Biosynthesis of Mycophenolic Acid

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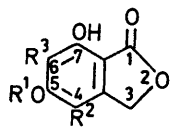
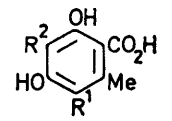
Incorporation experiments with labelled potential biosynthetic intermediates suggest that the methyl group at C-4 of the phthalide system in mycophenolic acid is introduced at the tetraketide stage. This then gives way to the aromatic system, which is further oxidised to 5,7-dihydroxy-4-methylphthalide. The isolation of 6-farnesyl-5,7-dihydroxy-4-methylphthalide from the culture and the high incorporation of this compound into mycophenolic acid indicate that the most important process for the biosynthesis of the side-chain is the introduction of a C_{15} terpene chain followed by oxidative fission at the appropriate double bond. ' Enzymic trap ' experiments confirm these results. Alternative pathways for the biosynthesis of the side-chain through 6-geranyl-5,7-dihydroxy-4-methylphthalide are discussed.

THE molecule of mycophenolic acid [1] (1) has been demonstrated to consist of an acetate-derived aromatic nucleus, a terpenoid side-chain, and two methionine-derived methyl groups (the 5-*O*-methyl group and that at C-4).[1] The incorporation of [2-^{14}C]mevalonate into compound (1) and the isolation of labelled acetone under these conditions led to the hypothesis that the terpenoid



(1) $R^1 = R^2 = Me$, $R^3 = HO_2C{\cdot}[CH_2]_2{\cdot}CMe{=}CH{\cdot}CH_2$
(4) $R^1 = R^2 = Me$, $R^3 = OHC{\cdot}CH_2$
(5) $R^1 = R^3 = H$, $R^2 = Me$
(6) $R^1 = R^2 = Me$, $R^3 = H$
(7) $R^1 = R^2 = R^3 = H$
(8) $R^1 = R^2 = H$, $R^3 = HO_2C{\cdot}[CH_2]_2{\cdot}CMe{=}CH{\cdot}CH_2$
(9) $R^1 = H$, $R^2 = Me$, R^3 = *trans,trans*-farnesyl
(10) $R^1 = H$, $R^2 = Me$, R^3 = geranyl
(11) $R^1 = R^2 = H$, R^3 = geranyl
(12) $R^1 = R^2 = H$, R^3 = *trans,trans*-farnesyl
(13) $R^1 = H$, $R^2 = Me$, $R^3 = HO_2C{\cdot}[CH_2]_2{\cdot}CMe{=}CH{\cdot}CH_2$
(14) $R^1 = R^2 = H$, $R^3 = MeO_2C{\cdot}[CH_2]_2{\cdot}CMe{=}CH{\cdot}CH_2$
(15) $R^1 = H$, $R^2 = Me$, $R^3 = MeO_2CMe{=}CH{\cdot}CH_2$



(2) $R^1 = R^2 = H$
(3) $R^1 = Me$, $R^2 = H$
(16) $R^1 = Me$, $R^2 = Br$

side-chain was derived from geraniol.[2] Orsellinic acid (2), postulated as an intermediate,[3] is incorporated into mycophenolic acid to a small extent (Table 1). The low incorporations of orsellinic acid noted by us were derived from a degradation–resynthesis pathway; ozonolysis of the mycophenolic acid thus obtained gave the aldehyde [4] (4) with loss of 30% molar activity originally at C-1 of the postulated intermediate.

TABLE 1

Incorporations of intermediates into mycophenolic acid

Intermediate	Molar inc. (%)	Total inc. (%)
4,6-Dihydroxy-2-methyl[6-^{14}C]benzoic acid (2)	1·2	0·3
4,6-Dihydroxy-2,3-dimethyl[1-^{14}C]benzoic acid (3)	54·8	83·5
5,7-Dihydroxy-4-methyl[7-^{14}C]phthalide (5)	39·22	36·5
7-Hydroxy-5-methoxy-4-methyl[7-^{14}C]-phthalide (6)	2·95	3·80
5,7-Dihydroxy[7-^{14}C]phthalide (7)	0·21	0·15
6-Farnesyl-5,7-dihydroxy-4-methyl-[7-^{14}C]phthalide (9)	38·6	33·6
6-Geranyl-5,7-dihydroxy-4-methyl-[7-^{14}C]phthalide (10)	7·9	5·2
[7-^{14}C]-(13)	97·62	78·32

High incorporations were on the contrary, obtained with 4,6-dihydroxy-2,3-dimethyl[1-^{14}C]benzoic acid (3), leading to the conclusion that the introduction of the 4-methyl group of structure (1) precedes the aromatic ring closure.[5] Ozonolysis of compound (1) derived from the feeding of 4,6-dihydroxy-2,3-dimethyl[1-^{14}C]benzoic acid afforded the aldehyde (4) having almost the same molar activity as the labelled acid (1), indicating direct incorporation.

