ACTA CHEMICA SCANDINAVICA 27 (1973) 2710–2716

The Biosynthesis of Nidulin and Trisdechloronornidulin

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In contrast to earlier suggested theories experimental evidence has been obtained for the formation of nidulin and trisdechloronornidulin from acetate-malonate and one carbon units. The incorporations of L-isoleucine-4,5-^{3}H, L-methionine-$^{13}CH_3$, and L-methionine-$^{14}CH_3$ have been studied. Degradation of radioactive trisdechloronornidulin to 4,6-dinitroevernic acid and analysis of ^{13}C-NMR spectra conclusively localize the positions of the one carbon units in the structure.

The experiments supporting the earlier suggested theories are discussed and a hypothetical formation sequence of trisdechloronornidulin is presented.

A new fungal depsidone has recently been isolated by us from *Aspergillus nidulans*.[1] The structure of this depsidone was shown to correspond to that of trisdechloronornidulin (Fig. 1). The same compound has been isolated from *Aspergillus unguis* by two other groups of investigators [2,3] independent of each other. They call the depsidone yasimin and unguinol, respectively. *A. unguis* is an organism closely related to *A. nidulans*, both being members of the *Aspergillus nidulans* group.[4]

The structure of nidulin (Fig. 1) has been known since 1960,[5] and in 1963 Beach and Richards [6] made the first experiments in establishing its biosynthesis. The results of feeding experiments with acetate-1-^{14}C and uniformly labeled L-isoleucine-^{14}C made them suggest that ring A of nidulin, *i.e.* orsellinic acid, is derived from acetate and malonate and, more interesting, that ring B and its side chains are derived from a five-carbon fragment of isoleucine, tentatively tiglyl-coenzyme A, three acetate (or malonate) units, and an additional one carbon unit combined as shown in Fig. 2.

In contrast to the suggestion of Beach and Richards, in a recent publication Kamal *et al.*[7] propose that the eleven carbon atoms of B ring and its side-chains originate from two isoprene units and an additional one carbon unit. They find support for their proposal from feeding experiments with DL-mevalonic-2-^{14}C acid and L-methionine-CH_3-^{3}H. It is not evident how these three units are joined together to form the B ring system.

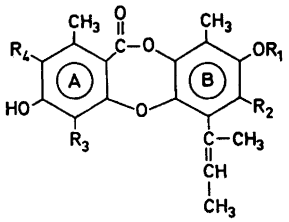

Fig. 1. $R_1 = CH_3$; $R_2 = R_3 = R_4 = Cl$, nidulin. $R_1 = R_2 = R_3 = R_4 = H$, trisdechloronornidulin.

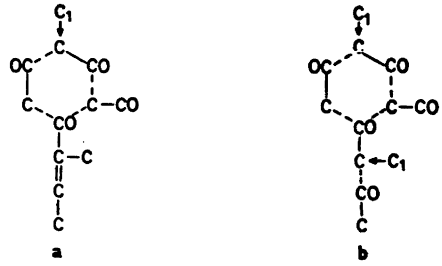

Fig. 2. Formation of the B ring system according to (a) Beach and Richards,[6] (b) "the acetate pathway".

The chemical structure represented by ring B and its side chains is rare among natural products. The skeletal feature of the 2-butenyl side chain is present in, *e.g.*, citrinin. In this case the side chain is derived from acetate-malonate and a one carbon unit.[8,9] The presented experimental results have not convinced us about the reliability of either of the suggested pathways of the biosynthesis of the B ring system so we have therefore tested the possibility of the formation of the system according to an "acetate pathway", *i.e.* from acetate-malonate and one carbon units as shown in Fig. 2. In our experiments we have studied the incorporation of L-isoleucine-4,5-^{3}H, L-methionine-$^{14}CH_3$, and L-methionine-$^{13}CH_3$ into trisdechloronornidulin and nidulin.

EXPERIMENTAL

Culture conditions. Aspergillus nidulans CMI 85, 473 (NRRL 2006) was grown at 28°C for 23 – 30 days as surface cultures in 1 l Fernbach's flasks each containing 350 ml of substrate medium ($NaNO_3$ 2.0 g, KH_2PO_4 1.0 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, yeast extract 1.0 g, glucose 40 g, and distilled water to 1 l).

