THE BIOSYNTHESIS OF THE MACROLIDE ANTIBIOTIC LUCENSOMYCIN

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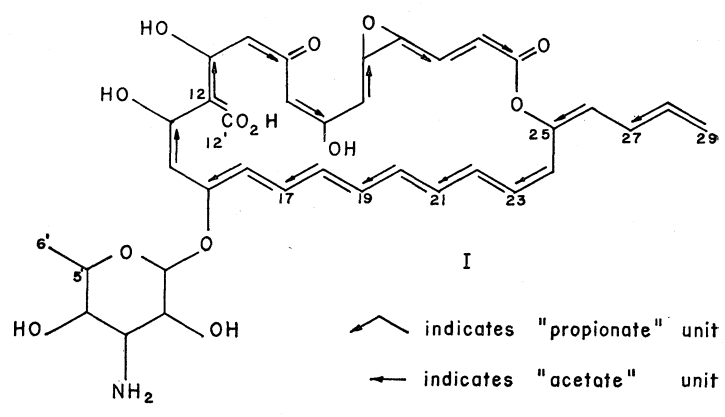
The biosynthesis of lucensomycin in cultures of *Streptomyces lucensis* has been studied using ^{14}C-labelled precursors. The aglycone arises from two propionate and twelve acetate units. One propionate unit provides the chain-initiating unit, whilst the second, involved in chain-extension, undergoes oxidation of its methyl group to form the free carboxyl function of the aglycone.

Although extensive studies have been made of the mode of biosynthesis of non-polyene macrolide antibiotics, *e.g.* erythromycin[1~3], methymycin[3,4] and magnamycin[2,3,5], only limited information[6] is available for one polyene macrolide, nystatin[6,7,8]. It appears, however, that throughout these two sub-groups of Streptomycete metabolites one-carbon branches in the aglycone skeleton represent the incorporation of propionate units (via methylmalonyl-coenzyme A) into the developing carbon chain[3], an event more frequent in the non-polyenes than in the polyenes characterised to date. This route of macrolide biosynthesis operates[6] even in an organism such as *Streptomyces noursei* which is known[9,10] to be capable of branched-chain synthesis by the more common route[11] of transmethylation of an acetate-derived polyketide skeleton.

To supplement the limited evidence relating to the polyene macrolide sub-group, we have studied the biosynthesis of lucensomycin[12] (I) in cultures of *S. lucensis*. The aglycone of this antibiotic clearly arises primarily from acetate units (incorporated via malonyl-coenzyme A[3]) as indicated in (I), but contains two features of particular interest, the *n*-butyl group at C25 and

Formula 1.



I

the secondary carboxyl function at C 12. One would anticipate that both these features involve the utilization of propionyl-coenzyme A units (cf. I), in the former case as a chain-initiating unit of propionyl-coenzyme A itself, and in the latter case as its carboxylation product, methylmalonyl-coenzyme A[3]. The amino-sugar mycosamine, 3-amino-3,6-dideoxy-D-mannose[13], which is commonly present in polyene macrolides, would be expected to arise directly from carbohydrate metabolism[3,14]. We present here evidence in support of these views.

Fermentation of *S. lucensis* in the presence of sodium [3-^{14}C]propionate and [1-^{14}C]acetate gave crude radioactive lucensomycin, with 1.1 % and 0.51 % isotope incorporation respectively. After dilution with carrier material and purification, the distribution of radioactivity was determined by degradation. Acid solvolysis afforded C 12′ as barium carbonate and the sugar moiety as methyl mycosaminide hydrochloride. Glyoxal, representing an average of the two-carbon segments C 17~18, C 19~20 and C 21~22, resulted from ozonolysis followed by reductive work-up and was assayed as the *bis*-2,4-dinitrophenylhydrazone. Controlled oxidation of lucensomycin with chromic acid gave *n*-valeric, *n*-butyric and propionic acids (this last in amount insufficient for assay) from the C-butyl group, together with acetic acid which arises from both C-methyl groups and is consequently of little diagnostic value. Isolation of C 25 was effected by SCHMIDT degradation of the *n*-valeric acid to barium carbonate and *n*-butylamine representing C 26~29. KUHN-ROTH oxidation of the *n*-valeric acid, followed by SCHMIDT degradation of the resulting acetic acid, afforded C 28 and C 29 as barium carbonate and methylamine respectively. Fatty acids were assayed as their crystalline *p*-bromophenacyl esters, amines as the corresponding N-alkyl-2, 4-dinitroanilines. Results, corrected where appropriate for dilutions with carrier material and expressed as percentages of molar radioactivity relative to that of lucensomycin are collected in the Table 1.

