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BIOSYNTHESIS OF TRICHOTHECIN AND RELATED COMPOUNDS

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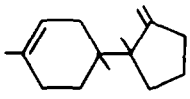
Abstract.--Trichothecolone biosynthesized in the presence of doubly labeled mevalonate, [4(*R*)-4-^{3}H,2-^{14}C]MVA, was degraded to show that the carbon atom at position 8 originated from $C_{(2)}$ of mevalonic acid. Two minor cytotoxic metabolites were isolated from *Trichothecium roseum* and their biogenetic relation to trichothecin is shown.

IN THE PRECEDING PAPER[1] we described the isolation and structural elucidation of trichodiene (**1**) and trichodiol (**2**).* The former has been considered as a possible precursor of trichothecin (**3**) and related substances.[3] During investigations on the biosynthesis of trichothecin and its congeners using doubly labeled mevalonate as a substrate, we have found that $C_{(10)}$ does not originate from $C_{(2)}$ of the mevalonate as reported previously,[4] but from $C_{(4)}$ of the substrate. Hanson *et al.*[5] reported experimental evidence that the hydrogen atom on $C_{(10)}$ of trichothecin was derived from pro-4(*R*)hydrogen of mevalonate. Here we report our own results on the same problem as well as the isolation of two minor metabolites (**12** and **13**) from the fermentation materials of *Trichothecium roseum*. Compounds **12** and **13** showed marked cytotoxic activity against HeLa cells in culture.



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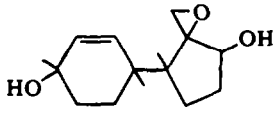

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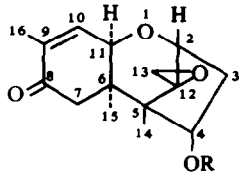

3 R = $CH_3CH{=}CHCO$

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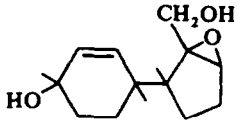
4 R = H



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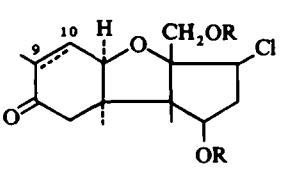
[4(*R*)-4-^{3}H,2-^{14}C]-Mevalonic acid was fed to a 24-hour-old culture of *Trichothecium roseum*. Incubation was continued aerobically for a further 6 days. To the non-saponifiable fraction of the broth extract were added pure samples of trichothecolone (**4**) and trichodiol-A (**5**).[1] These substances were carefully purified by column

* Trichodiol, previously reported,[2] has been found to be an artefact produced from compound **2**; we have given the name trichodiol to the compound having structure **2** (see preceding paper).

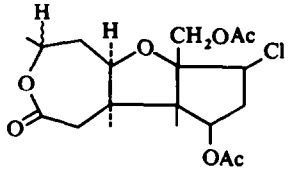
chromatography and recrystallization. Trichothecolone and trichodiol-A showed the following atomic ratio of radioactivities. Trichothecolone: $^3H:^{14}C$, 2·01:3; Trichodiol-A: $^3H:^{14}C$, 2·02:3. Ergosterol obtained from the mycelium showed the ratio ($^3H:^{14}C$, 2·87:5) as expected.

In order to confirm that one of the ^{14}C atoms is located at $C_{(8)}$, the following degradative work was carried out.

The radioactive trichothecolone (^{14}C-specific activity, 12,700 dpm/mmol) was treated with HCl to obtain the known chlorohydrin **6**.[6] Hydrogenation of **6** followed by acetylation gave **7**, which on treatment with *m*-chloroperbenzoic acid gave lactone **8**. Hydrolysis of **8** and successive acetylation afforded carboxylic acid **9** whose methyl ester (**10**) showed ($^3H:^{14}C$, 1·94:3). Compound **9** was allowed to react with Br_2 and HgO in CCl_4 to yield bromoderivative **11** which showed ($^3H:^{14}C$, 1·78:2, ^{14}C-specific activity, 7,600 dpm/mmol). $BaCO_3$ prepared from the CO_2 evolved in this reaction was radioactive.



6 $C_{(9)}$–$C_{(10)}$, unsat., R = H
7 $C_{(9)}$–$C_{(10)}$, sat., R = Ac



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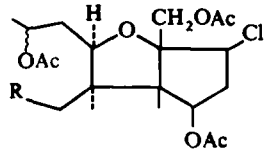
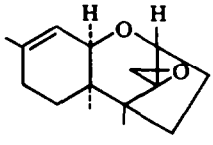


9 R = COOH
10 R = $COOCH_3$
11 R = Br

These results prove unequivocally that $C_{(8)}$ and $C_{(10)}$ of trichothecolone is derived from $C_{(2)}$ and $C_{(4)}$ of mevalonate, respectively, in disagreement with the result of Jones and Lowe[4] and in agreement with the result of Hanson *et al.*[5]

In connection with the biosynthetic studies described above, we have isolated two new compounds (**12** and **13**) which are considered as biogenetic intermediates of trichothecin, from *Trichothecium roseum*.



