Tetrahedron, 1963, Vol. 19, pp. 1001 to 1010. Pergamon Press Ltd. Printed in Northern Ireland

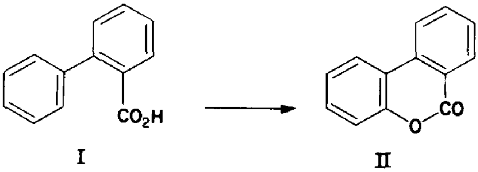
EXPERIMENTS RELATED TO THE BIOSYNTHESIS OF NOVOBIOCIN AND OTHER COUMARINS[1]

C. A. Bunton,* G. W. Kenner, M. J. T. Robinson and B. R. Webster
The Robert Robinson Laboratories, University of Liverpool
* William Ramsay and Ralph Forster Laboratories, University College, London

(*Received* 14 *December* 1962)

Abstract—The heterocyclic oxygen atom in the coumarin portion of novobiocin (III) comes from the carboxyl group of L-tyrosine added to the fermentation. Oxidation of 4,4'-dimethoxydiphenic acid with lead tetra-acetate gives the dimethyl ether of the dihydroxy 3,4-benzcoumarin (XIV), a natural product. These and other results are discussed in terms of the general concept of oxidative cyclization.

OXIDATIVE cyclization of biphenyl-2 carboxylic acid (I) and its derivatives leads to 3,4-benzcoumarin (II) or its derivatives.[2] Evidence was presented that cyclization can occur by intramolecular attack of a potential carboxylate cation or radical, depending on the reagents. In the meantime Corey *et al.*[3] have shown that cations can be generated by electrolysis, and it should be noted that the results of anodic cyclizations, which we tentatively interpreted by radical substitution, are consistent with the intervention of carboxylate cations, because, as we have already pointed out,[2] this very reactive species should undergo a rapid cyclization unaffected by substituents in the ring being attacked except in so far as they determine the conformation of the biphenyl system.



Our discovery of this oxidative cyclization prompted the speculation that some naturally occurring coumarins might be formed in an essentially similar way. Indeed the biological connection between cinnamic acids, which may be regarded as variants of structure (I), and coumarins is close.[4] The biosynthesis of coumarin itself has been the subject of extensive, careful investigations.[5,6] These have been hindered by the

---

[1] Part of this work was outlined by K. Chambers, G. W. Kenner, M. J. T. Robinson and B. R. Webster, *Proc. Chem. Soc.*, 291 (1960).

[2] G. W. Kenner, M. A. Murray and C. M. B. Tylor, *Tetrahedron* **1**, 259 (1957).

[3] E. J. Corey, N. L. Bauld, R. T. La Londe, J. Casanova and E. T. Kaiser, *J. Amer. Chem. Soc.* **82**, 2645 (1960).

[4] H. Grisebach and W. D. Ollis, *Experientia* **17**, 4 (1961).

[5] F. Weygand and H. Wendt, *Z. Naturforsch.* **14b**, 421 (1959); T. Kosuge and E. E. Conn, *J. Biol. Chem.* **234**, 2133 (1959); S. A. Brown, G. H. N. Towers and D. Wright, *Canad. J. Biochem. Physiol.* **38**, 143 (1960); F. Weygand, H. Simon, H. G. Floss and U. Mothes, *Z. Naturforsch.* **15b**, 765 (1960); S. A. Brown. *Ibid.* 768.

[6] T. Kosuge and E. E. Conn, *J. Biol. Chem.* **236**, 1617 (1961); S. A. Brown, *Canad. J. Biochem. Physiol.* **40**, 607, (1962).

difficulties of biosynthetic work with plants, but it has been clearly established that shikimic acid, phenylalanine, and cinnamic acid are precursors of coumarin. It has also been shown that cinnamic acid is rapidly converted by *Melilotus alba* and *Hireochloe odorata* into the β-glucoside of *o*-coumaric acid and that coumarin is probably produced by *trans* to *cis* isomerisation followed by enzymic hydrolysis of the glucoside.[6] Thus, although every step of biosynthesis has not yet been defined precisely, it is unlikely that an oxidative cyclization, analogous to that in the biphenyl series, is involved. However, before these investigations, our attention had been caught by three natural coumarins, which might have arisen by oxidative cyclization. Two were benzcoumarins, extracted from castoreum, and these will be discussed in the latter half of this paper. The third was novobiocin (III);[7] it appeared that the 3-aminocoumarin portion of this molecule might be formed from tyrosine (IV) by oxidative cyclization of a derivative. Novobiocin, a fungal metabolite, is also a much more attractive object of biosynthetic study than most coumarins, which are products of higher plants. Other aspects of its biosynthesis, including the introduction of the 8-methyl group of the coumarin portion, are being examined by Birch *et al.*[8]

