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Investigations of the biosynthesis of novobiocin

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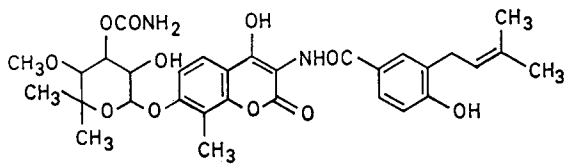
The biosynthesis of novobiocin was investigated using isotopically labelled compounds. The amino-group of the coumarin unit was found to be derived from tyrosine, other typical 7-oxycoumarin precursors were not incorporated into the coumarin unit of novobiocin, 4′-hydroxyphenylpyruvate was a better precursor of the substituted benzoic acid unit than was 4′-hydroxycinnamic acid. A biosynthetic route to this unit of novobiocin is suggested. Synthetic pathways for 2′,4′-dihydroxyphenylalanine-1-^{14}C and 4-hydroxybenzaldehyde-U-^{14}C are reported for the first time.

Novobiocin, (I) a clinically useful antibiotic produced by *Streptomyces niveus* and *S. spheroides*, is used infrequently because micro-organisms rapidly develop resistance to it (Findland & Nichols 1957). Attempts by synthetic methods to prepare analogues that retain antibiotic activity but to which microorganisms are not resistant, have been unsuccessful (Okumara 1960). It is possible that analogues could also be produced by feeding modified biosynthetic intermediates of novobiocin to cultures of *S. niveus*. A detailed knowledge of the biosynthesis of novobiocin would be an essential requirement for the production of analogues by this method.



I

The sugar unit of novobiocin has been shown to be derived from glucose (Birch, Cameron & others, 1960; Birch, Halloway & Rickards, 1962; Kominek, 1967), the aminocoumarin unit and the substituted benzoic acid unit from tyrosine (Kenner, Burton & others, 1960) and the heterocyclic oxygen of the aminocoumarin from one of the carboxyl oxygens of tyrosine (Kenner, Burton & others, 1963). Kenner & others (1963) therefore proposed that the aminocoumarin unit is derived from tyrosine by an oxidative cyclization mechanism via a substituted cinnamic acid intermediate. The intermediates from tyrosine to the aminocoumarin and substituted benzoic acid units have not been investigated. However biosynthetic pathways to 7-oxycoumarins in other organisms have been elucidated. All other microbial 7-oxycoumarins investigated have been shown to be derived from acetate (Brown, Spring & Stoker, 1971) but since acetate is not incorporated into the aminocoumarin of novobiocin (Birch & others, 1960) comparisons are unrewarding. Plant 7-oxycoumarins are derived from phenylpropanoid compounds (Brown, 1966). Since the coumarin unit of novobiocin and the 7-oxycoumarin found in monocotyledons are both derived from tyrosine these units could have a common biosynthetic pathway.

However, although the hetero atom of plant coumarins is thought to arise by *ortho*-hydroxylation and not by oxidative cyclization (Austin & Meyers, 1965b), Stoker (personal communication) could find no evidence for such a hydroxylating system. An alternative mechanism to oxidative cyclization for 7-oxycoumarin formation in *S. niveus* which would be consistent with previous results would be lactone formation tyrosine, or a cinnamic acid derivative of tyrosine, followed by hydrolysis to give the 2′,4′-dihydroxy compound. This route could also account for 7-oxycoumarin formation in monocotyledons.

We have attempted to elucidate the pathway from tyrosine to the aminocoumarin unit of novobiocin to find if the known biosynthetic pathways to plant 7-oxycoumarins are involved. Possible isotopically labelled intermediates of this pathway were prepared and the ability of these to act as precursors of the aminocoumarin unit was examined. Cell free extracts of *S. niveus* were also examined for enzymes involved in known pathways to 7-oxycoumarins. Since the substituted benzoic acid unit is also derived from tyrosine the intermediates from tyrosine to this unit of novobiocin were also investigated.

