The Biosynthesis of Phenols. Part XXIV.[1] The Conversion of the Anthraquinone Questin into the Benzophenone, Sulochrin, in Cultures of *Aspergillus terreus*

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It has been established that questin (2), with different levels of [^{14}C]-labelling at two centres, is transformed by cultures of *Aspergillus terreus* to sulochrin (1) with the same ratio of specific [^{14}C]-labelling at these centres. The significance of this result is discussed in relation to hypotheses concerning the biosynthesis of sulochrin.

THERE are two types of hypothesis concerning the biosynthesis of sulochrin (1) by fungi. According to the first,[2] this benzophenone is derived from two benzenoid compounds, each of which has been formed from acetate and malonate units. According to the second,[3] the compound is formed through an anthrone, or anthraquinone, which has been derived from a single polyketide chain. At the time when they were proposed, there was no *a priori* reason for favouring one of these hypotheses. The similarity of the structure of sulochrin to that of the only closely related fungal benzophenone, griseophenone Y (3),[4] does, as Richards and Hendrickson point out,[5] suggest that the ester function in the former compound may be an oxidized methyl group of an orsellinic acid residue, but there was no labelling evidence for this. The biosynthesis of no other fungal benzophenone had been investigated. Radioactive labelling studies relating to aromatic ketones derived from plants suggested that for such compounds as phloridzin (4),[6] and the presumed chalcone intermediates in anthocyanin biosynthesis, one aromatic nucleus is derived from shikimic acid and the other from acetate-malonate units.[7] However, other related plant metabolites, such as eleutherinol appear to be derived from acetate-malonate units, alone.[8]

Some studies of the labelling pattern of sulochrin, following incorporation of either [2-^{14}C]acetic acid, or [2-^{14}C]malonic acid in *Aspergillus terreus*, led us to favour the first hypothesis.[9] The differences in the degree of labelling of the carbon atoms of rings A and B of sulochrin indicated that both rings were derived from acetate and malonate units, but there were differences in the average labelling of the carbon atoms that suggested separate origins for the two rings. However, attempts to incorporate appropriate labelled monobenzenoid compounds into sulochrin were not successful.

In what follows, we shall describe [^{14}C]-labelling experiments which were designed to determine whether, in cultures of *A. terreus*, sulochrin is derived from the related anthraquinone, questin (2). This compound had been identified previously in cultures of *Penicillium frequentans* and *A. terreus* which produced sulochrin.[10,11]

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[3] S. Gatenbeck, *Svensk. kem. Tidskr.*, 1960, **72**, 188; T. Money, *Nature*, 1963, **199**, 592.
[4] W. J. McMaster, A. I. Scott, and S. Trippett, *J. Chem. Soc.*, 1960, 4628.
[5] J. H. Richards and J. B. Hendrickson, 'The Biogenesis of Steroids, Terpenes, and Acetogenins,' W. A. Benjamin, New York, 1964, p. 82.
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[7] H. Grisebach, *Z. Naturforsch.*, 1957, **12*b***, 227, 597; 1958, **13*b***, 335; 1959, **14*b***, 485.
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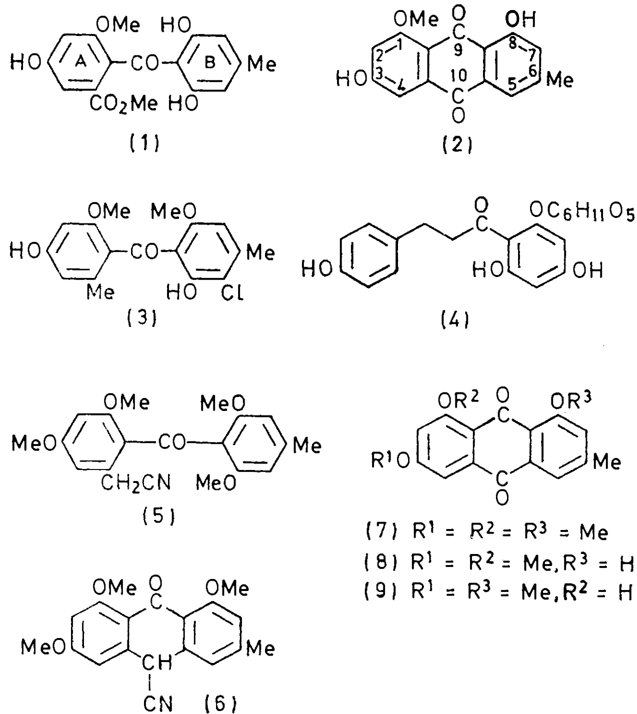
[11] P. C. Harries, Ph.D. Thesis, University of Wales, 1963, p. 79.