Our particular interest in the biosynthesis of novobiocin concerns the origin of the heterocyclic oxygen atom in the coumarin nucleus and clearly the requisite information can only be derived from experiments with ^{18}O-labelled compounds. However ^{14}C-labelling had also to be employed in determining the origin of the carbon skeleton of the coumarin portion, making parallel controls of carbon-incorporation during experiments with ^{18}O, and establishing the feasibility of the ^{18}O work. The insensitivity of mass spectrometric analysis, as compared with radio-active counting, demanded very efficient incorporation of tyrosine into the coumarin. In the event, we were fortunate in choosing novobiocin, and it is very unlikely that similar work could be carried out with higher plants.

L-[^{14}C]-Tyrosine (IV, generally labelled) was added to cultures of *Streptomyces niveus*, which Dr. P. E. Macey kindly grew for us. There was high incorporation in novobiocin (III), which was cleaved by acetic anhydride and pyridine at the amide link.[7a] The relative radio-activities of the two fragments showed that tyrosine had been an equally efficient precursor of the two aromatic sections of the antibiotic, supplying seven carbon atoms to the benzenoid section and nine to the coumarin. At this stage noviose was still attached to the coumarin, but acidic hydrolysis of the glycoside left all the activity in the coumarin.* The inactivity of the sugar was confirmed by an alternative degradation with hydrochloric acid in aqueous ethanol[7a] to ethyl novioside and cyclonovobiocic acid (V), which was the intermediate in all further work. Drastic hydrolysis with hydrochloric acid in acetic acid, an adaptation of a technique applied to dihydronovobiocic acid,[7b] yielded the phenacylamine hydrochloride (VII), carbon dioxide and 2,2-dimethylchroman-6-carboxylic acid (VIII) with the expected relative activities. Thus, without recourse to specific labelling of tyrosine,

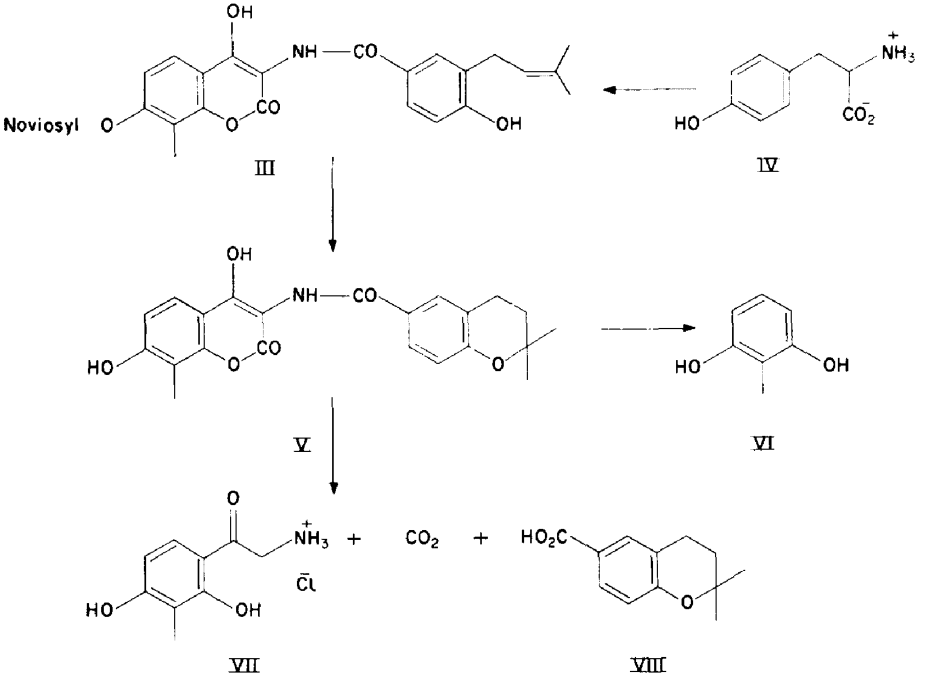
---

* The structures of these degradation products and their relative radio-activities are given in ref. 1. The activity of compound (VII) in that communication should have been 7.3, not 7.7.

[7a] J. W. Hinman, E. L. Caron and H. Hoeksema, *J. Amer. Chem. Soc.* **79**, 3789 (1957);

[7b] C. H. Stammer, E. Walton, A. N. Wilson, R. W. Walker, N. R. Trenner, F. W. Holly and K. Folkers, *Ibid.* **80**, 137 (1958); and papers cited therein.

