Structures of Some New Sesquiterpenoid Metabolites of *Marasmius alliaceus*

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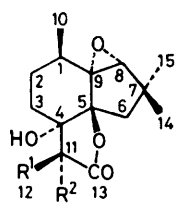
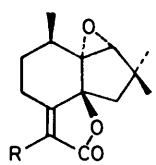
The sesquiterpenoids, alliacolide II (alliacol A) (2), 12-hydroxydehydroalliacolide (alliacol B) (3), 12-noralliacolide (5), 11- and 12-hydroxyalliacolide (6) and (7), respectively, and alliacide (8) have been isolated from *Marasmius alliaceus* and their structures established by a combination of their spectra and chemistry.

THE novel sesquiterpenoid, alliacolide (1), was isolated from *Marasmius alliaceus* during the examination of the Basidiomycetes for polyacetylenic fungal metabolites. The structure and relative stereochemistry of alliacolide were determined by *X*-ray analysis.[1] Subsequently, we have established[2] the absolute configuration by degradation and circular dichroism measurements. During biosynthetic studies of this unique carbon skeleton,[3] we have isolated some related sesquiterpenoid metabolites from the culture broth, the structures of which are reported herein. Very recently the abstract of a poster appeared describing structures (2) and (3) for alliacol A and B.[4] Comparison of the spectral data of alliacolide II (2) [1b] with that of alliacol A confirmed their identity.

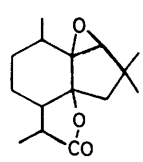
Careful chromatography of the neutral extract of the broth of *Marasmius alliaceus* afforded mellein (4),[5] alliacolide (1), alliacolide II (2),[1] 12-hydroxydehydroalliacolide (3), 12-noralliacolide (5), 11-hydroxyalliacolide (6), and 12-hydroxyalliacolide (7). Acetylation of the mellein fraction and further chromatography gave alliacide (8). The structures of these metabolites followed from an examination of their ^1H and ^{13}C n.m.r. spectra (see Tables 1 and 2), their mass spectra, and some inter-relationships. The metabolites gave characteristic signals in their ^1H and ^{13}C n.m.r. spectra which were assigned to a secondary : tertiary epoxide, a secondary methyl group, at least two tertiary methyl groups, a γ-lactone attached to a tertiary carbon atom and, in the ^1H n.m.r. spectra, an isolated methylene AB doublet. These suggested a relationship with the major metabolite, alliacolide (1). A major peak in the mass spectra of alliacolide (1) and the new 4-hydroxy-metabolites (2), (5), (6), and (7) occurs at 193 a.m.u which corresponds to the fragment (10). Hence these metabolites must differ only in the pendant group at C-4.



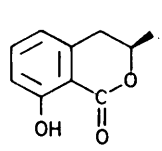
(1) R^1=Me, R^2=H
(2) R^1,R^2 = CH_2
(5) R^1=R^2=H
(6) R^1=Me, R^2=OH
(7) R^1 = CH_2OH, R^2=H



(3) R = CH_2OH
(9) R = Me



(8)



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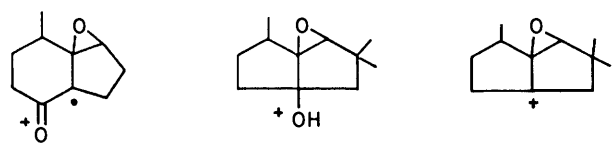
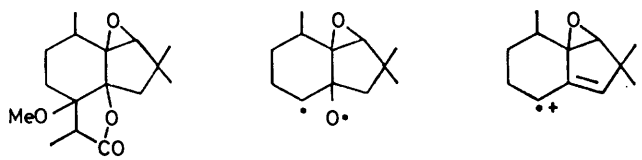


(10) (11) (12)



(13) (14) (15)

In addition to the spectroscopic data associated with the perhydroindane system, alliacolide II (2) contained a methylene-lactone (^1H n.m.r. δ 5.9 and 6.35; ^{13}C n.m.r. δ 168.8, 142.9, and 124.7 p.p.m.; $\nu_{max.}$ 1 775 and 1 680 cm^{-1}). The AB doublet assigned to the C-6 protons (δ 1.30 and 2.00) showed a similar downfield component to alliacolide indicating the same C-4 hydroxy-group stereochemistry. The isomeric metabolite (3) gave a ^1H n.m.r. signal (δ 4.43) characteristic of a primary alcohol attached to a fully substituted carbon atom. The ^{13}C n.m.r. spectrum contains signals associated with a tetra-substituted olefin. Comparison of their chemical