The preparation of double-labelled, [^{14}C]questin was based on a convenient new procedure for obtaining polysubstituted anthraquinones in good yield; [12] before this, no synthesis of this natural anthraquinone had been reported. [^{14}C]Carboxy-labelled, di-*O*-methyl-*p*-orsellinic acid was condensed with α-cyano-3,5-dimethoxytoluene in the presence of trifluoroacetic anhydride to give the benzophenone (5). This was converted into the anthraquinone, tri-*O*-methylemodin (7) through the cyano-anthrone (6), which was oxidised with hydrogen peroxide in alkali. Details of this and related anthraquinone syntheses have been described.[13]

The demethylation of tri-*O*-methylemodin with several reagents was investigated to allow the preparation of questin. When the fully methylated emodin was refluxed with sodium iodide in glacial acetic acid, it gave a mixture of two products (8) and (9); their structures were indicated by ^{1}H n.m.r. data for the mixture. A single compound was formed in almost quantitative yield by the action of boron tribromide in dichloromethane at −70°. This was identified as



1,3-dimethoxy-8-hydroxy-6-methylanthraquinone (8), by means of ^{1}H n.m.r. data and the fact that it has the same properties as the compound, m.p. 210°, which had been assigned this structure by Stickings.[10] Fusion of tri-*O*-methylemodin with pyridine hydrochloride for several hours gave complex mixtures but when the reaction was prolonged to 7 hr., good yields (95%) of emodin (10) were obtained. These results taken with the observation that preferential 3-*O*-benzylation (85%) could be achieved using a benzyl chloride–triethylamine mixture, led to a high-yielding synthesis of questin from emodin through 3-*O*-benzylemodin (11) and 3-*O*-benzyl-1,8-di-*O*-methylemodin, in turn. It was found, for the last step, that simultaneous removal of 3-*O*-benzyl and 8-*O*-methyl groups occurred with boron tribromide at −100° to give almost quantitative yields of questin. This synthesis of questin made it possible to introduce a second [^{14}C]-label into [9-^{14}C]-labelled emodin as the 1-*O*-[^{14}C]-methyl function. The ratio of the [^{14}C]-labelling at the two positions was determined by demethylation to [^{14}C]tetramethylammonium iodide and [9-^{14}C]emodin.

After this radioactive questin (12) had been incubated with cultures of *A. terreus* for 25 hr. the sulochrin was isolated and was shown by degradation to contain 18% of the radioactivity of the added questin. Furthermore, degradation of this [^{14}C]sulochrin (13) to [^{14}C]-*p*-orsellinic acid (14) and 3-hydroxy-5-[^{14}C]methoxybenzoic acid (15), as in the Scheme, showed that the ratio of the [^{14}C]-label at the two corresponding centres in sulochrin was the same as that in questin.

These experiments, and an independent investigation using an enzyme preparation from *Penicillium frequentans* for a similar transformation of questin,[14] have established that sulochrin is derived from this anthraquinone by an oxidative cleavage involving the 10-carbonyl group.

Our earlier studies of sulochrin prepared from cultures containing [^{14}C]acetic and [^{14}C]malonic acids provided evidence of small but significant differences in the average labelling of the carbon atoms of the separate rings. Similar differences must now be attributed to the [^{14}C]questin from which this [^{14}C]sulochrin was derived. There is no direct information on the degrees of labelling of each of the ring carbons of such an 'acetate-malonate'-derived anthraquinone but it has been generally assumed that, apart from 'starter group' effects, labelling is precisely uniform.[15,16] This anticipates that the [^{14}C]-labelled, two-carbon units are all incorporated into the polyketide chain at virtually the same time. For the most part, the labelling evidence is in accord with this but there are some other cases of acetate-malonate-derived aromatic compounds where there is variation in the labelling.[17,18] It seems likely in such cases that the final product is formed, as for certain fatty acids, through preformed acetate-malonate-derived groups with four or more carbon atoms; such groups may have somewhat different degrees of labelling, for each two-carbon fragment, from the acetate units which are added sequentially to form the polyketide. Moreover, there is evidence in some cases of more than

