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STUDIES IN TERPENOID BIOSYNTHESIS—I.

THE BIOSYNTHESIS OF METABOLITES OF *TRICOTHECIUM ROSEUM*

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Abstract—The incorporation of the pyrophosphates of geraniol, farnesol and the bicyclic alcohol (V) into the metabolites of *Tricothecium roseum* is described. Evidence is also presented for the biosynthesis of rosenonolactone from desoxyrosenonolactone.

TERPENOID biosynthesis has been the subject of considerable speculation. The biogenetic theories which have been summarized in the biogenetic isoprene rule[1] and extended to cover the various classes of terpene, have been reviewed on a number of occasions.[2] Despite a wealth of interest there has been relatively little experimental study utilizing post-mevalonoid precursors except in the case of the steroids and the gibberellins.[3] It is our purpose in this* and subsequent papers to present some experimental evidence to define various stages of post-mevalonoid terpenoid biosynthesis.

The diterpenes are formed by the sequential conversion of mevalonate through isopentenyl pyrosphosphate and dimethylallyl pyrophosphate to geranyl pyrophosphate, farnesyl pyrophosphate, and thence the C_{20} geranylgeranyl pyrophosphate. Cyclization of the latter is thought to occur in a number of discrete stages through bicyclic and tricyclic intermediates to the tetracyclic diterpenes. At the outset of this work mevalonic acid lactone had been shown to act as a precursor of the tricyclic diterpene, rosenonolactone and the tetracyclic diterpenes, gibberellic acid[5] and the kaurenolides.[6] Geranylgeranyl pyrophosphate has been shown to be converted[7] to (−)-kaurene and the latter to gibberellic acid.[6]

The fungus *Tricothecium roseum* Link has already been used for mevalonoid studies.[5,8] It has the advantage of producing both sesqui- and diterpenoid metabolites whose structures are well known.[9,10] The biogenetic proposals for rosenonolactone (VI) are summarized in

* Part of this work has been the subject of preliminary communications.[4]

[1] See *inter alia* L. RUZICKA, *Experientia* **9**, 357 (1953); *Proc. Chem. Soc.*, 341 (1959).

[2] CIBA Foundation Symposium, *Biosythesis of the Terpenes and Sterols* (edited by G. WOLSTENHOLME and M. O'CONNOR). Churchill, London (1959); J. H. RICHARDS and J. B. HENDRICKSON, *Biosynthesis of Terpenes, Steroids and Acetogenins*. Benjamin, New York (1964); P. BERNFELD, *Biogenesis of Natural Compounds*. Pergamon Press, Oxford (1963).

[3] R. B. CLAYTON, *Quart. Rev.* **19**, 168 (1965).

[4] J. R. HANSON and B. ACHILLADELIS, *Tetrahedron Letters* 1295 (1967); *Chem. & Ind.*, in press (1967).

[5] A. J. BIRCH, R. W. RICKARDS, H. SMITH, A. HARRIS and W. B. WHALLEY, *Tetrahedron* **7**, 241 (1959).

[6] B. E. CROSS, R. H. B. GALT and J. R. HANSON, *J. Chem. Soc.*, 295 (1964).

[7] J. E. GRAEBE, D. T. DENNIS, C. D. UPPER and C. A. WEST, *J. Biol. Chem.* **240**, 1847 (1965).

[8] E. R. H. JONES and G. LOWE, *J. Chem. Soc.*, 3959 (1960).

[9] J. FISHMAN, E. R. H. JONES, G. LOWE and M. C. WHITING, *J. Chem. Soc.*, 3948 (1960); W. D. GODTFREDSEN and S. VANGEDAL, *Proc. Chem. Soc.*, 185 (1964).

[10] A. HARRIS, A. ROBERTSON and W. B. WHALLEY, *J. Chem. Soc.* 1799, 1807 (1958); B. GREEN, A. HARRIS and W. B. WHALLEY, *Chem. & Ind.*, 1369 (1958); W. B. WHALLEY, B. GREEN, D. ARIGONI, J. J. BRITT and C. DJERASSI, *J. Am. Chem. Soc.* **81**, 5520 (1959); G. A. ELLESTAD, B. GREEN, A. HARRIS, W. B. WHALLEY and H. SMITH, *J. Chem. Soc.*, 7246 (1965); M. R. COX, G. A. ELLESTAD, A. J. HANNAFORD, I. R. WALLWORK, W. B. WHALLEY and B. SJOBERG, *J. Chem. Soc.*, 7257 (1965).

