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BIOSYNTHESIS OF PEBROLIDE

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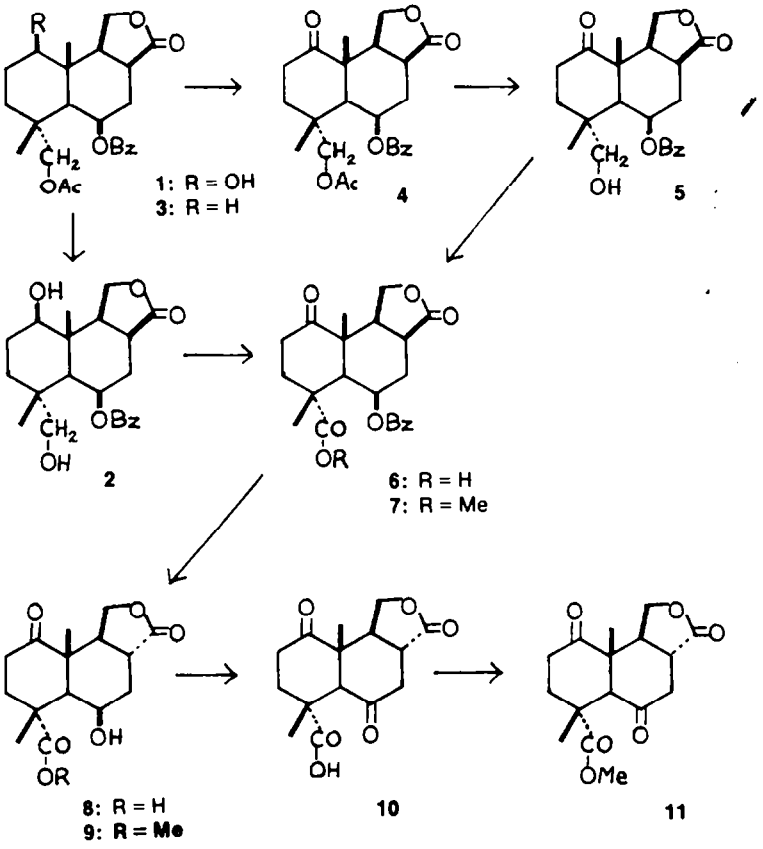
Abstract—In pebrolide biosynthesised from MVA-[2,2-$^3H_2$], 1, 1½ and 2 tritium atoms respectively are retained at C-1, C-15 and C-7.

We have reported the isolation and structural elucidation of pebrolide (**1**) and the closely related metabolites **2** and **3**.[1] These were the first simple drimane sesquiterpenes to be isolated from fungal sources. Determination of the pattern of incorporation of tritium from [2,2-$^3H_2$]mevalonate into **1**, using a simple degradative scheme suggested by the distribution of functionality, is now described.[2]

Samples of [^{14}C, ^{3}H]pebrolide were obtained by feeding an aqueous solution of MVA-[2-^{14}C, 2,2-$^3H_2$] (^{3}H: ^{14}C = 38:1) to 4 or 3 day-old surface cultures of *P. brevicompactum* and isolating the pebrolide after a further 2 and 1 days' growth respectively. Under these feeding conditions, incorporations into **1** were somewhat low, namely, w.r.t. ^{14}C, 0.014 and 0.002% respectively. Incorporation into **2** also isolated in the second experiment was even lower (0.0004%). However, these conditions were chosen to avoid persistent difficulties in isolation associated with separation from a cyclic depsipeptide ('brevigellin'[3]) produced from the 7th day onwards and from a highly radioactive contaminant thought to be ergosterol, produced from the 5th day onwards.

Stepwise removal of tritium from positions 1, 15 and 7 was carried out using the degradative sequence indicated in Scheme 1. The ^{3}H:^{14}C ratio of **1** obtained in each experiment was consistent with incorporation of 4⅓ tritium atoms as indicated in Table 1. In the first experiment, oxidation of **1** to **4**[1] was accompanied by loss of 17%



Scheme 1. Degradation of pebrolide-[^{14}C, ^{3}H].

Table 1.

