Studies in the Biosynthesis of Fungal Metabolites

3. THE BIOSYNTHESIS OF FUNGAL PERINAPHTHENONES*

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The existence of perinaphthenone derivatives as products of the fungal metabolism of sugars has recently been demonstrated for herqueinone ($C_{20}H_{20}O_7$), norherqueinone ($C_{19}H_{18}O_7$) and atrovenetin ($C_{19}H_{18}O_6$). Independent investigations of the colouring matters of *Penicillium herquei* led to the isolation of herqueinone by Stodola, Raper & Fennell (1951) and of herqueinone and norherqueinone by Galarraga, Neill & Raistrick (1955). A subsequent report of a study of the morphologically related species *P. atrovenetum* (Neill & Raistrick, 1956) described the isolation of the pigment atrovenetin.

In the course of these investigations, together with that of Barton, de Mayo, Morrison, Schaeppi & Raistrick (1956), the nature of the interrelationship of these pigments was recognized. Thus herqueinone is a monomethyl ether of norherqueinone, and this latter compound on treatment with zinc dust in acetic acid at room temperature yields deoxynorherqueinone, which is identical with atrovenetin. All three compounds were considered to be derivatives of 9-hydroxyperinaphthenone, but the first complete structure was not proposed until Barton, de Mayo, Morrison & Raistrick (1959) described chemical degradations which permitted the formulation of atrovenetin as (I), although the minor modification (II) remains a possible alternative (D. H. R. Barton, personal communication).

Although the structures of herqueinone and norherqueinone have not yet been fully determined, an essential step in arriving at a structure for atrovenetin involved its established identity with deoxynorherqueinone. Consequently, it is apparent that these three compounds possess a common carbon skeleton and it is therefore reasonable to assume that in each case this skeleton arises via the same biosynthetic mechanism.

Barton *et al.* (1959) suggested that the origin of the perinaphthenone nucleus (III) involves a poly- β -diketone intermediate (IV) (Fig. 1), as previously postulated for the genesis of certain other types of

phenolic substances (Birch & Donovan, 1953). The ethereal side chain was assumed to be derived from a mevalonic acid (V) precursor.

Many instances of this mode of synthesis of fungal phenols have been demonstrated in recent years (Birch, 1957; Gatenbeck, 1958; Thomas, 1959), involving a head-to-tail linkage of acetate units, although the nature of the intermediates has not yet been elucidated. An alternative scheme of aromatic synthesis, leading, for example, to the amino acids phenylalanine and tyrosine, has been studied in considerable detail in mutant strains of *Neurospora crassa* and *Escherichia coli* (cf. Srinivasan, Shigeura, Sprecher, Sprinson & Davis, 1956). Acetate is not directly involved in this latter pathway, which utilizes a complex series of intermediates, including shikimic acid and prephenic acid, which arise from glucose via the hexose monophosphate shunt.

The simultaneous operation of both mechanisms of aromatic synthesis has recently been shown to take place in the biosynthesis of the flavanoid and anthocyanin pigments, quercetin (Watkin, Underhill & Neish, 1957; Geissman & Swain, 1957), cyanidin (Grisebach, 1957) and rutin (Shibata & Yamakazi, 1958). In each case, the experimental evidence supported the view that the two constituent benzene rings arose, one through the acetate and the other via the shikimic acid pathway. Thus despite the attractive nature of the acetate hypothesis as an explanation of the origin of the perinaphthenone nucleus, it was of interest to consider alternative possibilities in which one or more of the three rings arose from shikimic acid.

One such scheme involving the $C_6-C_3-C_6$ precursor (VI), shown in Fig. 2, could, by alternative cyclizations of the three acetate units, give rise either to the flavonol morin (VII) or the stilbene oxyresveratrol (VIII), which occur together in the wood of the osage orange, *Toxylon pomiferum* (Barnes & Gerber, 1955), or alternatively atrovenetin via the perinaphthenone (IX).