It is clear (cf. Table 1) that radioactivity from the [3-^{14}C]propionate precursor is restricted to two positions in lucensomycin, C 12′ and C 29, marking propionate-derived segments as indicated in structure (I). As is frequently observed[1~3,11], the chain-initiating unit, arising directly from propionyl-coenzyme A itself, carries a higher

Table 1. Percentage molar radioactivity of fragments relative to lucensomycin

Compound	Origin	$^{14}CH_3CH_2CO_2H$	$CH_3{}^{14}CO_2H$
Lucensomycin		100.0	100.0
$BaCO_3$	C 12′	43.6	0.093
Methyl mycosaminide [a]	Sugar	—	0.41
Glyoxal [b]	C 17~18, 19~20, 21~22	—	7.62
n-Valeric acid [c]	C 25~29	60.2	9.21
n-Butyric acid [c]	C 26~29	—	3.17
Acetic acid [c]	C 5′~6′, 28~29	1.7	5.49
n-Butylamine [d]	C 26~29	59.2	—
$BaCO_3$	C 25	0	6.04 [e]
Methylamine [d]	C 29	58.3	—
$BaCO_3$	C 28	0	—

a) Assayed as the hydrochloride.
b) Assayed as the *bis*-2, 4-dinitrophenylhydrazone.
c) Assayed as the *p*-bromophenacyl ester.
d) Assayed as the N-alkyl-2, 4-dinitro-aniline.
e) Obtained by difference.

label (60.2 %) than the chain-extending unit (43.6 %) which is utilized only via methylmalonyl-coenzyme A. Oxidation of this latter unit has occurred at some stage, as was observed also in the polyene macrolide nystatin[6]. The low radioactivity of acetic acid formed on oxidation of the intact antibiotic is attributed to heavy dilution of acetate from C 28~29, itself obtained only in low yield together with higher fatty acids, with acetate produced in high yield from the readily-oxidized mycosamine. The specificity of labelling of C 12′ and C 29 indicate negligible randomization of isotope from the precursor.

The distribution of radioactivity from [1-^{14}C]acetate precursor (cf. Table 1) is in agreement with the expected presence of twelve acetate units in the aglycone, as depicted in structure (I). Thus the glyoxal *bis*-2,4-dinitrophenylhydrazone represents the average of the three acetate units between C 17 and C 22, and its activity (7.62 %) is in accord with the activity of barium carbonate from C 25 (6.04 %, determined by difference), which represents the carboxyl of an acetate unit. There is apparently some utilization of the acetate for propionate synthesis, as shown by the activity (3.17 %) of the *p*-bromophenacyl butyrate representing C 26~29. This presumably occurs via succinate and methylmalonate to [1-^{14}C]propionate, in accordance with the inactivity of C 12′ (which represents C 3 of a propionate unit) and with earlier results on erythromycin[15,16] and methymycin[4]. Allowing for two such propionate-derived units and twelve acetate units in the aglycone, together with minor activity (0.41 %) in the sugar, approximately 97 % of the total lucensomycin activity is accounted for. The activity of the acetic acid (5.49 %) from oxidation of the lucensomycin is anomalously high, particularly in comparison with the earlier [3-^{14}C]propionate result, but may perhaps be explained by the presence of an impurity yielding labelled acetate on oxidation.

Pimaricin[17] (I; Me instead of *n*-Bu) from *S. natalensis* represents a lower homologue of lucensomycin (I) in which the starter propionate unit is lacking and chain development is initiated by an acetyl-coenzyme A unit.

Experimental

General

Melting points were determined on a Kofler stage and are uncorrected. Compounds were purified to constant radioactivity and assayed as thin films 1 inch in diameter on a Beckman Lowbeta II low background Geiger flow counter. Radioactivities (p) and (a) refer to labelled lucensomycin and corresponding degradation products derived from sodium [3-^{14}C]propionate and [1-^{14}C]acetate respectively, and are expressed as counts per min per millimole, derived graphically from assay of several samples. Maximum percentage errors, estimated graphically, are given in parentheses.

Fermentations

S. lucensis[22] was grown in 300 ml Erlenmeyer flasks each containing a medium (50 ml) consisting of glucose, distillers' solubles, corn steep liquor, soybean oil and inorganic salts in tap water, for 144 hours at 27°C on a rotary shaker. The yield of lucensomycin varied between 10~20 mg per flask.

[^{14}C]Lucensomycin

Sodium [3-^{14}C]propionate (100 μC) and sodium [1-^{14}C]acetate (100 μC in duplicate runs)

were each added to two culture flasks after incubation for 24 hours. At harvest the mycelium was extracted thrice with aqueous methanol (75 %), and the combined extracts evaporated under reduced pressure. The residue was dissolved in aqueous methanol (20 ml, 50 %) and basified to pH 10 with dilute aqueous sodium hydroxide with stirring under nitrogen. Neutralization with phosphoric acid (0.05 M) in aqueous methanol (50 %) afforded crystalline lucensomycin, collected by centrifugation. Recrystallization by the same procedure gave solvated lucensomycin of 80~84 % purity. The propionate fermentation yielded 20.6 mg of this crude [^{14}C]lucensomycin (with 1.2 % incorporation of radioactivity), the acetate fermentations (where a higher-producing subculture was used) gave 42.8 and 42.9 mg respectively (1.2 and 0.8 % incorporations). Further purification after dilution with pure inactive material resulted in some loss of radioactivity, and afforded [^{14}C]lucensomycin suitable for degradation [Found: (p) 35.8×10^3 (5 %); (a) 162.6×10^3 (5 %)].