The next step in the biogenetic sequence is the oxidation of the benzoic acid (3) to 5,7-dihydroxy-4-methylphthalide [6] (5), which, although never isolated from the culture, was incorporated into mycophenolic acid (Table 1) to a significant extent and without any randomisation of radioactivity, as shown by degradation to the aldehyde (4).

Comparison of the incorporation data for the labelled phthalide (5) and its *O*-methyl derivative [6a,7] (6) (Table 1)

[1] A. J. Birch, R. J. English, R. A. Massy-Westropp, and H. Smith, *Proc. Chem. Soc.*, 1957, **233**; A. J. Birch, R. J. English, R. A. Massy-Westropp, and H. Smith, *ibid.*, p. 365; A. J. Birch, *Chem. Weekblad.*, 1960, **56**, 597; G. Jaureguiberry, G. Farrugia-Fougerose, H. Audie, and E. Lederer, *Compt. rend.*, 1964, **259**, 3108.

[2] A. J. Birch, ' Biosynthesis of Aromatic Compounds,' Pergamon, Oxford, 1966, p. 3; *Proc. Chem. Soc.*, 1962, 3; *Science*, 1964, **156**, 202; ' Biogenesi delle Sostanze Naturali,' Accademia Nazionale dei Lincei, Roma, 1964, p. 57.

[3] A. J. Birch, *Ann. Rev. Plant. Physiol.*, 1968, **19**, 321; *Chem. Weekblad*, 1960, **56**, 597 and references cited.

[4] J. H. Birkinshaw, A. Broken, E. N. Morgan, and H. Raistrick, *Biochem. J.*, 1948, **43**, 216.

[5] (*a*) L. Canonica, W. Kroszczynski, B. M. Ranzi, B. Rindone, and C. Scolastico, *Chem. Comm.*, 1971, 257; (*b*) C. T. Bedford, J. C. Foirlie, P. Knittel, T. Money, and G. T. Phillips, *ibid.*, p. 323.

[6] (*a*) L. Canonica, W. Kroszczynski, B. M. Ranzi, B. Rindone, and C. Scolastico, *Chem. Comm.*, 1970, 1357; (*b*) L. Canonica, B. Rindone, E. Santaniello, and C. Scolastico, *Tetrahedron Letters*, 1971, 2691.

[7] A. J. Birch and J. J. Wright, *Austral. J. Chem.*, 1969, **22**, 2635.

showed a 10 : 1 ratio, the aldehyde (4) obtained in each case having nearly the same molar activity as the starting material. This suggested that prenylation of (5) followed by the methylation step is the main biogenetic pathway. The low incorporation of compound (6) into mycophenolic acid can be explained as derived from a collateral path or from some enzymic adaptation due to the introduction of the substrate. This fact is also apparent from the feeding of 5,7-dihydroxy[7-^{14}C]-phthalide [5a] (7); this gave a compound to which structure (8) was assigned by comparison with an authentic specimen.[8] Under these conditions, the isolated mycophenolic acid was only feebly radioactive (Table 1), probably owing to a degradation–resynthesis process.

During the study concerning the introduction of the side-chain, feeding of 5,7-dihydroxy-4-methyl[7-^{14}C]-phthalide (5) and interruption of the fermentation when a minor amount of mycophenolic acid had been formed (2 days) led to the isolation of 6-*trans*,*trans*-farnesyl-5,7-dihydroxy-4-methylphthalide (9) (incorporation 9%), identified by comparison of its spectral and physical characteristics with those of an authentic specimen.[8] Feeding of labelled (9) to the culture rapidly gave mycophenolic acid (Table 1).

We considered that an 'enzymic trap' experiment might confirm the foregoing results. Introduction of 4,6-dihydroxy-2,3-dimethyl[1-^{14}C]benzoic acid (3) together with a large amount of unlabelled 5,7-dihydroxy-4-methylphthalide (5) to the culture, and interruption of the fermentation when only part of the phthalide (5) had been transformed, allowed the recovery of 39% of the radioactivity in (5), and 3·2 and 12% in compounds (9) and (1) respectively. This distribution of radioactivity can be ascribed to exchange through the cell wall of the labelled phthalide (5), biosynthesised from the benzoic acid (3), with unlabelled (5) present in excess in solution.