	Compound	^3H: ^{14}C ratio (dpm)	^3H: ^{14}C ratio (atomic)	No. of ^3H atoms
Expt (i)	MVA	38.0	6:3	6
	1	27.2	4.29:3	$4\frac{1}{3}$
	4	22.5	3.55:3	$3\frac{1}{3}$
	7	11.7	1.85:3	2
Expt (ii)	MVA	36.0	6:3	6
	1	25.8	4.30:3	$4\frac{1}{3}$
	6	11.6	1.93:3	2
	10	10.8	1.80:3	~2
	10*	0.21	0.035:3	0
	2†	24.2	4.03:3	$\sim 4\frac{1}{3}$
	6‡	11.9	1.99:3	2

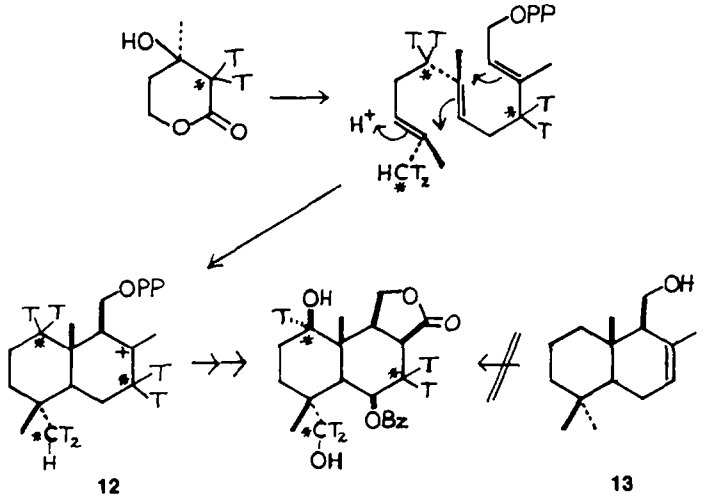
* After hydrolysis and re-esterification.

† Isolated from broth. ‡ From isolated 2.

of the ^3H activity indicating the presence of one tritium atom at C-1. Hydrolysis of **4** to the hydroxymethyl ketone **5**, followed by oxidation of this to the keto-acid **6** (counted in this case as its methyl ester **7**) was accompanied by further loss of ^3H indicating the presence of $1\frac{1}{3}$ tritium atoms at C-15 (*cf* Table 1). In this sequence, repeated crystallization was required to produce radiochemically pure **4** and the final yield of **6** was therefore low.

In the second experiment, a more convenient route to **6** was used, namely acid hydrolysis of **1** to give **2**,[1] followed by oxidation to **6**. The ^3H loss again corresponded to that of $2\frac{1}{3}$ tritium atoms (*cf* Table 1). The sequence was completed by basic hydrolysis of the benzoate group (accompanied by epimerization at C-8[1]) followed by oxidation to the diketone **10** which was counted as its methyl ester **11**. A small amount of ^3H was lost in this sequence (**6**→**11**) corresponding to loss of ^3H located at C-2, C-6 and C-8 and/or partial exchange of ^3H located at C-7. Treatment of **11** with aqueous base and re-esterification resulted in almost complete loss of ^3H. Unless ^3H were considered to be located in unprecedented fashion at C-5, 2 tritium atoms must be located at C-7. Desacetyl pebrolide isolated in the second experiment was of low radioactivity and results can be considered only approximate. However ^3H:^{14}C ratios for **2** itself and the derived **6** are in fair agreement with the above values.

These results are consistent with the biogenetic scheme indicated in Scheme 2. Formation of a bicyclofarnesyl cation **12** by stereospecific cyclization of farnesyl pyrophosphate would be expected to result in



Scheme 2. Biogenesis of pebrolide.

retention of all six H atoms derived from C-2 of MVA. Hydroxylation at C-1 and C-15 would result in removal of one of these atoms from C-1 and statistically, $\frac{2}{3}$ of an atom from C-15. In fact, $4\frac{1}{3}$ atoms were found to be retained in **1**. The results of degradation indicate clearly that of the C-4 substituents only C-15 had ^3H atoms attached (*ca* $1\frac{1}{3}$ atoms). The terminal Me groups of the prenyl pyrophosphate thus retain their individuality in the formation of **1** as has been demonstrated for analogous diterpenes[4] and triterpenes.[5] Recently the Δ^7 compound (–)-drimenol (**13**) has been isolated from the Basidiomycete *Lactarius uvidus* together with oxygenated derivatives.[6] Formation of the *cis* lactone system of **1** is likely to involve such an olefinic intermediate. However, since the results here indicate that both hydrogen atoms at C-7 in **1** are derived from C-2 of MVA, this intermediate would have to be a Δ^8- or $\Delta^{8(12)}$- rather than a Δ^7-bicyclofarnesol derivative.