[8] A. J. Birch, D. W. Cameron, P. W. Holloway and R. W. Rickards, *Tetrahedron Letters* Nos. 25, 26 (1960); A. J. Birch, P. W. Holloway and R. W. Rickards, *Biochim. Biophys. Acta* **57**, 143 (1962).



there is no doubt that it is rationally incorporated in both aromatic sections of novobiocin.

Turning to ^{18}O-labelling, the prime requirement was a degradation product, containing preferably only one oxygen atom and at most three, which did not suffer oxygen-exchange during the degradation. The best, accessible compound appeared to be 2-methylresorcinol (VI), and in fact it was obtained from cyclonovobiocic acid (V) in satisfactory yield by fusion with potassium hydroxide. Moreover, fusion with K^{18} OH gave isotopically normal 2-methylresorcinol. The preceding, fairly mild acidic degradation to cyclonovobiocic acid may quite likely exchange the carbonyl oxygen of the coumarin, but it could not disturb the heterocyclic oxygen atom. During isotopic analysis of 2-methylresorcinol, the method of Rittenberg–Ponticorvo[9] gave high, but reproducible results when applied to isotopically normal samples. Provided that a correction was made for the spuriously high "normal" abundance this method gave results agreeing qualitatively with those obtained by another, more reliable method, but, except for one control experiment, we quote only the results obtained by the latter method. This consists in heating the sample with a mixture of guanidine hydrochloride and *o*-phenylene diamine hydrochloride at 350° in a vacuum for 4–5 hours; the evolved carbon dioxide is analysed mass-spectrometrically. This method does not eliminate oxygen from a single phenolic group, but it works with 2-methylresorcinol, presumably *via* the keto-form.

Carboxyl-[^{18}O]-tyrosine was prepared by hydrogen bromide-catalysed exchange with $H_2{}^{18}O$. A technique was developed for 99% recovery of the water. Degradation to isotopically normal anisic acid (Rittenberg–Ponticorvo analysis) confirmed that

---

[9] D. Rittenberg and L. Ponticorvo, *Int. J. Applied Radiation Isotopes* **1**, 208 (1956).

the phenolic oxygen atom had not been exchanged. For fermentations, 5·4 g of L-tyrosine, carboxyl-[^{18}O] and generally-[^{14}C]-labelled, was prepared; the abundance of ^{18}O in the carboxyl group was 15·2 atom% excess (guanidine hydrochloride method). Preliminary work with greatly diluted [^{14}C]-tyrosine had shown that relatively massive quantities could be added to a fermentation without drastically lowering the efficiency of incorporation. The doubly labelled tyrosine was added to six 4 l. fermenters, which yielded 12 g of novobiocin. ^{14}C-Counting showed that 3% of the coumarin section had been formed from the added tyrosine, Hence, on the hypothesis of oxidative cyclization and allowing for only half the oxygen in the 2-methylresorcinol coming from the heterocyclic ring of the coumarin, this degradation product should have contained 0·22 atom % excess of ^{18}O, provided that there was no exchange of oxygen between the carboxyl group and the environment prior to cyclization. The experimental result was 0·078 atom % excess and this clearly signifies inclusion of carboxylate oxygen in the heterocyclic ring.* A similar amount of environmental exchange was observed in double labelling experiments on the incorporation of acetate into orsellinic acid in *Chaetomium cochliodes*.[10] We considered carefully the possibility of checking its extent by a separate degradation to a coumarin, which would include the carbonyl oxygen, but this did not seem to be feasible with any useful accuracy and the results could all too easily be vitiated by carbonyl exchange *after* ring-closure, either during fermentation or degradation.

The results of the foregoing tracer work are in accord with our speculation concerning the biosynthesis of novobiocin and at variance with involvement of *ortho*-hydroxylation, which is the path to coumarin.[6] It is well recognised that coumarin itself is exceptional, amongst natural products containing the ring system, in lacking an oxygen atom at the 7-position.[11] Therefore the biosynthesis of novobiocin may well be more typical despite the presence of a nitrogen atom and other unusual structural features. For simplicity, the following discussion is in terms of cyclization of cinnamic acids, although the three-carbon side chain might be at a different level of oxidation. Two hypotheses allocate a specific function to a *p*-hydroxyl group which becomes the 7-hydroxyl substituent in coumarin, and both are consistent with our data. Haworth[12] suggested formation of hydroxy dienone (IX). The mechanism of its cyclization was not specified but presumably addition of carboxylate anion to the α,β-unsaturated ketone and subsequent elimination of water was envisaged.† The second hypothesis[4,13] is more akin to the general concept of oxidative cyclization. The spiro-dienone (X) might be produced by dehydrogenation of the *p*-hydroxycinnamic acid and it could rearrange to the 7-hydroxycouramin (or, by carbon migration, to the 6-hydroxycoumarin). Indeed spiro-dienones without the double bond in the five-membered ring have been produced from *p*-hydroxyphenylpropionic acids,

---

* Incidentally it confirms that tyrosine is rationally incorporated in the coumarin section of novobiocin.

