# Studies in Terpenoid Biosynthesis. Part 27.[1] The Acetate and Mevalonate Labelling Patterns of the Sesquiterpenoid, Alliacolide

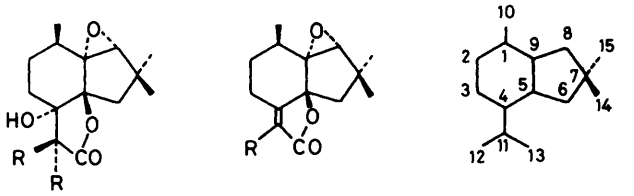
**A. Peter W. Bradshaw and James R. Hanson** *
*The School of Molecular Sciences, University of Sussex, Brighton, Sussex BN1 9QJ*
**Ian H. Sadler**
*Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ*

The enrichment and labelling patterns of alliacolide biosynthesized by *Marasmius alliaceus* from [1-$^{13}$C]-acetate and [1,2-$^{13}C_2$]acetate, have been used to define the isoprene units. The sites of labelling of alliacolide by [2-$^{2}H_3$]acetate and by [2-$^{2}H_2$]-, [4-$^{2}H_2$]-, and [5-$^{2}H_2$]-mevalonates have been determined. The occurrence of hydrogen rearrangements to C-1 and from C-5 to C-6 during the biosynthesis has been demonstrated by $^{2}$H n.m.r. experiments.

Alliacolide (1) [2] and its relatives (2)—(6) [3,4] are metabolites of the Basidiomycete, *Marasmius alliaceus*. Alliacols A (2) and B(6) have cytotoxic properties [4] and are inhibitors of DNA synthesis. The sesquiterpenoid alliacane skeleton (7) contains a number of features of biosynthetic interest. Firstly there are several plausible ways exemplified by (8) to (10), by which this carbon skeleton may be derived from three isoprene units each involving either a non-head to tail linkage or a rearrangement. Secondly whilst the substitution pattern of the six-membered ring is reminiscent of that of a cadinane [*cf.* the biosynthesis of the fungal metabolite, avocettin (11) [5]], the *gem*-dimethyl substitution of the five-membered ring suggests a relationship to the illudanes [1,6] [*cf.* illudin M, (12)] which are also metabolites of the Basidiomycetes. Thus, there are two possible starting points for incorporating the sesquiterpenoid farnesyl pyrophosphate chain both of which require a rearrangement to generate the alliacane skeleton. Thirdly, the structure contains secondary methyl groups which, in terpenoid biosynthesis, are often derived by hydrogen rearrangements. Through the courtesy of Dr. T. G. Halsall and Dr. V. Thaller (Oxford), we have begun a study of the biosynthesis of alliacolide as part of our programme to seek common features of fungal sesquiterpenoid biosynthesis. The results of feeding $^{2}$H and $^{13}$C labelled acetate and $^{2}$H, $^{3}$H, and $^{14}$C labelled mevalonates to *Marasmius alliaceus* form the subject of this paper.[7]

The optimum time (14 days) of feeding substrates and period (21 days) of further incubation of *M. alliaceus* for incorporation into alliacolide were established by trial experiments using sodium [1-$^{14}$C]acetate. The $^{13}$C n.m.r. signals of alliacolide (1) have been assigned previously in the course of our structural work on the related metabolites.[3] Sodium [1-$^{13}$C]-acetate was fed in one pulse on day 14 to *M. alliaceus* whilst sodium [1,2-$^{13}C_2$]acetate was diluted and fed in four aliquots on days 14, 17, 19, and 21 to the fungus. The former regime would favour the multiple labelling of prenyl precursors which could, by the generation of $^{13}$C–$^{13}$C couplings, be used to detect rearrangements or abnormal prenyl couplings. The centres which were enriched and coupled are given in Table 1.