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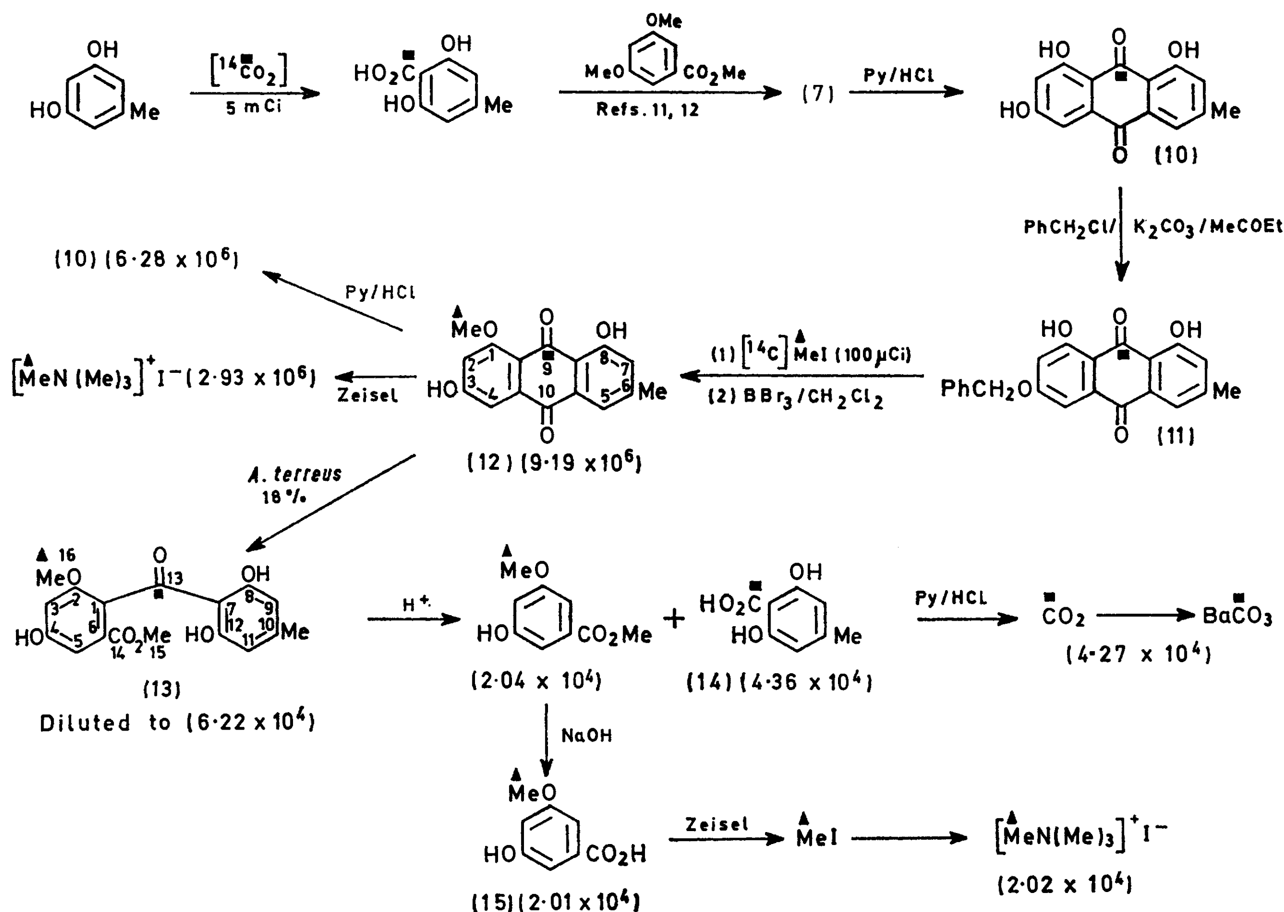
one polyketide chain being involved in the biosynthesis of a single molecule.[19,20,21] More detailed evidence of the stages involved in the formation of the polyketide precursor is necessary before a satisfactory explanation can be offered for the irregularities reported for the [^{14}C]-labelling pattern of sulochrin.