EXPERIMENTAL

The hydroxymethyl ketone **5**. The ketone **4** (69 mg) in acetone (25 ml) was refluxed with 6N H_2SO_4 (5 ml) for 1 hr. Upon cooling, extraction with $CHCl_3$ gave the *hydroxymethyl ketone* **5** (50 mg, 80%), m.p. 208–210° from $CHCl_3$–pet. ether; IR KBr) 3500, 1704, 1596, 1580, 704 cm^{-1}; IR ($CHCl_3$) 1775 cm^{-1} (γ-lactone, ε 530), 1705 (ketone, benzoate, ε 620); ^1H NMR ($CDCl_3$) 1.0 δ(3H, s, 4β-Me), 1.6 (3H, s, 10-Me), 3.22 and 3.68 (ea. 1H, AB q, J = 10 Hz, CH_2OH), 2.40 (2H, m, H-11), 5.70 (1H, m, H-6), 7.50 and 7.39 (3H and 2H, m, benzoate); MS *m/e* 386 (1%, M^+), 281 (10, M-105), 264 (8, M-122), 263 (5), 234 (11), 233 (5, M-122-31), 216 (5), 192 (5), 105 (100) with m* corresponding to 386→264. (Found: C, 68.2; H, 6.8. $C_{22}H_{26}O_6$ Requires: C, 68.4; H, 6.7%).

Carboxyketone **6**. (i) The diol **2** (166 mg) in acetone was treated with a slight excess of CrO_3 in conc H_2SO_4 at room temp for 5 min. After addition of ice water, extraction with $CHCl_3$ gave the *carboxyketone* **6** (160 mg, 97%), m.p. 123–125° from aq. MeOH; IR (KBr) 1786, 1755, 1717, 1606, 1582, 723 cm^{-1}; IR ($CHCl_3$) 1784 cm^{-1} (γ-lactone, ε 800), 1718 (ketone, CO_2H and benzoate, ε 1150); MS *m/e* 400.1518 (0.1%, $C_{22}H_{24}O_7$ Requires: 400.1522), 295 (1%, M-105), 278.1155 (24, M-122), 234 (25, M-122-44), 232 (22, M-122-18-28), 122 (100%) with m* corresponding to 400→278.

(ii) The carboxyketone **6** was similarly obtained by oxidation of **5**.

Carbomethoxyketone **7**. Esterification of **6** (33 mg) in MeOH with an excess of CH_2N_2 in ether gave the *carbomethoxyketone* **7** (29 mg, 82%), m.p. 227–232° from $CHCl_3$–pet. ether; IR (KBr) 1779, 1731, 1712, 1604, 1585, 723 cm^{-1}; IR ($CHCl_3$) 1785, 1722 cm^{-1}; ^1H NMR ($CDCl_3$) 1.48 δ (3H, s, 4β-Me), 1.68 (3H, s, 10-Me), 3.77 (3H, s, OMe), 4.40 (1H, dd, J = 5, 10 Hz, H-11α), 4.68 (1H, d, J = 10 Hz, H-11β), 5.54 (1H, m, H-6), 7.56 and 8.04 (3H and 2H, m, benzoate); MS *m/e* 414 (1% M^+), 383 (1, M-31), 355 (1, M-59), 309 (48, M-105), 292 (50, M-122), 277 (30, M-105-32), 260 (5, M-122-32), 259 (15), 249 (70, M-105-32-28), 233 (40, M-122-59), 232 (45), 231 (48), 209 (60) with m* corresponding to 414→309, 309→277 and 277→249. (Found: C, 66.7; H, 6.5. $C_{23}H_{26}O_7$ Requires: C, 66.7; H, 6.3%).

Carbomethoxyketol **9**. The carboxyketone **6** (63 mg) was refluxed with 6N NaOH (5 ml) for 4 hr, and then neutralised with dil HCl aq. Ether extraction gave a solid which, after washing with hot ether to remove benzoic acid, and crystallization from $CHCl_3$–pet. ether gave **8**, (30 mg, 63%), m.p. 251–254°. Esterification of this in MeOH with CH_2N_2 in ether gave the *carbomethoxyketol* **9** as plates (30 mg, 95%), m.p. 175–179° from ether-pet. ether; IR (KBr) 3550, 1768, 1712, 1692 cm^{-1}; IR ($CHCl_3$) 1770 cm^{-1} (γ-lactone), 1709 (ketone and ester); ^1H NMR (60 MHz, $CDCl_3$) 1.50 δ (3H, s, 4β-Me), 1.72 (3H, s, 10-Me), 3.75 (3H, s, OMe), 4.29 (1H, m, H-6), 4.25 (1H, m, J = 8, 12 Hz, H-11α), 4.81 (1H, m, J = 7, 8 Hz, H-11β); MS *m/e* 310 (1%, M^+), 295 (1, M-15), 292 (1, M-18), 260 (20), 251 (100, M-59), 233 (25, M-59-18), 232 (25), 205 (20), 187 (20), 184 (50) with m* corresponding to 310→292 and 251→233 (Found: C, 62.0; H, 7.2. $C_{16}H_{22}O_6$ Requires: C, 61.9; H, 7.2%).

