BIOSYNTHESIS OF STREPTOLIDINE MOIETY OF STREPTOTHRICINS BY *STREPTOMYCES NOURSEI* JA 3890b

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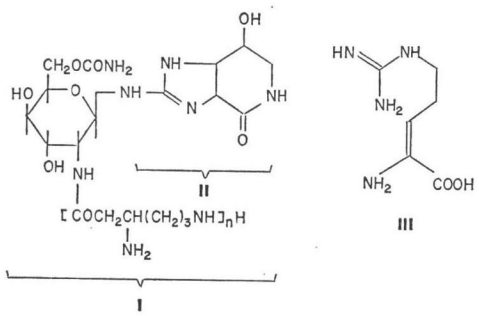
The incorporation of uniformly ^{14}C-labeled compounds into the streptothricin-type antibiotic nourseothricin was studied with a strain of *Streptomyces noursei* JA 3890b. 6.5% of radioactivity from U-^{14}C-L-arginine was incorporated into the antibiotic, while glutamic acid, aspartic acid, alanine, proline, glycine and leucine displayed much lower incorporations. Furthermore, 95% of the activity incorporated from arginine was located in the streptolidine moiety supporting the suggestion that this subunit of streptothricin antibiotics is formed *via* the dehydroarginine pathway.

In order to contribute to the understanding of regulatory mechanisms in the biosynthesis of streptothricin-type antibiotics, we reported on the stimulatory effect of *o*-aminobenzoic acid (OAB, anthranilic acid) on the production of nourseothricin by *Streptomyces noursei* JA 3890b and corresponding alterations of enzymic activities[1,2].

It is commonly accepted that studies on the regulation of biosynthesis of secondary metabolites require knowledge of the individual biosynthetic steps. At present, there is a lack of information about the complete biosynthetic pathway of streptothricin antibiotics (**I**). According to VORONINA *et al.*[3] and CARTER *et al.*[4] β-lysine is formed in streptomycetes *via* isomerization of α-lysine. On the other hand, it is likely that the gulosamine moiety is biosynthesized from glucose as a precursor.[5,6] Although streptolidine (**II**), the third constituent of streptothricins, has been prepared by chemical methods,[7] only limited information is available concerning the pathway of its biogenesis. Hence, we studied the biosynthesis of this streptothricin moiety by means of incorporation of ^{14}C-labeled amino acids.



Experimental

Material:

U-^{14}C-L-Arginine, U-^{14}C-L-proline, U-^{14}C-L-glutamic acid, U-^{14}C-L-aspartic acid, U-^{14}C-L-alanine, U-^{14}C-glycine and U-^{14}C-L-leucine were purchased from UVVVR Prague (Czechoslovakia), U-^{14}C-D-glucose from The Radiochemical Centre (Amersham/England), cation-exchanger resin type Wofatit CP and anion-exchanger resin type Wofatit L 150 from VEB Farbenfabrik Wolfen (DDR), thin-layer plates (20×20 cm) precoated with cellulose (0.1 mm layer) from E. Merck (Darmstadt, BRD), chromatography paper 2043b Mgl from Schleicher and Schuell (Dassel/BRD).

Methods:

For liquid scintillation studies, a LKB-WALLAC 81000 liquid scintillation system (LKB-Instruments AB, Bromma/Sweden) was used. The liquid scintillation fluid was prepared by mixing 1 liter toluene with 5 g PPO and 0.3 g POPOP. Samples to be counted were extracted within the scintillation vials with water (0.4 ml) and ethanol (5 ml). After that 8 ml of scintillation fluid were added.

Cultivation of *S. noursei* JA 3890b:

S. noursei JA 3890b from the strain collection of Central Institute of Microbiology and Experimental Therapy, Jena, was cultivated on a complex medium that contained[1]: glucose 2.9 %, soybean meal 1.3 %, NaCl a.g. (analytical grade) 0.5 %, $CaCO_3$ a.g. 0.3 %, pH 6.5 (prior to sterilization). A suitable production of antibiotic by this strain was achieved by adding OAB (7.5 mM) to the medium prior to addition of 3 ml of inoculum[1]. Each 100 ml of culture, grown for 44 hours in 500 ml shaking flasks, was transferred into 500 ml special flasks that were aerated with sterilized air (1:1). All cultivations were performed at 25°C on rotary shakers at 240 r.p.m. Under these conditions the production of nourseothricin started at nearly the 45th hour and rose to the maximum value of 150~200 mg/liter by the 96th hour. If *o*-aminobenzoic acid was omitted only 20~30 mg/liter antibiotic were produced.[8] Labeled compounds were added to 44-hour cultures and the antibiotic was isolated from 96-hour fermentations.

