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PREPARATION OF LABELLED TERPENYL PYROPHOSPHATES USING EXTRACTS OF BARLEY SEED EMBRYOS

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Abstract—A cell-free system derived from seed embryos of barley (*Hordeum vulgare* cv. Zephyr) grain has been used to prepare substrate quantities of radioactively-labelled C_5–C_{20} intermediates of terpenoid biosynthesis. The purification and characterization of high specific activity all-*trans* farnesyl-[4, 8, 12-^{3}H] and all-*trans* geranylgeranyl-[4, 8, 12, 16-^{3}H] pyrophosphates, suitable for use in studies of sterol and carotenoid biosynthesis, are described in detail. The effects of the plant growth retardant AMO 1618 on the system are reported.

INTRODUCTION

It is well known that C_5–C_{20} terpenyl pyrophosphates are intermediates in the biosynthesis of higher terpenoids such as the gibberellins, squalene and sterols, carotenoids and polyprenols. Detailed studies of the later stages of the formation of these higher terpenoids require the provision of substrate quantities of the appropriate terpenyl pyrophosphates, such as farnesyl pyrophosphate (FPP, C_{15}) and geranylgeranyl pyrophosphate (GGPP, C_{20}), which have high specific radioactivities (^{14}C and/or ^{3}H). Although synthetic methods have been used in the preparation of such substrates [1], biosynthetic methods have the advantages that stereospecifically-labelled species may more readily be obtained and that more than one compound (e.g. both FPP and GGPP) may be prepared in the same incubation and these therefore have the same specific radioactivity (per isoprenoid unit). To date, terpenyl pyrophosphates have been prepared using enzyme systems derived from rat liver [2–5], pig liver [5–7], tomato fruit plastids [8], carrot root [6], immature seeds of the wild cucumber *Echinocystis macrocarpa* Greene [9], pumpkin seeds [10], baker's yeast [11, 12] and the carotenogenic bacterium *Micrococcus lysodeikticus* [13].

This report describes the production of substrate amounts of labelled FPP and GGPP using an enzyme preparation from barley seed embryos; the system has a number of advantages over others described previously. In common with some of the others, it employs the plant growth retardant AMO 1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine carboxylate methochloride) which has been shown to block the cyclization of GGPP to kaurene (and thus any potential gibberellin formation) in *E. macrocarpa* [14] and in *Fusarium moniliforme* Sheld [15].

In addition, a complete series of terpenyl intermediates has been characterized in the barley embryo system; these compounds conform to the normal [16] terpenyl pyrophosphate pathway of terpenoid biosynthesis.

RESULTS

The barley embryo system was incubated with either DL-mevalonic acid (MVA)-[2-^{14}C] or DL-MVA-[2-^{3}H] as substrate in phosphate buffer and

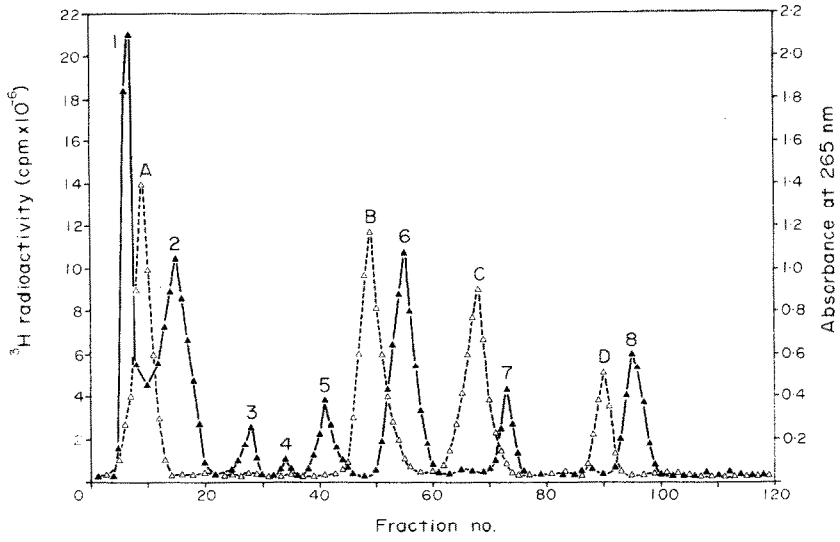

Fig. 1. Separation of incubation mixture components on DE 52 cellulose column developed with 0–0·2 M $(NH_4)_2CO_3$ (5 ml fractions). Column effluent was monitored for 3H-radioactivity (▲——▲) and absorbance at 265 nm (△- - - -△). Alternate baseline points have been omitted. 1, MVA; 2, IP; 3, FP; 4, MVAP; 5, IPP; 6, FPP; 7, MVAPP; 8, CGPP; A, adenosine; B, AMP; C, ADP; D, ATP.