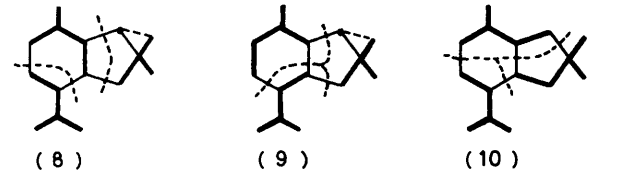
The acetate labelling pattern derived from these results is shown in Figure 1. This pattern suggests that the structure is made up of a combination of isoprene units as shown in (10). The enrichment of the alliacolide (7.35%, calculated on the basis of the C-14 incorporation) derived from the [1-$^{13}$C]-acetate was sufficiently high to enable the induced coupling ($J$ 41 Hz) between C-6 and C-7 which arose from the multiple enrichments of the prenyl chain, to be detected. This also is in accordance with the combination of isoprene units shown in (10). The carbonyl group of the lactone ring (C-13) is coupled



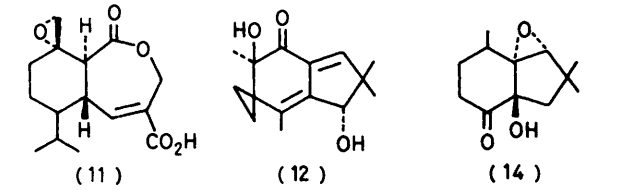
(1) $R^1$ = Me, $R^2$ = H
(2) $R^1$, $R^2$ = CH
(3) $R^1$, $R^2$ = $H_2$
(4) $R^1$ = Me, $R^2$ = H
(5) $R^1$ = $HOCH_2$, $R^2$ = H

(6) R = $CH_2OH$
(13) R = Me

(7)



(8) (9) (10)



(11) (12) (14)

to C-11, *i.e.* the carbonyl group must originate from the methyl group of mevalonic acid. Consequently the lactone ring may not have its origin in the cleavage of the isoprene chain. This result could suggest that the isopropyl residue (C-11 to C-13) rather than the *gem*-dimethyl group (C-14 to C-15) marks the starting point of the prenyl (farnesyl) chain.

In order to distinguish between these possibilities, [1,2-$^{13}C_2$,2-$^{14}$C]farnesyl pyrophosphate was fed to *M. alliaceus*. Although the alliacolide showed an incorporation of *ca.* 0.1% carbon-14, no unique coupling pattern was observed. At high resolution a number of centres showed a low intensity coupling indicating prior degradation of the farnesyl pyrophosphate to acetate followed by resynthesis and consequent scrambling of the label. Repetition of the experiment in the presence of a phosphatase inhibitor, potassium fluoride, gave no improvement. Although at present this precludes a definitive conclusion on the manner in which the isoprene units are linked

**Table 1.** Incubation of $^{13}C$ labelled acetates with *M. alliaceus*

| Carbon atom | [1-$^{13}$C]Acetate experiment: Alliacolide δ | Alliacolide Enrichment | 12-Hydroxy-alliacolide δ | 12-Hydroxy-alliacolide Enrichment | [1,2-$^{13}$C]Acetate experiment alliacolide: Coupling constant (Hz) |
|---|---|---|---|---|---|
| 1 | 31.4 | 8.36 | 31.4 | 20.7 | 36.6 |
| 2 | 25.3 | 1.00 | 25.5 | 1.12 | en [d] |
| 3 | 28.2 | 9.42 | 28.9 | 19.5 | 36.6 |
| 4 | 77.2 | 1.03 | 77.1 | 0.78 | 36.6 |
| 5 | 92.6 | 8.51 | 93.1 | 21.4 | 38.1 |
| 6 | 40.8 | 0.99 | 40.8 | 1.21 | 38.1 |
| 7 | 38.5 | 7.59 [a] | 38.6 | 14.0 [b] | 35.1 |
| 8 | 68.2 | 4.87 [a] | 68.1 | 14.7 [b] | 10.7 |
| 9 | 68.6 | 1.32 | 68.3 | *c* | 10.7 |
| 10 | 24.3 | 1.14 | 24.3 | 1.31 | 36.6 |
| 11 | 45.0 | 8.89 | 51.7 | 18.9 | 50.4 |
| 12 | 7.6 | 0.96 | 57.6 | 0.65 | en |
| 13 | 176.1 | 0.63 | 174.5 | 1.53 | 50.4 |
| 14 | 17.4 | 1.06 | 17.5 | 1.24 | 35.1 |
| 15 | 23.9 | 0.94 | 24.0 | 1.24 | en |

The enrichment = $\frac{\text{enriched sample peak height}}{\text{natural abundance sample peak height}} \times$ normalization factor

Normalization factor = $\frac{\text{total peak heights unlabelled signals nat. abund. spectrum}}{\text{total peak heights unlabelled signals enriched spectrum}}$

[a] Coupled, *J* 41.2 Hz. [b] Coupled, *J* 41.2 Hz. [c] Signal not cleanly resolved. [d] Enriched.