Rings B and C of the perinaphthenone would thus be required to arise from the precursor (VI), 2-(2:4-dihydroxyphenylpyruvoyl)-3:5-dioxohexanoic acid, by condensation of the carbon atoms 2 and 5

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Fig. 1. Structure of atrovenetin and postulated biosynthesis. * Denotes acetate-carboxyl-derived carbon atoms.

of the hexanoic acid moiety with the phenyl group. However, the former condensation, giving rise to ring B, would be unlikely to proceed by direct oxidative coupling, owing to the unfavourable orientation of the two phenolic hydroxyl groups (Barton & Cohen, 1957). A more plausible coupling mechanism would involve oxidation of the resorcinol ring to a substituted phoroglucinol, which could then condense as its keto tautomer with the appropriate active methylene group. A subsequent decarboxylation step would leave only two acetate-carboxyl-derived carbon atoms in the perinaphthene nucleus as shown, compared with seven if the pathway involved acetate exclusively (Fig. 1).

This difference provided a simple means of distinguishing between the alternative schemes. Thus if only acetate is involved, then a Kuhn-Roth oxidation of norxanthoherquein (X) would yield 1 mole equivalent of acetic acid containing one-seventh of its total activity; however, the alternative $C_6-C_3-C_6$ pathway would yield acetic acid containing approximately half the activity of the parent norxanthoherquein, assuming negligible incorporation of acetate into the phenylpyruvoyl

moiety, as was shown to be the case for quercetin (Watkin *et al.* 1957).

The experimental approach was the same as that described in previous papers (Chaplen & Thomas, 1960; Thomas, 1961). Norherqueinone was selected in preference to herqueinone because of the relative ease of its isolation. Atrovenetin was considered to be less suitable, since its C_6 side chain is not readily cleaved on acid hydrolysis and the presence of two additional carbon-methyl groups would unnecessarily complicate the interpretation of the Kuhn-Roth oxidation data.

Accordingly, *P. herquei* was grown on a Raulin-Thom medium in surface culture as described by Galarraga *et al.* (1955) and sodium $[1-^{14}C]$ acetate was added after incubation for 13 days at 24° . After a further 7 days' growth, the mycelium was harvested, washed and then dried. In a parallel experiment DL-[2- ^{14}C]mevalonic acid lactone was fed to the mould. Both precursors gave rise to radioactive mycelia, which were continuously extracted with ether in a Soxhlet apparatus, yielding deep-red crystalline deposits in the extraction flasks, consisting of almost pure norherqueinone.

*, Denotes acetate-carboxyl-derived carbon atoms.

Fig. 3. Norherqueinone degradation scheme. Values shown in parentheses indicate the relative activities of the degradation products, calculated on the basis of a theoretical incorporation of nine acetate units into norherqueinone.

Table 1. *Activities of norherqueinone and its degradation products*

Precursor	Compound assayed	Norherqueinone-derived carbon atoms*	Activity (μC /mole)	
			Found	Theory
$CH_3 \cdot ^{14}CO_2H$	Norherqueinone	1-14, 1'-5'	1186	$9 \times 132^\dagger$
	Norxanthoherquein	1-14	908	922
	Methyl isopropyl ketone	1'-5'	276	264
	dinitrophenylhydrazone			
	p -Bromophenacyl acetate	13, 14	129	132
	$BaCO_3$	14	0	0
	$BaCO_3$	1-12	64	66
$[2-^{14}C]$ Mevalonic acid lactone	$BaCO_3$	1'	128	132
	Norherqueinone	1-14, 1'-5'	116	—
	Norxanthoherquein	1-14	0	0
	Methyl isopropyl ketone	1'-5'	114	116
	dinitrophenylhydrazone			

* Arbitrary numbering system as in (II) (Fig. 1).

† Based on the uniform incorporation of nine acetate units.

norherqueinone, was found to contain all the activity of the parent compound, the residual norxanthoherquein being completely unlabelled (Table 1). This confirmed the true isoprenoid nature of the side chain, the mevalonate precursor probably arising from acetate via β -hydroxy- β -methylglutaryl-coenzyme A.

