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Biosynthesis of Polyketomycin Produced by *Streptomyces diastatochromogenes* Tü 6028†

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The biosynthesis of polyketomycin was investigated by feeding ^{13}C-labeled acetate and propionate to the growing cultures of *Streptomyces diastatochromogenes* Tü 6028. ^{13}C NMR spectral analysis demonstrated the polyketide origin of the aglycone and the dimethylsalicyloyl moieties. The *O*-methyl group and 6-CH_3 of the aglycone as well as 3B-CH_3 of L-axenose and 3C-CH_3 of the salicyloyl residue were labeled by feeding L-[methyl-^{13}C]methionine. Both deoxysugars emerged from D-glucose. The biosynthesis of the aglycone and the assembly of the glycoside are discussed. The polyketomycin producing strain may be a candidate for further exploration in combinatorial biosynthesis.

Polyketomycin is a novel tetracyclic quinone glycoside with antibacterial and cytotoxic activity. It was isolated independently from *Streptomyces* sp. MK 277-AF1[2,3] and *Streptomyces diastatochromogenes* Tü 6028, which was isolated from a soil sample collected in Iguaçu, Argentina. The structure as a result of spectroscopical analysis, chemical degradation and X-ray analysis is shown in Fig. 1[3]. This paper deals with the biogenetic origin of all carbon atoms of polyketomycin.

Fermentation and Isolation

Batch fermentations of *S. diastatochromogenes* Tü 6028 were carried out in a 20-litre fermenter using a complex medium that consisted of mannitol 2% and soybean meal 2% (pH 7.5). Production of polyketomycin started at about 24 hours and reached a maximum after 143 hours with a concentration of 4.3 g/litre polyketomycin in the mycelium. For feeding experiments the strain was cultivated in shaking flasks for 72 hours, under these conditions the yield of polyketomycin was only about 100 mg/litre. Polyketomycin was isolated from the mycelium by extraction with MeOH - acetone (1 : 1). The extract was concentrated, extracted with ethyl acetate and subjected to a silica gel column, followed by a chromatography step using Sephadex LH-20. Finally, pure polyketomycin was obtained after preparative reversed-phase HPLC using Nucleosil C-18 material with 0.5% formic acid-MeOH gradient elution, resulting in a orange powder after concentration to dryness.

A minor metabolite, SEK 15, was excreted into the culture medium and reached a concentration of 18 mg/liter after 90 hours. The compound was isolated from the culture filtrate by ethyl acetate extraction, followed by consecutive silica gel and Sephadex LH-20 chromatography.

Feeding Experiments

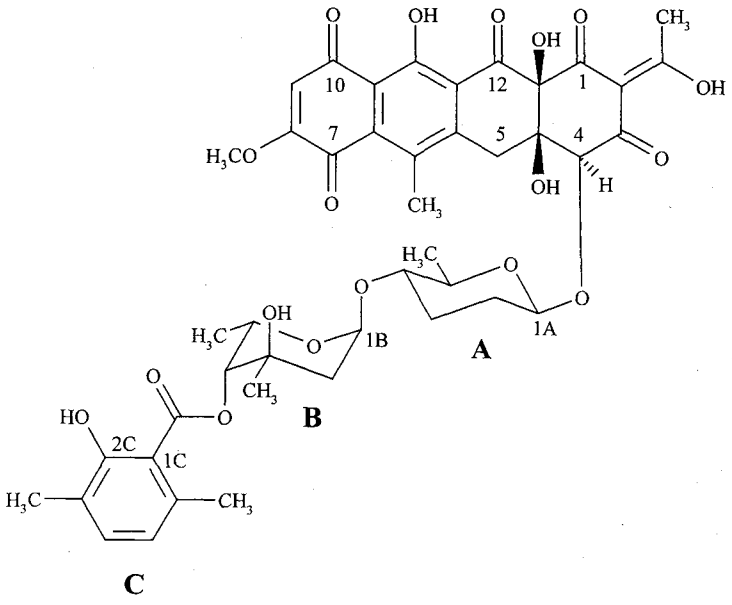
Polyketomycin from strain Tü 6028 was completely characterized by verifying all spectroscopical data. Especially the NMR signals were assigned unambigously by 2D-NMR mesurements (HSQC, HMBC, COSY, TOCSY, NOESY). Using CD_2Cl_2 instead of $CDCl_3$ as solvent allowed a much better separation of the proton signals (Table 1 and 2).