Fig. 1. 1-^{14}C-Geranyl pyrophosphate (I) was prepared by literature methods.[11] The lithium salt in aqueous solution, was distributed between three shake flasks (100 ml) of *T. roseum* (CM1. 50, 660) after 6 days' growth. The fermentation was harvested after a total of 32 days. The metabolites were isolated by chromatography and crystallized to constant activity. Rosenonolactone (VI) showed an incorporation of 0·15 per cent and rosololactone (VII) 0·34 per cent. Insufficient material was isolated for degradation. However, geranyl pyrophosphate is a specific precursor in steroid biosynthesis[12] and there is little reason to suspect

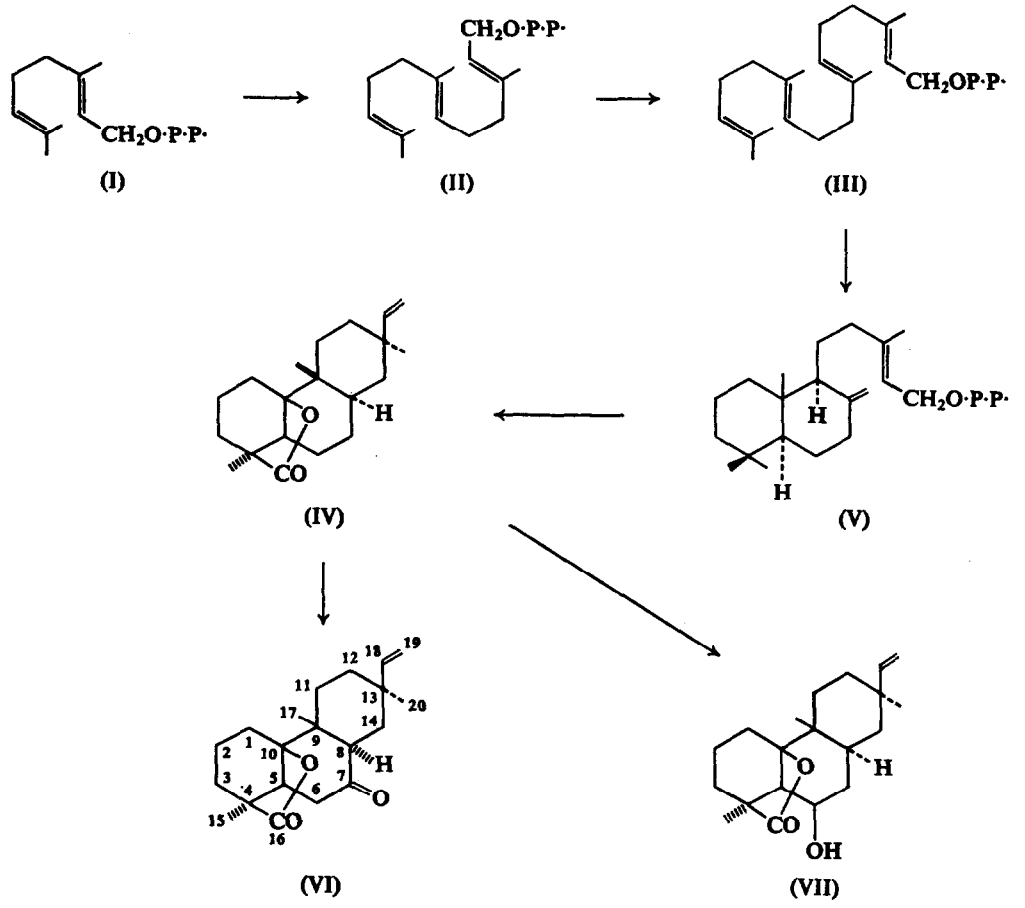


FIG. 1.

any difference in this instance, although this is the first record of its incorporation into a diterpene.