Despite the fact that the acetate origin of the norxanthoherquein nucleus is based only on the results of Kuhn-Roth oxidation, it would seem reasonable to regard this as adequate confirmation of the postulated pathway, in view of the considerable number of fungal phenols now known to conform to this mechanism. A corresponding assumption was made by Birch, Ryan & Smith (1958) in relation to the Kuhn-Roth degradation of labelled helmintho-

sporin (4:5:8-trihydroxy-2-methylanthraquinone) obtained on growth of *Helminthosporium gramineum* in the presence of sodium $[1-^{14}C]$ acetate. Confirmatory evidence of the acetate origin of this class of fungal pigments was provided independently by Gatenbeck (1958), who carried out more extensive degradations of the labelled dianthraquinone skyrin.

The results in Table 1 indicate that the incorporation of acetate into the aromatic nucleus and the isoprenoid side chain occurs to approximately the same extent. A similar finding was previously obtained by Birch, English, Maesy-Westropp & Smith (1958) in an analogous study of mycophenolic acid, a product of *Penicillium brevicompactum*.

As is also the case for the other acetate-derived

phenols described thus far, the sequence of condensation of acetate units required to form the nor-xanthoherquein skeleton is unknown. Although, as pointed out by Barton *et al.* (1959), this ring system could arise by suitable folding of the linear poly- β -oxo acid (IV), there is at present no evidence which favours this precursor over the alternative of a branched-chain keto acid, as has been proposed for several other C_{14} fungal phenols (Thomas, 1961). The unusual nature of the C_5 side chain was discussed by Barton *et al.* (1959), who noted the existence of an identical side chain in the naphthaquinone dunnione (Price & Robinson, 1939). In both compounds the aromatic nucleus is linked to the tertiary carbon atom of the parent mevalonate unit, the original hydroxymethyl substituent of which is reduced to a methyl group. As regards the alternative structures (I) or (II) for atrovenetin, the mode of attachment of the C_5 side chain in the latter would be biogenetically preferable, as it is in accord with the normal head-to-tail linkage of acetate units. Furthermore, (II) also contains more β -orientated oxygen substituents, which is a characteristic of compounds formed in this manner.

In view of the existence of a common carbon

skeleton in herqueinone, norherqueinone and atrovenetin, it is reasonable to assume that these fungal perinaphthenones arise through the same mechanism involving acetate. However, this may not be the only means of biosynthesis of this tricyclic-ring system. Thus the aglycone of the plant colouring matter haemocerin (XIV) (Cooke, Johnson & Segal, 1958), which is at present the only other known naturally-occurring perinaphthenone, contains two sets of *ortho*-orientated oxygen substituents, which is unusual among acetate-derived phenols. A more attractive biosynthetic scheme for this product would appear to involve simply the ring closure of an intermediate (XII) related to the plant substance *p*-hydroxycinnamoylferuloylmethane (XIII), isolated from the rhizome of *Curcuma longa* (turmeric) by Srinivasan (1953).

The required intermediate (XII) is postulated as arising from two C_6-C_3 units (cinnamic acid and 3:4-dihydroxyphenylpyruvic acid), which substitute into the methyl group of an acetate unit before decarboxylation. Both of these C_9 compounds would be expected to originate through shikimic acid and prephenic acid by analogy with known pathways of aromatic amino acid formation.

Fig. 4. Scheme for the biosynthesis of haemocerin.

On this basis, the known plant and fungal perinaphthenones would be biogenetically unrelated, despite their structural similarity.

EXPERIMENTAL

All melting points are uncorrected.

Culture and medium. The culture of *Penicillium herquei* used in these studies was the London School of Hygiene and Tropical Medicine strain no. P219. It was grown in surface culture on Raulin-Thom solution as described by Galarraga *et al.* (1955).

Radioactive assay. Samples mounted on 1.5 cm.-diam. planchets at infinite thickness were assayed for ^{14}C content with a thin end-window Geiger-Müller counter, as described, by Chaplen & Thomas (1960).

