaOH (20 ml) was added slowly under N_2 and the mixture refluxed ($\frac{1}{2}$ hr). The solution was chilled with ice, acidified with H_2SO_4 (50%) and the product (orsellenic acid) was isolated from ethyl acetate, and esterified with excess of diazomethane (15 min). The solvent was removed and the residue was taken up in a mixture of HNO_3 (1 ml) and glacial acetic acid (1 ml) and left overnight. Acetic acid was removed under reduced pressure and the residue was dissolved in ethyl acetate, washed with water, aqueous $NaHCO_3$ and finally extracted with aqueous 2N NaOH three or four times. The combined NaOH extract was acidified with 0.1N HCl and methyl orsellinate isolated from ethyl acetate, m.p. 140°C, 70 mg (r.m.a. 2.24×10^5, $4 \times$ C*).

Kuhn–Roth Oxidation of Methyl Orselinate Derived from Sodium (1-C^{14})-*Acetate.*—Methyl orsellinate (r.m.a. 2.24×10^5, 55 mg, 0.003 mole) was oxidised with CrO_3 aqueous (2N, 10 ml) in presence of concd H_2SO_4 (0.6 ml) under O_2 bubbling through the solution (3 hr). The excess of the oxidant was decomposed with hydrazine hydrate solution (aq. 50%), basified with 2N NaOH, acidified with orthophosphoric acid, steam distilled into 2N NaOH (2 ml) till 150 ml distillate was collected.

Schmidt Degradation.—The above sodium acetate solution (150 ml) was evaporated and the residue was thoroughly dried in an oven at 110°C. It was dissolved in concd H_2SO_4 (0.5 ml), cooled in ice and sodium azide (100 mg) was added. The reaction mixture was heated in N_2 gas at 65°C and the generated CO_2 was collected as $BaCO_3$ which was purified as described earlier (50 mg, r.m.a. 0.6×10^5, calc. 0.56×10^5, $1 \times$ C*).

The residue remaining after removal of CO_2 was basified and steam distilled into 2N HCl (100 ml). *N*-ethyl-2,4-dinitroaniline was obtained from the resulting methylamine-HCl as described earlier (m.p. 170°C, 40 mg, r.m.a. 0.00).

Degradation of Yasimin Labeled from Sodium (2-C^{14})-*Acetate*

Isolation of Orcinol Derived from Sodium (2-C^{14})-*Acetate from Yasimin.*—Yasimin (r.m.a. 4.5×10^5, 326 mg) when hydrolysed and decarboxylated as described earlier gave $BaCO_3$ (180 mg, r.m.a. 0.00). The remaining product, on reaction with a mixture of HI and concd HNO_3, yielded orcinol which was recrystallised several times from hexane to constant activity (80 mg, m.p. 106°C, r.m.a. 2.35×10^5, $4 \times$ C*).

Kuhn–Roth Oxidation ana Schmidt Degradation of Orcinol.—orcinol (r.m.a. 2.35×10^5, 62 mg) was oxidised with 2N CrO_3 (10 ml) and concd. H_2SO_4 (0.5 ml). On steam distillation, the acetic acid formed was collected as sodium acetate as usual which was decarboxylated with H_2SO_4 (0.5 ml) and sodium azide (150 mg) at 65°C. CO_2 evolved was collected as $BaCO_3$ (70 mg, r.m.a. 0.00). The remaining solution was basified

and steam distilled into 2N HCl to obtain methylamine-HCl characterised as *N*-methyl-2,4-dinitroaniline (50 mg, m.p. 170°C, r.m.a. 0.77×10^5, calc. 0.58×10^5, $1 \times C^*$).

Isolation of Methyl Orsellinate Derived from Sodium (2-C^{14})-Acetate from Yasimin

Yasimin (r.m.a. 12.4×10^5, 326 mg) was hydrolysed with 2N NaOH (20 ml) and the resulting acid was esterified with excess of diazomethane. The ether linkage was cleaved with concd HNO_3 (1 ml) in presence of acetic acid (1 ml). On working up the reaction product in the usual manner, pure methyl orsellinate was obtained (110 mg, m.p. 140°C, r.m.a. 4.56×10^5, $4 \times C^*$)

Kuhn–Roth Oxidation and Schmidt Degradation of Methyl Orsellinate Derived from Sodium (2-C^{14})-Acetate.—The above methyl orsellinate (90 mg) was oxidised with 2N CrO_3 (10 ml) in presence of concd H_2SO_4 (0.5 ml). On steam distillation of reaction mixture, sodium acetate (30 mg) was obtained which was treated with concd H_2SO_4 (1 ml) and sodium azide (150 mg) and decarboxylated at 65°C. The evolved CO_2 was trapped as $BaCO_3$ (70 mg, r.m.s. 0.00). The reaction mixture was basified and steam distilled into 2N HCl, giving methylamine hydrochloride solution (100 ml). Removal of water from the distillate gave methylamine hydrochloride which was reacted with 2,4-dinitrochlorobenzene to give *N*-methyl-2,4-dinitroalinine derivative (40 mg, m.p. 170°C, r.m.a. 1.2×10^5, against 1.1×10^5 for $1 \times C^*$).

Degradation of Yasimin Labeled from Sodium (1-C^{14})-Malonate

Methyl Orsellinate Derived from Sodium (1-C^{14})-Malonate.—Yasimin (r.m.a. 30.2×10^5, 326 mg) was hydrolysed with 2N NaOH (20 ml) and esterified with excess of diazomethane and the ether linkage was cleaved with concd HNO_3 in presence of acetic acid. Methyl orsellinate so obtained was recrystallised from a mixture of ethyl acetate and petroleum ether to constant activity (115 mg, m.p. 140°C, r.m.a. 10.4×10^5, $4 \times C^*$).

Kuhn–Roth Oxidation of the Above Methyl Orsellinate.—Methyl orsellinate (r.m.a. 10.4×10^5, 90 mg) was oxidised with 2N CrO_3 (10 ml) and concd H_2SO_4 (0.3 ml) for 3 hr with O_2 bubbling through the reaction mixture in the usual manner. The reaction mixture was treated with hydrazine hydrate (50%), basified, acidified and steam distilled into 2N NaOH solution giving sodium acetate (25 mg).

Formation of p-*Bromophenacyl Ester of Sodium Acetate.*—The above sodium acetate was dissolved in ethanol (95%) and HCl was added till acidic. *p*-bromophenacyl bromide (100 mg) was added and refluxed for 2 hr. The reaction mixture was filtered, the filtrate evaporated to dryness and the *p*-bromophenacyl ester was recrystallised several times from acetone (75 mg, m.p. 86°C, r.m.a. 2.11×10^5 against 2.59×10^5, $1 \times C^*$).

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