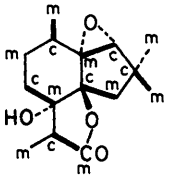



**Figure.** The acetate labelling pattern of alliacolide

together, nevertheless some biosynthetic conclusions can be drawn from the mevalonoid hydrogen labelling pattern.

The numbers of mevalonoid hydrogen atoms which were incorporated into alliacolide (1) by *M. alliaceus* were established by conventional $^{3}H$ : $^{14}C$-ratio studies and are summarized in Table 2.

Two of the three centres (C-4 and C-9) which, on the basis of the division into isoprene units established by the $^{13}C$ studies, would be expected to be labelled by 4(*R*)-[4-$^{3}$H]-mevalonate, are fully substituted and hence to account for the incorporation of two labels, one *pro*-4(*R*)-mevalonoid hydrogen atom must be involved in a rearrangement. Secondly one of the centres (C-5) which would be expected to be labelled by a [5-$^{3}$H]mevalonate is also fully substituted and another (C-8) bears only one hydrogen atom. In order to account for the incorporation of the fourth [5-$^{3}$H]mevalonoid label, it must also be involved in a rearrangement.

Bearing in mind the location of a [5-$^{3}$H]mevalonoid hydrogen atom in the isopropyl group of avocettin (1),[5] we expected that the [5-$^{3}$H]mevalonoid hydrogen atom involved in the rearrangement might be located at C-11 in alliacolide (1). However, simple dehydration of each of the MVA labelled samples to give dehydroalliacolide (13) (see Table 2) showed, surprisingly, that there was no 2-, 4- or 5-mevalonoid label

**Table 2.** Incorporation of $^{3}H$ : $^{14}C$ mevalonates into alliacolide and dehydroalliacolide

| | Mevalonate: 2-$^{3}H_2$,2-$^{14}$C | 4(*R*)-4-$^{3}$H,2-$^{14}$C | 5-$^{3}H_2$,2-$^{14}$C |
|---|---|---|---|
| 3H : $^{14}$C Ratio in mevalonate | 9.62 : 1 | 12.98 : 1 | 22.6 : 1 |
| Atom ratio | 6 : 3 | 3 : 3 | 6 : 3 |
| Quantity $^{14}$C fed (μC) | 11.5 | 11.8 | 13.15 |
| 3H : $^{14}$C ratio in (1) | 8.33 : 1 | 8.22 : 1 | 14.93 : 1 |
| Atom ratio | 5.2 : 3 | 1.90 : 3 | 3.96 : 3 |
| % Incorporation | 1.29 | 0.99 | 1.59 |
| $^{3}H$ : $^{14}C$ Ratio in (13) | 8.44 : 1 | 8.14 : 1 | 15.09 : 1 |
| Atom ratio | 5.3 : 3 | 1.9 : 3 | 4 : 3 |

at C-11. Subsequently we were able to show that dehydroalliacolide (13) is a very effective precursor of alliacolide (1) (32.7% incorporation) and alliacolide II (2) (5.03%) suggesting that the hydroxy-group at C-4 may be the result of a hydration rather than a hydroxylation process. The alliacolide ($^{3}H$ : $^{14}C$, 8.4 : 1; atom ratio 5.25 $^{3}H$ : 3 $^{14}C$) labelled from [2-$^{3}H_2$,2-$^{14}$C]-MVA, was degraded *via* dehydroalliacolide (13) (specific activity 277016 d.p.m./mmol) to the hydroxy-ketone (14) ($^{3}H$ : $^{14}C$, 8.85 : 1; atom ratio 3.68 $^{3}H$ : 2 $^{14}C$, specific activity 173 250 d.p.m./mmol $^{14}C$) with the loss of one third of the carbon-14 specific activity thus establishing that C-12 bore two [2-$^{3}$H]mevalonoid hydrogen labels.