in the presence of the appropriate cofactors and AMO 1618 (see Experimental for details). Each anaerobic incubation was terminated after 2 hr, the protein precipitated and the supernatant chromatographed on a Whatman DE 52 cellulose column using increasing concentrations of aq $(NH_4)_2CO_3$ as the solvent. Column fractions (5 ml) were monitored for UV absorbance at 265 nm and for radioactivity. Identical elution patterns were obtained irrespective of whether MVA-[2-^{14}C] or MVA-[2-^{3}H] was the substrate.

Table 1. PC behaviour of DE 52 cellulose column fraction components

Bulked sample number	R_f × 100* in system (1)	(2)	(3)	Tentative identification
1	69†	75†	100	MVA
2	76†	96†	100	IP
3	0	15	91	FP
5	12†	51†	100	IPP
6	0	0	87	FPP
7	9	32	94	MVAPP
8	0	0	70	GGPP

* Systems [17]: (1) *n*-BuOH–HCOOH–H_2O (77:10:30); (2) *n*-PrOH–aq. NH_3(sp. gr. 0·88)–H_2O; (3:1:1) (3) 2-PrOH–*iso*-BuOH–aq. NH_3 (sp. gr. 0·88)–H_2O (40:20:1:39).

† Co-chromatographs with a known standard.

Figure 1 shows a typical separation on the DE 52 cellulose column of the products from an incubation in which MVA-[2-^{3}H] was used as the substrate. The components were identified, some only tentatively, by comparisons of their elution properties and sequence with those reported for similar separations on DEAE-cellulose columns [17] and by paper chromatography. The latter analyses (Table 1) identified the eluted compounds as follows: 1, MVA-[^{3}H]; 2, isopentenyl phosphate (IP)-[^{3}H]; 3, farnesyl phosphate (FP)-[^{3}H]; 5, isopentenyl pyrophosphate (IPP)-[^{3}H]; 6, FPP-[^{3}H]; 7, MVA-5-pyrophosphate (MVAPP)-[^{3}H]; 8, GGPP-[^{3}H]. Compound 4 corresponded in its elution behaviour on DE 52 cellulose to mevalonic acid-5-phosphate (MVAP)-[^{3}H] while dimethylallyl pyrophosphate (DMAPP)-[^{3}H] may have been a contaminant of the IPP-[^{3}H].

Since the primary objective of this study was to develop a system for the preparation of substrate quantities of labelled FPP and GGPP, further analyses were performed only on the two bulked samples comprising, respectively, the high activity fractions corresponding to elution peaks 6 (FPP) and 8 (GGPP). Unambiguous identifications of these compounds were based on investigations of the terpenol moieties released from each by treatment with bacterial alkaline phosphatase.

Table 2. GLC and TLC of standard and unknown terpenols

Compound	Relative retention time* on GLC†	R_f × 100 on TLC‡
Geraniol	0·19	89
Linalool	0·11	85
Cis farnesol	0·96	63
All-*trans* farnesol	1·00	
Cis nerolidol	0·61	57
All-*trans* nerolidol	0·68	
Cis geranylgeraniol	1·65	37
All-*trans* geranylgeraniol	1·69	
Geranyl-linalool	1·47	23
Phytol	1·60	8
Alkaline phosphatase hydrolysis products§ of bulked sample:		
6	1·00	63
8	1·69	37

* Relative to all-*trans* farnesol; R_t all-*trans* farnesol = 11·80 min.

† 2% SE-52 on Gas Chrom Q (80–100 mesh); 3 min at 100° then 6°/min increase to 225°.

‡ Reversed-phase TLC on paraffin-impregnated Kieselguhr G; see text for details.

§ Detected by radioactivity; see text for details.