Incorporation studies:

The culture liquids were separated from the mycelia by suction-filtration and added to columns (1.5 cm diameter, 30 cm length) that contained 30 ml of Na^+-loaded cation-exchanger CP according to the modified procedure of VORONINA *et al.*[3]. A flow rate of approximately 75 ml/hour was established. After washing the resin with 1 liter of 0.1 N acetic acid and then with 150 ml distilled water, the nourseothricin complex was eluted with 0.05 N HCl. When pH 1 was reached, the washing was continued with a further 100 ml of 0.05 N HCl. The HCl-eluate was immediately neutralized by adding OH^--loaded anion-exchanger type L 150. The resin was filtered off, and after addition of 40 mg of unlabeled nourseothricin complex, the eluate was evaporated under vacuum to dryness (40°C, rotary evaporator). The residue was dissolved in methanol and brought to 5 ml. Paper disks (30 cm diameter) were each loaded with 50 µl of these solutions and circular chromatograms were developed overnight in *n*-propanol - pyridine - acetic acid - water (15:10:3:12, v/v). The zones containing streptothricins F, E and D were cut into small pieces and transferred into scintillation vials. On the chromatograms, these zones were detected by spraying parallel blanks with ninhydrin-collidine reagent[9]. To assure an adequate level of antibiotic in all experiments, the nourseothricin in the culture liquids was assayed by the agar plate diffusion technique with *Bacillus subtilis* ATCC 6633 as the test organism.

Acidic hydrolysis of nourseothricin:

Labeled samples of nourseothricin were precipitated from the methanolic solution by adding dry acetone (25~30 ml). The precipitates were removed by centrifugation, washed with acetone and dried in a vacuum desiccator. After that, the products were dissolved in 10 ml 6 N HCl and heated for 24 hours at 110°C in sealed glass tubes according to BORDERS *et al.*[10] The hydrolysed solutions were evaporated in vacuum to dryness using a rotary evaporator (40°C), dissolved in 20 ml water and heated for a few minutes with charcoal. After the charcoal was filtered off, the filtrate was neutralized, evaporated in vacuum to dryness, and dissolved in 2 ml of water.

Chromatographic separation of hydrolysates:

Thin-layer plates, each charged with 20~50 µl solution of hydrolysate, were developed in methanol - chloroform - 25 % aqueous ammonia (2 : 1 : 1, v/v). The zones to be measured were detected by spraying parallel blanks with ninhydrin-collidine[9]. Each chromatogram showed three clearly separated zones of gulosamine (R_f 0.74), β-lysine (R_f 0.55) and streptolidi-

dine (Rf 0.25)[8]. The layers of adequate areas that remained unstained were transferred into scintillation vials and measured.

Results

Although washing the ion-exchange resin with acetic acid and water removed the major part of radioactive non-nourseothricin products and salts, more basic impurities were partially retained in the resin and were finally eluted together with the antibiotic. Therefore, prior to the determination of incorporation, it was advantageous to separate the nourseothricin components from labeled by-products by means of paper chromatography. Furthermore, according to our experience, during the elution procedure with 0.05 N HCl some inactivation of the antibiotic may occur that is due to partial hydrolysis of streptolidine moiety[11] (10~15 %). Hence, it seemed to be unreasonable to calculate specific radioactivities of isolated nourseothricin samples. On the other hand, on the paper chromatograms the Rf values of streptothricin D and the inactivation product of streptothricin F, the major constituent of nourseothricin[1], were identical THRUM, unpublished results). For that reason, we can assume that the radioactivity from partially hydrolysed nourseothricin was measured together with the streptothricin D.

In Table 1 the results of the incorporation studies are given. U-^{14}C-L-Arginine showed the best incorporation (6.5 %) of added radioactivity. The other amino acids and U-^{14}-C-D-

Table 1. Incorporation of ^{14}C-labeled amino acids and D-glucose into the nourseothricin complex.