Feeding of sodium [1-^{13}C]acetate resulted in signal enhancements in the aglycone (C-1, C-3, C-4a, C-5a,

† Art. No. 12 on 'Biosynthetic Capacities of Actinomycetes'. Art. No. 11: See ref. 1.

Fig. 1. Structure of polyketomycin.

The aglycone represents the relative configuration only, derived from the X-ray analysis[3].



C-6a, C-8, C-10, C-11, C-12 and C-13) and the dimethylsalicyloyl moiety (1C-CO, C-2C, C-4C, and C-6C) as shown in Table 1. The remaining ambiguity, concerning the folding of the polyketide chains, was removed by a sodium [1,2-$^{13}C_2$]acetate feeding experiment leading to ten intact acetate units in the aglycone by strong couplings of the following pairs: C-13/13-CH_3, C-2/C-1, C-12a/C-12, C-11a/C-11, C-10a/C-10, C-9/C-8, C-7/C-6a, C-6/C-5a, C-5/C-4a and C-4/C-3. Additionally, four coupling pairs were detected in the dimethylsalicyloyl moiety: 6C-CH_3/C-6C, C-5C/C-4C, C-3C/C-2C and C-1C/1C-CO. The coupling pairs were named in direction of the biosynthetic pathways beginning in each case with the starter unit. All other carbon atoms of polyketomycin were seen as non-enhanced singlets. Thus there are two different polyketide building blocks as part of polyketomycin.

In order to establish the origin of the not acetate-derived *C*-methyl groups feeding experiments were performed using sodium [1-^{13}C]propionate and L-[methyl-^{13}C]methionine. No label at all in the frist case and a high enrichment of 6-CH_3 and 3C-CH_3 in the second indicated a *C*-methylation of the polyketide moieties. Additionally, the *O*-methyl group of the aglycone and the *C*-methyl group of sugar B (L-axenose) were labeled. A final feeding experiment with [U-$^{13}C_6$]-D-glucose resulted in a complete coupling pattern for the C_6-frame of both deoxysugars (Table 2). From the coupling pattern of C-3A/C-4A and C-3B/C-4B (dd and d) can be detected that besides a complete incorporation of glucose a second pathway exists, which bases on a C-3/C-4 splitting of glucose. A similar effect could be seen for C-2B indicating an additional C-2/C-3 splitting of glucose. The comparable doublet coupling of C-2A is visibel but the signals were weak only. The carbon atoms of the aglycone and the salicyloyl moiety were labeled too, due to a scrambling by glycolysis pathways.

Discussion

The biogenetic origin of all carbon atoms of polyketomycin has been proved. The results indicated that *Streptomyces diastatochromogenes* needs two different polyketide synthases of the iterative type II for establishing the decaketide of the aglycone, polyketomycinone, and the tetraketide of the salicyloyl moiety. The first resulted in a tetracyclic linear hydroxyquinone with a *C*-methylation at C-6 similar to that of the tetracyclines[4]. The second leads to 6-methylsalicylic acid (6-MSA), which becomes methylated at C-3. While the later biosynthetic pathway is more typical for fungi and has been established in detail[5], the folding mode of the decaketide is similar as that assumed for terramycin X[4]. The condensation starts with a C-7/C-12 cyclisation and aromatisation of the first ring and one may suggest a tetracyclic aromatic intermediate

Table 1. ^{1}H NMR and ^{13}C NMR signals of polyketomycin (aglycone and dimethylsalicyloyl residue) in CD_2Cl_2 together with specific incorporations and coupling constants after feeding with [1-^{13}C]acetate (I), [1,2-$^{13}C_2$]acetate (II) and L-[methyl-^{13}C]-methionine (III).