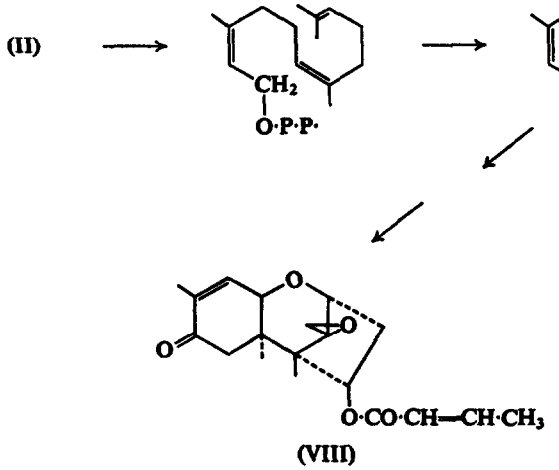
1-^{14}C-Farnesyl pyrophosphate (II) was also prepared by literature methods.[11] This was fed again as its lithium salt to a 10-day surface culture of *T. roseum* and the fermentation then harvested on the 42nd day. Rosenonolactone showed an incorporation of 0·19 per cent and rosololactone 0·17 per cent. The sesquiterpenoid metabolite tricothecin (VIII) was also

[11] F. CRAMER and W. BOHM, *Angew. Chem.* **71**, 775 (1959); G. POPJAK, J. W. CORNFORTH, R. H. CORNFORTH, R. RYHAGE and D. S. GOODMAN, *J. Biol. Chem.* **237**, 56 (1962).

[12] F. LYNEN, H. AGRANOFF, H. EGGERER, U. HENNING and K. MOSLEIN, *Angew. Chem.* **71**, 657 (1959).

isolated from this fermentation, and showed an incorporation of 1·51 per cent. Degradation to tricothecolone by cold methanolic 1 N KOH demonstrated that the radioactivity (97 per cent) was confined to the terpenoid portion of the molecule. Previous workers have shown[8] that the tricothecolone moiety incorporated three molecules of mevalonate in such a way that a double 1:2 methyl migration was involved in its biosynthesis (Fig. 2). Although farnesol pyrophosphate has been shown to act as an intermediate in the biosynthesis of squalene[11, 13] the triterpene eburicoic acid[14] and the steroids,[15] this represents the first demonstration of its intervention in the biosynthesis of a sesquiterpene and diterpenes. In the case of the former it does not of course specify which double bond isomer acts as an intermediate immediately prior to cyclization.

Geranylgeranyl pyrophosphate has already been shown[7] to act as a precursor of (−)-kaurene and attention was therefore directed to the cyclic intermediate (V). The parent alcohol was prepared from sclareol by dehydration and allylic rearrangement.[16] It was oxidized to the aldehyde with manganese dioxide and reduced with sodium borohydride-T.



(VIII)

FIG. 2.

The labelled alcohol, solubilized in Tween 80, was then fed to a 5-day surface culture of *T. roseum*. The fermentation was harvested after a further 13 days and the rosenonolactone isolated. It showed an incorporation of 0·13 per cent. The alcohol was phosphorylated[11] using di-(triethylamine) hydrogen phosphate and trichloroacetonitrile in acetonitrile and the pyrophosphate precipitated as its lithium salt. This was fed in aqueous solution to a 12-day surface culture of *T. roseum*. After a further 28 days the fermentation was harvested and the metabolite isolated. Rosenonolactone showed an incorporation of 0·16 per cent and rosolo-lactone 0·13 per cent. In both instances the specificity of incorporation into rosenonolactone was confirmed by ozonolysis. The formaldehyde was isolated as its dimedone derivative and retained 97·5 per cent and 99 per cent of the activity respectively. The C_{19}-fragments were inactive.

[13] F. LYNEN, H. EGGERER, U. HENNING and I. KOSSEL, *Angew. Chem.* **70**, 738 (1958).
[14] W. LAWRIE, J. MCLEAN, P. L. PAUSON and J. WATSON, *Chem. Comm.*, 623 (1965).
[15] B. SAMUELSON and D. S. GOODMAN, *J. Biol. Chem.* **239**, 98 (1963).
[16] G. OHLOFF, *Ann.* **617**, 134 (1958).