Source of label	Spec. activity (mc/mmole)	Added activity (dpm)	Incorporated activity (dpm)	Incorporation (%)
U-^{14}C-L-Arginine	187	21.6×10^6	$1,400\times10^3$	6.5
U-^{14}C-L-Proline	125	160×10^6	164×10^3	0.103
U-^{14}C-L-Glutamic acid	178	73.4×10^6	56×10^3	0.0764
U-^{14}C-L-Aspartic acid	140	88×10^6	60×10^3	0.0682
U-^{14}C-D-Glucose	335	111×10^6	65×10^3	0.059
U-^{14}C-L-Alanine	105	88×10^6	41×10^3	0.046
U-^{14}C-Glycine	74	60×10^6	16.1×10^3	0.0268
U-^{14}C-L-Leucine	50.8	82.5×10^6	6.7×10^3	0.0081

Table 2. Distribution of radioactivity within the products of acidic hydrolysis of nourseothricin (abbreviations: G=gulosamine, L=β-lysine, S=streptolidine).

Labeled precursor	Activities in 50 μl solution of hydrolysate (dpm)			Percentage of whole activity G+L+S			dpm ratio S/L
	G	L	S	G	L	S	
U-^{14}C-L-Arginine	108	2,740	57,700	0.2	4.5	95.3	21.05
U-^{14}C-L-Proline	68	319	2,009	2.8	13.3	83.8	6.29
U-^{14}C-L-Glutamic acid	78	410	453	8.3	43.5	48.1	1.10
U-^{14}C-Glycine	26	183	135	7.6	53.1	39.3	0.74
U-^{14}C-L-Alanine	33	418	235	4.8	60.9	34.3	0.56
U-^{14}C-L-Aspartic acid	10	283	123	2.4	68.0	29.5	0.43
U-^{14}C-D-Glucose	468	560	245	36.8	44.0	19.2	0.44

glucose were used much less efficiently for antibiotic biosynthesis. Among the products of hydrolysis of nourseothricin, the streptolidine moiety was identified by its reaction with nitroprusside reagent giving a red color[12]. Gulosamine was detected by its reducing properties towards TOLLENS' reagent[12], and β-lysine by cochromatography with α-lysine[8]. When the plates were sprayed with ninhydrin-collidine reagent, the streptolidine stained green, while gulosamine and β-lysine displayed blue-violet spots.

In Table 2 the radioactivities found in the products of hydrolysis and the percentage of individual radioactivities of gulosamine, β-lysine and streptolidine are shown. U-^{14}C-L-Arginine was a superior precursor of the streptolidine moiety. In comparison with U-^{14}C-L-proline, the ratio of radioactivity incorporated into streptolidine/β-lysine was elevated more than three times. However U-^{14}C-L-proline and U-^{14}C-L-glutamic acid also showed preferred incorporation into streptolidine. If the molar ratio of streptothricins F, E and D in the nourseothricin complex[1] and the resulting streptolidine/β-lysine ratio of approximately 0.6 are taken into account, the incorporation of U-^{14}C-L-alanine and U-^{14}C-glycine was almost equal between β-lysine and streptolidine. U-^{14}C-L-Aspartic acid and U-^{14}C-D-glucose were mainly incorporated into the β-lysine moiety of the antibiotic.

Discussion

According to BYCROFT and KING,[13] streptolidine (**II**) may be formed *via* an intermediate derivative of capreomycin involving an intramolecular rearrangement of the guanidino group from C-atom 5 to C-atom 3. This proposal also has been supported by stereochemical results from X-ray studies of the crystal structure of streptolidine.[13] As has been expressed by BYCROFT,[14] in this biosynthetic conception the arginine-derived dehydroarginine (**III**) was assumed to be the common precursor not only of streptolidine but also of a part of the molecules of capreomycin, viomycin and stendomycin, respectively. Direct experimental evidence confirming this hypothesis has been presented by CARTER *et al.*[4] who established an excellent incorporation of arginine into the antibiotic viomycin in *Streptomyces* species. Although RACZYNSKA-BOJANOWSKA[18] proved the involvement of a transamidinase in viomycin biosynthesis, the true mechanism of rearrangement of the guanidino group remains to be elucidated. The results presented in this paper is compatible with BYCROFT's hypothesis[13,14] concerning the involvement of dehydroarginine (**III**) as an intermediate of streptolidine biosynthesis. Of all the precursors tested, arginine showed the highest incorporation into both the nourseothricin complex and the streptolidine moiety. The relatively high incorporation of U-^{14}C-L-proline into this constituent of streptothricins may be due to weak catabolism of this amino acid and its possible conversion to ornithine. The incorporation of proline into the antibiotic was much lower than that of arginine suggesting that there is no separate pathway of streptolidine biosynthesis. The unexpected low incorporation obtained with glutamic acid, aspartic acid and glucose may be due to intensive turnover of these compounds during primary metabolism.

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