Position	δ_H (ppm, J in Hz)	δ_C (ppm)	I[a]	II ($^{1}J_{CC}$/Hz)	III[a]
Aglycone					
1		190.9	1.9	57.5	
2		110.9	0.6	57.5	
3		196.5	5.6	45.0	
4	4.54 (s)	73.8	0.4	45.0	
4a	2.89 (s, br, 4a-OH)	75.9	4.3	39.0	
5	3.04 (d, 17.5)	35.2	0.1	39.0	
	3.78 (d, 17.5)				
5a		151.0	2.7	57.5	
6		132.9	0.0	57.5	
6-CH_3	2.58 (s)	16.8	0.3		24.0
6a		132.8	1.4	57.5	
7		181.6	−0.3	57.5	
8		161.6	1.2	69.5	
8-OCH_3	3.91 (s)	57.3	−0.1		11.7
9	6.13 (s)	108.9	−0.2	69.5	
10		191.3	4.0	54.0	
10a		114.1	0.2	54.0	
11	14.18 (s, 11-OH)	162.3	2.8	67.5	
11a		123.6	0.3	67.5	
12		192.5	5.6	45.0	
12a	4.87 (s, br, 12a-OH)	81.5	1.0	45.0	
13	18.1 (s, br, 13-OH)	201.5	5.6	43.5	
13-CH_3	2.71 (s)	27.0	1.0	43.5	
3,6-Dimethyl-salicyloyl residue					
1C		111.3	−0.2	72.5	
1C-CO		171.9	2.2	72.5	
2C	11.63 (s, br, 2C-OH)	162.0	2.9	67.5	
3C		124.8	0.0	67.5	
3C-CH_3	2.20 (s)	15.9	0.4		31.9
4C	7.19 (d, 7.5)	135.7	2.3	54.5	
5C	6.66 (d, 7.5)	122.6	0.1	54.5	
6C		139.0	2.0	43.5	
6C-CH_3	2.54 (s)	24.7	0.2	43.5	

[a] Relative enrichments were normalized to the peak intensity of the C-1B signal.

similar to that of other decaketides[6] (Scheme 1). The left side of this intermediate will then be *O*-methylated and oxidized to the quinone, while the right side will be changed by oxygenase and hydration reactions similar to that proved for tetracenomycin C[7]. The polyketide synthase of the aglycone and the enzymes of the late biosynthesis combines different activities known from pathways of other strains in a new manner. Thus our strain, which shows a remarkable productivity of polyketomycin (4.3 g/litre) may be a good candidate for the exploration of combinatorial biosynthesis[8]. As a shunt product the decaketide SEK 15 has been isolated[9]. One may speculate that the missing methyl group at C-6 of the decaketide reduces the substrate specifity for the next cyclisation, the non-fitting intermediate undergoes other catalytically or thermodynamically controlled cyclisation and dehydratation steps[9].

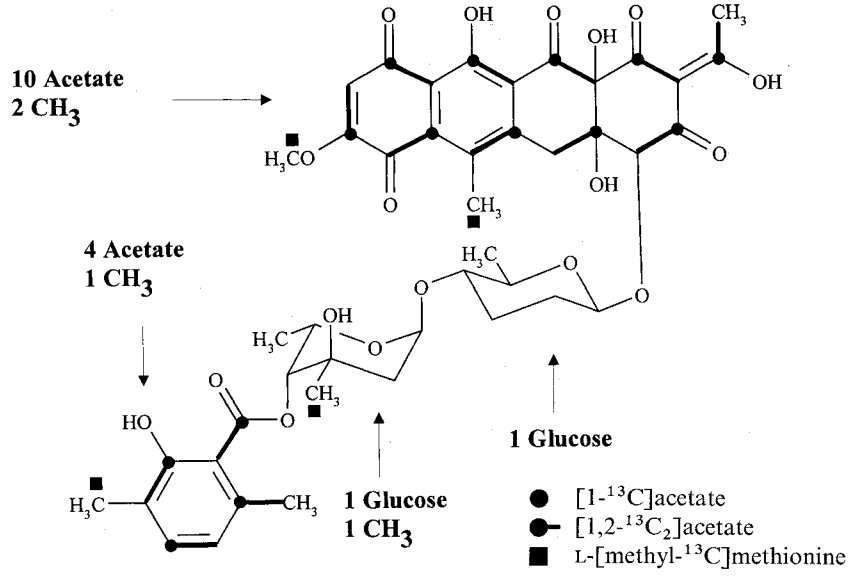
The biosynthesis of the deoxysugars will follow the pathway established for the different types of microbial glycosides[10]. While D-amicetose is more widespread among microbial metabolites, L-axenose has been found in axenomycin A and B[11] and dutomycin[12] only. The biosynthetic pathway seems to be different from that described for axenose in the axenomycin[11], because the