Decarboxylation of [^{14}C]lucensomycin

Decarboxylation of lucensomycin (150 mg) with sulphuric acid as described by GAUDIANO, BRAVO and QUILICO[18] gave carbon dioxide, collected as barium carbonate (15 mg) [Found: (p) 15.6×10^3 (3 %); (a) 0.15×10^3].

Methanolysis of [^{14}C]lucensomycin

Lucensomycin (20 mg) in methanolic hydrogen chloride (10 ml, 3 N) was refluxed for 2 hours. After cooling and dilution with water, the mixture was centrifuged to remove solid material, extracted several times with *n*-butanol, and then evaporated to dryness. The residual methyl mycosaminide hydrochloride (3 mg) was identified by paper chromatography (R_F 0.5 in *n*-butanol : acetic acid : water, 4 : 1 : 5, detected by ninhydrin spray) in comparison with authentic material [Found: (a) 0.67×10^3].

Ozonolysis of [^{14}C]lucensomycin

Ozonolysis of [^{14}C]lucensomycin (100 mg) as described[18] afforded glyoxal, isolated as the *bis*-2, 4-dinitrophenylhydrazone (70 mg), m. p. 319°C after two crystallizations from dimethylformamide containing a few drops of acetic acid [Found: (a) 12.4×10^3 (6 %)].

Chromic acid oxidation of [^{14}C]lucensomycin

[^{14}C]Lucensomycin (600 mg) and potassium dichromate (3.0 g) in sulphuric acid (100 ml, 2 N) were refluxed for 30 minutes. The volatile acids were separated by steam distillation and neutralised to phenolphthalein with lithium hydroxide (0.5 N). The solution was then concentrated under reduced pressure (to 5 ml) and just acidified with hydrochloric acid (0.1 N). *p*-Bromophenacyl bromide (500 mg) was added, together with sufficient ethanol to produce solution at reflux temperature. After refluxing for 2 hours the solution was cooled, and unreacted phenacyl bromide (100 mg) which separated was removed by filtration. The filtrate was diluted with water and extracted with chloroform, affording semi-solid material (520 mg) shown by thin-layer chromatography on silica gel to contain the *p*-bromophenacyl esters of acetic, propionic (a trace), butyric and valeric acids, together with *p*-bromophenacyl bromide. Separation by preparative gas-liquid chromatography (using an Aerograph A 350 instrument fitted with a 1.5 m × 0.64 cm SE 52 column at 190°C with helium as carrier gas) gave *p*-bromophenacyl acetate (25 mg), propionate (a trace), butyrate (10 mg), and valerate (32 mg). Crystallization from light petroleum gave *p*-bromophenacyl *n*-valerate (8 mg), m. p. 74°C [Found: (p) 21.5×10^3 (2 %); (a) 17.6×10^3 (6 %)], *p*-bromophenacyl *n*-butyrate (3 mg), m. p. 63°C [Found: (a) 5.13×10^3 (6 %)], and *p*-bromophenacyl acetate (10 mg), m. p. 86°C [Found: (p) 0.61×10^3 (8 %); (a) 8.94×10^3 (8 %)]. Insufficient propionate derivative was obtained for assay.

Degradation of *n*-valeric acid

(a) SCHMIDT degradation. *p*-Bromophenacyl *n*-valerate (p above, 2.21 mg) was diluted with inactive material (to 9.02 mg) [Found: (p) 5.28×10^3 (5 %)] and hydrolyzed in refluxing methanol (2 ml) and aqueous sodium hydroxide (5 ml, 0.2 N) for 2.5 hours under nitrogen. After removal of the alcohols by steam distillation, the residue was acidified with dilute sulphuric acid and steam distilled further. The valeric acid was isolated by titration of

the distillate with aqueous sodium hydroxide and evaporation to dryness. SCHMIDT degradation of this salt by the method of PHARES[19] gave carbon dioxide and *n*-butylamine, assayed respectively as barium carbonate (5 mg) [Found : (p) 0] and N-(*n*-butyl)-2,4-dinitroaniline (5 mg), m. p. 90~91°C (lit.[20] records m. p. 90°C) after preparative thin-layer chromatography and sublimation at 100°C/0.2 mm [Found : (p) 5.23×10^3 (5 %)].

(b) KUHN-ROTH oxidation. *p*-Bromophenacyl *n*-valerate (p above, 2.02 mg) was diluted with inactive material (to 28.76 mg) [Found : (p) 1.45×10^3 (5 %)], and a portion (15 mg) was subjected to KUHN-ROTH oxidation. SCHMIDT degradation[19] of the resulting acetic acid, isolated as sodium acetate, yielded carbon dioxide and methylamine. These fragments were assayed as barium carbonate [Found : (p) 0], and N-methyl-2,4-dinitro-aniline, m. p. 177~178°C (lit.[21] quotes 178°C) after chromatography on neutral alumina and sublimation at 110°C/0.05 mm [Found : (p) 1.40 (7 %)].

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