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus. Details of the radiochemical equipment are given in Part X.[9] Light petroleum had b.p. 60—80°. I.r. spectra (P.E. 257, in KBr discs), u.v. spectra (Unicam SP 800, in ethanol), n.m.r. spectra (P.E. R10, 60 Hz, $CDCl_3$ with $SiMe_4$), and mass spectra (MS9, 70 e.v.) were determined in the usual way. T.l.c. was Kieselgel G developed with (system 1) benzene–methanol–acetic acid (10 : 2 : 1, v/v) and (system 2) chloroform–ethyl acetate (1 : 1, v/v). Chromatograms were examined under light of 2537 Å.

(a) *Cultures and Media.*—Cultures of *A. terreus* (60-ml. per flask) were prepared as in Part X [9] and after incubation at 25° on a shaker for 50 hr. double-labelled [^{14}C]questin (see below) (30 mg.) in ethanol (7·5 ml.) was distributed equally between 15 flasks. After 25 hr. at 25°, the combined broths were filtered, and the filtrate was acidified with 4N-hydrochloric acid to pH 1 and shaken with ether (4 × 400 ml.). The ether extract was concentrated (300 ml.), shaken with saturated sodium hydrogen carbonate and then 0·5N-sodium carbonate (3 × 100 ml.); this was then acidified and re-extracted with ether. The extract was washed, dried, and evaporated to give sulochrin (32 mg.). Authentic sulochrin (800 mg.) was then added. Recrystallisation from ethyl acetate–light petroleum gave pure sulochrin (760 mg.) m.p. 261°, recrystallised to constant radioactivity. The incorporation of radioactivity was 18·0%.



SCHEME R.m.a. values in d.p.m./mmole

Ratio of ■C : ▲C in question = 6·28 : 2·93 = 2·14   Ratio of ■C : ▲C in sulochrin = 4·27 : 2·02 = 2·12

(b) *Radioactive Assays.*—The method described in Part X [9] was used. All samples were counted to 10^4 counts and to constant activity; at least three determinations were made for each sample.

(c) *Synthesis of* [^{14}C]*Questin*.[11]—(i) p-*Orsellinic* [^{14}C]-*acid*. Orcinol (2·5 g.) and anhydrous potassium carbonate (7·5 g.) were mixed as a fine powder and transferred to a high-pressure vessel (80 ml.) in a rocking autoclave, which was flushed with dry nitrogen. Barium [^{14}C]carbonate

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(5 mCi; diluted to 3·6 g.) was treated with sulphuric acid (*d*, 1·84; 50 ml.) in a vacuum system and the carbon dioxide which was evolved was trapped in a test-tube in liquid nitrogen. The test-tube was removed from the vacuum line and transferred at liquid N_2 temperature to the autoclave which was sealed and rapidly heated with shaking to 210°. The pressure rose to 10 atmos. and gradually fell during 20 hr. after which time the autoclave was allowed to cool for 24 hr. The granular product was dissolved in 4N-hydrochloric acid (100 ml.) and extracted with ether (2 × 100 ml.) which was then shaken with 0·5N-sodium hydrogen carbonate (2 × 100 ml.). Acidification and work-up in the usual way gave *p*-orsellinic [^{14}C]-acid (2·9 g.) as colourless prisms from benzene–methanol, m.p. 176° (lit.,[22] m.p. 176°). When this sample was decarboxylated as described below no radioactivity was detected in the recovered orcinol.

(ii) 1,3,8-*Trimethoxy*-6-*methyl*[9-^{14}C]*anthraquinone* (7). The preceding *p*-orsellinic [^{14}C]acid was converted, as described previously,[12,13] into 1,3,8-trimethoxy-6-methyl[9-^{14}C]anthraquinone, m.p. 163° (lit.,[23] m.p. 163°).