Trisdechloronornidulin (I) from 4,5-^{3}H-L-isoleucine. After 22 days of growth 150 μCi (0.02 μmol) of 4,5-^{3}H-L-isoleucine were equally distributed between two flasks. The mycelia were filtered off and dried at 70°C over P_2O_5 after 16 h exposure to the radioactive precursor. Extraction with diethyl ether in a Soxhlet apparatus and repeated recrystallizations from hexane/diethyl ether of the solid residue obtained on evaporation of the diethyl ether solution yielded 200 mg (8.15×10^5 dpm/mmol) of labeled trisdechloronornidulin. The yield of incorporation of added radioactivity was 0.2 %.

O-*Methyl-trisdechloronidulin-^{3}H* (II). Tritium labeled I (200 mg) was methylated with an excess of diazomethane in methanol at room temperature for 3 h. The methanol was driven off *in vacuo* and the residue redissolved in ether. After washing with 2 M NaOH and water, the product crystallized on addition of hexane. Yield 150 mg, m.p. 136 – 139°C, specific radioactivity 7.5×10^5 dpm/mmol. Before degradation, II was diluted with 100 mg of cold *O*-methyl-trisdechloronidulin to a specific radioactivity of 4.2×10^5 dpm/mmol.

Methyl O-*methyl-trisdechloronidulinate-^{3}H* (III). 200 mg of II was dissolved in 20 ml of absolute methanol containing 20 mg of Na and heated for 15 min at 60 – 65°C under nitrogen. After cooling to 0°C the pH of the solution was adjusted to 2.5 with 1 M HCl. The precipitate was filtered off, washed with water and dried *in vacuo*. Yield 203 mg, m.p. 136 – 137°C, *m/e* 386. (Found: C 68.1; H 7.01. $C_{22}H_{26}O_6$ requires: C 68.4; H 6.79).

ν_{max} (KBr) 3400, 1708, 1605, and 1585 cm^{-1}; NMR (acetone-d_6) at δ 6.50, 6.40, 6.15 (aromatic protons), 5.76 (vinyl proton), 3.95, 3.86, 3.67 (*O*-methyls), 2.37, 2.07 (aromatic methyls), 1.93, 1.67 (vinyl methyls). UV: λ_{max} 280 nm (ε 4180), $\lambda_{infl.}$ 245 nm (ε 16700) Specific radioactivity 4.4×10^5 dpm/mmol.

Degradation of III to methyl 4,6-dinitroeverinate-^3H (IV). 100 mg of III were dissolved in 1 ml of glacial acetic acid at 60 – 65° and 125 µl of HNO_3 (*d*, 1.40) was added. After 2 min the reaction mixture was diluted with 50 ml of water followed by extraction with ether. The ether solution was washed several times with water and IV was then extracted with several small portions of aqueous $NaHCO_3$. The aqueous solution was extracted with ether after acidification with conc. HCl. The ether phase was washed with water, dried over anhydrous Na_2SO_4 and then evaporated to dryness. The residue was recrystallized from hexane/chloroform and finally from diethylether. Yield 30 mg, m.p. 155°C, *m/e* 286. (Found: C 41.86; H 3.42; N 9.94. $C_{10}H_{10}N_2O_8$ requires: C 41.94; H 3.52; N 9.79). ν_{max} (KBr) 1670, 1605, 1570, 1535, 1368, 1330, 1240 cm^{-1}; NMR (acetone-d_6) at δ 4.03 (ester methyl), 3.97 (ether methyl), 2.45 (aromatic methyl), no aromatic protons. UV: λ_{max} 306 nm (ε 2800), $\lambda_{infl.}$ 238 nm (28 000). Specific radioactivity 1.5×10^5 dpm/mmol.