METHODS

Microbiological

The strain of *Streptomyces niveus* used was kindly supplied by Dr. Macey (Boots Pure Drug Co. Ltd) as a freeze dried powder of spores. The organisms were maintained on agar slants consisting of (% w/v) glucose, 1; potassium chloride, 0·4; calcium chloride, 0·1; agar, 2 and tap water to volume, stored at 4° under liquid paraffin. Spores from these slopes were suspended in distilled water and used as inoculum for a vegetative medium of the following composition, (% w/v) glucose, 10; dried skimmed milk, 5; NZ Amine B, 5; Yeast-O-Lac, 5 and tap water to volume. The inoculated medium was incubated at 29° on a reciprocating shaker for 48 h the production medium used was that of Walton, Woodruff & McDaniel (1962). This medium (200 ml in sterile 1 litre flasks) was inoculated with the vegetative growth (20 ml) and incubated at 29° on a rotary shaker. The production of novobiocin followed the same pattern as that reported by Hoeksema & Smith (1961). Potential precursors were therefore fed 48 h after inoculation of the production medium and novobiocin was extracted after 144 h.

Extraction

The novobiocin content of the culture (600 ml) was determined (Smith, Perry & others, 1958). The culture was then centrifuged, the supernatant retained, the mycelium extracted with methanol: water, 1 : 1, (100 ml) and the extract added to the supernatant; this was adjusted to pH 5 and extracted with ethyl acetate ($3 \times \frac{1}{3}$ volumes). The extracts were bulked, dried over anhydrous magnesium sulphate and then evaporated to dryness under reduced pressure. The crude extract was recrystallized from ethanol–water (2 : 1) and light petroleum (60–80°)–acetone (10 : 1) to give a pale yellow powder of crude novobiocin (50–60% yield). A fraction of the crude extract (20%) was purified to constant radioactivity by chromatography using the following systems consecutively until constant values for specific activity (μCi/mM) were obtained.

(1) The system of Rangone & Sferruzza (1962). (2) The system of Korachagin (1966). (3) T.l.c. with chloroform–formic acid–water–methanol (100:0.8:8:12·5). (4) The system of Given & Colbar (1967). (5) The system of Trenner (personal communication). (6) T.l.c. with chloroform–acetic acid (9:1).

Degradation of novobiocin. The remaining novobiocin was degraded according to Hoeksema, Hinman & Carron (1957) to compounds containing the sugar unit, the coumarin unit and the benzoic acid unit. The coumarin and benzoic acid units were purified to constant specific activity by chromatography using systems 2, 3 and 6 above.

Preparation and administration of precursors. L-Tyrosine-U-^{14}C and L-phenylalanine-U-^{14}C were obtained from the Radiochemical Centre, Amersham. L-Tyrosine-^{15}N was obtained from Schwartz Bio-Research Corporation N.Y. The remaining precursors were prepared.

Compounds tested for their ability to act as precursors of novobiocin are given in Table 1 together with their specific radioactivity and the total activity administered to cultures of *S. niveus*.

Table 1. *Activity of compounds fed to cultures of* S. niveus.

Compound fed		Specific activity as mCi/mM	Activity fed μCi ^{14}C	^{15}N
L-Tyrosine-U-^{14}C		475	9·3	
L-Phenylalanine-U-^{14}C	(1)	405	10	
	(2)	405	10	
L-Tyrosine-U-^{14}C-^{15}N		0·183	9·9	95 atom % excess
		0·0448	10·7	95 atom % excess
2′,4′-Dihydroxyphenylalanine--1-^{14}C	(1)	0·06	11·65	
	(2)	0·06	24	
4′-Hydroxycinnamic acid -2-^{14}C		0·046	12·85	
Methyl-4′-hydroxycinnamic acid -2-^{14}C		0·05	9·9	
4′-Hydroxycinnamic acid 3,1′,2′,3′,4′,5′,6′-^{14}C	(1)	0·09	8·3	
	(2)	0·048	4·1	
4′-Hydroxyphenylpyruvic acid -U-^{14}C		3·5	12·8	
4-Hydroxybenzoic acid -G-^{3}H		23·2	1·2mCi	
4-Hydroxybenzoic acid -G-^{3}H + 3-Amino-7-hydroxy-8-methylcoumarin (20 mg/flask)		23·2	1·2mCi	

G = Generally labelled with ^{3}H.

The compounds were fed to cultures of *S. niveus* in aqueous solutions of their sodium salts or of their hydrochlorides except for methyl-4′-hydroxy-cinnamate-2-^{14}C which was fed as a solution in ethanol–water (1:1). Precursor solutions were sterilized by filtration through a Gelman membrane filter, 0·45 nm pore size, on a Swinnex support before addition to the medium.