TABLE 1

^{1}H N.m.r. signals of alliacolide and its related compounds (in $CDCl_3$)

Hydrogen atom	Compound (1) [a]	(2)	(3)	(5) [b]	(6)	(7) [a]	(8)	(9) [a]	(13)
1	1.85	n.o. [c]	n.o.	n.o.	n.o.	1.83	n.o.	1.90	n.o.
6 [d]	1.29	1.30	1.20	1.47	1.50	1.29	n.o.	1.10	n.o.
	1.96	2.00	1.87	2.37	2.00	1.98		1.71	
8	3.22	3.20	3.25	3.25	3.22	3.23	3.25	3.32	3.22
10 [e]	1.14	1.20	1.00	1.18	1.18	1.20	1.30	0.81	1.25
11	2.69 [f]			2.75 [g]		2.91	n.o.		2.78 [f]
				3.15					
12	1.18	5.9	4.43		1.42	4.05	1.50	1.79	1.25
		6.35							
14 }	1.12	1.20	1.20	1.13	1.18	1.12	1.18	1.16	1.18
15 }	1.12	1.20	1.20	1.09	1.18	1.12	1.18	1.08	1.18

[a] Determined at 360 MHz. [b] In pyridine. [c] n.o. = Not observed. [d] $J_{6:6'}$ 14 Hz. [e] Doublet, J 7 Hz. [f] Quartet, J 7 Hz. [g] Doublets, J 17 Hz.

TABLE 2

^{13}C N.m.r. signals of alliacolide and its relatives (in $CDCl_3$; p.p.m. from Me_4Si)

Carbon atom	Compound (1)	(2)	(3)	(5) [a]	(6)	(7)	(8)	(9)	(13) [b]
1	31.4	31.5	31.4	32.4	31.5	31.4	31.6	29.9	31.5
2	25.3	26.3	23.1	27.0	25.3	25.5	26.7	22.6	23.0
3	28.2	38.6	35.3	32.4	28.9	28.9	19.7	34.9	25.7
4	77.2	76.5	170.4	75.2	76.6	77.0	31.6	166.4	81.2
5	92.6	95.1	94.7	96.3	94.1	93.1	90.6	93.9	93.6
6	40.8	41.7	43.8	42.1	42.6	40.8	46.8	43.8	41.0
7	38.5	39.2	41.4	39.1	38.6	38.6	38.6	41.1	38.6
8	68.2	67.1	67.8	67.1	67.3	68.1	69.5	68.1	67.6
9	68.6	69.6	68.2	69.1	68.6	68.3	67.9	72.1	68.1
10	24.3	24.4	24.2	24.9	24.2	24.3	24.3	24.6	24.5
11	45.0	142.9	127.0	44.4	74.4	51.7	42.5	124.4	44.6
12	7.6	124.7	54.8		17.4 [c]	57.6	9.6	8.7	10.0
13	176.1	168.8	173.6	174.2	174.9	174.5	178.0	173.2	176.0
14 }	23.9	24.0	23.1	24.2	24.1	23.9	23.8	23.1	23.1
15 }	17.4	19.4	19.2	19.2	18.0 [c]	17.5	17.0	19.2	18.3

[a] In [^{2}H]pyridine. [b] OMe 52.2. [c] These resonances may be interchanged.