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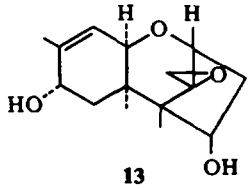

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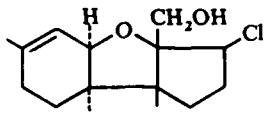
Compound **12** was isolated from the mycelium as an oil in extremely minute amount. Its mass spectrum showed a molecular ion peak at 234. The NMR spectrum (Experimental) revealed the presence of two tertiary methyl groups, one olefinic methyl group, two protons of oxirane methylene, and two protons adjacent to ether oxygen. As these data suggested that the structure should be 12,13-epoxytrichothec-9-ene (**12**), basic skeleton of the trichothecane type sesquiterpene, chemical correlation of this compound with trichodiol was undertaken. Compound **12** was rearranged by the action of HCl to the chlorohydrin (**14**) which was identical, in all respects, with

the chlorohydrin[1] obtained from trichodiol-A. This rearrangement is a common reaction for trichothecane type compounds. The stereochemistry was assigned as shown by comparing the NMR signals, especially the splitting patterns of the methine protons adjacent to the oxygen, of this compound with those of trichodermol.[7]

Compound **13** was isolated from the nonsaponifiable fraction of the broth extract as colourless crystals, m.p. 190–191° (from acetone–hexane), $[\alpha]_D$ −53·4°. The molecular formula was determined as $C_{15}H_{22}O_4$ by mass spectrum (M^+, 266) and elemental analysis. The NMR spectrum was similar to that of trichothecolone: the major differences were that the signal of olefinic proton of compound **13** appeared at 5·52 whereas that of trichothecolone appeared at 6·46 and that a broad doublet (4·08, 1H, J = 5 Hz) newly appeared in addition to the other five —CH—O— protons. From these data we have postulated structure **13**, 4β,8α-dihydroxy-12,13-epoxy-trichothec-9-ene, for this compound. The structure was ascertained by its transformation into trichothecodione (**15**), a known compound[6] derived from trichothecolone and also by the formation of the natural substance **13** by reduction of trichothecolone with $NaBH_4$. The configuration of $C_{(8)}$—OH was determined by the coupling constant of $C_{(8)}$—H which appeared as a broad doublet (J = 5 Hz). Inspection of the Drieding model revealed that the dihedral angles between the protons at position 7 and 8 are as shown below and has provided strong evidence in favour of the α-configuration for the OH at position 8.



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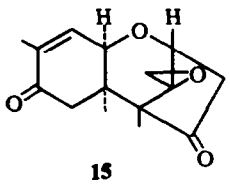

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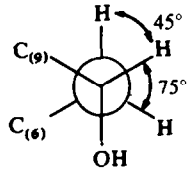
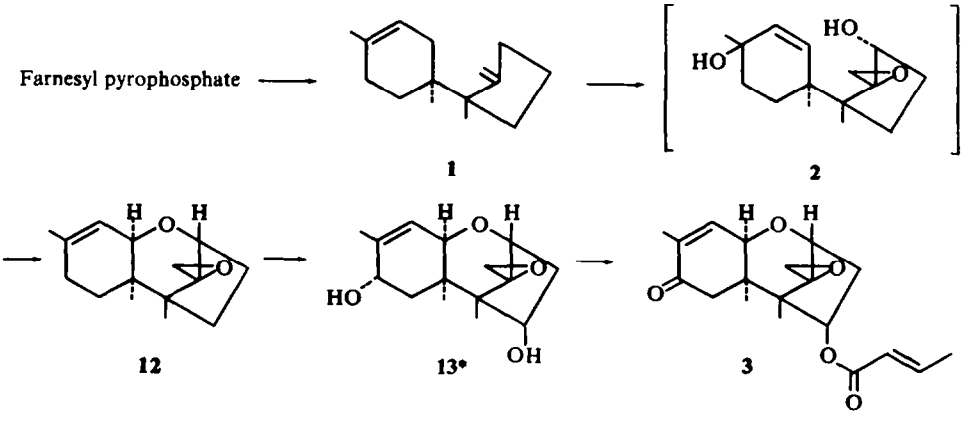


Inspection of the structures described herein and in the preceding paper suggests their biogenetic relationship and a hypothetical scheme may be formulated as shown below.