A portion of the alkaline phosphatase hydrolysis product of sample 6 was mixed with standard carrier terpenols and chromatographed, together with individual standards, by a reversed-phase procedure on a liquid paraffin-impregnated Kieselguhr G thin-layer plate. The terpenols were located with iodine vapour or by spraying with solutions of Rhodamine 6G or anisaldehyde. Scanning the plate for radioactivity (in the case of a run in which MVA-[2-^{14}C] had been the original substrate) showed that the label co-chromatographed with farnesol (Table 2). Successive areas of the thin-layer chromatogram were eluted with Et_2O and the solutions assayed for radioactivity; this procedure confirmed that the label was located in the farnesol fraction. In a similar way, the radioactivity of sample 8 was shown, after hydrolysis, to migrate on TLC with geranylgeraniol.

Portions of the alkaline phosphatase hydrolysis products of sample 6 and 8 were also analysed by GLC after the addition of standard carrier terpenols. The chromatograph was equipped with a 10:1 mainstream splitter which permitted the trapping of 90% of each solute component of the effluent in collecting tubes immersed in an ice bath. As each GLC peak was recorded, a sample was taken of the eluting solute and assayed for radioactivity. Successive samples were taken continuously throughout each GLC analysis. As Fig. 2 and Table 2 show, the majority of the radioactivity extracted as free terpenol after hydrolysis of sample 6 co-chromatographed with all-*trans* farnesol. The terpenol moiety of the sample 8 component behaved as all-*trans* geranylgeraniol on GLC (Table 2).

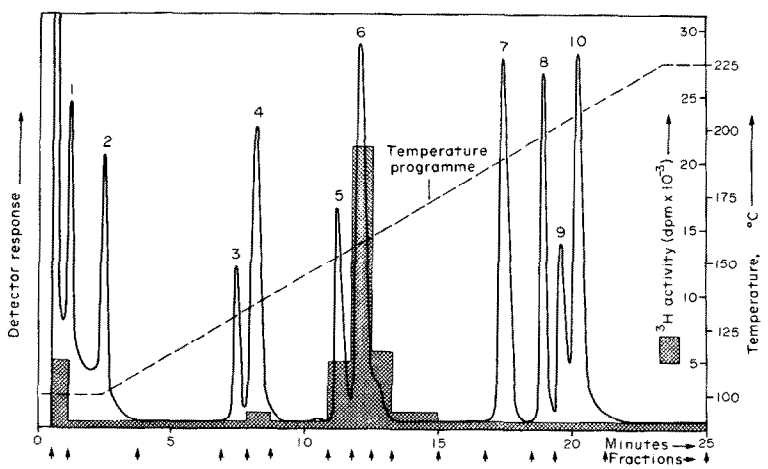

Fig. 2. Preparative GLC of a mixture of standard terpenols with the alkaline phosphatase hydrolysis product of farnesyl-[^{3}H]-pyrophosphate (Sample 6, Fig. 1). Fractions, as denoted by vertical arrows, were assayed for radioactivity. Further details are given in the text. 1, Linalool; 2, geraniol; 3, *cis*-nerolidol; 4, all-*trans* nerolidol; 5, *cis*-farnesol; 6, all-*trans*-farnesol; 7, geranyl-linalool; 8, phytol; 9, *cis*-geranylgeraniol; 10, all-*trans*-geranylgeraniol.

When a portion of sample 6 was subjected to acid rather than to alkaline phosphatase hydrolysis, the major portion of the radioactivity chromatographed on GLC and on reversed-phase TLC with nerolidol, while less radioactivity was associated with the farnesol; this was consistent with the identification of sample 6 as labelled FPP, for acids not only hydrolyse the pyrophosphate but also induce an allylic rearrangement of the free terpenol [3].

Some 20% of an FPP-[^{3}H] sample was hydrolysed using alkaline phosphatase; the radioactivity of the FPP-[^{3}H] was determined before, and that of the farnesol-[^{3}H] after hydrolysis in order to determine the efficiency of the reaction. Acid hydrolysis was also carried out on a known aliquot of the FPP-[^{3}H]; Et_2O-extractable radioactivity and the activity of the C_{15} alcohols (nerolidol and farnesol) were compared in order to determine the percentage of FPP-[^{3}H] in the original sample.

GLC was also used to determine the specific activity of the FPP-[^{3}H] synthesized by the barley embryo system. The alkaline phosphatase hydrolysis product was applied to the GLC column without the addition of any carrier. In this analysis, the preparative GLC system was prequantified with known amounts of farnesol and was modified (1:10 mainstream splitter) so that 90% of any eluted compound passed through the detector (FID) while 10% was collected quantitatively. This procedure enabled simultaneous determinations to be made of the amount of farnesol in the hydrolysis product and of its radioactivity; the sp. act. of the farnesol could thus be calculated.