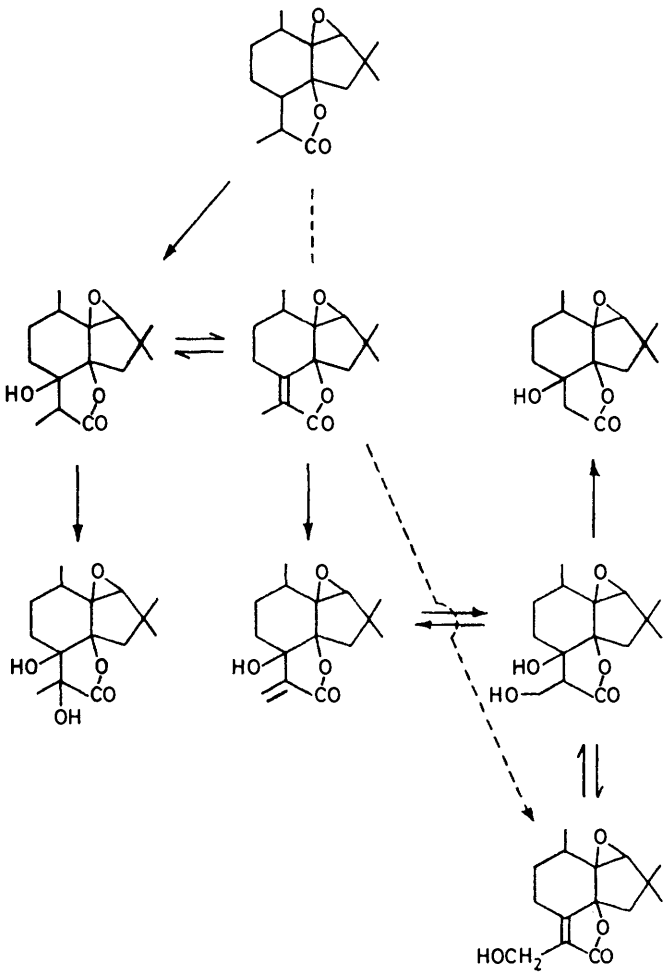
shifts with those of dehydroalliacolide (9) shows that the primary alcohol had replaced the olefinic methyl group. The olefinic-methyl proton-resonance of dehydroalliacolide (9) was also absent. 12-Noralliacolide (5) co-chromatographed with 12-hydroxydehydroalliacolide and its ^{1}H n.m.r. spectrum, whilst similar to that of alliacolide, lacks a methyl doublet and contains an additional AB quartet ($\delta_{pyridine}$ 2.75 and 3.15) which was assigned to a CH_2CO group. The C-6 AB quartet appeared at $\delta_{pyridine}$ 1.47 and 2.37 p.p.m. Like the other metabolites, the mass spectrum shows strong ions at 193, 182, and 165 a.m.u. corresponding to the fragments (10)—(12). The possibility cannot be discounted that this compound is an artefact arising from the retro-aldol cleavage of 12-hydroxyalliacolide.

Comparison of the ^{1}H and ^{13}C n.m.r. spectra of the 11- and 12-hydroxyalliacolides (6) and (7) with those of alliacolide (1) reveals the presence of an additional tertiary and a primary alcohol, respectively. In the case of the former metabolite, the 11-H signal is absent and the 12-H_3 signal, now a singlet, is shifted to lower field. In the case of the latter metabolite, the 11-H signal (δ 2.91) is coupled to the primary alcohol (δ 4.05) in accord with the proposed structure. These diols are co-related with dehydroalliacolide (9) and alliacolide II (2), respectively. Dehydration of alliacolide with phosphorus oxychloride in pyridine gave dehydroalliacolide (9) which, on osmylation, gave 11-hydroxyalliacolide (6), identical (i.r. and n.m.r. spectra) with the natural product. Molecular models show that osmylation must occur from the α-face of the molecule. This glycol was also oxidized with methanolic periodic acid to form a pyruvate ester [δ 2.40 (3 H, s)] consistent with a *cis*-relationship of the hydroxy-groups. During an attempt to prepare a derivative of 12-hydroxyalliacolide (7) with an optically active reagent, dehydration occurred in the presence of bromocamphorsulphonyl chloride to give alliacolide II (2), thus establishing a link between these two metabolites.

The absolute stereochemistry of alliacolide II (2) and alliacolide (1) was correlated biosynthetically. An interesting aspect of alliacolide biosynthesis [3] is that the 11-H is not labelled by mevalonate. Similar isopropyl groupings in, for example, the fungal metabolites avocettin and sativene carry a 5-mevalonoid hydrogen, originally at C-1 in farnesyl pyrophosphate.[6] A possible explanation of this was that dehydroalliacolide (9) was involved in the biosynthesis of alliacolide although we have, hitherto, been unable to detect its presence in the fungus. [^{14}C]Dehydroalliacolide (9) was prepared by the dehydration of [^{14}C]alliacolide, biosynthetically labelled from [2-^{14}C]mevalonate. The labelled dehydro-

alliacolide was incorporated by *Marasmius alliaceus* into alliacolide (1) to the extent of 32.7% and into alliacolide II (2) to the extent of 5.03%. In this context it is interesting to note that dehydroalliacolide (9) undergoes a facile nucleophilic addition of the methoxide ion at C-4 to generate a methyl ether (13). However, the stereochemistry of the adduct was not determined.