O-Methyl-trisdechloronidulin from L-metionine-$^{14}CH_3$. Trace amounts of methionine-$^{14}CH_3$ (25 µCi) were added to two 25 days old cultures. After another 5 days the cultures were harvested and the dried mycelia were extracted with ether. The crude extract was recrystallized once from petroleum ether and then methylated with methyl iodide in acetone as described earlier.[1] The yield of crude labeled *O*-methyl-trisdechloronidulin was 115 mg. 34 mg of this labeled compound was recrystallized from methanol-water to constant specific radioactivity with 100 mg cold carrier substance. Yield 107 mg, specific radioactivity 2.5×10^6 dpm/mmol. Incorporation of ^{14}C-labeling 5.8 %. Degradation of *O*-methyl-trisdechloronidulin-^{14}C was performed as described above. The obtained methyl 4,6-dinitroeverinate contained practically no radioactivity.

Trisdechloronornidulin from L-methionine-$^{13}CH_3$. L-Methionine-$^{13}CH_3$ was prepared from $^{13}CH_3I$ (purchased from Wilmad Glass Company, N.J., USA) and L-homocystine as described by Jackman *et al.*[10] To each of two 25 days old cultures 10 mg/day of L-methionine-$^{13}CH_3$ was added for 5 days. The trisdechloronornidulin was isolated as described above. Yield 160 mg.

Nidulin from L-methionine-$^{14}CH_3$. Nidulin-^{14}C was prepared as described for *O*-methyl-trisdechloronidulin from L-methionine-$^{14}CH_3$ using two cultures with the substrate supplemented with KCl. The radioactive nidulin (specific radioactivity 4.5×10^6 dpm/mmol) was demethylated according to Zeisel.[11] The formed methyl iodide was trapped in trimethylamine and isolated as tetramethylammonium iodide (specific radioactivity 1.4×10^6 dpm/mmol.

Radioactive measurements. All radioactive determinations were performed in a Packard Scintillation Spectrometer (model 3375) using appr. 1 mg of substance dissolved in 0.1 ml of methanol and 20 ml of toluene containing standard amounts of PPO and POPOP.

RESULTS AND DISCUSSION

In one of their feeding experiments Beach and Richards used uniformly ^{14}C-labeled L-isoleucine.[6] They found that 13 % of the radioactivity incorporated into nidulin were localized to the orsellinic acid part of the molecule, which means that 87 % of the radioactivity was concentrated to the B ring system. Without further experimental evidence Beach and Richards suggest that most of this radioactivity is situated in the 2-butenyl side chain. If "the acetate pathway" is true one would expect an almost equal labeling of the orsellinic acid part and the B ring system from uniformly labeled isoleucine. This is on the assumption that the metabolic degradation of isoleucine follows the known route to acetyl-coenzyme A and propionyl-coenzyme A. On the other hand, if uniformly labeled isoleucine besides labeled acetyl CoA could deliver labeled one carbon units the experimental results of Beach and Richards could still support the formation of the B ring according to "the acetate

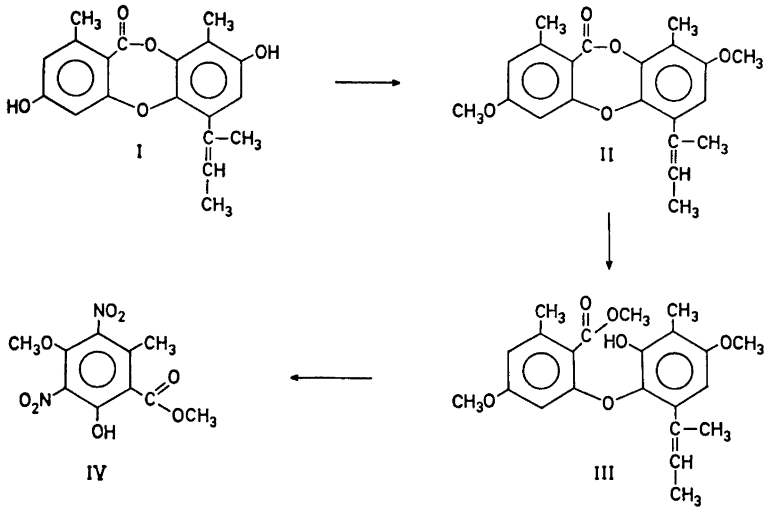

Fig. 3. Degradation scheme of trisdechloronornidulin.