The remaining sites of the labels were determined using [2-$^{2}H_3$]acetate and the [2-$^{2}H_2$]-, [4-$^{2}H_2$]-, and [5-$^{2}H_2$]-mevalonates with the aid of deuterium n.m.r. spectroscopy. The proton signals (determined at 360 MHz) on ring A of alliacolide were assigned as follows. Proton (1-H) appeared as a sextet (δ 1.85) coupled to the methyl group (10-H) (δ 1.14, *J* 7.5 Hz). This sextet was also coupled to multiplets (2-H) at δ 2.02 and 1.63 which were, in turn, coupled to partially obscured multiplets (3-H) at δ 1.95 and 1.28. Superimposed on these latter resonances were two geminally coupled doublets (6-H) (δ 1.96 and 1.29, *J* 14 Hz). In a shift reagent experiment with Eu(fod)$_3$ the lower field of these two resonances showed the greater shift and was thus *cis* to the epoxide and hydroxy-groups. The 11-H signal appeared, as anticipated, as a quartet (δ 2.69, *J* 7.2 Hz) whilst the 8-H signal was a singlet (δ 3.23). The lower field of the two methyl group doublets (δ 1.18) showed the greater shift with Eu(fod)$_3$ and was assigned to 12-H. In the case of dehydroalliacolide (13) the proton resonances were better resolved and were assigned as follows. The signal for 1-H appeared at δ 1.90 as a pentuplet (*J* 7.5 Hz) with an additional small coupling ($J \sim 1$ Hz). Decoupling experiments established that this signal was coupled to the methyl signal (δ 0.81, *J* 7.5 Hz) and to signals (2-H) at δ 1.33 ($J \sim 1$ Hz) and δ 2.42 (*J* 7.5 Hz). Irradiation at δ 1.33 and at 2.42 established that the allylic protons (3-H) were multiplets at δ 2.55 and 2.65. Irradiation at the latter positions confirmed the couplings to 2-H. The 6-H signals appeared as doublets (*J* 12.5 Hz) at δ 1.71 and 1.10.

Although the deuterium n.m.r. signals (determined at 55.28 MHz) for alliacolide were not particularly well-resolved except in the case of material labelled by [5-$^{2}H_2$]mevalonate, a satisfactory set of $^{2}H$ n.m.r. spectra were obtained for the degradation product, dehydroalliacolide (13). These results, which were superimposed on the natural abundance spectrum, are summarized in Table 3.

The following conclusions may be drawn from these results. The hydrogen atom at C-1 is derived from the 4-position of mevalonate and thus alliacolide represents a further example

**Table 3.** Labelling of dehydroalliacolide (13) by $^2H$ labelled substrates: resonances in p.p.m. from $Me_4Si$; approx. relative integral in brackets

| Carbon atom | $^1H$ Signal | Substrate | | | |
|---|---|---|---|---|---|
| | | $[^2H_3]$-$CH_3CO_2Na$ | [2-$^2H_2$]-MVA [b] | [4-$^2H_2$]-MVA [c] | [5-$^2H_2$]-MVA [d,e] |
| 1 | 1.90 | 1.93 (0.5) | | 1.83 (0.8) | |
| 2 | 1.33 | 1.37 (1) | 1.34 (1) | | |
| | 2.42 | 2.44 (1) | 2.42 (1) | | |
| 3 | 2.55 | | | | 2.62 (1.8) |
| | 2.65 | | | | |
| 6 | 1.71 | | | | 1.71 (1.1) |
| | 1.10 | 1.10 [a] | | 1.03 (1) | |
| 8 | 3.32 | | | | 3.34 (1) |
| 10 | 0.81 | 0.83 (3) | | | |
| 12 | 1.79 | 1.82 (2) | 1.78 (1.7) | | |
| 14 | 1.08 | 1.10 [a] | | | |
| 15 | 1.16 | 1.20 (2) | 1.17 (2.3) | | |
| Enrichment % | | 0.35 | 0.35 | 0.48 | 0.25 |

[a] Total relative integral 3.8. [b] Small signals (rel. int. *ca.* 0.3) were also observed in the methyl signals at 0.83 and 1.10 possibly due to some degradation and resynthesis from acetate. [c] Small signals (rel. int. *ca.* 0.3) were also observed at 0.8, 1.12(sh), 1.3, 1.7, and 2.4. [d] The alliacolide showed $^2H$ signals at 1.91 (2.2) (3-H and 6-H), 1.58 (1) (3-H), and 3.21 (1) (8-H). [e] The dehydroalliacolide from the [5-$^2H_2$,4-$^{13}C$]-MVA experiment showed signals at 3.36 (1), 2.64 (2), 1.73 (doublet, *J* 20.9 Hz), and small signals at 1.81, 1.20, 1.13, and 0.85.