A similar experiment involving addition to the culture of 5,7-dihydroxy-4-methyl[7-^{14}C]phthalide (5) and excess of unlabelled farnesyl derivative (9) yielded recovery of 30% of the label in (9) and 45% label in (1). These results confirm those already obtained.

Table 2 shows the incorporation of 5,7-dihydroxy-4-methyl[7-^{14}C]phthalide (5) into compounds (9) and (1)

TABLE 2

Incorporations of 5,7-dihydroxy-4-methyl[7-^{14}C]phthalide into compounds (9) and (1) at various times

	Total inc. (%) 2 days	Molar inc. (%) 2 days	Total inc. (%) 7 days	Molar inc. (%) 7 days
(9)	9·62	73·36	0·52	6·65
(1)	62·51	96·64	36·45	37·03

after various fermentation times. The rate of formation of (9) seems to depend more on the biosynthesis of the phthalide (5) than on that of farnesol: introduction of compound (5) to the culture medium heightens the rate of production of mycophenolic acid and its absolute yield. Table 2 indicates that the endogenous biosynthesis of (5) occurs only after the second day; in the first two days compound (1) is formed only if the phthalide (5) is fed. Furthermore, Table 2 indicates that, under the experimental conditions used, the rate for the step (9) → (1) is greater than that of the synthesis of (9) from (5).

The results so far indicate that the main biosynthetic route involves the attachment of a farnesyl unit to the aromatic precursor (5). It seemed possible that a minor pathway might involve formation of a geranyl derivative. However, 6-geranyl-5,7-dihydroxy-4-methylphthalide (10) was not detected in the chromatographic fraction containing the farnesyl derivative either by n.m.r. or by g.l.c.–mass spectroscopy [these systems were able to reveal small amounts of (10) in synthetic mixtures]. It was considered that compound (10) might be transformed as soon as formed; however introduction of synthetic 6-geranyl-5,7-dihydroxy-4-methyl[7-^{14}C]phthalide [8] (10) yielded only 5·2% incorporation into mycophenolic acid after 7 days. The molar activity of the aldehyde (4) obtained from this sample of mycophenolic acid ruled out a degradation–resynthesis pathway, leading to the conclusion that either compound (10) takes part in a minor pathway or it is feebly incorporated owing to some adaptability of the enzymic complex. The latter conclusion is reinforced by the fact that synthetic 6-geranyl-5,7-dihydroxy[7-^{14}C]phthalide (11) [5a] shows 0·63% incorporation into the acid (8), whereas the labelled farnesyl derivative (12) shows 30% incorporation.

With respect to the sequence of the methylation and the side-chain degradation, there is some indication that the oxidative process precedes the methylation: the synthetic labelled acid [8,9] (13) is methylated by the culture to mycophenolic acid with 78·6% yield in 24 h.

The labelled compounds used were prepared as follows. Ethyl 2-methyl-4,6-dioxo[6-^{14}C]cyclohexanecarboxylate,[10] prepared from the condensation of ethyl [3-^{14}C]acetoacetate with ethyl crotonate, was transformed into ethyl 3,5-dibromo-4,6-dihydroxy-2-methylbenzoate.[11] This dibromide was hydrolysed to the corresponding acid with conc. sulphuric acid at 0°; catalytic hydrogenolysis on palladium–charcoal then afforded the acid (2) in high yield. In an analogous way ethyl 2,3-dimethyl-4,6-dioxo[1-^{14}C]cyclohexanecarboxylate, obtained from the condensation between diethyl [2-^{14}C]malonate and 3-methylpent-3-en-2-one, gave ethyl 5-bromo-4,6-dihydroxy-2,3-dimethyl[1-^{14}C]-benzoate on treatment with bromine. Hydrolysis as for the preparation of the acid (2), gave the corresponding bromo-acid (16), hydrogenolysed subsequently to the acid (3).

[8] L. Canonica, B. Rindone, and C. Scolastico, *Tetrahedron Letters*, 1971, 2689.

[9] D. F. Jones and S. D. Mills, *J. Medicin. Chem.*, 1971, **14**, 305.

[10] A. Schilling and F. Vorlander, *Annalen*, 1899, **308**, 1952.

[11] A. Sonn, *Ber.*, 1928, **61**, 926.