pathway" as the one carbon units are entirely localized to the B ring system. In order to avoid possible formation of labeled one carbon units an incorporation experiment was performed with L-isoleucine-4,5-^{3}H, which should only give rise to acetyl-coenzyme A as a labeled catabolic product. Isolation of radioactive trisdechloronornidulin (specific radioactivity 4.4×10^5 dpm/mmol) and its subsequent degradation to 4,6-dinitroeverinate (specific radioactivity 1.5×10^5 dpm/mmol) indicated that the labeling from L-isoleucine was not specifically incorporated into the B ring system. The distribution of the radioactivity between the orsellinic acid part and the B ring system was in accordance with a degradation of isoleucine to labeled acetyl-coenzyme A and a formation of the ring B system as proposed in "the acetate pathway". The degradation reactions of isoleucine in the organism have apparently, as judged from the experiment of Beach and Richards, involved one carbon units that have effectively been transferred to methyl groups.

As seen from Fig. 2, two methyl groups are introduced into the B ring system during the biosynthesis of the depsidone according to "the acetate pathway". One of the methyl groups is attached to the aromatic nucleus and the other one is found in the 2-butenyl side chain on the carbon atom nearest the aromatic ring.

In their biosynthetic experiments Kamal *et al.*[7] have used L-methionine-CH_3-^{3}H as labeled precursor. They found that the orsellinic acid part did not contain any labeling from the one carbon metabolism and they claimed that only one of the three methyl groups of the B ring system is derived from methionine and they suggest the methyl group on the aromatic ring to be the labeled one. This is not in accordance with "the acetate pathway". On the other hand, we found that when methionine-$^{13}CH_3$ (60 % excess) is fed to *A. nidulans* the isolated trisdechloronornidulin contains an excess (3 %) of ^{13}C.

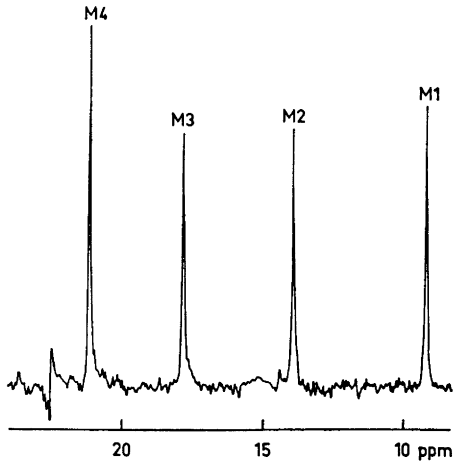

Fig. 4. ^{13}C-NMR spectra of trisdechloronornidulin (natural abundance). Varian X L-100 ^{13}C (25.2 MHz); sample 200 mg/3.0 ml CD_3OD; s.w. 1000 Hz; p.w. 60 μsec (30°); repetition 2 sec × 6800; lock = CD_3OD internal.

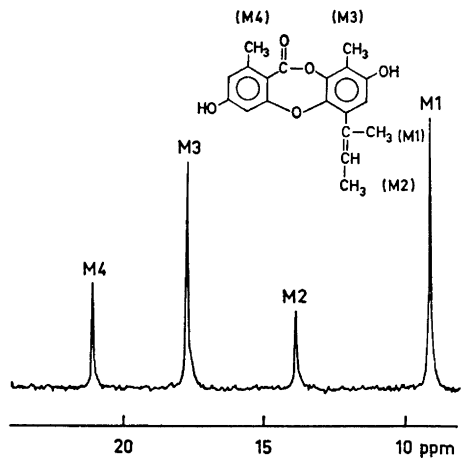

Fig. 5. ^{13}C-NMR Spectra of trisdechloronornidulin (^{13}C-enriched from methionine-$^{13}CH_3$). Varian X L-100 ^{13}C (25.2 MHz); sample 100 mg/3.0 ml CD_3OD; s.w. 1000 Hz, p.w. 60 μsec (30°); repetition 2 sec × 8300; lock = CD_3OD internal.