In one run, for example, a sp. act. of 1624 μCi/μmol was recorded for the FPP-[^{3}H] and, by using the results from the acid hydrolysis, it could be shown that FPP-[^{3}H] represented 97·7% of the radioactivity of sample 6. The total radioactivity of the seven high activity 5 ml fractions which were bulked as sample 6 was 79·3 μCi and the incorporation of the biologically-active isomer of DL-MVA-[2-^{3}H] (400 μCi of racemate) was thus 39·7%. The amounts of GGPP-[^{3}H] isolated were too low to allow accurate determinations of specific activity (the calc. sp. act. is 2165 μCi/μmol), but the amount of radioactivity associated with GLC-purified geranylgeraniol-[^{3}H] obtained by hydrolysis of sample 8 (five 5 ml high activity fractions from the DE 52 cellulose column) showed an incorporation of 3% of the active isomer of MVA into GGPP in the same incubation.

In a series of preliminary experiments, it had been shown that a number of factors markedly influenced the production of FPP and GGPP by the barley embryo system. The most critical of these was the concentration of AMO 1618 in the incubation mixture. If this exceeded 1 mM, both FPP and GGPP formation were dramatically reduced; indeed, terpenyl pyrophosphates were barely detectable. If, on the other hand, the AMO 1618 concentration was less than 100 μM, the formation of FPP and, more particularly, of GGPP was again decreased. Phosphate, rather than Tris–HCl (0·1 M, pH 7·5), buffer was used in production runs since the latter favoured the formation of intermediates and degradation products (MVAPP, IPP, FP etc.) at the expense of FPP and GGPP. The inclusion of KF in the incubation mixture was also necessary in order to achieve the highest yields of FPP and GGPP, presumably because of the presence in the embryo extracts of active phosphatases.

As other workers have reported [17], monitoring the elution sequence from the DE 52 cellulose column of ATP and its hydrolysis products by their UV absorbance at 265 nm and comparing this with the pattern of radioactivity elution is useful in tentatively identifying the terpenyl pyrophosphates. On paper chromatographic analysis of the fractions comprising the high absorbance peaks (A–D in Fig. 1), it was shown that ATP (D) undergoes hydrolysis of all its ester bonds in the incubation since not only ATP (D), ADP (C) and AMP (B), but also free adenosine (A) were detected.

DISCUSSION

This use of a barley seed embryo system for the production of substrate amounts of labelled FPP and GGPP is the latest of an extensive series of trials which have been made in this laboratory. Other systems we have investigated (B. H. Davies, P. M. Bramley, A. F. Rees, J. M. Hill, A. Warren and R. A. London, unpublished studies) have included those derived from the seeds of *Echinocystis macrocarpa* Greene [9] and pumpkins (*Cucurbita pepo*, L.) [10], pea fruits (*Pisum sativum*) [18], tomato fruit (*Lycopersicon esculentum*) [19] and seedlings of castor bean (*Ricinus communis*, vars. *cambodgensis* and *zanzibariensis*), pea [20] and

barley; systems from the fungi *Fusarium moniliforme* Sheld [15] and *Phycomyces blakesleeanus* (C2 mutant) have also been examined. The system from barley embryos has the advantages of rapidity, ready availability of the natural material (as commercial barley grain) and therefore convenience over all the others examined. Although the yields of GGPP we have obtained are much lower than those reported for e.g. the *E. macrocarpa* [9] and tomato systems [19], the barley embryo system is, in our experience, the most reproducible of all the systems tested.

The ability of the system to form significant amounts of FPP and GGPP from MVA is dependent upon the inclusion in the incubation mixture of AMO 1618. This plant growth retardant has been shown to block the cyclization of GGPP to kaurene (and therefore gibberellin formation) in the fungus *F. moniliforme* [15, 21], in *E. macrocarpa* [13] and in pea fruits [18]. It is notable that in contrast to the fungal system (which requires only 5 μM AMO 1618), the barley embryo system requires a higher concentration (1 mM) of AMO 1618 to inhibit GGPP cyclization. Although we have not tested the effect of a wide range of inhibitor concentrations on the barley embryo system, we have noted little accumulation of the terpenyl pyrophosphates when concentrations lower than 100 μM are used. This relatively high requirement for AMO 1618 is consistent with those (1 and 3 mM respectively) reported for other higher plant systems from *E. macrocarpa* and pea fruit [9, 18]. The reduction in FPP and GGPP formation by the barley system in the presence of higher concentrations of AMO 1618 is presumably due to a further inhibitory effect of the plant growth retardant on prenyl transferase activity.