Syntheses

4′-hydroxycinnamic acid-2-^{14}C was prepared according to Austin & Meyers (1965a) and the methyl ester by methylation of the acid with diazomethane.

4'-Hydroxyphenylpyruvic acid-U-^{14}C was prepared (Greenstein & Winitz, 1961) from L-tyrosine-U-^{14}C by enzymic oxidative deamination using a crude L-amino-acid oxidase isolated from the venom of *Crotalus adamanteus*.

4'-Hydroxycinnamic acid-3,1',2',3',4',5',6'-^{14}C was prepared (Austin & Meyers, 1965a) using 4-hydroxybenzaldehyde-U-^{14}C obtained from 4'-hydroxyphenylpyruvate-U-^{14}C by alkaline oxidation.

2',4'-Dihydroxyphenylalanine-1-^{14}C. 2,4 Dimethoxyphenylacetaldehyde bisulphate (600 mg) (Clemo, Duxbury & Swan, 1952), ammonium bicarbonate (600 mg), sodium cyanide-^{14}C (250 μCi) diluted with unlabelled sodium cyanide to 127 mg and 50% aqueous ethanol (3 ml) were heated at 60° for 12 h with stirring. The crude hydantoin (250 mg) was filtered dried and heated in a tube, sealed under vacuum, with 37% hydrochloric acid (2 ml) at 150–160° for 2 h. The product was diluted with water (10 ml) and evaporated to dryness under reduced pressure. The residue was dissolved in water (2 ml) and neutralized with ammonia before preparative paper chromatography using butanol–acetic acid–water (1:1:1) as developing solvent. The band corresponding to 2',4'-dihydroxyphenylalanine was eluted with water and the eluate evaporated to dryness under reduced pressure. The residue was recrystallized from water to give 2',4'-dihydroxyphenylalanine-1-^{14}C (87 mg 19%) m.p. = 220–222° (223–224° Hirai, 1926). A sample prepared by this method but excluding the sodium cyanide-^{14}C gave the following analytical data. Calculated for $C_9H_{11}NO_4$ C, 54·9; H, 5·59, N, 7·1. Found C, 54·6; H, 5·5, N, 6·7. The ethyl ester had a molecular ion at *m/e* 225 (11·4% of base peak *m/e* 123) on mass spectral analysis.

All prepared precursors were shown to be radioactively pure by co-chromatography with authentic samples followed by scanning of the chromatograms using a scintillation chromatogram scanner.

Determination of isotopic content was made using an I.D.L. liquid scintillation counter and a toluene-Triton X 100 scintillator (Patterson & Greene, 1965) or a toluene scintillator of composition PPO 0·6 g, POPOP 0·5 g, toluene to 100 ml.

The ^{15}N content of samples was determined from the ratio of the P + 1 : P peaks of the enriched compounds compared with that of the isotopically normal compound (analysis by the Physico-Chemical Measurement Unit, Harwell).

Examinations of mycelia for non-oxidative deaminating system. Cells from production cultures of *S. niveus*, harvested at 48, 72 and 96 h after inoculation of the production medium, were used to prepare a cell free system which was examined for non-oxidative deaminating ability (Moore, Towers & Subba Rao, 1968).

A second series of cell-free systems were obtained from cultures to which L-tyrosine (25 mg/200 ml of cultures) had been fed 48 h after inoculation. These cultures were also examined for non-oxidative deaminating ability.

Preparation of tritiated compounds. Tritiated compounds were prepared by the method of Garnett (1962).

RESULTS AND DISCUSSION

For each feeding experiment the radioactivity of the purified novobiocin and its degradation products was determined and expressed as specific activity (mCi/mM). The percentage incorporation of each precursor into novobiocin, the aminocoumarin and benzoic acid units was calculated from these values. These results are given in Tables 2 and 3. Since the yields of novobiocin were consistent (0·3–0·4 mg/ml), the values of percentage incorporation can be used to compare the ability of compounds