The procedure of Allison and Newbold [12] was applied to ethyl 3,5-dibromo-4,6-dihydroxy-2-methyl[6-^{14}C]-benzoate to prepare 5,7-dihydroxy[7-^{14}C]phthalide (7), from which 5,7-dimethoxy-4-methyl[7-^{14}C]phthalide was obtained.[13] Selective hydrolysis of the dimethyl ether to 7-hydroxy-5-methoxy-4-methyl[7-^{14}C]phthalide (6) was achieved with boron trichloride in dichloromethane. Complete hydrolysis to the dihydroxy-derivative (5) was performed with boron tribromide in the same solvent.

Compounds (9)—(12), (14), and (15) were obtained by reactions of allylic bromides with the appropriate 5,7-dihydroxyphthalides in dioxan in the presence of silver oxide.[7,8] The methyl esters (14) and (15) were hydrolysed to the acids (8) and (13) with sodium hydroxide in ethanol–water.

EXPERIMENTAL

Microanalyses were performed on a Perkin-Elmer 240 Elemental Analyser. I.r. spectra were measured with a Perkin-Elmer 257 spectrophotometer. N.m.r. spectra were recorded with a Perkin-Elmer R10 spectrometer with tetramethylsilane as internal reference. M.p.s were determined on a Buchi apparatus. Mass spectrometric investigations were conducted on a LKB 9000 (70 eV) g.l.c.–mass spectrometer system.

Mycophenolic acid was obtained from the strain of *Penicillium brevi-compactum* Dierx ATCC 11885.

Cultural Conditions.—Erlenmeyer flasks (750 ml) containing 150 ml of potato-glucose medium or Czapek-Dox [14] were inoculated with a suspension of *Penicillium brevicompactum* prepared from 2—3 week potato agar slope cultures and incubated at 27° on a shaker. After 10 h incubation, the following labelled compounds (20—30 mg per flask) were added to the cultures as solids if water-soluble or in acetone solution: 4,6-dihydroxy-2-methyl-[6-^{14}C]benzoic acid (2), 4,6-dihydroxy-2,3-dimethyl[1-^{14}C]-benzoic acid (3), 5,7-dihydroxy[7-^{14}C]phthalide (7), 7-hydroxy-5-methoxy-4-methyl[7-^{14}C]phthalide (6), and 5,7-dihydroxy-4-methyl[7-^{14}C]phthalide (5). After interruption of the transformation at a suitable time, the mycelium of every flask was filtered, crushed with Celite, and extracted with ethyl acetate (5 × 50 ml). The cultural broth was acidified to pH 1 with conc. hydrochloric and extracted with ethyl acetate (5 × 100 ml). The organic extracts were washed with water until neutral, dried (Na_2SO_4), and evaporated to dryness under vacuum. The residue was chromatographed over silica gel (Merck, 0·05—0·2 mm), with heptane–ethyl acetate (1 : 1), ethyl acetate, and ethyl acetate–chloroform–acetic acid (55 : 45 : 1) as eluants. Fractions eluted with the last eluant contained mycophenolic acid, and were collected and evaporated to dryness under vacuum. The product was crystallised from heptane–ethyl acetate. Table 3 reports the amounts of labelled compounds incubated and of the mycophenolic acid obtained.

After incubation of 5,7-dihydroxy[7-^{14}C]phthalide (7) a new compound was indicated by t.l.c. on silica gel G HF 254 + 366 (Merck) with ethyl acetate–chloroform–acetic acid 45 : 55 : 1 as eluant. In this case the column chromatography was performed by adsorbing on silica gel (0·05—0·2 mm Merck; 160 g) the mixture of compounds (400 mg) dissolved in ethyl acetate (5 ml) and eluting with ethyl acetate–chloroform–acetic acid (45 : 55 : 1; 10 ml fractions). Fractions 2—4 gave the phthalide (7) (6 mg); fractions 5—9 gave mycophenolic acid (1) (55 mg); fractions 10—16 gave compound (8) (27 mg). 6-(*4,6-Dihydroxy-3-oxophthalan-5-yl*)-*4-methylhex-4-enoic acid* (8) (from heptane–ethyl acetate) had m.p. 193—194°, $\nu_{max.}$ (Nujol) 1710, 1670, and 1620 cm^{-1}; for n.m.r. see Table 6 (Found: C, 61·5; H, 5·6. $C_{15}H_{16}O_6$ requires C, 61·65; H, 5·5%).

TABLE 3

Compd.	Number of flasks	mg per flask	Acid (1) isolated (mg)	Activity * Precursor	Activity * (1)
(2)	2	25	34	170,581	1124
(3)	3	25	201	25,505	7945
(5)	1	30	53	176,200	36,335
(6)	2	20	51	161,617	3732
(7)	2	20	55	195,614	11,550

* Disint. min^{-1} mg^{-1}.