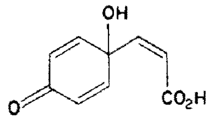
† An alternative, which is inconsistent with the results of the tracer work unless the two carboxylates became more or less equivalent during rearrangement of dienone to phenol, is lactonization to the spiro-compound (X).

[10] S. Gatenbeck and K. Mosbach, *Acta Chem. Scand.* **13**, 1561 (1959).

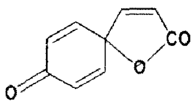
[11] F. M. Dean, *Fortschr. Chem. org. Naturstoffe* **9**, 225 (1952).

[12] R. D. Haworth, *J. Chem. Soc.* 448 (1942).

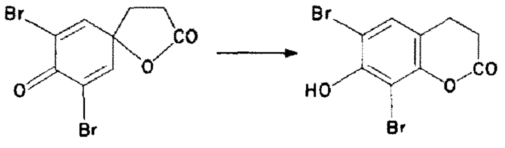
[13] A. I. Scott, *Proc. Chem. Soc.* 207 (1962).



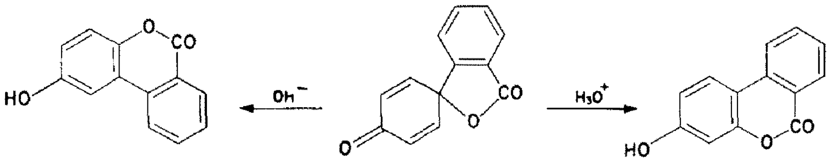
IX



X



XI XII



XIII

including derivatives of tyrosine,[14] and acid-catalysed rearrangement of the dienone (XI) yields the 7-hydroxydihydrocoumarin (XII).[14b] Still more relevantly, the spiro-dienone (XIII) rearranges under acidic catalysis to 7-hydroxy-3,4-benzcoumarin but under alkaline catalysis to 6-hydroxy-3,4-benzcoumarin.[15] Dr. A. I. Scott has suggested to us that, by analogy with formation of spiro-dienones during anchimerically assisted solvolysis of aralkyl sulphonates containing a *p*-oxygenated substituent, attack by a potential carboxylate cation is more likely to produce the spiro-compound than proceed by substitution *meta* to the hydroxyl group. However, the transition state in the acid-catalysed dienone-phenol rearrangement might be virtually identical with that of direct *meta* substitution by a carboxylate cation, presumably an extremely indiscriminate reagent, and hence, depending on the nature of the reagent, the distinction between the two mechanisms may be rather fine. Radical substitution would probably be direct. Preparation of labelled compounds of types IX and X should permit testing of their status as intermediates in biosynthesis. Until such experiments have been made, it appears to us simplest to envisage biosynthesis of 7-hydroxycoumarins as a process of oxidative cyclization, possibly involving spiro-dienones (X).

The two natural benzcoumarins, to which we have already alluded, were isolated by Lederer[17,18] from castoreum, the dried scent gland of the Canadian beaver. Structures XIV and XV were assigned to pigments I and II respectively, partly on account their positive reaction in the Griessmayer test (red colour with mixed nitrous and

---

[14a] E. J. Corey and L. F. Haefele, *J. Amer. Chem. Soc.* **81**, 2225 (1959);

[14b] G. L. Schmir, L. A. Cohen and B. Witkop, *Ibid.* 2228.

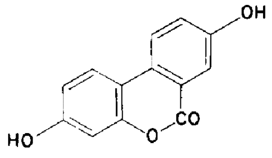
[15] J. A. Leonard, Ph.D. Thesis, London (1962); we are indebted to Professor D. H. Hey for drawing our attention to this work and permitting us to quote it.

[16] R. Baird and S. Winstein, *J. Amer. Chem. Soc.* **84**, 788 (1962); and earlier papers.

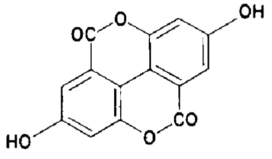
[17] E. Lederer, *Bull. Soc. Chim. Biol.* **24**, 1155 (1942).

[18] E. Lederer, *J. Chem. Soc.* 2115 (1949).