* Recently Adams and Hanson[8] reported that epoxide crotocin or similar epoxide might be a precursor of trichothecin. They presented some experimental support for an NIH type shift which caused isomerization of the epoxide to the ketone.

Biogenetical intermediacy of compound **2** in the formation of compound **12** is obscure since there might be a possibility that trichodiol (**2**) is a metabolite of compound **12**.

EXPERIMENTAL

Radioactive assays were carried out with a Packard TRI-CARB Liquid Scintilation Spectrometer 2311 using a soln of 2,5-diphenyloxazole (4 g) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (0·1 g) in toluene (1·1) and corrections were made for background and efficiencies. For other general remarks see preceding paper.[1]

Incubation of [4(*R*)-4-^{3}H,2-^{14}C]-*mevalonic acid* (MVA)

Trichothecium roseum was inoculated in the potato medium (10 ml) containing sugar (2%), $MgSO_4$ (2·5%), KH_2PO_4 (5%), and peptone (5%). After 24 hr incubation at 27°, the doubly labeled MVA (^{3}H:^{14}C, 12·07:1 (dpm ratio), ^{14}C, 20 μCi) was added to the medium and fermentation continued for 6 days. The nonsaponifiable fraction of the broth extract was obtained as described in the preceding paper, to which cold trichothecolone (300 mg) and trichodiol-A (46 mg) were added. The mixture was separated by column chromatography (30% ether–benzene elution for trichothecolone and 60% ether–benzene elution for trichodiol-A) and recrystallizations (acetone–hexane for trichothecolone and ether–hexane for trichodiol-A). Trichothecolone gave 800 dpm/mg (^{14}C, 1·2% incorporation); ^{3}H:^{14}C, 8·07:1 (dpm ratio), 2·01:3 (atomic ratio). Trichodiol-A gave 180 dpm/mg (^{14}C, 0·04% incorporation); ^{3}H:^{14}C, 8·14:1 (dpm ratio), 2·02:3 (atomic ratio).

Degradation procedure of trichothecolone

Compound **7**. Compound **6** (1·000 g) was hydrogenated over 5% Pd-C (200 mg) in MeOH (35 ml) and the crude product acetylated in the usual manner. Column chromatography of the product (3% ether–benzene elution) afforded a pure sample of **7** (982 mg), $C_{19}H_{27}O_6Cl$; mass spectrum: 386 (M^+); NMR ($CDCl_3$): 0·85 (3H, s), 0·98 (3H, d, J = 6 Hz), 1·08 (3H, s), 2·07 (3H, s), 2·09 (3H, s), 3·72 (1H), 4·09 (1H, q, J = 12 Hz, 6 Hz), 4·43 (2H, s), 5·08 (1H, q, J = 10 Hz, 5·5 Hz); IR: 1720, 1745 cm^{-1}.

Compound **8**. To a soln of **7** (960 mg) in $CHCl_3$ (10 ml) was added a soln of 2 eq of *m*-chloroperbenzoic acid in $CHCl_3$ (20 ml) with stirring. After standing overnight at room temp the mixture was washed with aq. $NaSO_3$ and water, dried, and the solvent evaporated. Column chromatography of the residue (2% ether–benzene elution) gave **8** (510 mg), $C_{19}H_{27}O_7Cl$; mass spectrum: 402 (M^+); NMR ($CDCl_3$): 0·97 (3H, s), 1·10 (3H, s), 2·06 (3H, s), 2·11 (3H, s), 1·34 (3H, d, J = 7 Hz), 2·23, 3·18 (2H, ABq, J = 13 Hz), 3·75 (1H), 4·10 (1H, q, J = 12 Hz, 6 Hz), 4·35 (2H, s), 4·71 (1H, m), 5·08 (1H, q, J = 10 Hz, 5·5 Hz); IR: 1735, 1200–1250 cm^{-1}.

Compound **10**. A soln of **8** (130 mg) in 10% ethanolic KOH was heated under reflux for 3 hr and worked up as usual. Acetylation of the product followed by column chromatography (10% ether–benzene elution) gave **9** (51 mg), $C_{21}H_{31}O_9Cl$; IR: 3540, 2500–3000, 1740, 1200–1250 cm^{-1}. Esterification of this compound with CH_2N_2 gave quantitative yield of **10**, m.p. 114–116° (from acetone–hexane); $C_{22}H_{33}O_9Cl$; mass spectrum: 434 (M-42), 416 (M-60); NMR ($CDCl_3$): 1·10 (3H, s), 1·13 (3H, s), 2·02 (3H, s), 2·04 (3H, s), 2·12 (3H, s), 3·62 (3H, s), 1·23 (3H, d, J = 6 Hz), 3·55 (1H), 4·02 (1H, q, J = 12 Hz, 6 Hz), 4·15, 4·36 (2H, ABq, J = 12 Hz), 5·05 (2H, m); IR: 1740, 1200–1250 cm^{-1}.

