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The Mechanism of Clavatol Formation in *Aspergillus clavatus*

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In the formation of clavatol (2,4-dihydroxy-3,5-dimethylacetophenone) in *Aspergillus clavatus* the resacetophenone part was shown to be derived from acetate (the acetyl group) and malonate (the aromatic nucleus), whereas the methyl groups in 3 and 5 positions originate from methionine.

Evidence has been obtained for an introduction of the methyl groups into the structure on a pre-aromatic stage. Using washed cells of *Streptomyces rimosus*, however, a direct methylation of resacetophenone and 3-methylresacetophenone to clavatol has been demonstrated.

During the work on the isolation of patulin (clavatin) from *Aspergillus clavatus* Bergel *et al.*[1] obtained as a byproduct a phenolic substance lacking antibacterial activity. The structure of the phenolic compound was later elucidated by Hassall and Todd.[2] They found that the new phenolic compound was identical with 2,4-dihydroxy-3,5-dimethylacetophenone.

In the course of our work on C-methylation, clavatol was thought to be an exellent object for experimental studies providing that the nuclear C-methyl groups originated from the C_1-metabolism. If this were true the easily available substances, resacetophenone, 3-methylresacetophenone or 5-methylresacetophenone, could be used as C-methyl acceptors in cellfree reactions.

There have been only a few acetophenone derivatives isolated from microorganisms. Allport and Bu'Lock[3] identified a series of substances from *Daldinia concentrica* that from structural considerations showed biogenetic relationships with each other. Two of these substances were 2,6-dihydroxyacetophenone and 2,6-dihydroxybutyrophenone. Since these two compounds as well as the others in the series investigated could be built up by linear condensations of acetate derived C_2 units, Allport and Bu'Lock tested and confirmed this hypothesis with the butyrophenone. Clavatol, too, fits very well into the acetate theory if the nuclear C-methyl groups are derived from the C_1 metabolism, *i.e.*, the acetophenone skeleton would then be formed from one acetate unit (the acetyl group) and three malonate units (the aromatic ring). Of seven strains of *A. clavatus* obtained from the Commonwealth Mycological Institute only one produced substantial amounts of clavatol when grown in Czapek-Dox medium with molasses as an additional substrate. In addition

to clavatol this strain also produced minor amounts of 6-methylsalicylic acid, gentisic acid, and patulin, all identified by paper chromatography. However, when molasses was omitted from the culture medium these three substances were the main products and clavatol occurred only in small amounts. The acetate-malonate origin and the close biogenetic relationship between 6-methylsalisylic acid and patulin has been established in the *Penicillium* series by several authors.[4-6] Gentisic acid has also been shown to be derived from C_2 units, but its immediate relationship to 6-methylsalisylic acid and patulin is still obscure.

In order to investigate the origins of the C atoms in clavatol, cultures of *A. clavatus* were incubated with methionine-^{14}C (methyl) or sodium malonate-2-^{14}C. The isolated ^{14}C-labelled clavatol was diluted with synthetic, nonlabelled clavatol and degraded. The labelling pattern shown in Fig. 1 indicated

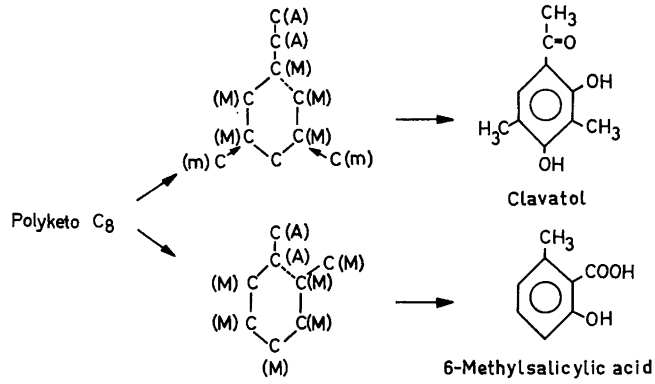

Fig. 1. The isotope distribution in clavatol and a proposed scheme for its biosynthesis in relation to 6-methylsalicylic acid formation. (A), (M), and (m) indicate the origin of the C atoms from acetate, malonate, and methionine, respectively.

that a simple degradation series with Kuhn-Roth oxidation should furnish all the information of the isotope distribution needed. It turned out, however, that in the Kuhn-Roth oxidation a significant cleavage was obtained between the methyl group and the carbonyl group and there was migration of the nuclear methyl groups during the reaction.