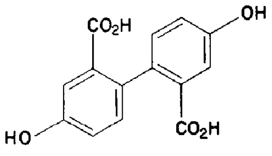
nitric acids),[19] which was held to be confined to those 3,4-benzcoumarins containing two hydroxyl groups in the positions shown. Lederer was of the opinion that the pigments arose by deoxygenation of ellagic acid occurring in the beaver's diet. An alternative, to us more plausible hypothesis is that *m*-hydroxybenzoic, also a constituent of castoreum, is oxidatively coupled[20] to 4,4′-dihydroxydiphenic acid (XVI), which then yields pigment II (XV) by a double cyclization and pigment I (XIV) by a single cyclization with ejection of carbon dioxide. In this way the unusual, unsymmetrical structure of pigment I is explained.



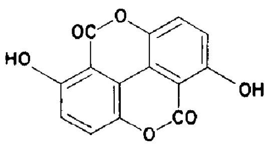
XIV



XV



XVI



XVII

We have not made any experiments on the oxidative coupling of *m*-hydroxybenzoic acid, but it may be noted that gentisic acid is coupled by manganese dioxide, yielding after *normal* lactonization, a benzcoumarin (XVII).[21] Earlier[2] we reported that diphenic acid itself underwent oxidative cyclization with loss of carbon dioxide when treated with peracetic acid. It has now been found that lead tetra-acetate in benzene is a much more efficient reagent, and indeed it gives a better yield of 3,4-benzcoumarin (II) from diphenic acid than from biphenyl-2 carboxylic acid (I). Applied to 4,4′-dimethoxydiphenic acid, this technique gives the dimethyl ether of pigment I (XIV) in 8% yield. This ether was identical with material prepared from resorcinol and 2-bromo-5-hydroxybenzoic acid following Lederer's instructions[22] and previously identified with the ether of the natural product. A careful search for the dimethyl ether of pigment II in the reaction product was fruitless. More deliberate attempts to synthesize this compound by two-stage cyclizations were also unsuccessful.[23] Thus our hypothesis needs further substantiation, but it seems to us the most likely explanation.

While our attention has been mainly on the possible role of oxidative cyclization in biosynthesis, we have made some experiments intended to widen the variety of oxidizing agents and structures accessible by this route. Sodium bismuthate yielded a little 3,4-benzcoumarin from diphenic acid, but none from biphenyl-2 carboxylic acid.

---

[19] V. Griessmayer, *Liebigs Ann.* **160**, 51 (1871).

[20] D. H. R. Barton and T. Cohen, *Festschrift Arthur Stoll* p. 117. Birkhäuser, Basel (1957); H. Erdtman and C. A. Wachtmeister, *Ibid.* p. 144.

[21] V. Juch, *Monatsh.* **26**, 839 (1905).

[22] E. Lederer and J. Polonsky, *Bull. Soc. Chim. Fr.* 831 (1948).

[23] K. Chambers, Ph.D. Thesis, Liverpool (1959).

Iodine pentoxide merely converted diphenic acid into its anhydride. The action of bromine on silver diphenate[24] gave a minute yield of 3,4-benzcoumarin. Frémy's salt, potassium nitrosyldisulphonate, a well-known source of free radicals,[25] did not touch either diphenic acid or biphenyl-2 carboxylic acid. Oxidative cyclizations of *o*-carboxycinnamic acid,[26] benzylidene malonic acid, 2-phenylcinnamic acid, 2-phenylpropionic acid, *cis*-*o*-methoxycinnamic acid, and N-acetylphenylalanine were tried unsuccessfully under various conditions, principally electrolysis, peracid oxidation, and chromic acid oxidation.[27] Evidently the biphenyl system, in which we discovered oxidative cyclization, is particularly favourable to it.

EXPERIMENTAL

M.p.'s are uncorrected. Together with the specific milli-molar activities of the degradation products of ^{14}C labelled novobiocin, the activities related to novobiocin = 16*C (since 16 of the carbon atoms of novobiocin come from tyrosine) are quoted.

Novobiocin (III)

The fermentations of *Streptomyces niveus* and the isolation of the crude antibiotic were carried out by Boots Pure Drug Company. The novobiocin was purified by counter-current distribution between borax buffer (pH 8·6) and ethyl acetate (*K* approximately 1·0).

Incorporation of tyrosine in novobiocin

L-[^{14}C]-Tyrosine (generally labelled; 50 μC, 1·08 mg) was added to a fermentation of *Streptomyces niveus* and the resulting pure novobiocin was found to be of specific activity 0·92 μC/mmole (~10% incorporation).