It is interesting to note that the sp. act. determined for the FPP-[4, 8, 12-^{3}H] in the example quoted (1624 μCi/μmol) is higher than would be expected (1500 μCi/μmol) on the basis of the formation of each molecule from three of MVA-[2-^{3}H] (500 μCi/μmol). This discrepancy is within the limits of the experimental error of the determination of the sp. act. of the MVA (Dr. J. P. Kitcher, personal communication) and merely emphasizes the need to determine the sp. act. of products from preparative biosynthetic systems if these compounds are to be used as substrates in further studies. It is clear that the radioactivity of the terpenyl pyrophosphates is not diluted by any endogenous compounds.

The presence in the barley embryo system of not only FPP and GGPP but also of MVAPP, IPP and possibly MVAP and DMAPP indicates that terpenoid biosynthesis is proceeding along the normal pathway [16]. The occurrence of FP and IP in the incubation mixture may be taken to indicate that although phosphate buffer is used for, and KF is included in the incubations, there is still an appreciable level of phosphatase activity.

EXPERIMENTAL

Standard compounds. Geraniol, linalool, farnesol, nerolidol and phytol were obtained commercially. Geranyl-linalool and geranylgeraniol were generous gifts from F. Hoffmann–La Roche & Co. Ltd., Basel, Switzerland. A sample of IP-[4-^{14}C] was obtained by the partial hydrolysis of IPP-[4-^{14}C].

Preparation of cell-free extracts. Barley seeds (*Hordeum vulgare* Zephyr) were obtained locally as grain. The seeds were soaked in tap H_2O for 2 hr at room temp before the husks were removed and the swollen embryos excised. An average run used some 500 embryos (wet wt *ca* 5 g), which were ground by pestle in a cold mortar with 5 ml cold 0·1 M KH_2PO_4/Na_2HPO_4 buffer, pH 7·5, for 5 min to yield a thick, smooth paste. This was centrifuged at 10000 *g* for 20 min and the resulting cell-free supernatant (*ca* 3 ml), which contained an average of 46·5 mg protein/ml as estimated by a standard biuret reaction [22], was used as the enzyme mixture in subsequent experiments.

Incubation system. Incubation systems contained either 200–400 μCi DL-MVA-[2-^{3}H] lactone (sp. act. 500 mCi/mmol) or 10–30 μCi DL-MVA-[2-^{14}C] lactone (sp. act. 10 mCi/mmol; both from the Radiochemical Centre, Amersham) which was converted to its sodium salt prior to the the addition of any other incubation components with a 2:1 molar excess of aq NaOH. Cofactors and inhibitors were added in 0·1 M KH_2PO_4–Na_2HPO_4 buffer, pH 7·5, to give a vol. of 2·5 ml. The reactions were initiated by adding 2·5 ml (*ca* 100 mg protein) of the barley embryo supernatant. The final vol. was 5 ml and the concentrations of cofactors and inhibitors were: ATP 10 mM, GSH 10 mM, Mg^{2+} 6 mM, AMO 1618 1 mM and KF 5 mM. Incubations were carried out in daylight at 24° in an atm. of argon and were terminated after 2 hr by heating the mixture at 80° in a H_2O bath for 2 min or until a protein ppt. formed. The mixture was centrifuged and the ppt. washed 2× with 2 ml vol. dist. H_2O. These washings were combined with the original supernatant and the bulked soln was subjected to column chromatography.

Separation of terpenyl intermediates. The incubation mixture supernatant and washings were immediately applied to a 1·8 × 12 cm column of Whatman DE 52 cellulose which had been washed, packed and pre-equilibrated at 4° with glass-redistilled H_2O. After application of the sample, the column was eluted at 4° with a concave gradient (600 ml) of 0–0·2 M $(NH_4)_2CO_3$ and 5 ml fractions were collected. The column fractions were first examined for absorbance at 265 nm and then their radioactivity was determined by assaying 0·5 ml aliquots each in 10 ml NE 260 scintillation fluid for 10 min in an NE 8310 Automatic Liquid Scintillation Spectrometer. Fractions of high activity in the same elution peak (Fig. 1) were bulked for further analysis.