Two possible pathways may then be considered. The bicyclic alcohol (V) may cyclize to a pimaradiene (IX) either directly or through a manöol intermediate and the tricyclic pimaradiene subsequently undergo a methyl shift comparable to the acid-catalysed rearrangement and lactonization of the diterpenoid acids.[17] Alternatively methyl migration and cyclization may be concerted (Fig. 3, pathway B). Although we shall return to the discussion of this sequence of events in a later publication, some evidence is available. Tritiated pimaradiene (IX) was prepared[15] from pimaric acid. The methyl ester of pimaric acid was reduced to the alcohol which was in turn cautiously oxidized to the aldehyde. The semicarbazone was shaken with T_2O and then subjected to a Wolff-Kishner reduction in the presence of T_2O. The tritiated pimaradiene solubilized in Tween 80, was fed to a 12-day surface culture of *T. roseum* and the fermentation harvested after 32 days. However, the rosenonolactone was

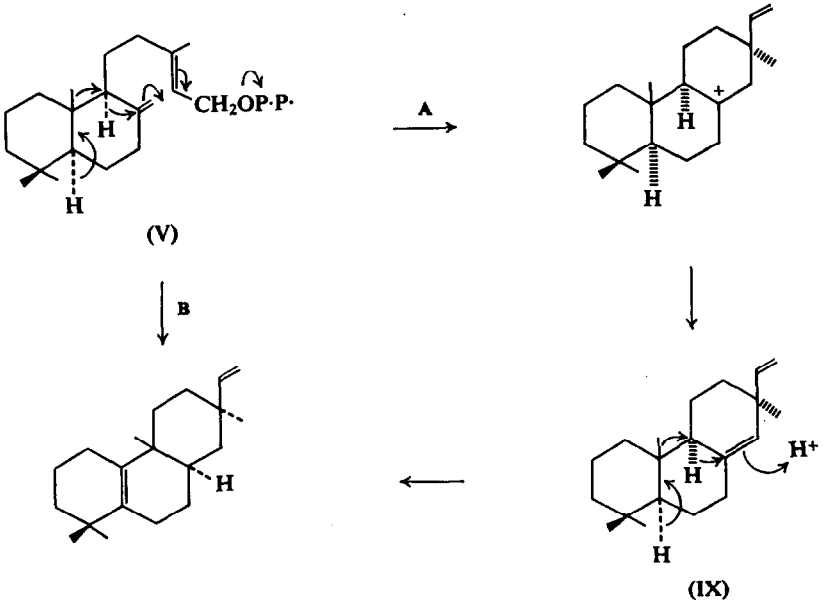


FIG. 3.

inactive. Despite the imponderable doubts associated with negative evidence in this field, nevertheless this suggests that pathway B may be operative.

The co-occurrence of small amounts of desoxyrosenonolactone[10] (IV) together with rosenonolactone (VI) and rosololactone (VII) suggests that (IV) may act as the precursor of the more oxygenated metabolites. This relationship was clarified by feeding 2-^{14}C-mevalonic acid lactone to *T. roseum* and studying the distribution of the label at different time intervals. In order to facilitate isolation and purification, the desoxyrosenonolactone was isolated by dilution with inactive material and the results corrected for the normal level of the metabolite. Rosololactone was not always isolated. The results are shown in Table 1. It is clear that the maximum label in desoxyrosenonolactone appears before that in rosenonolactone and rosololactone, suggesting a continuous channelling of mevalonate through these metabolites. This sequence of oxygenation is the subject of further work.

[17] L. A. SUBLUSKEY and T. F. SANDERSON, *J. Am. Chem. Soc.* **70**, 3512 (1954).

TABLE 1. INCORPORATION OF DL-MEVALONIC ACID-2-^{14}C-LACTONE

Time of harvest (days)	21	31	43	50	70	86
Desoxyrosenonolactone, dps/mg	0	24	77	107	38	7
Rosenonolactone, dps/mg	0	1·90	93	90	114	62
Rosololactone, dps/mg	0	1·09	—	—	102	54

EXPERIMENTAL

Melting points were determined on a Kofler block. I.r. spectra were recorded on a Perkin Elmer 237 or Unicam SP. 200 Spectrometer. NMR spectra were determined in $CDCl_3$ on a Varian Associates HA.100 spectrometer with T.M.S. as internal standard. The radioactive compounds were counted by liquid scintillation counting using a solution of *p*-terphenyl (4 g) and 2-*p*-phenylene-bis-(5-phenyl oxazole) (0·1 g) in AnalaR toluene (1 l.) and an I.D.L. counter type 6012. Count rates were corrected for background and the efficiency of the counter. Alumina for chromatography was Woelm (neutral); silica gel was supplied by B.D.H.; light petroleum refers to the fraction, b.p. 60–80°.