In a later experiment L-[^{14}C]-tyrosine (generally labelled; 50 μC, 1·08 mg) was diluted with inactive L-tyrosine to a mass of 211 mg of specific activity 46·2 μC/mmole. Part (180 mg) of this labelled tyrosine was added in one lot to a fermentation of *Streptomyces niveus* yielding pure novobiocin (434 mg) of specific activity 1·90 μC/mmole (2·2% incorporation). This represents an isotopic dilution into the coumarin portion of novobiocin of 43 fold.

A further quantity (1 g) of L-[^{14}C]-tyrosine (2·90 μC/mmole) was added in one lot to a 4 l. fermentation yielding a total of 1·4 g of pure novobiocin with specific activity 0·11 μC/mmole (1·8% incorporation), which represents an isotopic dilution into the coumarin portion of novobiocin of 46 fold.

Degradation of ^{14}C*-novobiocin*

(a) *Reaction of novobiocin with acetic anhydride.* Novobiocin (149 mg, 0·92 μC/mmole, 16*C) was treated in pure dry pyridine with acetic anhydride,[7a] affording 4-acetoxy-3-(3-methyl-2-butenyl)-benzoic acid (39 mg, 60%), m.p. 113–114°, specific activity 0·36 μC/mmole (6·3*C) and 7-[4-(carbamoyloxy)-tetrahydro-3-acetoxy-5-methoxy-6,6-dimethylpyran-2-yloxy]-2,6-dimethyl-4H-(1)-benzopyrano-(3,4-d)-oxazol-4-one (53 mg, 72%), m.p. 169–170°, specific activity 0·51 μC/mmole (8·7*C).

(b) 3-*Amino*-4,7-*dihydroxy*-8-*methylcoumarin.* The coumarin from the previous reaction was treated with 0·63N methanolic hydrogen chloride[7a] yielding 3-amino-4,7-dihydroxy-8-methylcoumarin (5 mg), specific activity 0·52 μC/mmole (9·1*C). An authentic sample of this coumarin was available[28] for comparison.

---

[24] Cf. C. V. Wilson, *Org. Reactions* **9,** 332 (1957).

[25] H. J. Teuber and G. Jellinek, *Chem. Ber.* **85,** 95 (1952); and many subsequent papers by H. J. Teuber *et al.*

[26] A coumarin-like smell during the preparation of this acid from α-naphthol has been observed by J. Boeseken and M. Lockmann von Königsfeldt, *Rec. Trav. Chim.* **54,** 313 (1935).

[27] B. R. Webster, Ph.D. Thesis, Liverpool (1962).

[28] Sample kindly provided by Upjohn Co., Kalamazoo, U.S.A.

(c) *Cyclonovobiocic acid* (V) *and ethyl* 3-0-*carbamyl-4-0-methyl-5,5-dimethyl*-L-*lyxoside.* A solution of novobiocin (870 mg; 0·171 μC/mmole, 16*C) in ethanol was treated with conc. hydrochloric acid[7a] yielding cyclonovobiocic acid (475 mg, 87%), m.p. 287–289°, specific activity 0·167 μC/mmole (15·6*C), and the sugar (11 mg, 18%), m.p. 170–174°, activity negligible.

(d) 2,4-*Dihydroxy*-3-*methylphenacylamine hydrochloride* (VII) *and* 2,2-*dimethylchroman*-6-*carboxylic acid* (VIII). Cyclonovobiocic acid (450 mg; 0·167 μC/mmole) in acetic acid was treated with conc. hydrochloric acid for 48 hr (cf. Ref. 7*b*) giving the phenacylamine hydrochloride (37·6 mg, 18%), m.p. 250–260° (dec), specific activity 0·0824 μC/mmole (7·9*C), and the chroman carboxylic acid (82 mg, 42%), m.p. 176–177°, specific activity 0·0781 μC/mmole (7·3*C).

The carbon dioxide liberated was collected from a similar reaction of cyclonovobiocic acid (498 mg; 0·325 μC/mmole) in a sealed tube as barium carbonate (129 mg, 76%), specific activity 0·0180 μC/mmole (0·91*C).

Preliminary experiments using $H_2{}^{18}O$ *of low enrichment*

Figures for ^{18}O content refer in each case to the excess above the isotopically normal compound.

(a) *Equilibration of tyrosine with enriched water* (HBr). Hydrogen bromide was bubbled into enriched water (∼1 atom % excess, 9·745 g) until it was half molar. This acid was then boiled for 48 hr with tyrosine (850 mg) after which the dilute acid was slowly sublimed (∼−10°, 0·02 mm) from the reaction mixture onto a 'cold-finger' type condenser (solid carbon dioxide–acetone). The recovered dil. acid was then titrated to a phenolphthalein end-point with lithium metal and the enriched water recovered by subliming (∼−10°, 0·02 mm) it from the lithium bromide, the trace of lithium hydroxide and the phenolphthalein. The water recovered represented 99% of the original mass of water used. The tyrosine hydrobromide was converted to tyrosine (813 mg) by dissolving it in a slight excess of sodium bicarbonate solution and then back titrating with acetic acid.