Table 2. *Results of feeding experiments.*

Precursor	S.A. of novobiocin	% Incorp. into novobiocin	S.A. of amino-coumarin	% Incorp. into amino-coumarin	S.A. of benzoic acid unit	% Incorp. into benzoic acid unit
L-Tyrosine-U-^{14}C	$1{\cdot}7 \times 10^{-3}$	7·4	$9{\cdot}5 \times 10^{-4}$	4·12	5×10^{-4}	2·12
L-Phenylalanine-U-^{14}C	$2{\cdot}8 \times 10^{-5}$	0·11	$7{\cdot}4 \times 10^{-6}$	0·03	0	
	$3{\cdot}2 \times 10^{-5}$	0·18	9×10^{-6}	0·05	0	
4′-Hydroxycinnamic acid -2-^{14}C	$5{\cdot}2 \times 10^{-4}$	0·92	$1{\cdot}98 \times 10^{-4}$	0·35	0	
Methyl-4′-hydroxy cinnamate-2-^{14}C	$2{\cdot}1 \times 10^{-4}$	0·41	$8{\cdot}3 \times 10^{-5}$	0·18	0	
2′,4′-Hydroxyphenylalanine-1-^{14}C	4×10^{-5}	0·014	0		6×10^{-6}	0·002
	7×10^{-5}	0·012	0		0	
4′-Hydroxycinnamic acid 3,1′,2′,3′,4′,5′,6′-^{14}C	$3{\cdot}4 \times 10^{-4}$	1·66	$9{\cdot}9 \times 10^{-5}$	0·6	8×10^{-5}	0·48
	$4{\cdot}3 \times 10^{-4}$	2·37	$1{\cdot}6 \times 10^{-4}$	0·88	$1{\cdot}26 \times 10^{-4}$	0·7
4′-Hydroxyphenylpyruvic acid -U-^{14}C	$3{\cdot}68 \times 10^{-3}$	9·59	7×10^{-4}	1·83	2×10^{-3}	5·2
4-Hydroxybenzoic acid -G-^{3}H	$5{\cdot}2 \times 10^{-3}$	0·1				
4-Hydroxybenzoic acid -G-^{3}- & 3-Amino-7-hydroxy-8-methyl coumarin	$4{\cdot}1 \times 10^{-3}$	0·099				

S.A. = Specific Activity expresses as mCi/mM.
G = Generally labelled with ^{3}H.

Table 3. *Results of* L-*tyrosine-U-*^{14}*C-*^{15}*N experiments.*

Precursor	S.A. of novobiocin	% Incorp. into novobiocin	S.A. of amino-coumarin	% Incorp. into amino-coumarin	^{15}N Atom % excess of amino-coumarin	Specific Incorp. of ^{15}N into* amino-coumarin
L-Tyrosine-U-^{14}C-^{15}N	$2{\cdot}8 \times 10^{-3}$	6·7	$1{\cdot}59 \times 10^{-3}$	3·8	0·193	0·2
	$2{\cdot}8 \times 10^{-3}$	11·6	$1{\cdot}55 \times 10^{-3}$	6·3	0·55	0·58

Ratio ^{15}N:^{14}C		Exp. 1	Exp. 2
	For precursor =	550	2120
	For aminocoumarin =	121	350

$$* = \frac{^{15}\text{N content of aminocoumarin}}{^{15}\text{N content of novobiocin}} \times 100$$

Specific Activity expressed as mCi/mM.

to act as precursors of novobiocin, assuming that all compounds can penetrate the cell membrane.

The preliminary experiment with L-tyrosine-U-^{14}C confirmed that tyrosine was an excellent precursor of the aminocoumarin unit of novobiocin. In contrast L-phenylalanine-U-^{14}C, an excellent precursor of plant 7-oxycoumarins, was a poor precursor of novobiocin. Since in monocotyledons the conversion of tyrosine to 4′-hydroxycinnamic acid is catalysed by tyrase, a non-oxidative deaminating enzyme (Neish, 1961), it was expected that, if a similar pathway operated to the 7-oxycoumarin unit of novobiocin, a similar enzyme would be present in *S. niveus*. However, cell free preparations did not show such ability suggesting that the biosynthetic pathway to the coumarin unit of novobiocin differs from that of similar units in monocotyledons. Also, if the pathway to the coumarin unit was similar to that in plants then 4′-hydroxycinnamic acid would be expected to be a better precursor of the coumarin unit than tyrosine, but we found 4′-hydroxycinnamic acid-2-^{14}C and its more lipophilic methyl ester were less efficient precursors of the coumarin unit than tyrosine.