Isolation of 6-trans,trans-*Farnesyl-5,7-dihydroxy-4-methylphthalide* (9).—After 10 h incubation 5,7-dihydroxy-4-methyl[7-^{14}C]phthalide (5) (25 mg; 7560 disint. min^{-1} mg^{-1}) was added to each flask of culture of *P. brevicompactum*. Interruption of the fermentation after a further 48 h and the usual work-up of the material from four flasks yielded a residue which was chromatographed over silica gel (0·05—0·2 mm, Merck; 80 g). Hexane–ethyl acetate (8 : 2) eluted *compound* (9) (28 mg), m.p. 98—100° (from methanol–water), specific activity 2600 disint. min^{-1} mg^{-1}, $\nu_{max.}$ ($CHCl_3$) 1730 and 1632 cm^{-1} (Found: C, 74·9; H, 8·45. $C_{24}H_{32}O_4$ requires C, 74·95; H, 8·4%). With heptane–ethyl acetate (3 : 7) and ethyl acetate as eluants, 5,7-dihydroxy-4-methylphthalide (5) (19 mg; 2650 disint. min^{-1} mg^{-1}) was obtained; finally chloroform–ethyl acetate–acetic acid (55 : 45 : 1) eluted mycophenolic acid (115 mg; 4110 disint. min^{-1} mg^{-1}).

An analogous experiment with a 7 day fermentation time yielded compound (9) (16·7 mg; 236 disint. min^{-1} mg^{-1}) and compound (1) (175 mg; 1575 disint. min^{-1} mg^{-1}). Compound (5) was not present.

Isolation of 6-trans,trans-*Farnesyl-5,7-dihydroxyphthalide* (12).—To ten flasks of culture, 5,7-dihydroxy[7-^{14}C]-phthalide (7) (30 mg per flask; 19,629 disint. min^{-1} mg^{-1}) was added. After 48 h fermentation, the usual work-up gave a residue which was chromatographed over silica gel (0·05—0·2 mm, Merck; 80 g) with hexane–ethyl acetate (8 : 2) as eluant. The 6-*trans,trans*-farnesyl-5,7-dihydroxyphthalide (12) thus obtained (42 mg; 3780 disint. min^{-1} mg^{-1}), m.p. 101—103° (from ethanol–water) was identical with authentic specimen (Found: C, 74·6; H, 8·2. Calc. for $C_{23}H_{30}O_4$: C, 74·55; H, 8·15%).

'Enzymic-trap' Experiment with 4,6-*Dihydroxy*-2,3-*dimethyl*[1-^{14}C]*benzoic Acid* (3) *and* 5,7-*Dihydroxy*-4-*methylphthalide* (5).—4,6-Dihydroxy-2,3-dimethylbenzoic acid (3) (50 mg; 25,505 disint. min^{-1} mg^{-1}) and unlabelled 5,7-dihydroxy-4-methylphthalide (5) (200 mg) were added simultaneously in a single portion to two flasks of culture after 10 h incubation. After a further 48 h fermentation, the usual work-up gave a residue which did not show the presence of compound (3) on t.l.c. Methylation of the material in ethyl acetate with ethereal diazomethane for

[12] W. R. Allison and G. T. Newbold, *J. Chem. Soc.*, 1959, 3335.

[13] W. R. Logan and G. T. Newbold, *J. Chem. Soc.*, 1957, 1946.

[14] A. E. Oxford and H. Raistrick, *Biochem. J.*, 1948, **42**, 323.

1 min at 0° followed by evaporation to dryness under vacuum yielded a residue which was chromatographed over silica gel (0·05—0·2 mm. Merck: 140 g). Elution with heptane–ethyl acetate (4 : 1) gave 6-*trans,trans*-farnesyl-5,7-dihydroxy-4-methylphthalide (9) (25 mg; 1733 disint. min^{-1} mg^{-1}). Further elution with the same solvent mixture (1 : 1 ratio) yielded mycophenolic acid methyl ester (79 mg; 1890 disint. min^{-1} mg^{-1}); heptane–ethyl acetate (1 : 4) and ethyl acetate eluted 5,7-dihydroxy-4-methylphthalide (5) (108 mg; 4630 disint. min^{-1} mg^{-1}).