Table 2. ^{1}H NMR and ^{13}C NMR signals of the sugar moieties of polyketomycin in CD_2Cl_2 together with the coupling constants after feeding with D-[U-$^{13}C_6$]glucose.

Position	δ_H (ppm, J in Hz)	δ_C (ppm)	$^{1}J_{CC}$ (Hz)
β-D-Amicetoside	(sugar A)		
1A	4.69 (dd, 9.0, 1.5)	102.3	d, 41
2A	1.64 (m_c, H_a)	30.5	dd, 41, 36
	2.06 (m_c, H_e)		d, weak
3A	1.56 (m_c, H_a)	29.6	dd, 36, 38
	2.20 (m_c, H_e)		d, weak
4A	3.17 (ddd, 10.0, 5.0, 4.5)	80.4	dd, 38, 40
			d, 40
5A	3.06 (dq, 10.0, 6.0)	74.7	dd, 40, 41
5A-CH_3	0.62 (d, 6.0)	17.4	d, 41
α-L-Axenoside	(sugar B)		
1B	5.05 (d, 3.5)	100.4	d, 40
2B	1.71 (d, 14.5, H_a)	37.4	dd, 40, 38
	1.96 (dd, 14.5, 3.5, H_e)		d, 40
3B	3.90 (s, br, OH)	68.7	dd, 38, 40
			d, 38
3B-CH_3[a]	1.08 (s)	26.0	—
4B	5.04 (s, br)	76.3	dd, 40, 41
			d, 41
5B	4.48 (q, 6.5)	62.6	dd, 41, 40
5B-CH_3	1.12 (d, 6.5)	16.9	d, 40

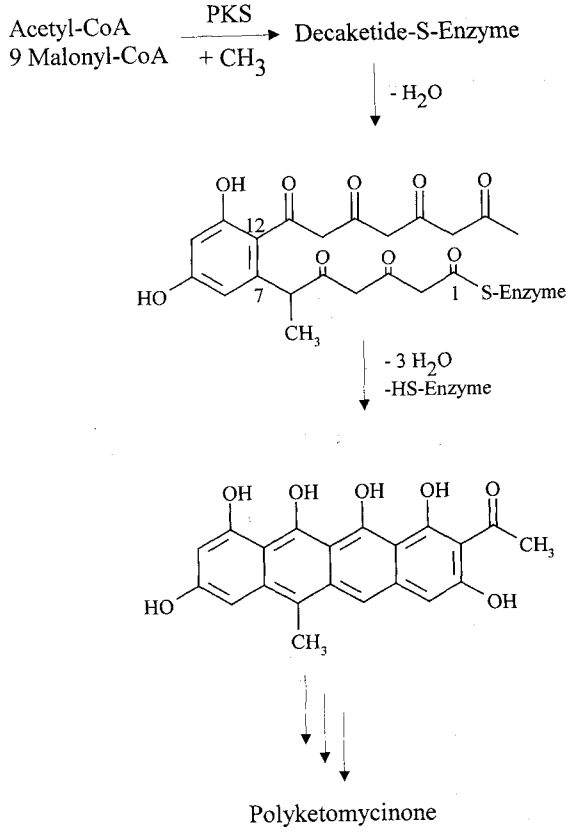
[a] Enrichment from feeding with L-[methyl-^{13}C]methionine: 25.4%.