This biosynthetic transformation prompted a search for dehydroalliacolide amongst the metabolites. Trial experiments showed that it would co-chromatograph with mellein (4) which was also produced by the fungus. However, acetylation of the mellein fraction and careful further chromatography gave the saturated γ-lactone ($\nu_{max.}$ 1 770 cm^{-1}), alliacide (8). The third oxygen atom of this metabolite was present as an epoxide (δ 3.30).



SCHEME Relationship between the metabolites of *Marasmius alliaceus*

The mass spectrum shows fragments which corresponded to the loss of Me and CO and contains ions at 195, 194, 182, 177, and 165 a.m.u. to which the structures (14), (14 +H), (11), (15), and (12) are assigned, respectively. The ^1H and ^{13}C n.m.r. spectra are in accord with structure (8). *R*-(−)-Mellein (4) was identified by its ^{13}C n.m.r. spectrum and by the ^1H n.m.r. spectrum of the acetate.

A plausible biogenetic relationship between this group of metabolites in terms of the facile dehydration–hydration of methylene-lactones and the oxidation of dehydroalliacolide (9) is shown in the Scheme. In this context it is interesting to note that the methylene-lactone (2) and 12-hydroxydehydroalliacolide (3) show tumour inhibitory activity.[4]

EXPERIMENTAL

General experimental details have been described previously.[7] ^1H and ^{13}C N.m.r. data for compounds (1)—(3), (5)—(9), and (13) are given in Tables 1 and 2.

Isolation of the Metabolites.—*Marasmius alliaceus* (obtained from CBS, Baarn) was grown in shake culture (100 ml per flask) on a 3% malt medium (8 l) for 39 d. The mycelium was filtered off and the broth was acidified to pH 2 with dilute hydrochloric acid and then extracted with ethyl acetate. The organic phase was extracted with aqueous sodium hydrogencarbonate and aqueous sodium chloride, dried over sodium sulphate, and the solvent was then evaporated to afford a semi-crystalline gum. Preliminary chromatography on silica in chloroform–ethyl acetate–acetic acid (40 : 10 : 1) gave four main fractions (t.l.c. control). Fraction 1 contained mellein (4) and alliacide (8), fraction 2 contained alliacolide (1) and alliacolide II (2), fraction 3 contained 11-hydroxyalliacolide (6) and 12-noralliacolide (5), whilst fraction 4 contained 12-hydroxyalliacolide (7).

Alliacolide (1) (100 mg l^{-1}) was obtained from fraction 2 by repeated chromatography on silica and sublimation (140 °C, 0.3 mmHg). It crystallized from ethyl acetate as needles, m.p. 193 °C, $[\alpha]_D$ −33° (*c* 0.8, $CHCl_3$) (lit.,[1] 192—194 °C, $[\alpha]_D$ − 35°) (Found: C, 67.8; H, 8.3. Calc. for $C_{15}H_{22}O_4$: C, 67.6; H, 8.3%); $\nu_{max.}$ (CCl_4) 3 590, 3 450, and 1 785 cm^{-1}; *m/e* 266 (25%), 238, (15), 211 (11), 210 (11), 193 (100), 182 (46), 151 (26), 142 (42), and 125 (39). Alliacolide II (2) was conveniently separated from alliacolide (1) by dehydration of the latter. Fraction 2 was treated with phosphorus oxychloride (2 ml) in pyridine (5 ml) at 60 °C overnight. The mixture was cooled, poured onto ice, and extracted with ethyl acetate. The extract was washed thoroughly with dilute hydrochloric acid and aqueous sodium chloride, dried, and then evaporated to give a semi-crystalline residue which was purified by chromatography on silica. Elution with chloroform–ethyl acetate–acetic acid (40 : 10 : 1) gave dehydroalliacolide (9) (*vide infra*) (320 mg) which was further purified by sublimation at 110 °C (0.3 mmHg). Further elution gave *alliacolide II* (2) (43 mg) which was purified by sublimation at 140 °C (0.3 mmHg). It crystallized from ethyl acetate–light petroleum as needles, m.p. 156—158 °C, $[\alpha]_D$ +10° (*c* 0.95, $CHCl_3$) (Found: C, 68.3; H, 7.6. $C_{15}H_{20}O_4$ requires C, 68.2; H, 7.6%); $\nu_{max.}$ (CCl_4) 3 580, 3 450, 1 775, 1 680, 1 125, and 900 cm^{-1}; *m/e* 264 (30%), 249 (29), 247 (20), 236 (45), 193 (70), 151 (75), and 83 (100).