'Enzymic-trap' Experiment with 5,7-*Dihydroxy*-4-*methyl*[7-^{14}C]*phthalide* (5) *and* 6-trans,trans-*Farnesyl*-5,7-*dihydroxy*-4-*methylphthalide* (9).—Compound (5) (50 mg; 35,500 disint. min^{-1} mg^{-1}) and unlabelled (9) (350 mg), each dissolved in acetone (5 ml) were added to two flasks of culture after 10 h incubation. After 48 h fermentation, the usual work-up gave a residue which was chromatographed over silica gel (0·05—0·2 mm, Merck; 300 g) with hexane–ethyl acetate (4 : 1) as eluant to afford compound (9) (170 mg; 3130 disint. min^{-1} mg^{-1}). Further elution with chloroform–ethyl acetate–acetic acid (55 : 45 : 1) gave mycophenolic acid (140 mg; 5705 disint. min^{-1} mg^{-1}).

Incorporation of 6-*Geranyl*-5,7-*dihydroxy*-4-*methyl*[7-^{14}C]-*phthalide* (10) *and* 6-*Farnesyl*-5,7-*dihydroxy*-4-*methyl*[7-^{14}C]-*phthalide* (9) *into the Acid* (1).—To one flask of culture, after 10 h incubation, compound (10) (15 mg; 9882 disint. min^{-1} mg^{-1}) in acetone (4 ml) was added. After 7 days fermentation, work-up as usual gave a residue which was chromatographed over silica gel (0·05—0·2 mm, Merck) to give the acid (1) (10 mg; 768 disint. min^{-1} mg^{-1}) (incorporation 5·2%).

A similar experiment involving feeding 6-*trans,trans*-farnesyl-5,7-dihydroxy-4-methylphthalide (26 mg) in acetone (3 ml) gave the acid (1) (19 mg; 1194 disint. min^{-1} mg^{-1}) (total incorporation 33·6%; molar inc. 38·26%).

Incorporation of 6-*Geranyl*-5,7-*dihydroxy*[7-^{14}C]*phthalide* (11) *and* 6-trans,trans-*Farnesyl*-5,7-*dihydroxy*[7-^{14}C]*phthalide* (12) *into Compound* (8).—To one flask of culture, after 10 h incubation, compound (11) (30 mg; 37,757 disint. min^{-1} mg^{-1}) was added. After 7 days fermentation, work-up as usual gave a residue which was chromatographed on silica gel (0·05—0·2 mm, Merck) to give starting material (25 mg; 37,700 disint. min^{-1} mg^{-1}), the acid (1) (33 mg; 66 disint. min^{-1} mg^{-1}), and compound (8) (1 mg) which, diluted with an unlabelled specimen (10·6 mg) and crystallised to constant activity had a specific radioactivity of 655 disint. min^{-1} mg^{-1}.

A similar experiment involving feeding compound (12) (30 mg; 2090 disint. min^{-1} mg^{-1}) gave a residue from which, after chromatography over silica gel (0·05—0·2 mm, Merck), compound (1) (25 mg; 17 disint. min^{-1} mg^{-1}) and compound (8) (11 mg; 1650 disint. min^{-1} mg^{-1}) were isolated.

Incorporation of De-O-*methylmycophenolic Acid* (13) *into the Acid* (1).—To one flask of culture, after 10 h incubation, [7-^{14}C]-(13) (30 mg; 421 disint. min^{-1} mg^{-1}) was added. After 24 h fermentation, the usual work-up and chromatography gave the acid (1) (25 mg; 396 disint. min^{-1} mg^{-1}).

4,6-*Dihydroxy*-2-*methyl*[6-^{14}C]*benzoic Acid* (2).—A solution of ethyl 3,5-dibromo-4,6-dihydroxy-2-methyl[6-^{14}C]benzoate (1 g) in conc. sulphuric acid (30 ml) was left in the dark for 5 days at −5°, then poured on ice (150 g) and extracted with diethyl ether (3 × 50 ml). The extracts were washed with brine, dried (Na_2SO_4), and evaporated under vacuum. The residue was dissolved in saturated aqueous sodium hydrogen carbonate (5 ml), water (10 ml), and palladium–charcoal (50 mg) were added, and the mixture was hydrogenated at ambient pressure and temperature. After consumption of 2 mol. equiv. of hydrogen, filtration and acidification with 20% sulphuric acid gave a precipitate which was filtered off and crystallised twice from methanol–water to yield the *acid* (2), m.p. 155—158° (Found: C, 57·1; H, 4·9. $C_8H_8O_4$ requires C, 57·15; H, 4·8%).