Fig. 2. Biosynthesis of the carbon atoms of polyketomycin.



total incorporation of glucose is observed and there is no striking uneven distribution of the enrichments in the two halves of the sugar molecules. Worth mentioning is the result that there exist three pathways at least to convert the carbon frame of glucose into that of deoxyhexoses. The *C*-methylation of deoxysugars by methionine has been recently confirmed in the case of L-chromose B as a part of chromomycin A_3[13]. One may expect a stepwise glycosyl transfer starting with D-amicetose to the aglycone and a final acylation with

Scheme 1. Proposed intermediates of the polyketomycinone biosynthesis.



3,6-dimethylsalicyloyl-CoA. Intermediates of this pathway have not been isolated yet.

Experimental

General

^{1}H, ^{13}C and 2D NMR spectra of pure polyketomycin were recorded with a Varian VXR 500 and a Varian Inova 500 by using standard Varian software. Chemical shifts are expressed in δ values (ppm) using the solvent as internal reference (CD_2Cl_2: $\delta_H = 5.32$, $\delta_C = 53.8$).

Labeled Compounds

^{13}C-labeled compounds were 99% ^{13}C atom purity. 10.9 mmol/litre sodium [1-^{13}C]acetate (Chemotrade), 5.6 mmol/litre sodium [1,2-$^{13}C_2$]acetate (Chemotrade), 7.5 mmol/litre sodium [1-^{13}C]propionate (Chemotrade), 1.1 mmol/litre L-[methyl-^{13}C]methionine (Chemotrade) and 5.7 mmol/litre D-[U-$^{13}C_6$]glucose (Cambridge) were feeded.

Incorporation of Isotope-labeled Compounds to Polyketomycin

S. diastatochromogenes Tü 6028 was grown as shaking cultures in 500 ml-Erlenmeyer flasks with one intrusion and 100 ml medium consisting of: mannitol 2% and soybean meal 2% in tap water (pH 7.6, adjusted with 5 N NaOH). The cultures were inoculated with 5 vol-% of a pre-culture grown for 48 hours on a rotary shaker at 120 rpm and 27°C. The precursors were added to each culture following the pulse feeding method in 1 ml-portions at 20, 23, 25 and 27 hours after incubation.

Isolation of Polyketomycin

Three 100 ml-shaking cultures were harvested after 72 hours of incubation by centrifugation. The supernatant was discarded and the polyketomycin containing mycelium was extracted twice with 150 ml of MeOH-acetone (1 : 1). The combined extracts were concentrated *in vacuo* to the aqueous residue. Water was added to a final volume of 300 ml, adjusted to pH 7, and lipophilic compounds were extracted with 150 ml petroleum benzene. The aqueous layer was adjusted to pH 5 (1 M HCl) and extracted three times with 150 ml ethyl acetate. After concentration of the organic extract to an oily residue, it was applied to a silica gel column (23 × 3 cm; silica gel SI 60, Merck) with CH_2Cl_2 as eluent. The labeled compounds were desorbed in a step gradient from CH_2Cl_2 to CH_2Cl_2 - MeOH (98 : 2), CH_2Cl_2 - MeOH - acetic acid (95 : 5 : 0.1) and CH_2Cl_2 - MeOH - acetic acid (90 : 10 : 0.1). After Sephadex LH-20 chromatography (26 × 21.5 cm; MeOH) the labeled polyketomycin was obtained in the following amounts: 1.7 mg ([^{13}C-methyl]methionine), 23.2 mg ([1-^{13}C]acetate), 11.6 mg ([1,2-$^{13}C_2$]acetate), 30.5 mg ([U-$^{13}C_6$]glucose) and 21.0 mg ([1-^{13}C]propionate).

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