Production of labelled norherqueinone. Three 1 l. flasks, each containing sterile Raulin-Thom medium (350 ml.), were inoculated with a mycelial suspension of *P. herquei* and incubated at 24° in the dark. After 13 days, 3.2 mg. of $\text{CH}_3\cdot^{14}\text{CO}_2\text{Na}$ (200 μC) was added in sterile aqueous solution (5 ml.) to each flask, so as to pass directly under the mycelial pad.

Growth was continued for a further 7 days and the cultures were harvested after a total incubation period of 20 days. The metabolism solution (pH 5.9) was orange-brown; the mycelium was deeply folded and bright brownish red on the underside. The mycelia from the three flasks were combined, washed with water and dried (yield 13.33 g.).

A simplified extraction procedure was used, consisting of direct continuous extraction (90 hr.) with ether in a Soxhlet apparatus. An intense reddish-brown crystalline precipitate separated from the dark-green ether extract. This was collected by filtration and proved to be almost pure norherqueinone (0.547 g.). It was found to be highly active (19.6 μC /g.), corresponding to a minimum incorporation of approx. 1.8% of the original labelled acetate; no attempt was made to extract further product from the mother liquors.

The labelled norherqueinone (0.540 g.) was mixed with inactive pure product (2.749 g.) and recrystallized from boiling acetic acid to yield very small red needles, m.p. 279° (decomp.), of specific activity 3.31 μC /g. (1186 μC /mole). The inactive norherqueinone used for dilution purposes was obtained in a separate production experiment with 44 flasks (yield, 6.00 g.).

Addition of 9.8 mg. of DL-[2- ^{14}C]mevalonic acid lactone (50 μC) to a single flask under precisely parallel conditions yielded norherqueinone (143 mg.), whose specific activity (2.64 μC /g.) corresponded to an incorporation of 0.75%. This was diluted with inactive product (0.876 g.) as described above to give recrystallized norherqueinone of activity 116 μC /mole.

Degradation of acetate-derived norherqueinone

Acid hydrolysis of norherqueinone. Norherqueinone (0.493 g.) was suspended in a mixture of 2N- H_2SO_4 (75 ml.) and acetic acid (75 ml.) and gently refluxed in a stream of N_2 for 12 hr. The effluent gases were passed through bubblers containing Brady's reagent (0.32% 2,4-dinitrophenylhydrazine in 2N-HCl), giving rise to a yellow precipitate consisting of the 2,4-dinitrophenylhydrazone of methyl isopropyl ketone. This was collected by filtration (0.219 g.) and crystallized from ethanol, giving orange needles, m.p. 123° (activity 276 μC /mole).

The residue remaining after acid hydrolysis was orange, compared with the dark red of the starting product. This was filtered off, washed and dried (0.353 g.) and purified by vacuum sublimation at 270° , yielding a yellow-orange crystalline sublimate of norxanthoherquein, m.p. $> 360^\circ$ (activity, 908 μC /mole).

In a further experiment, norherqueinone (2.20 g.) was hydrolysed with a mixture of conc. H_2SO_4 (60 ml.), acetic acid (60 ml.) and water (120 ml.). The resulting methyl isopropyl ketone was retained in the mixture with the aid of an efficient reflux condenser. After 18 hr., the cooled solution was treated with sodium acetate (40 g.) in water (1 l.), thereby precipitating the norxanthoherquein, recovery and purification of which was carried out as before. The methyl isopropyl ketone fraction was collected by steam-distillation and oxidized directly with an alkaline solution containing a mixture of I_2 (10 g.) and KI (20 g.). A precipitate of iodoform rapidly formed, which was separated by filtration (0.60 g.) and crystallized from ethanol. This was then oxidized with chromic acid solution under van Slyke-Folch conditions and the resulting CO_2 was assayed as BaCO_3 (300 mg. of iodoform gave 141 mg. of BaCO_3 , 128 μC /mole).

An attempt to recover isobutyric acid from the alkaline iodine oxidation filtrate as its *p*-bromophenacyl ester was abandoned because of the low yield of crude product.