4,6-*Dihydroxy*-2,3-*dimethyl*[1-^{14}C]*benzoic Acid* (3).—To a solution of sodium ethoxide [from sodium (0·549 g) and absolute ethanol (8·1 ml)] were added diethyl [2-^{14}C]-malonate (4·125 g; 0·05 mCi) and, after 20 min, 3-methylpent-3-en-2-one (2·295 g) during 45 min. The mixture was refluxed with stirring for 2 h, then cooled in ice, acidified with dil. sulphuric acid (Congo Red), and extracted with diethyl ether (3 × 50 ml). The extracts were washed with water until neutral, dried (Na_2SO_4), and evaporated to dryness. The residue which was dissolved in saturated aqueous sodium hydrogen carbonate and extracted with diethyl ether to eliminate some neutral material. Acidification of the aqueous layer with dil. sulphuric acid and extraction with diethyl ether yielded ethyl 2,3-dimethyl-4,6-dioxo[1-^{14}C]cyclohexanecarboxylate (3·4 g) as a viscous yellow oil, $\nu_{max.}$ ($CHCl_3$) 1735, 1720, 1650, and 1560 cm^{-1}. A solution of bromine (3·39 g) in acetic acid (3 ml) was added with stirring at −5° to this product (2 g) dissolved in acetic acid (5 ml). After 12 h at room temperature, the precipitate obtained filtered off and crystallised from acetic acid–water to yield *ethyl* 5-*bromo*-4,6-*dihydroxy*-2,3-*dimethyl*[1-^{14}C]*benzoate* (2·9 g), m.p. 67—69° (Found: C, 45·8; H, 4·6. $C_{11}H_{13}BrO_4$ requires C, 45·7; H, 4·5%). Hydrolysis as for the preparation of 4,6-dihydroxy-2-methyl[6-^{14}C]benzoic acid (2) afforded 5-*bromo*-4,6-*dihydroxy*-2,3-*dimethyl*[1-^{14}C]*benzoic acid* (80%), m.p. 160—162° (from hexane–diethyl ether) (Found: C, 42·1; H, 5·35. $C_9H_9BrO_4$ requires C, 41·95; H, 3·55%). Hydrogenation as before yielded 4,6-*dihydroxy*-2,3-*dimethyl*[1-^{14}C]*benzoic acid* (3) (52%), m.p. 167—168° (from methanol–water) (Found: C, 59·15; H, 5·35. $C_9H_{10}O_4$ requires C, 59·35; H, 5·55%).

7-*Hydroxy*-5-*methoxy*-4-*methyl*[7-^{14}C]*phthalide* (6).—5,7-Dimethoxy-4-methyl[7-^{14}C]phthalide (1 g) dissolved in dichloromethane (300 ml) was cooled to −10°, and boron trichloride (25 ml) (cooled to −60°) was added with stirring.

TABLE 4

Yields and reaction times for prenylation of compounds (5) and (7)

Allylic bromide	Compound (5)	Compound (7)
$MeO_2C{\cdot}[CH_2]_2{\cdot}CMe{=}CH{\cdot}CH_2Br$	30 min; 36%	120 min; 36%
Farnesyl bromide	30 min; 28%	120 min; 30%
Geranyl bromide	30 min; 34%	120 min; 32%

The solution was left at room temperature for 10 days. Decomposition with water and separation of the organic layer followed by washing with water, drying (Na_2SO_4), and evaporation to dryness afforded a residue which, crystallised from methanol, gave the *hydroxy-phthalide* (6) (810 mg), m.p. 216—218° (transition 160—180°), $\nu_{max.}$ ($CHCl_3$) 3450, 1730, and 1635 cm^{-1} (Found: C, 61·95; H, 5·2. $C_{10}H_{10}O_4$ requires C, 61·9; H, 5·2%).

5,7-Dihydroxy-4-methyl[7-^{14}C]*phthalide* (5).—5,7-Dimethoxy-4-methyl[7-^{14}C]phthalide (1 g) in dichloromethane (150 ml) was cooled to −10° and boron tribromide (6 ml) was added with stirring. After 8 days at room temperature, work-up as before yielded *compound* (5), m.p. 252—254° (from methanol–water) (65%), $\nu_{max.}$ (Nujol) 3420, 3330, 1725, and 1630 cm^{-1}, δ 2·17 (3H, s, CH_3Ar), 5·17 (2H, s, $ArCH_2O$), and 6·96 p.p.m. (1H, s, ArH) (Found: C, 60·15; H, 4·7. $C_9H_8O_4$ requires C, 60·0; H, 4·5%).