The localization in the structure of this excess of ^{13}C is seen from the ^{13}C-NMR spectrum in Fig. 4 which also shows the ^{13}C-NMR spectrum of the non-enriched compound. In these spectra the signals of the four methyl groups are found at δ 9.2 ppm, 14.0 ppm, 18.0 ppm, and 21.4 ppm from TMS. The two high-field signals are easily assigned to the methyl groups M1* (9.2 ppm) and M2 (14.0 ppm), respectively. The other two signals arise from the two aromatic methyl groups (M3, M4) and the assignments of their individual chemical shifts are not quite evident to us. Apparently two of the four methyl groups contain ^{13}C in excess of the natural abundance and are consequently derived from methionine. One of the ^{13}C-enriched methyl groups is attached to an aromatic nucleus. The other one is the methyl group designated Ml in Fig. 5 which is in full accordance with "the acetate pathway".

In order to be able to definitively establish which one of the aromatic methyl groups that is derived from a one carbon unit the incorporation of methionine-$^{14}CH_3$ was studied. After degradation of the radioactive trisdechloronornidulin to 4,6-dinitroeverinate the latter was shown to contain no radioactivity. From this follows that the methyl group M4 is not derived from the one carbon metabolism.

The described results all support the hypothesis that trisdechloronornidulin is synthesized from two polyketide units, (a) the orsellinic acid derived from one acetate unit and three malonate units, (b) the B ring system formed from one acetate unit and four malonate units and in addition two one carbon units.

* For designation of the methyl groups (M1, M2, M3, M4), see Fig. 4.

Even if there should be no doubt that nidulin is biosynthesized in a similar way, we have studied the incorporation of methionine-$^{14}CH_3$ into nidulin. The radioactive nidulin (specific radioactivity 4.5×10^6 dpm/mmol) was demethylated in a Zeisel reaction and the formed methyl iodide trapped as tetramethylammonium iodide (specific radioactivity 1.4×10^6 dpm/mmol).

From the specific activities can be calculated that the radioactive nidulin contained 3.2 labeled methyl groups, which indicates that "the acetate pathway" is valid also for the nidulin biosynthesis.

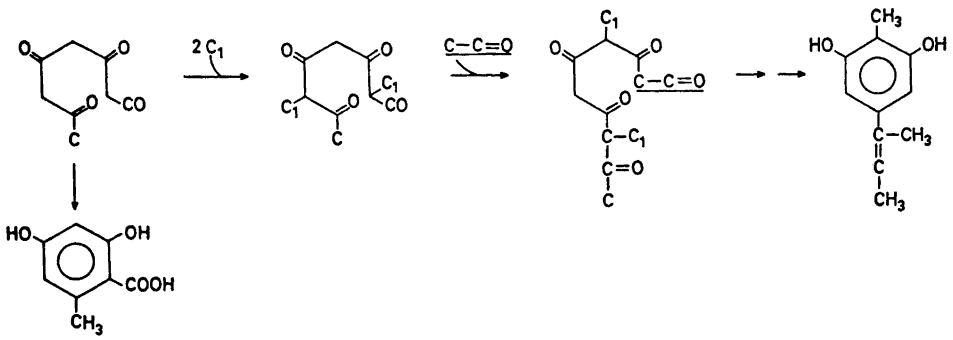

Fig. 6. Hypothetical formation sequence of the B ring system.

From the obtained results it is possible to speculate that in the formation of orsellinic acid and the B ring system a common intermediate is involved. This intermediate (a C-8 polyketomethylene chain) is in some cases methylated at positions that hinder the formation of a benzene derivative of orsellinic acid type or phloroglucinol type. If we assume that the products can only be released from the enzyme protein after aromatization, the methylated C-8 polyketomethylene chain has to be extended with one malonate unit to be able to form a benzoid compound. As seen from Fig. 6 this compound is easily transformed to the B ring system of trisdechloronornidulin.

Acknowledgements. This investigation was supported by a grant from the *Swedish Natural Science Research Council.* The authors are also grateful to Dr. T. Drakenberg, Chemical Centre, Lund, for running the ^{13}C-NMR spectra.

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Received April, 26 1973.