Another degradation route was developed involving the formation of the oxime of clavatol (Fig. 2). The acetylated oxime was condensed to form a benzoxazole derivative which subsequently underwent a Beckmann rearrangement. The obtained anilide was hydrolyzed and oxidized to 2-hydroxy-3,5-dimethyl-*p*-benzoquinone. The reaction series was performed without isolation or purification of any of the intermediates. The identity of the benzoquinone was established by comparison with an authentic product synthesized according to the method described by Smith *et al.*[8] using 2,4-dimethylresorcinol as starting material. The benzoquinone was further degraded by Kuhn-Roth oxidation and the radioactivity of the formed carbon dioxide measured as well as that of the individual C atoms in the isolated acetic acid after Schmidt

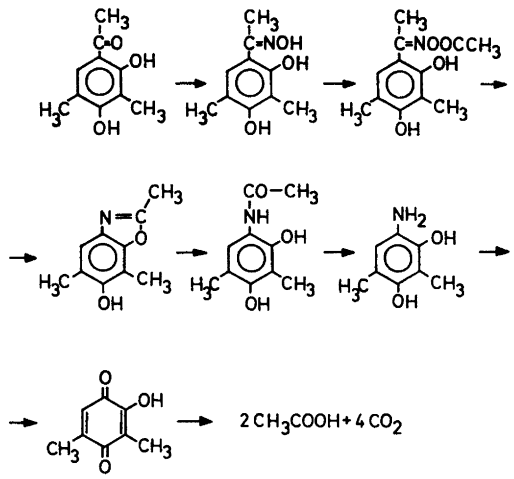

Fig. 2. Degradation reactions of clavatol.

degradation. The isotope content of the acetyl group of clavatol was obtained as the difference between the total radioactivities of clavatol and the benzoquinone. In Table 1 are listed the figures obtained from the radioactivity measurements. The experimental results are in full accordance with the proposed labelling pattern shown in Fig. 1. Furthermore, the low labelling in the acetyl group as compared to the labelling in the carboxyl group of the acetic acid formed in the Kuhn-Roth oxidation of the malonate-2-^{14}C labelled benzoquinone indicates the position of the terminal acetate unit in the linear condensations of acetate-malonate.

The simultaneous production of 6-methylsalicylic acid and clavatol in the same culture is noteworthy as they could theoretically be derived from a common C_8-polyketo intermediate. This C_8-intermediate could be cyclisized in two ways as shown in Fig. 1 On the one hand cyclization could occur between positions 2 and 7 giving rise to 6-methylsalicylic acid while on the other hand condensation between position 1 and 6 would give rise to the resaceto-

Table 1. Isotope distribution in clavatol from ^{14}C-substrates.

Material	Methionine-^{14}C(methyl) cpm/mmole clavatol	Malonate-2-^{14}C cpm/mmole clavatol
Clavatol	2.43×10^5	7.90×10^4
2-Hydroxy-3,5-dimethyl-benzoquinone	2.52×10^5	7.08×10^4
Acetyl group	0	0.82×10^4
Pos. 1, 2, 4 and 6 (Kuhn-Roth ox. CO_2)	0.17×10^5	2.26×10^4
Pos. 3 and 5 (Schmidt degr. COOH group)	0.03×10^5	4.32×10^4
Nuclear CH_3 groups (Schmidt degr. CH_3 group)	2.35×10^5	0.31×10^4

phenone structure. When speculating on the factors that might direct the condensation one way or the other the attention is drawn to the nuclear methyl groups in the clavatol structure. If the methyl groups, especially the one in position 3, are introduced on a prearomatic stage during the clavatol biosynthesis, position 2 in the C_8 intermediate will be blocked and the cyclization would take place between position 1 and 6.

In the next step of the investigation ^{14}C-carbonyl labelled resacetophenone was prepared and added to a producing culture. The isolation of clavatol was performed as in the other isotope experiments. The isolated clavatol contained radioactivity initially which disappeared after a few recrystallisations. This experiment was repeated with the labelled resacetophenone present in the culture medium for different lengths of time. The labelled resacetophenone was taken up by the cells and metabolized, as was shown by the presence of radioactivity in the respired carbon dioxide and the disappereance of the resacetophenone from the culture medium. These experiments were also performed with ^{14}C-carbonyl labelled 3-methylresacetophenone as substrate. The conclusion we would like to draw from these experiments is that C-methylations during the biosynthesis of clavatol in *A. clavatus* occur before the aromatic structure is formed, giving some support to the speculation on the directing mechanism for the biosynthesis of 6-methylsalicylic acid and clavatol as outlined above.