6-Prenylation of Compounds (5) *and* (7).—To a solution of the appropriate allylic bromide (1·3 mmol) in dioxan (20 ml) were added the phthalide (1 mmol) and silver oxide (1·5 mmol), and the mixture was stirred at room temperature (see Table 4). Filtration and evaporation under vacuum yielded a residue which was chromatographed over silica gel (0·05—0·2 mm, Merck; 60 g) with hexane–ethyl acetate (4 : 1). Fractions (15 ml) were monitored by t.l.c. [eluant heptane–ethyl acetate (7 : 3)], which showed that *C*-alkylation products were eluted first. Further fractions gave *O*-alkyl derivatives, and subsequently traces of starting material. Tables 5 and 6 give physical and n.m.r. data.

Hydrolysis of the Methyl Esters (14) *and* (15).—To a solution of methyl ester (14) (1·2 mmol) in ethanol–water (2 : 1; 20 ml) was added N-sodium hydroxide (2 ml). After 12 h at room temperature the mixture was acidified with acetic acid, evaporated to dryness under vacuum, and extracted with ethyl acetate. Compound (8), thus obtained in 90% yield, was identical with the sample from the culture. *6-(4,6-Dihydroxy-7-methyl-3-oxophthalan-5-yl)-4-methylhex-4-enoic acid* (13), similarly prepared (88% yield), had m.p. 147—149° (from heptane–ethyl acetate) (Found: C, 62·65; H, 6·0. $C_{16}H_{18}O_6$ requires C, 62·75; H, 5·9%).

TABLE 5

Physical data

Compound	M.p. (°C)	$\nu_{max.}$/cm^{-1} ($CHCl_3$)	Found (%) C	Found (%) H	Required (%) C	Required (%) H
(9) *	98—100	3440, 2920, 1735, 1635	74·9	8·4	74·95	8·4
(10) *	98—100	3440, 2930, 1735, 1635	72·15	7·75	72·1	7·6
(11) *	140—142	3400, 2930, 1727, 1640	71·55	7·4	71·5	7·35
(12) *	101—103	3390, 2940, 1730, 1637	74·6	8·25	74·55	8·15
(8) † ‡	193—194	1710, 1670, 1620 §	61·6	5·6	61·65	5·5
(15) *	107—110	3440, 2940, 1735, 1635	62·75	6·0	62·75	5·9

* From methanol–water. † From heptane–ethyl acetate. ‡ Condensation performed with methyl ester. Reaction product characterised as acid. § In Nujol.

TABLE 6

N.m.r. data (δ in p.p.m.; *J* in Hz)

Compd.	Solv.	$-[CH_2]_n-$	$CH_3{\cdot}C{=}CH$	HC=C<	ArH	OMe	ArMe	$ArCH_2{\cdot}O$	$ArCH_3{\cdot}CH{=}$
(1)	$CDCl_3$	2·38(m) $n = 2$	1·81(s)	5·18 [c]		3·76(s)	2·15(s)	5·18(s)	3·37 (d, *J* 7)
(1)	C_5D_5N	2·62(m) $n = 2$	1·95(s)	5·7(t) *J* 7		3·73(s)	2·00(s)	5·08(s)	3·69 (d, *J* 7)
(9)	$CDCl_3$	2—2·16(m) $n = 4$	1·60(s) [a] 1·68(s) [b] 1·87(s) [b]	4·95—5·3			2·06(s)	5·18(s)	3·47 (d, *J* 7)
(10)	$CDCl_3$	2·06(m) $n = 2$	1·60(s) [b] 1·69(s) [b] 1·83(s) [b]	5·12 [c]			2·06(s)	5·14(s)	3·46 (d, *J* 7)
(15)	$CDCl_3$	2·41(m) $n = 2$	1·85(s)	5·2 [c]		3·66(s)	2·07(s)	5·16(s)	3·44 (d, *J* 7)
(8)	C_5D_5N	2·63(m) $n = 2$	2·03(s)	5·89(t) *J* 7	6·69(s)			5·08(s)	3·86 (d, *J* 7)
(11)	$CDCl_3$	2·06(m) $n = 2$	1·6(s) [b] 1·67(s) [b] 1·83(s) [b]	5·1—5·4 (2H, m)	6·45(s)			5·2(s)	3·44 (d, *J* 7)
(11)	C_5D_5N	2·05(m) $n = 2$	1·53(s) [b] 1·63(s) [b] 2·03(s) [b]	5·84—6·06 (2H, m)	6·74(s)			5·11(s)	3·91 (d, *J* 7)
(12)	$CDCl_3$	2·04(m) $n = 4$	1·58(s) [a] 1·66(s) [b] 1·82(s) [b]	5·02—5·38 [b]	6·44(s)			5·2(s)	3·44 (d, *J* 7·8)

[a] 6H. [b] 3H. [c] Observed.

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