The aminocoumarin isolated from cultures to which L-tyrosine-U-^{14}C-^{15}N had been fed, had a higher content of ^{15}N than control samples suggesting that the amino-group

of tyrosine is retained in the aminocoumarin unit. Although the $^{15}N:^{14}C$ ratio of the aminocoumarin was lower than that of the L-tyrosine-U-^{14}C-^{15}N, the results indicate that 20% of the administered tyrosine was incorporated into the aminocoumarin as an intact unit. A larger dilution of ^{15}N compared with ^{14}C was expected because of transamination reactions in the mycelium. Other evidence for the occurrence of transamination reactions was the high incorporation of 4′-hydroxyphenylpyruvic acid-U-^{14}C into the aminocoumarin unit. It was concluded that the aminocoumarin unit of novobiocin is derived from tyrosine, with retention of the amino-group, by a novel biosynthetic pathway to 7-oxycoumarins.

The preliminary experiment with L-tyrosine-U-^{14}C also confirmed that tyrosine is an excellent precursor of the benzoic acid unit of novobiocin. There are two major biosynthetic pathways in micro-organisms for the conversion of tyrosine to 4-hydroxybenzoic acid. These are (a) tyrosine → 4′-hydroxycinnamic acid → β-oxidation → 4-hydroxybenzoic acid, (Moore & others, 1968), (b) tyrosine → 4′-hydroxyphenylpyruvic acid → 3(4′-hydroxyphenyl)-3-hydroxyproprionic acid → 4′-hydroxycinnamic acid → β-oxidation → 4-hydroxybenzoic acid (Ommorri, Yasuis & Yamamoto, 1966). Since non-oxidative deaminating ability could not be demonstrated in *S. niveus* it is unlikely that route (a) is in operation. Also 4′-hydroxycinnamic acid should be incorporated more efficiently into the benzoic acid unit than into the coumarin unit but it was not.

4′-Hydroxyphenylpyruvic acid-U-^{14}C was a more efficient precursor of the benzoic acid than of the coumarin unit. If its incorporation was solely by conversion to tyrosine a higher incorporation into the coumarin unit would be expected. This result suggests that route (b) outlined in Fig. 1 could be involved in the formation of the benzoic acid unit. The low incorporation of 4′-hydroxycinnamic acid which is involved in this route at a later stage than 4′-hydroxyphenylpyruvate may be due to introduction of the isopentenyl group occurring before degradation of the side-chain

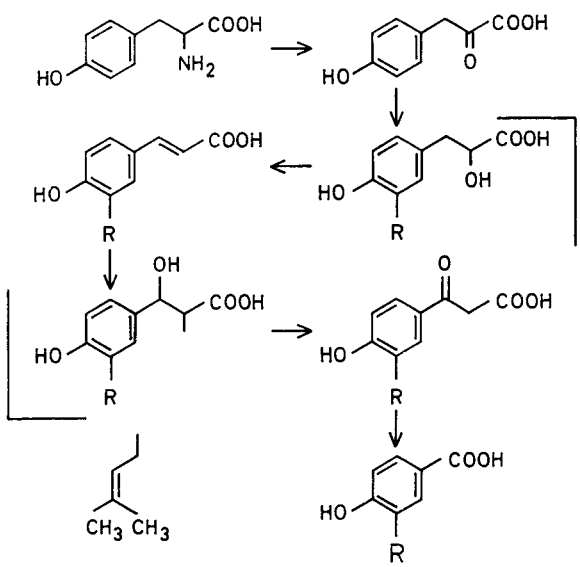


FIG. 1. Proposed biosynthetic pathway from tyrosine to the substituted benzoic acid unit of novobiocin.

is complete. That alkylation does occur before complete degradation was also suggested by the failure of tritiated 4-hydroxybenzoic acid to be incorporated into novobiocin. This experiment was repeated in the presence of excess aminocoumarin unit in the fermentation, a condition favourable for amide bond formation (Kominek, 1967), but the isolated novobiocin was not labelled in the benzoic acid unit. Therefore the substituted benzoic acid unit of novobiocin is derived from tyrosine by the biosynthetic pathway outlined in Fig. 1, alkylation occurring at some point before side-chain degradation is complete.

We conclude that the aminocoumarin unit of novobiocin is derived from tyrosine by a novel biosynthetic pathway to 7-oxycoumarins. The substituted benzoic acid unit of novobiocin is derived from tyrosine via 4'-hydroxyphenylpyruvate with alkylation occurring before degradation is complete.

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