(iii) [9-^{14}C]*Emodin* (10). 1,3,8-Trimethoxy-6-methyl-[9-^{14}C]anthraquinone (1·85 g.) was heated with pyridine hydrochloride (30 g.) at 175—180° (oil bath) for 7 hr. The product was cooled, water (100 ml.) was added to it and the precipitated [9-^{14}C]emodin was collected and then dissolved in N-sodium carbonate (250 ml.). The dark red solution was filtered and acidified with hydrochloric acid; the yellow precipitate was collected, washed, dried, and recrystallised from aqueous pyridine to give [9-^{14}C]-emodin (1·39 g.) as yellow needles, m.p. 256° (lit.,[24] 256—257°), R_F (system 1, 0·52; 2, 0·50).

Attempts to carry out specific demethylation with aluminium trichloride in benzene,[25] lithium iodide in collidine,[26] sodium or lithium iodide in acetic acid,[27] boron trichloride in dichloromethane at 0°,[28] and hydrogen bromide in acetic acid,[29] all gave varying mixtures of dimethyl ethers of emodin; physcion, and emodin, which could be easily distinguished by t.l.c.; there was no evidence of questin. The sodium iodide–acetic acid reagent gave a 1 : 1 mixture of (8) and (9); τ, (pair of equal peaks) 7·55 $ArCH_3$ of (8), and 7·60 $ArCH_3$ of (9); four equal peaks, 5·99, 6·01, 6·05, 6·15 (2 × $2OCH_3$); four pairs of peaks 2·60—3·36 2 × 4 ArH. With boron tribromide in dichloromethane at −70° (ref. 30) raised to 0° over 1 hr., 8-hydroxy-2,3-dimethoxy-6-methylanthraquinone,[9] m.p. 210°, identical with that obtained from questin by methylation with diazomethane, was produced.

(iv) 3-*Benzyloxy*-1,8-*dihydroxy*-6-*methyl*[9-^{14}C]*anthraquinone* (11). [9-^{14}C]Emodin (0·8 g.), freshly distilled benzyl chloride (7 ml.), and triethylamine (7 ml.), were dissolved in dry ethyl methyl ketone (140 ml.) and heated under reflux for 18 hr. with continuous stirring. The product was poured into N-hydrochloric acid (150 ml.) which was shaken with ether (500 ml.). The ether extract was shaken with 0·5N-sodium carbonate and then with N-sodium hydroxide (6 × 100 ml.). Acidification of alkaline extract and work up by ether extraction in the usual way gave the *benzyl* ether (11) (0·9 g.), m.p. 195° [Found: C, 73·1; H, 4·6%; *M* (mass spectrometry), 360. $C_{22}H_{16}O_5$ requires C, 73·3; H, 4·5%; *M*, 360], $\nu_{max.}$ 3100 (OH), 1670 (OH bonded CO), and 1475—1460 (OH); $\lambda_{max.}$ 260, 268, 292, and 440 nm.; τ, 2·41 (1H), 2·6 (5H), 2·96 (1H), 3·30 (1H, Ar), 4·84 (2H, CH_2), and 7·57 (3H, $ArCH_3$); R_F (system 2, 0·75).

(v) 3-*Benzyloxy*-1,8-[$^{14}C_2$]*dimethoxy*-6-*methyl*[9-^{14}C]*anthraquinone*. [^{14}C]Methyl iodide (100 μCi) was transferred *via* a vacuum line to a 250-ml. flask cooled in liquid nitrogen. Cooled, dry acetone (150 ml.) was added followed by the preceding benzyl ether (0·9 g.), anhydrous potassium carbonate (2·0 g.), and methyl iodide (2·4 g.). The solution was heated under reflux with magnetic stirring for 40 hr. and monitored by t.l.c. The cooled solution was filtered and the filtrate evaporated; the residue was shaken with chloroform (200 ml.) which was filtered and evaporated. The residue crystallised from ethyl acetate–light petroleum to give the 3-*benzyloxy*-1,8-*dimethoxy-derivative* (0·88 g.) as yellow prisms, m.p. 172° [Found: C, 74·3; H, 5·5%, *M* (mass spectrometry) 388. $C_{24}H_{30}O_5$ requires: C, 74·2; H, 5·2%], $\nu_{max.}$ 3060 (OH) and 1655 (OH bonded CO); $\lambda_{max.}$ 279 and 406 nm; R_F (system 1), 0·58 and (system 2) 0·45.