Conditions of Growth of Tricothecium roseum, Link (*CM*1. 50, 660)

The medium used for fermentation was NH_4NO_3 2 per cent; $MgSO_4.7H_2O$, 0·05 per cent; K_2HPO_4, 0·1 per cent; KCl, 0·05 per cent; $FeSO_4,7H_2O$, 0·001 per cent; glucose, 5 per cent; corn steep liquor 1 per cent. Surface cultures were grown in Roux bottles (200 ml) at 24°; shake cultures in Erlenmeyer flasks (100 ml) at 20°. The pH of shake cultures fell from 5 to 4·5–4·6 during the first 4 days and then climbed steadily to 7·6–8·2 until the fermentation was harvested. For surface culture the pH fell for the first 7 days and then rose steadily. The best yields of metabolites were obtained after about 4 weeks on shake culture and about 6 weeks' surface culture.

Extraction Procedure

The mycelium was filtered, dried at 50° for 10 hr, and extracted in a Soxhlet with $CHCl_3$ for 15 hr. The solvent was dried over Na_2SO_4 and evaporated to give a semi-solid residue from which rosenonolactone (VI) (m.p. 212–215°) was obtained by trituration with petrol and crystallization from methanol. The broth was extracted with $CHCl_3$ and rosenonolactone obtained as above.

The combined residues were chromatographed on alumina. Elution with 2 per cent ethyl acetate:light petroleum gave desoxyrosenonolactone (IV) which crystallized from light petroleum as prisms, m.p. 116–118° (approx. yield 60 mg/l.). Elution with 10 per cent ethyl acetate:light petroleum gave tricothecin (VIII) which crystallized from light petroleum as needles, m.p. 117–119° (approx. yield 75 mg/l.). Elution with 15 per cent ethyl acetate:light petroleum gave rosenonolactone (total yield approx. 350 mg/l.). Elution with 20–30 per cent ethyl acetate:light petroleum gave rosololactone (VII) which crystallized from ethyl acetate:light petroleum as needles, m.p. 184–185° (approx. yield 210 mg/l.). The metabolites were identified by their i.r. and NMR spectra.

Incorporation of 1-^{14}*C-Geranyl Pyrophosphate*

The lithium salt of 1-^{14}C-geranyl pyrophosphate prepared according to the literature method[11] gave $1{\cdot}56 \times 10^3$ dps/mg. Its purity was checked by TLC on silica gel chromatogram sheets (Eastman Kodak K301 R2) with isopropanol (6):0·880 ammonia (3):water (1) as a developing solvent.

The salt (50 mg) in water (30 ml) was sterilized by filtration and distributed between three shake flasks each with 100 ml medium 6 days after inoculation. After 32 days the fermentation was harvested and the metabolites isolated.

Rosenonolactone (41 mg) gave 2·52 dps/mg (0·15 per cent incorporation)
Rosololactone (22 mg) gave 5·56 dps/mg (0·35 per cent incorporation)

Incorporation of 1-^{14}*C-Farnesyl Pyrophosphate*

The lithium salt of 1-^{14}C-farnesyl pyrophosphate ($1{\cdot}23 \times 10^3$ dps/mg) was prepared by the literature method[11] and its homogeneity checked by TLC. The salt (120 mg) in water (60 ml) was sterilized by filtration and distributed between six Roux bottles 10 days after inoculation. The surface culture was harvested after a further 32 days and the metabolites isolated.

Rosenonolactone (95 mg) gave 3·02 dps/mg (0·19 per cent incorporation)
Rosololactone (14 mg) gave 2·70 dps/mg (0·17 per cent incorporation)
Tricothecin (24 mg) gave 22·5 dps/mg (1·5 per cent incorporation)

Hydrolysis of Tricothecin

Tricothecin (15 mg) in alcoholic 1 N KOH solution (2 ml) was left to stand overnight. Water was added, the methanol removed *in vacuo* at 40° and the residue extracted with $CHCl_3$ to give tricothecolone which crystallized from ethyl acetate:light petroleum, m.p. 184° (29·17 dps/mg=97 per cent activity of tricothecin).

Preparation of the Tritiated Alcohol (V)

The corresponding aldehyde was prepared from sclareol and characterized as its semicarbazone, m.p. 175° (lit.[16] 176–178°). The aldehyde (1·0 g) was treated with a mixture of sodium borohydride-T (1·388 mc/mg) (18 mg) and sodium borohydride (48 mg) in methanol (50 ml). Water (1 ml) and 10 per cent NaOH (0·2 ml) were added and the reaction mixture stirred for 2 hr. The methanol was evaporated, water added and the product recovered in ether and distilled to give the tritiated alcohol (660 mg, b.p. 175°/0·1 mm, $4·3\ 10^4$ dps/mg).