(b) *Anisic acid from tyrosine.* Dimethyl sulphate (1·5 ml) slowly added over 30 min to an ice-cooled solution of tyrosine [500 mg; 0·59 atom % excess (Rittenberg method) 0·89 atom % excess (phenylenediamine method)] in aqueous sodium hydroxide (1·9 g in 25 ml). After this mixture had been boiled for 4 hr, potassium permanganate (2·5 g) was added and the boiling was continued for 5 hr. Sulphur dioxide was bubbled through the cool reaction mixture and, after the pH was adjusted to pH 3, it was ether extracted (3x). The ethereal solution was dried (Na_2SO_4) and the ether removed by evaporation leaving impure anisic acid (57 mg, 11%), which was purified by sublimation giving pure anisic acid (43 mg, 8%).

^{18}O analysis:	
anisic acid (Rittenberg method)	0·00 atom % excess

(c) 2-*Methylresorcinol.* Oxygen-labelled potassium hydroxide was prepared by adding a solution of water (∼1 atom % excess, 3 ml) in sodium-dried liquid ammonia (10 ml) to a solution of potassium metal (6 g) in sodium-dried liquid ammonia (excess). After the mixture had been stirred for 1 hr the excess ammonia was allowed to evaporate, leaving the hydroxide as a finely-divided white solid.

A solution of cyclonovobiocic acid (300 mg) in warm ethanol (2 ml) was added to a stirred melt of $K^{18}OH$ (3 g) and the temp was rapidly raised to 250° while a stream of pure nitrogen was passed over the mixture. After 30 min the reaction mixture was poured into water (25 ml), acidified and the resulting solution was extracted with ether (3 × 20 ml). The ethereal solution was washed with sodium bicarbonate solution, water and then dried (Na_2SO_4). The ether was removed by evaporation and the residue sublimed (120°, ∼760 mm) to yield 2-methylresorcinol (23 mg) as fine white needles m.p. 118°.

^{18}O analyses:	
$H_2{}^{18}O$	1·25 atom % excess
2-methylresorcinol (Rittenberg method)	0·002 atom % excess

Experiments with highly enriched $H_2{}^{18}O$

Using the procedure described, L-[^{14}C]-tyrosine (generally labelled; 5·4 g, specific activity 2·90 μC/mmole) was equilibrated with $H_2{}^{18}O$ (ca. 20 atom % excess) using hydrobromic acid as the catalyst. The doubly-labelled tyrosine was then divided equally between six 4 l. fermentations of

Streptomyces niveus. The resulting novobiocin was purified, counted and degraded to 2-methylresorcinol. The activity of the novobiocin (12 g) was 0·17 μC/mmole (4% incorporation) which represents an isotopic dilution, into the coumarin portion, of 32 fold.

^{18}O analyses:

tyrosine	15·2 atom % excess
2-methylresorcinol	0·078, 0·078 atom % excess

The exact agreement between these independent determinations on separate batches is fortuitous. Earlier determinations on one batch, using guanidine hydrochloride alone, gave 0·064, 0·058 atom % excess; without *o*-phenylenediamine hydrochloride the elimination of CO_2 was comparatively inefficient.

In order to confirm that the incorporation had proceeded normally a small quantity of the novobiocin was degraded[7a] to 2,6-dimethyl-7-acetoxy-4H-(1)-benzopyrano-[3,4-d]-oxazol-4-one (0·098 μC/mmole; 9·4*C) and 2,2-dimethylchroman-6-carboxylic acid (0·073 μC/mmole; 6·9*C).

Experiments in the biphenyl series

(a) *Biphenyl-2-carboxylic acid.* This acid was obtained by the potassium hydroxide fusion of fluorenone[29] and crystallized from carbon tetrachloride or aqueous ethanol as colourless prisms (77%), m.p. 112–113°.

(b) *Diphenic acid.* A solution of phenanthrene (50 g) and hydrogen peroxide (87%, 28 ml) in glacial acetic acid (230 ml) was warmed until effervescence commenced. All external heating was then stopped; the mixture slowly turned orange and it boiled for 20 min. When the reaction had subsided, a further quantity (25 ml) of hydrogen peroxide (87%) was added and the mixture was, reheated until the reaction recommenced. This reaction continued, without further heat, for about 18 min and then allowed to stand at room temp overnight whereupon a mass of pale-yellow, prismatic crystals deposited. This product was collected, washed with a little benzene and dried in air (42·2 g, 63%), m.p. 226–229°. This acid crystallized from boiling water (4½ l.), using animal charcoal (5 g), as shining white prisms (37·1 g, 55%), m.p. 229–230°.