(vi) [1-*Me*O-^{14}C,9-^{14}C]*Questin* (12).—The preceding benzyl dimethyl ether (VII) (200 mg.) in dry dichloromethane (80 ml.) was cooled to −100° in liquid nitrogen. Boron tribromide (2·5 g.) in dichloromethane (100 ml.) was added dropwise during 30 min. with magnetic stirring, at −100°. Cooling was removed and the temperature was allowed to rise slowly during 1 hr. to 5° when dichloromethane (40 ml.) and N-hydrochloric acid (40 ml.) were added. The aqueous layer was separated, shaken with dichloromethane (20 ml.), and the combined organic phases were extracted with 0·5N-sodium carbonate (2 × 100 ml.). Acidification gave a flocculent orange precipitate which was purified by preparative t.l.c. (40 cm., 25-mg. per plate) developed with ethyl acetate–chloroform (1 : 1). The major component was isolated and crystallised from ethyl acetate–light petroleum to give [1-*Me*O-^{14}C,9-^{14}C]-questin (12) (124 mg.) as orange needles, m.p. 303°, (lit.,[10] 302°), showing no depression with natural material and identical in two t.l.c. systems (Found: C, 67·9; H, 4·0. Calc. for $C_{16}H_{12}O_5$: C, 67·6; H, 4·25%), R_F (system 1), 0·55 and (system 2) 0·35.

(d) *Degradation Methods*.—(i) *Degradation of* [1-*Me*O-^{14}C,9-^{14}C]*questin* (12). Doubly labelled questin (38 mg.) was heated under reflux with phenol (2·0 g.) and hydriodic acid (*d*, 1·7; 5 ml.) in a micro-Zeisel apparatus under a stream of nitrogen. Methyl iodide which was evolved was collected in two traps containing dry trimethylamine (1 ml.) in dry acetone (9 ml.). Tetramethylammonium iodide (17 mg.) was collected, washed with dry acetone, dried, and assayed by total combustion to barium carbonate.

A further sample of doubly labelled questin (30 mg.) was heated with pyridine hydrochloride (15 g.) at 170° for 5 hr. Work-up as described above gave [9-^{14}C]emodin (11) (23 mg.), m.p. 255°, identical with the earlier sample. After recrystallisation to constant activity this was assayed by total combustion to barium carbonate.

(ii) *Degradation of* [13,16-$^{14}C_2$]*sulochrin* (13). Material

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[24] R. A. Jacobson and R. Adams, *J. Amer. Chem. Soc.*, 1924, **46**, 1312.
[25] M. Kulka, *J. Amer. Chem. Soc.*, 1954, **76**, 5469.
[26] I. T. Harrison, *Chem. Comm.*, 1969, 616.
[27] T. L. V. Ulbricht, *J. Chem. Soc.*, 1961, 3345.
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[29] L. Lang, jun. and A. Burger, *J. Org. Chem.*, 1941, **6**, 852.
[30] J. F. W. McOmie and M. L. Watts, *Chem. and Ind.*, 1963, 1658.

from the incorporation experiment (above) (2 × 380 mg.) was degraded to *p*-orsellinic [^{14}C]acid (14) (204 mg.) and methyl 3-hydroxy-5-methoxybenzoate (261 mg.) and thence to the acid (15) as previously described [3]; samples were then assayed by combustion to barium carbonate.

(iii) *Degradation of* p-*orsellinic* [^{14}C]*acid* (14). Pyridine hydrochloride (25 g.) was heated at 150° for 3 hr. under a stream of nitrogen and cooled to 110°; [^{14}C]-*p*-orsellinic acid (140 mg.) was then added to it and the mixture was heated to 150° under a stream of nitrogen. Carbon dioxide which was evolved was collected as barium carbonate (82 mg.); this was assayed for radioactivity.

(iv) *Degradation of* 3-*hydroxy*-5-[^{14}C]*methoxybenzoic acid* (15). The acid (30 mg.) was demethylated as for questin (above) to yield tetramethylammonium iodide (25 mg.).

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