of a terpenoid in which a secondary methyl group is generated as a result of a hydrogen rearrangement during the biosynthesis. The hydrogen atoms 2-H, 3-H, one of those at 6-H, and 8-H are derived, as anticipated from the Figure, from the 2-, 5-, 4-, and 5-positions of mevalonate respectively. However the lower field (δ 1.71) of the two 6-H signals was enriched by a 5-labelled mevalonate indicative of a hydrogen rearrangement. This rearrangement may involve a 1,2-shift (C-5 to C-6) or a 1,3-shift (C-8 to C-6). The former occurs within an isoprene unit whilst the latter is between two isoprene units. These may be distinguished by the use of [5-$^2H_2$,4-$^{13}C$]mevalonate. In the case of a 1,2-shift a [$^2H$ : $^{13}C$]-coupling will be generated since the rearrangement occurs within an isoprene unit. However in the case of a 1,3-shift, provided there is sufficient dilution, the rearrangement will occur between labelled and unlabelled isoprene units and no such coupling will result. Since carbon-13 has a spin of ½ and deuterium has a spin of 1, such a coupling is more easily observed in the deuterium n.m.r. spectrum. In practice dehydroalliacolide (13) derived from [5-$^2H_2$,4-$^{13}C$]mevalonate showed a doublet (*J* 21 Hz) in the $^2H$ n.m.r. spectrum in place of a singlet at δ 1.71 in accordance with a 1,2-shift rather than a 1,3-shift. In agreement with the $^3H$ results, the H-11 signal in alliacolide derived from both [5-$^2H_2$]- and [5-$^2H_2$,4-$^{13}C$]-MVA was not enriched. Whilst it is possible to write a number of plausible overall biosynthetic schemes which would accommodate these $^{13}C$ and $^2H$ labelling patterns, the absence of experimental evidence for the manner in which the $C_{15}$ chain is folded precludes a definitive conclusion in favour of any one scheme.

## Experimental

General experimental details have been described previously.[8] N.m.r. spectra were determined in chloroform or deuteriochloroform; $^{13}C$ n.m.r. spectra were determined at 25 MHz on a JEOL PFT-100; $^2H$ and highfield $^1H$ n.m.r. spectra were determined on a Bruker WH 360 at 55.58 and 360 MHz respectively.

*General Fermentation Conditions.—Marasmius alliaceus* (obtained from CBS, Baarn and Dr. V. Thaller, Oxford) was grown in shake culture (100 ml medium per flask) on a 3% malt medium for 14 days. The substrates were then added (see Table 4). The mevalonates were treated with 1 equiv. of 1M-potassium hydroxide, taken up in ethanol (5 ml), and diluted to 20 ml with distilled water. After the substrates were added, the cultures were allowed to grow for a further 21 days. 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 hydrogen carbonate and aqueous sodium chloride, dried over sodium sulphate, and the solvent evaporated to afford a semicrystalline gum. The alliacolide was isolated by preparative layer chromatography on silica in chloroform–ethyl acetate–acetic acid (40 : 10 : 1) followed by sublimation (140 °C/0.3 mmHg). It was crystallized from ethyl acetate as needles, m.p. 193 °C, $[\alpha]_D$ −33° (*c* 0.8 in $CHCl_3$) (lit.,[2] m.p. 192—194 °C, $[\alpha]_D$ −35°). The yields obtained in the labelled fermentations are given in Table 4.

*Preparation of Dehydroalliacolide* (13).—Alliacolide (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 extract was washed with dilute hydrochloric acid, aqueous sodium hydrogen carbonate, water, and then dried over sodium sulphate. The solvent was evaporated to afford dehydroalliacolide (340 mg) which was further purified by sublimation at 80 °C (0.3 mmHg). Dehydroalliacolide (13) crystallized from light petroleum as needles, m.p. 154—156 °C (lit.,[3] 154—156 °C).