Fraction 3 was further purified by preparative thin layer chromatography on silica in chloroform–methanol (20 : 1) to afford (a) 11-*hydroxyalliacolide* (6) (75 mg), which crystallized from ethyl acetate in polymorphic forms, m.p. 199—201.5 and 228—229 °C (identical n.m.r. spectra); $[\alpha]_D$ −8.6° (*c* 0.56, $CHCl_3$) (Found: C, 63.7; H, 7.75. $C_{15}H_{22}O_5$ requires C, 63.8; H, 7.85%); $\nu_{max.}$ (Nujol) 3 470, 3 350, and 1 755 cm^{-1}; *m/e* 282 (10%), 211 (16), 205 (7), 193 (100),

179 (18), 165 (20), 151 (26), 147 (15), and 143 (35), and (b) 12-*hydroxydehydroalliacolide* (3) (34 mg), m.p. 95 °C (broad) (Found: C, 67.8; H, 7.3. $C_{15}H_{20}O_4$ requires C, 68.2; H, 7.6%); $\nu_{max.}$ (Nujol) 3 520, 3 440, 1 735, and 1 665 cm^{-1}. On some occasions 12-*noralliacolide* (5) was also isolated from this fraction. It crystallized as needles, m.p. 198 °C (Found: C, 66.6; H, 8.4%; M^+ 252.135. $C_{14}H_{20}O_4$ requires C, 66.6; H, 8.0%; M^+ 252.136); $\nu_{max.}$ 3 410 and 1 755 cm^{-1}; *m/e* 237 (11%), 210 (10), 193 (25), 182 (37), 167 (18), 151 (19), 142 (46), 140 (42), 125 (23), 113 (25), 109 (18), 97 (31), and 83 (100).

Fraction 4 was further purified by preparative thin layer chromatography and sublimation as above to afford 12-hydroxyalliacolide (7) (198 mg) which crystallized from ethyl acetate–light petroleum as needles, m.p. 191—193 °C, $[\alpha]_D$ −32° (*c* 0.47, $CHCl_3$) (Found: C, 63.7; H, 7.8. $C_{15}H_{22}O_5$ requires C, 63.8; H, 7.85%); $\nu_{max.}$ (Nujol) 3 470 and 1 745 cm^{-1}; *m/e* 282 (1%), 267 (5), 249 (5), 211 (20), and 193 (100).

Fraction 1 (from a total of 18 l of broth) was treated with acetic anhydride (5 ml) in pyridine (10 ml) at room temperature for 3 d. The product was poured onto ice and extracted with ethyl acetate. The extract was washed with dilute hydrochloric acid, dried over sodium sulphate, and the solvent was then evaporated. The residue was chromatographed on silica in chloroform–ethyl acetate (4 : 1) to afford *R*-(−)-mellein acetate (1.2 g), m.p. 126 °C, $[\alpha]_D$ −14.5° (lit.,[5] 126—127 °C); δ 1.45 (3 H, d, *J* 7 Hz), 2.30 (3 H, s), 2.90 (2 H, d, *J* 7 Hz), 4.6 (1 H, sextet, *J* 7 Hz), 7.05 (1 H, d, *J* 8 Hz), 7.15 (1 H, d, *J* 8 Hz), and 7.55 (1 H, t, *J* 8 Hz). Mellein (4) had m.p. 56 °C (lit.,[5] 58 °C); δ(^{13}C) 20.8 (q), 34.6 (t), 76.0 (s), 108 (s), 116 (d), 118 (d), 136 (d), 139.5 (s), 162.2 (s), and 170 (s). A slightly less polar fraction was rechromatographed on silica. Elution with 10% ethyl acetate–light petroleum gave *alliacide* (8) (20 mg) which crystallized from light petroleum as needles, m.p. 168—171 °C (Found: C, 72.1; H, 8.7%; M^+ 250.1571. $C_{15}H_{22}O_3$ requires C, 72.0; H, 8.9%; M^+ 250.1569); $\nu_{max.}$ 1 770 cm^{-1}; *m/e* 250 (27%), 235 (25), 222 (12), 195 (50), 194 (36), 193 (21), 182 (36), 180 (34), 179 (32), 177 (69), 165 (100), 125 (55), and 97 (63).