The Lithium Salt of the Pyrophosphate of Alcohol (V)

Di(triethylamine)hydrogen phosphate (0·54 g) in acetonitrile (15 ml) was added dropwise over 4 hr to a solution of the alcohol (220 mg) in trichloroacetonitrile (0·8 g) at 25°. After a further 2 hr, the solution in ether (150 ml) was extracted with 0·1 N NH_3 (3 × 50 ml) and the combined aqueous extracts washed with ether. These aqueous extracts were concentrated *in vacuo* at 40° (frothing) to 20 ml and cyclohexylamine (1 ml) added. After further concentration to 10 ml the solution was left at 4° overnight. The monophosphate (45 mg) was then removed by filtration, the solution treated with 0·880 ammonia (1 ml) and extracted with ether (100 ml). Aqueous 1 M LiCl (4 ml) was then added to the aqueous phase and this solution concentrated to 8 ml and stood at 4° for 24 hr. The lithium salt of the pyrophosphate which separated (125 mg) was filtered, dried with acetone, ether, and over P_2O_5. It was shown to be chromatographically pure by TLC as above and gave $2·55 \times 10^4$ dps/mg.

Incorporation Experiments

(*a*) A 7-day surface culture of *T. roseum* (5 × 200 ml) was inoculated with the tritiated allylic alcohol (30 mg) in ethanol (10 ml). After a further 14 days the fermentation was harvested. The metabolites were inactive.

(*b*) A 5-day surface culture of *T. roseum* (4 × 200 ml) was inoculated with the tritiated allylic alcohol (38 mg) solubilized in Tween 80. After a further 16 days the fermentation was harvested and the rosenonolactone (98 mg) crystallized to constant activity (21·7 dps/mg) (0·13 per cent incorporation).

(*c*) A 12-day surface culture of *T. roseum* (5 × 200 ml) was inoculated with the lithium salt of the pyrophosphate of the alcohol (40 mg) in water (50 ml). After a further 28 days the fermentation was harvested. Rosenonolactone (135 mg) gave 60·15 dps/mg (0·16 per cent incorporation) and rosololactone (38 mg) gave 52·20 dps/mg (0·14 per cent incorporation).

Degradation of the Rosenonolactone

Rosenonolactone (35 mg) (from fermentation (*b*)) in acetic acid (5 ml) was treated with a 250 per cent excess of ozonized oxygen. The solution was divided into two equal parts.

The first portion was treated with Zn dust (125 mg) and water (0·5 ml) for 4 hr and then steam distilled. The distillate (2 ml) was collected in 10 per cent ethanolic dimedone solution. The formaldehyde dimedone derivative (11 mg), m.p. 185–189°, gave 25·6 dps/mg which represented 97·5 per cent of the activity of the rosenonolactone. In the case of fermentation (*c*) the dimedone derivative gave 63·8 dps/mg which represented 99·0 per cent of the activity of the rosenonolactone.

The second portion was treated with 30 per cent H_2O_2 (0·5 ml) overnight. The solution was separated into acidic and neutral fractions. The acidic fraction gave the 19-nor carboxylic acid (5 mg), m.p. 252° (lit.[10] 258°) which was inactive.

Tritiated Pimaradiene

Pimaradiene was prepared from pimaric acid according to the literature method.[18] The final Wolff–Kishner reduction was carried out in the presence of T_2O to give the tritiated pimaradiene (110 mg) ($4·2 \times 10^4$ dps/mg).

Incorporation Experiment

The tritiated pimaradiene (100 mg) solubilized in Tween 80, was distributed between five Roux bottles of *T. roseum* (200 ml) after 12 days' incubation. The fermentation was harvested after a further 20 days. Rosenonolactone and rosololactone were isolated and repeatedly recrystallized until they showed no activity.

Incorporation of DL-*Mevalonic Acid-2-*^{14}*C-Lactone*

DL-Mevalonic acid-2-^{14}C-lactone (0·1 mc) was distributed between fifteen Roux bottles of *T. roseum* after 12 days' incubation. Groups of bottles were harvested at various time intervals (see Table 1) and the metabolites isolated.

[18] R. E. IRELAND and P. W. SCHIESS, *J. Org. Chem.* **28**, 6 (1963).