(c) 4,4′-*Dimethoxydiphenic acid.* A solution of *m*-methoxybenzoic acid (50 g) in glacial acetic acid (300 ml) was treated with bromine (53 g) in glacial acetic acid (150 ml) and then with water (300 ml).[30] The mixture was heated to b.p. and allowed to cool, when 2-bromo-5-methoxybenzoic acid (61 g, 79%) separated as fine needles, m.p. 158–160°. This bromo-acid on treatment with methanolic hydrogen chloride afforded methyl 2-bromo-5-methoxybenzoate as a colourless oil which was used without further purification.

A mixture of methyl 2-bromo-5-methoxybenzoate (60 g) and copper bronze (30 g) was heated to 220–250° for 1½ hr. The cooled mass was extracted several times with hot benzene and the extracts were filtered. Removal of the solvent gave *dimethyl* 4,4′-*dimethoxydiphenate* (47·5 g) as an oil, which crystallized on standing and recrystallized from benzene–petroleum ether (b.p. 40–60°) as colourless needles, m.p. 78° (Found: C, 65·7; H, 5·7. $C_{18}H_{18}O_6$ requires C, 65·5; H, 5·5%). Alkaline hydrolysis yielded 4,4′-dimethoxydiphenic acid (31·1 g), m.p. 250°.

(d) *Oxidations with lead tetra-acetate.* A solution of lead tetra-acetate (3·6 g) and the carboxylic acid (4 mmole) in dry benzene was warmed on a steam bath for 4 days. Water (8 ml), acetic acid (2 ml) and ethylene glycol (4 ml) were added and the resulting mixture was heated on a steam bath for 4 hr. Thorough ether extraction of the neutral product afforded the benzcoumarin which was purified by sublimation (100°/30 mm). With biphenyl-2-carboxylic and diphenic acids, this method gave 3,4-benzcoumarin as fine colourless needles, m.p. 91–92°, in yields of 12% and 35% respectively; 68% and 44% respectively of the original acids were recovered.

4,4′-Dimethoxydiphenic acid gave the lactone of 2′-hydroxy-4,4′-dimethoxybiphenyl-2-carboxylic acid (8%) as minute colourless needles, m.p. 158° (undepressed when mixed with authentic specimen[22]) (Found: C, 70·2; H, 4·7. Calc. for $C_{15}H_{12}O_4$: C, 70·3; H, 4·7%) and a minute amount of a compound of uncertain structure, m.p. 248–249°, The recovery of starting acid was 11%.

(e) *Oxidations with sodium bismuthate.* Sodium bismuthate (2·4 g, 8 mmole) was added to a

---

[29] C. Graebe and A. S. Rateanu, *Liebigs Ann.* **279**, 257 (1894); G. W. Kenner, M. J. T. Robinson, C. M. B. Tylor and B. R. Webster, *J. Chem. Soc.* 1756 (1962).

[30] A. Horeau and J. Jacques, *Bull. Soc. Chim. Fr.* 53 (1948).

solution of diphenic acid (1 g, 4 mmole) in dry carbon tetrachloride (20 ml) and the resulting suspension was boiled for 48 hr. The filtered reaction mixture afforded 3,4-benzcoumarin (26 mg, 3 %) together with starting acid (203 mg, 20·3 %). No similar reaction occurred with biphenyl-2-carboxylic acid.

(f) *Hunsdiecker reaction of silver diphenate.* A solution of bromine (2·5 ml, 1 equivalent) in dry carbon tetrachloride (20 ml) was slowly added over 2 hr to a warm, stirred suspension of silver diphenate (21 g) in dry carbon tetrachloride (180 ml). When the addition was complete the reaction mixture was boiled for 1 hr, filtered and the filtrate was washed with aqueous sodium thiosulphate solution (3x). The remaining colourless solution was separated into acidic and neutral fractions using aqueous sodium bicarbonate solution. The product was found to consist of 3,4-benzcoumarin (4·3 mg, 0·05 %) and an unidentified lactonic compound (3·8 mg), m.p. 198–199°, from methanol. The recovery of diphenic acid was 9·6 g (80 %).

Acknowledgements—We thank Dr. M. Lumb and Dr. P. E. Macey for all the fermentations, Dr. R. W. Rickards for co-operation in this section of the work, and Dr. K. Chambers for preliminary experiments in the biphenyl series. We are also grateful to Professors A. J. Birch and A. I. Scott for discussions.