Dehydroalliacolide (9).—Alliacolide (1) (400 mg) in dry pyridine (5 ml) was treated with phosphorus oxychloride (2 ml) at 60 °C overnight. The product was poured onto ice and recovered in ethyl acetate. The extracts were washed successively with dilute hydrochloric acid, aqueous sodium hydrogencarbonate, and water, and then dried and evaporated to afford dehydroalliacolide (9) (343 mg) which was purified by sublimation at 80 °C (0.3 mmHg). *Dehydroalliacolide* (9) crystallized from light petroleum as needles, m.p. 154—156 °C, $[\alpha]_D$ −1.4° (*c* 0.5, $CHCl_3$) (Found: C, 72.6; H, 8.1. $C_{15}H_{20}O_3$ requires C, 72.55; H, 8.1%); $\nu_{max.}$ (Nujol) 1 747 and 1 668 cm^{-1}; *m/e* 248 (15%), 180 (35), 151 (10), and 83 (100).

Preparation of 11-*Hydroxyalliacolide* (6).—Dehydroalliacolide (9) (115 mg) in tetrahydrofuran (10 ml) was treated with osmium tetraoxide (5 mg) followed by sodium metaperiodate (148 mg) in portions during 40 min. The mixture was stirred overnight at room temperature. The solvent was evaporated and the residue was extracted with ethyl acetate. The extract was washed with aqueous sodium chloride, dried, and then evaporated to give 11-hydroxyalliacolide (6) (110 mg) which crystallized as needles, m.p. 228—229 °C, identical (n.m.r.) with a sample of the natural product.

Oxidation of 11-*Hydroxyalliacolide* (6).—The diol (6) (100 mg) was treated with an excess of periodic acid in methanol (10 ml) overnight. The solution was poured into water and the product was recovered in ethyl acetate to afford the *pyruvate ester* (70 mg) which crystallized from ethyl acetate–light petroleum as needles, m.p. 121—124 °C (Found: C, 64.6; H, 7.1. $C_{15}H_{20}O_5$ requires C, 64.3; H, 7.2%); $\nu_{max.}$ 1 740 and 1 720 cm^{-1}; δ 1.12 (6 H, s), 1.35 (3 H, d, *J* 7 Hz), 2.40 (3 H, s), and 3.3 (1 H, s).

Alliacolide Methyl Ether (13).—Dehydroalliacolide (9) (50 mg) in methanol (2 ml) was treated with a solution of sodium (40 mg) in methanol (3 ml). Acetic acid (1 ml) and water (10 ml) were added and the methanol was removed under reduced pressure. The product was recovered in ethyl acetate to afford the *methyl ether* (50 mg) as an oil, b.p. 70 °C at 0.3 mmHg, $[\alpha]_D$ −34° (*c* 0.9, $CHCl_3$) (Found: C, 68.7; H, 8.6. $C_{16}H_{24}O_4$ requires C, 68.5; H, 8.6%).

Dehydration of 12-*Hydroxyalliacolide* (7).—12-Hydroxyalliacolide (7) (20 mg) in pyridine (0.5 ml) was treated with α-bromocamphor-π-sulphonyl chloride (35 mg) overnight. Water was added and the product was recovered in ethyl acetate to afford alliacolide II (2) (8 mg) which was identified by its ^{1}H n.m.r. spectrum. It was further purified by sublimation (90 °C, 0.3 mmHg) and recrystallized from ethyl acetate–light petroleum as needles, m.p. 156—157 °C.

Incorporation of Dehydroalliacolide (9) *into Alliacolide* (1).—Dehydroalliacolide (50 mg, ^{14}C 1 356 dpm mg^{-1}; ^{3}H : ^{14}C, 15.09 : 1, prepared from [5-$^3H_2$,2-^{14}C]mevalonic acid and dehydration of the alliacolide as above) in ethanol (2.5 ml) was distributed between five flasks (100 ml each) of *Marasmius alliaceus* 17 d after inoculation. The fermentation was harvested after a further 26 d and the metabolites were isolated as above to afford the recovered dehydroalliacolide (9) (8 mg, ^{14}C 1 319 dpm mg^{-1}; ^{3}H : ^{14}C 15.7 : 1; 15.6% recovery), alliacolide II (2) (2.7 mg; ^{14}C 1 262 dpm mg^{-1}; ^{3}H : ^{14}C, 15.5 : 1; 5.03% incorporation), and alliacolide (1) (18 mg; ^{14}C 1 231 dpm mg^{-1}, ^{3}H : ^{14}C, 15.2 : 1, 32.7% incorporation).

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