Carbomethoxy diketone **10**. The carbomethoxyketol **9** (24 mg) in acetone (0.5 ml) was treated with a slight excess of CrO_3 in conc H_2SO_4 at room temp for 2 min. After addition of ice water (2 ml), extraction with $CHCl_3$ gave the *diketone* **10** (20 mg, 80%), m.p. 180–185° from $CHCl_3$–pet. ether; IR (Nujol) 1770, 1700, 1230, 1000 cm^{-1}; ^1H NMR (60 MHz, $CDCl_3$) 1.27 δ (3H, s, 4β-Me), 1.72 (3H, s, 10-Me), 3.70 (3H, s, OMe), 4.30 (1H, m, J = 8, 12 Hz, H-11α), 4.96 (1H, m, J = 7, 8 Hz, H-11β); MS *m/e* 308.1261 (10%, $C_{16}H_{20}O_6$ requires 308.1260), 276 (30, M-32), 249.1125 (100, M-59), 248 (30, M-32-28), 220 (90), 208 (10), 203 (10) with m* corresponding to 308→276 and 276→248.

Feeding/degradative experiments. (i) A soln of MVA-[2-^{14}C, 2,2-$^3H_2$] (0.05 mCi of ^{14}C, ^3H: ^{14}C = 38:1) in sterile water (10 ml) was added to 4 day-old cultures of a strain of *P. brevicompactum* (5 Roux bottles). After a further 48 hr., the neutral fraction of the broth metabolites isolated as previously described.[1] To a soln of this in $CHCl_3$, inactive **1** (187 mg) was added. After pptn by addition of pet, ether, crystallization gave **1** (150 mg), homogeneous by tlc. Crystallization (×7) gave material of constant radioactivity, namely 3.2×10^4 and 8.7×10^5 dpm/mmol for ^{14}C and ^3H respectively.

Oxidation of **1** (100 mg of material from intermediate crystallizations still carrying traces of radioactive impurities) gave, after 2 crystallizations **4** (50 mg) which was chemically pure but contained a radioactive impurity (tlc radioscan). This was removed by repeated preparative tlc (silica gel HF_{254}) and further crystallizations. The ketone **4** was successively hydrolyzed (to **5**), oxidised (giving **6**) and esterified (giving **7**) as described above. Although samples of **7** were obtained from successive crystallizations showing unchanged ^3H:^{14}C ratio (*cf* Table 1), insufficient material remained to carry out further degradation.

Feeding/degradative experiments. (ii) A soln of MVA-[2-^{14}C, 2,2-$^3H_2$] (0.09 mCi of ^{14}C, ^3H: ^{14}C = 36:1) in sterile water (24 ml) was added to 3 day-old surface cultures of *P. brevicompactum* (11 Roux bottles). After a further 20 hr, the neutral fraction of the broth metabolites was isolated as before. A soln of this in $CHCl_3$ was diluted with inactive **1** (205 mg) and **2** (140 mg) and chromatographed on a column of silica gel (40 g). Elution with $CHCl_3$ gave crude **1** which was purified by preparative tlc on silica gel HF_{254} followed by repeated crystallization to constant radioactivity *viz* 1.37×10^4 and 3.53×10^5 dpm/mmol for ^{14}C and ^3H respectively. Elution of the column with 5% MeOH in $CHCl_3$ gave crude **2** which was crystallized from $CHCl_3$–pet. ether to constant radioactivity, *viz* 2.55×10^3 and 6.16×10^4 dpm/mmol for ^{14}C and ^3H respectively.

Using the degradative sequence **1**→**2**→**6**→**10** described above samples of **6** and **10** were obtained and purified to constant radioactivity by repeated crystallization (*cf* Table 1). The diketo ester **10** (9 mg) was refluxed with 5N NaOH (5 ml) for 4 hr. The soln was then neutralized with dil HCl aq and extracted with $CHCl_3$. Evaporation gave **11** which was re-esterified in MeOH with ethereal CH_2N_2. Successive crystallizations of the resulting **10** from $CHCl_3$-pet. ether gave samples with the same radioactivity (*cf* Table 1).

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