Kuhn-Roth oxidation of norxanthoherquein. Norxanthoherquein (0.750 g.) was refluxed with a mixture of 5N-chromic acid (250 ml.) and conc. H_2SO_4 (75 ml.) for 3 hr. in a stream of N_2 and the effluent CO_2 was trapped as BaCO_3 (activity 64 μC /mole). The residual acetic acid was recovered by steam-distillation and neutralized with 0.1N-LiOH (yield, 88% theory for 1 molequiv.). This was partially converted into *p*-bromophenacyl acetate, m.p. $84.5\text{--}85.5^\circ$ (activity, 129 μC /mole), and the remainder pyrolysed as the dry lithium salt to give acetone, which was subsequently converted into iodoform as described by Thomas (1961). The iodoform, after oxidation to BaCO_3 , was found to be inactive.

Degradation of mevalonate-derived norherqueinone

Acid hydrolysis of norherqueinone. Norherqueinone (0.500 g.) was hydrolysed with a mixture of 2N- H_2SO_4 (75 ml.) and acetic acid (75 ml.) and the resulting volatile methyl isopropyl ketone was removed in a stream of N_2 and precipitated as its 2,4-dinitrophenylhydrazone as described above. This product was filtered off (0.175 g.) and purified by crystallization from ethanol (activity 114 μC /mole).

The residual norxanthoherquein was recovered (0.256 g.) and, after purification by sublimation in high vacuum, was found to be devoid of activity.

SUMMARY

1. Possible alternative pathways of biosynthesis of the fungal perinaphthenones atrovenetin ($\text{C}_{19}\text{H}_{18}\text{O}_6$), herqueinone ($\text{C}_{20}\text{H}_{20}\text{O}_7$) and norherqueinone ($\text{C}_{19}\text{H}_{18}\text{O}_7$) are discussed.

2. *Penicillium herquei* was found to incorporate sodium [1- ^{14}C]acetate and DL-[2- ^{14}C]mevalonic acid lactone into norherqueinone, in a manner consistent with the derivation of the perinaphthenone nucleus from acetate units and the C_5 side chain from acetate or mevalonate.

3. A scheme is proposed for the biosynthesis of

the aglycone $C_{20}H_{14}O_4$ of the plant pigment haemocorin, the only other known perinaphthenone from natural sources. This requires the coupling of one acetate and two shikimic acid-derived C_6-C_3 units and involves an intermediate related to the plant product *p*-hydroxycinnamoylferuloylmethane ($C_{20}H_{18}O_5$), which then undergoes cyclization.

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Equilibrium Constant of Phosphoryl Transfer from Adenosine Triphosphate to Galactose in the Presence of Galactokinase

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The free energy of hydrolysis of adenosine triphosphate has been calculated as the sum of the free energies of phosphoryl transfer from this compound to $C_{(6)}$ of D-glucose and from $(\alpha + \beta)$ -D-glucose 6-phosphate to water (Vladimirov, Vlassova, Kolotilova, Lyzlova & Panteleyeva, 1957*a, b*; Robbins & Boyer, 1957). The equilibrium constant of the first reaction, catalysed by hexokinase, is so large that accurate estimation of the minor components of the equilibrium mixture is difficult. Colowick & Sutherland (1942) found the equilibrium constant of the phosphoglucomutase reaction,

to be 17 at pH 7.5. An α -hexose 1-phosphate thus has a more negative free energy of hydrolysis than

the corresponding hexose 6-phosphate, and the equilibrium constant of the reaction

is correspondingly smaller than that of the hexokinase reaction. Galactokinase catalyses formation of α -D-galactose 1-phosphate from adenosine triphosphate and D-galactose (Trueco, Caputto, Leloir & Mittelman, 1948; Wilkinson, 1949). It seemed probable that at pH 7.0 and 25°, in the presence of more than 10 mM-Mg²⁺, the equilibrium constant of the galactokinase reaction would be more than 10 and less than 100.

Addition of adenylate kinase to a solution containing adenosine diphosphate and adenosine triphosphate catalyses formation of adenosine monophosphate, and ensures that

$$(\text{ADP})^2/(\text{AMP})(\text{ATP})$$