Identification of adenosine derivatives. Fractions having a high UV absorbance at 265 nm (Samples A–D, Fig. 1) were examined by PC on Whatman No. 1 paper in butyric acid aq NH_3 (sp. gr. 0·88)–H_2O (66 : 1 : 3) and butyric acid–HOAc–H_2O (100 : 1 : 50). Descending chromatograms were run for 10–15 hr. Adenosine derivatives were detected as dark spots against a light background under short-wave UV light. The R_f values of standard adenosine, AMP, ADP and ATP in the two systems were 0·83, 0·56, 0·39 and 0·30; and 0·79, 0·43, 0·20 and 0·13 respectively.

PC of terpenyl intermediates. Aliquots of bulked column fractions containing suspected terpenyl intermediates were chromatographed on Whatman no. 1 paper in the three separate solvent systems of which details are given in Table 1. Radioactive compounds (^{14}C-labelled) were located on the resulting chromatograms by using an Actigraph III Paper Radiochromatography Scanner.

Hydrolysis of terpenyl pyrophosphates. Suspected terpenyl pyrophosphates (FPP and GGPP) were hydrolysed by either enzymic or acid procedures. For enzymic hydrolysis, an aliquot (1 ml) of the bulked column sample was added to an incubation mixture consisting of 1 ml 2 M Tris–HCl buffer, pH 8·0, which contained 12 μM Mg^{2+} and bacterial alkaline phosphatase (Type III-S, Sigma) equivalent to 6 mg protein [23]. The mixture was incubated for 4 hr at 37° and then the reaction was terminated by the addition of 2 ml EtOH. The mixture was extracted 3× with 5 ml vol. of peroxide-free glass-redistilled Et_2O and the combined extracts were dried (Na_2SO_4) and taken to dryness *in vacuo*. The resulting residue was analysed by TLC and GLC.

Acid hydrolysis was carried out by acidifying (to pH 1) an aliquot (1 ml) of the bulked column sample by the dropwise addition of 1 M HCl. The acidic soln was heated in a stoppered vial for 15 min in a H_2O bath (55–60°). The soln was made alkaline (pH 10) with 1 M NaOH and diluted to 5 ml with H_2O. After a 2% aliquot had been removed for radioassay, the remaining soln was extracted with Et_2O as described above. The Et_2O extract and the aq. hydrolysate residue were made up to known vol. and 2% aliquots were each radioassayed to determine the % Et_2O-extractable activity. The remainder of the Et_2O extract was concentrated to dryness *in vacuo* and analysed by TLC and GLC.

TLC of terpenols. Reversed-phase TLC of terpenols was carried out on 0·25 mm Kieselguhr G (Merck) plates which had been dipped into a 5% soln of liquid paraffin in light petrol (40–60°) and dried immediately prior to sample application. The chromatograms of hydrolysis products and known standards were developed in closed glass tanks, which had been pre-equilibrated for at least 1 hr, with Me_2CO–H_2O–liquid paraffin (65:35:0·5 v/v., lower phase). The terpenols were located by exposure to iodine vapour or by spraying with solns containing Rhodamine 6G or anisaldehyde [24]. ^{14}C-Labelled components were located on the layers by using a Berthold TLC Scanner. These positions were confirmed, and those of ^{3}H-labelled substances determined, by scraping the layer off in sections, extracting the portions of kieselguhr with Et_2O, concentrating the Et_2O solns in separate scintillation vials using a stream of N_2 and radioassay by liquid scintillation counting (see above).

GLC of terpenols. GLC of terpenols [25, 26] was carried out in a preparative manner on a gas chromatograph equipped with a FID and using the temperature programme described in Table 2. The column (glass, 1·52 m × 6·35 mm o.d., packed with 2% SE-52 on Gas Chrom Q, 80–100 mesh) was coupled to a 10 : 1 mainstream splitter which enabled simultaneous detector recording and radioactive sample collection in tubes cooled in an ice bath. The samples were dissolved in Et_2O, transferred to scintillation vials, concentrated to dryness under a stream of N_2 and radioassayed (see above).

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