*Oxidation of Dehydroalliacolide to the Hydroxy-ketone* (14). —Sodium periodate (5.3 g) and potassium permanganate (28 mg) were dissolved in water (250 ml). Dehydroalliacolide (100 mg, 1 036 d.p.m. $mg^{-1}$ $^{14}C$, $^3H$ : $^{14}C$ 8.94 : 1) in t-butyl alcohol (15 ml) was added to a mixture of this solution (25 ml), t-butyl alcohol (20 ml), water (90 ml), and potassium carbonate (100 mg) during 1.5 h. The mixture was then stirred at room temperature for a further 2 h. Aqueous sodium hydrogen sulphite was then added, and the solution was acidified and extracted with ethyl acetate. The solvent was dried over sodium sulphate and evaporated to afford a gum which was chromatographed on silica. Elution with chloroform–ethyl acetate–acetic acid (40 : 10 : 1) gave the hydroxy-ketone (14) (30 mg, 825 d.p.m. $mg^{-1}$ $^3H$ : $^{14}C$, 8.85 : 1) which crystallized from ether as needles, m.p. 111—114 °C (lit.,[3] 111—114 °C).

**Table 4.** Feeding experiments to *Marasmius alliaceus*

| Substrate | Amount fed | Volume of fermentation (litres) | Mass of alliacolide (mg) | $^{14}C$ Incorp. d.p.m. $mg^{-1}$ | % Incorp. |
|---|---|---|---|---|---|
| Sodium [1-$^{13}$C]acetate | 1.4 g, 20 μC | 1.4 | 220 | 6 786 | 3.53 |
| | | | 75 [a] | 11 359 | 1.92 |
| Sodium [1,2-$^{13}C_2$]acetate | 300 mg, 20 μC | 1.4 | 266 | 11 519 | 6.9 |
| [2-$^{3}H_2$,2-$^{14}$C]-MVA | 11.5 μC $^{14}$C | 1.4 | 317 | 1 035 | 1.29 |
| [4(*R*)-4-$^{3}$H,2-$^{14}$C]-MVA | 11.8 μC $^{14}$C | 1.4 | 230 | 1 126 | 0.99 |
| [5-$^{3}H_2$,2-$^{14}$C]-MVA | 13.15 μC $^{14}$C | 1.4 | 378 | 1 231 | 1.59 |
| Sodium [2-$^{2}H_3$,2-$^{14}$C]acetate | 2.3 g, 47.2 μC [b] | 8 [c] | 311 [d] | 9 380 | 2.78 |
| [2-$^{2}H_2$,2-$^{14}$C]-MVA | 400 mg, 26.6 μC | 5 | 823 | 778 | 1.08 |
| [4-$^{2}H_2$,2-$^{14}$C]-MVA | 430 mg, 25 μC | 4.2 | 982 | 913 | 1.63 |
| [5-$^{2}H_2$,2-$^{14}$C]-MVA | 380 mg, 25 μC | 4.2 | 1 033 | 543 | 1.02 |
| [5-$^{2}H_2$,4-$^{13}$C]-MVA | 400 mg | 5 | 103 [d] | *f* | *f* |

[a] 12-Hydroxyalliacolide. [b] As [$^{2}H_4$]acetic acid, neutralized with 1M-sodium hydroxide and evaporated to dryness. [c] Sodium acetate was dissolved in water (200 ml) and distributed in 5 aliquots over the period 14—28 days from inoculation. [d] Converted directly into dehydroalliacolide. [e] Distributed in two portions 14 and 21 days from inoculation. [f] No $^{14}$C result.

*Incubation of Dehydroalliacolide with* Marasmius alliaceus. —Dehydroalliacolide (50 mg) (1 425 d.p.m. $mg^{-1}$ $^{14}$C, prepared biosynthetically from [2-$^{14}$C]-MVA and dehydration of the resultant alliacolide) was fed to 5 flasks (100 ml medium) of *M. alliaceus* 14 days after inoculation. The fermentation was harvested after a further 21 days. The metabolites were recovered as above and separated by preparative layer chromatography on silica in chloroform–ethyl acetate–acetic acid (40 : 10 : 1) to afford dehydroalliacolide (8 mg) (1 319 d.p.m. $mg^{-1}$, $^{14}$C), alliacolide (18 mg) (1 232 d.p.m. $mg^{-1}$) and alliacolide II (2.7 mg, 1 262 d.p.m. $mg^{-1}$, $^{14}$C).

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