Compound **11**. To a stirred soln of red HgO (45 mg) was added dropwise a mixture of **9** (106 mg), Br_2 (60 mg), ether (0·5 ml), and CCl_4 (4·5 ml) at room temp under N_2. The temp was raised to 35° and stirring continued for 2 hr. HgO (23 mg) and Br_2 (30 mg) were added and the reaction was continued for a further 5 hr. After solvent removal, the residue was adsorbed on a silica gel column and eluted with 2% ether–benzene to obtain **11**. Repeated column chromatography gave pure **11** (13 mg), $C_{20}H_{30}O_7ClBr$; mass spectrum: 496, 498 (M^+, both); NMR (CCl_4): 1·03 (3H, s), 1·23 (3H, s), 2·04 (3H, s), 2·08 (3H, s), 2·12 (3H, s), 1·26 (3H, d, J = 6 Hz), 3·59 (2H, s), 3·70 (1H, m), 4·08 (1H, q, J = 12 Hz, 6 Hz), 4·22, 4·39 (2H, ABq, J = 12 Hz), 5·15 (2H, m); IR: 1740, 1200–1250 cm^{-1}.

Structures of compounds **12** *and* **13**

Isolation of compound **12**. This metabolite was isolated from the EtOAc extract of the mycelium by repeated column chromatography (10% ether–benzene). $C_{15}H_{22}O_2$; mass spectrum: 234 (M^+); NMR (CCl_4): 0·70 (3H, s), 0·75 (3H, s), 1·67 (3H, bs), 2·72, 3·00 (2H, ABq, J = 4 Hz), 3·53 (1H, d, J = 4·5 Hz), 3·57 (1H, d, J = 4·5 Hz), 5·29 (1H, bd, J = 4·5 Hz); IR: no OH or C=O bands.

Isolation of compound **13**. This substance was isolated from the nonsaponifiable fraction of the broth extract by column chromatography (70% ether–benzene elution) and crystallization from acetone–hexane, m.p. 190–191°, $[\alpha]_D$ −53·4° ($CHCl_3$); $C_{15}H_{22}O_4$; mass spectrum: 266 (M^+); NMR ($CDCl_3$): 0·79 (3H, s), 0·99 (3H, s), 1·82 (3H, bs), 2·80, 3·06 (2H, ABq, J = 4 Hz), 3·55 (1H, bd, J = 6 Hz), 3·775 (1H, d, J = 5 Hz), 4·085 (1H, bd, J = 5 Hz), 4·30 (1H, q, J = 2·5 Hz), 5·52 (1H, bd, J = 6 Hz); IR: 3600 cm^{-1}.

Compound **14**. Compound **12** was treated with HCl in MeOH (0·5 ml, conc HCl: MeOH, 1:10) at room temp for 30 min. MeOH was removed, water added, and the product isolated by ether extraction. Column chromatographic purification (20% ether–benzene elution) gave a chlorohydrin identical in all respects with the chlorohydrin obtained from trichodiol-A.

$NaBH_4$-*reduction of trichothecolone*. $NaBH_4$ (50 mg) was added portionwise to a stirred soln of **4** (100 mg) in MeOH (5 ml) and stirring continued for 3 hr at room temp. The mixture was worked up as usual and the product separated by column chromatography. Elution with 70% ether–benzene gave a diol (50 mg), identical in all respects with natural compound **13**.

Compound **15**. A 5% soln of CrO_3 in 95% AcOH (1 ml) was added to **13** (30 mg) in AcOH (0·3 ml) and the mixture stirred for 1 hr. Pouring it into cold water followed by ether extraction and appropriate work up afforded an almost quantitative yield of **15**. Recrystallization from acetone–hexane gave colourless crystals identical in all respects with trichothecodione obtained from trichothecolone.

Acknowledgement—We are indebted to Professor S. Okuda for providing us the strain of *Trichothecium roseum*, to Dr. Y. Nakayama, Nippon Kayaku Co., Ltd., for supplying us the fermentation materials, to Dr. W. O. Godtfredsen, Leo Pharmaceutical Products, for the generous gift of a sample of trichodermin, and to Hoansha for financial support.

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