In a previous publication we have described the isolation and some properties of an O-polyphenol methyltransferase from *Streptomyces rimosus*.[9] When resacetophenone was tested as a substrate for the purified enzyme it was O-methylated, using S-adenosylmethionine as methyl donor. However, when resacetophenone and ^{14}C-methyl labelled methionine were added to replacement cultures of *S. rimosus* a formation of clavatol as well as of the 4-methylether of resacetophenone was observed. The clavatol synthesis was more pronounced when resaceptophenone was substituted with 3-methylresacetophenone as substrate. In the latter case clavatol was isolated by paper chromatography, the radioactive area was cut out, and clavatol eluted from the paper and recrystallized to constant specific radioactivity with nonlabelled clavatol. In the best experiments about 1 % of the added radioactivity was incorporated into clavatol. The clavatol formation, however, was not consistent and in most experiments no clavatol was obtained.

Normally *S. rimosus* does not produce clavatol but evidently contains an enzyme system for nonspecific C-methylation of an aromatic nucleus. The only group of substances in *S. rimosus* known to be formed with C-methylation is the tetracyclines. The investigations of tetracycline biosynthesis by McCormick *et al.*[10] using mutants of *S. aurefaciens*, however, indicate that an aromatic C-methylation is unlikely in this case but would involve a mechanism compared to that suggested above for the clavatol synthesis in *A. clavatus*.

EXPERIMENTAL

Culture conditions. Aspergillus clavatus CMI 54399 was grown in 500 ml erlenmeyer flasks, each containing 150 ml of medium with the following composition: $NaNO_3$ 2.0 g, KH_2PO_4 1.0 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.01 g, glucose 50 g, yeast extract 1.0 g, molasses 10 g, agar 1.0 g and distilled water to 1 liter. The incubations were performed on a rotary shaker (250 rpm) at 26°C for 3—4 days.

Isolation of clavatol. ^{14}C(methyl)-methionine, and 250 μC of malonate-2-^{14}C were each evenly distributed into two flasks containing 3 days old cultures of *A. clavatus*. After 24 h incubation the mycelia were filtered off and the filtrates acidified with HCl and then extracted with ether. The acid substances were removed from the ether solution by extraction with aqueous $NaHCO_3$. After washing with distilled water, the ether phase was evaporated to dryness. The residue was sublimed in vacuum at 120°C. To the sublimate 200 mg of synthetic clavatol were added and the mixture repeatedly recrystallized from ligroin to a constant specific radioactivity.

Synthesis of clavatol. Clavatol was synthesized [8] in good yield via 4-formyl-2-methylresorcinol. Reduction of the latter gave 2,4-dimethylresorcinol which on treatment with acetic acid and anhydrous $ZnCl_2$ formed the acetate that rearranged during the reaction to clavatol.

Degradation of clavatol. The total radioactivity of clavatol was determined after combustion of a small sample according to the method of van Slyke and Folch.[11] All radioactivity measurements were performed in a liquid scintillator measuring CO_2 trapped as $BaCO_3$. The latter (*ca.* 10 mg) was suspended in a gel of Carbosil (4 %) in 10 ml of a toluene solution of diphenyloxazole (0.5 %). In the further degradation of clavatol 100 mg were mixed with 250 mg of H_2NOH HCl in 1.5 ml of water and 1.25 ml of 2 M NaOH. After the addition of enough of ethanol to obtain a clear solution the mixture was refluxed for 90 min. A small volume of water was added to the reaction solution and the oxime of clavatol crystallized on cooling. The yield of crude oxime dried at room temperature was 80.9 mg. The oxime was treated without further purification with 0.15 ml of acetic anhydride. The oxime dissolved easily and after a few minutes a precipitate of the acetyl derivative appeared. The acetyl derivative dissolved after adjustment with 2 M NaOH to a slightly alkaline reaction. A new precipitate was then formed which went into solution on further addition of aqueous NaOH. The clear yellow solution was acidified with H_2SO_4 and boiled for a few minutes to hydrolyze the formed anilide. The reaction product was oxidized with $FeCl_3$ and the formed 2-hydroxy-3,5-dimethyl-*p*-benzoquinone was continuously steam distilled off. After extraction of the distillate with ether and evaporation of the extract, the benzoquinone was recrystallized from cyclohexane, m.p. 102°C. The yields varied from 10 to 20 mg in different runs. The radioactive benzoquinone was diluted with 85 mg of synthetic substance and its total radioactivity determined as described for clavatol. 45.0 mg of the benzoquinone were oxidized to acetic acid and CO_2 in a Kuhn-Roth degradation. The radioactivity of the CO_2 was determined as above. The acetic acid was isolated by steam distillation and the distillate evaporated to dryness after neutralization of the acid. The sodium acetate was further degraded by a Schmidt reaction [12] and the CO_2 from the carboxyl was trapped as $BaCO_3$ and its radioactivity measured. The methylamine was isolated as the hydrochloride which was subsequently converted to CO_2 by wet combustion and its radioactivity determined.

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