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Short communication

Isolation and structural characterization of polymyxin B components

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Abstract

Polymyxin B is a peptide antibiotic complex present as sulphate. The components were separated preparatively on a poly(styrene–divinylbenzene) (PLRP-S), 1000 Å, 8 μm, 250×12.5 mm I.D. stationary phase maintained at 60°C and using 215 nm detection. Elution was carried out with acetonitrile–sodium sulphate solution (0.7%, m/v; pH adjusted to 2.5 with trifluoroacetic acid)–water (18:50:32, v/v) at a flow-rate of 4.0 ml/min. Seven polymyxin B components were isolated and characterized using ^{1}H and ^{13}C NMR. The molecular masses were confirmed by mass spectrometry. The structures of two components were determined for the first time. Polymyxins B_5 and B_6 were identified as having the same composition as polymyxin B_1 except that the fatty acid moiety was nonanoic acid and 3-hydroxy-6-methyloctanoic acid, respectively.

Keywords: Preparative chromatography; Polymyxins; Antibiotics; Polypeptides; Peptides

1. Introduction

Polymyxin B is a complex mixture of closely related polypeptides isolated from various strains of *Bacillus polymyxa* and related species and is active against a wide spectrum of Gram-negative bacteria [1]. Many attempts have been made to characterize the complex commercial product, polymyxin B sulphate. Polymyxins are cyclic polypeptides containing characteristic constituents such as α,γ-diaminobutyric acid, L-threonine and a fatty acid. They differ by the presence or absence of additional amino acid as well as the nature of the fatty acid. These structural features were established by amino acid and fatty acid analysis after partial hydrolysis. Volger and Studer [2] and Studer [3] reviewed the structural elucidation of polymyxins. Polymyxin B is subdivided into at least four components, polymyxins B_1, B_2, B_3 and B_4. They differ from each other only in the fatty acyl moiety: B_1 contains 6-methyloctanoic acid, B_2 6-methylheptanoic acid, B_3 octanoic acid and B_4 heptanoic acid [4]. Recently, the total synthesis of polymyxin B_1, identical to the naturally occurring antibiotic, was described [5].

Various methods for isolating single components from the complex polymyxin antibiotic have been reported. Counter-current distribution, a technique with low separation power, was reported first [6]. A preparative-scale separation of polymyxins B_1 and

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B_2 was established using displacement chromatography on a LiChrosorb RP-18, 5 μm, 250×4.6 mm I.D. stationary phase [7]. Polymyxins B_1 and B_2 and also a new compound, named polymyxin B_0, were separated with a linear gradient elution. The structure of polymyxin B_0 was deduced from the results of amino acid and fatty acid analysis. The fatty acyl moiety of B_0 was not elucidated [8]. Preparative isolation of polymyxin B components was achieved on LiChrosorb Si 100 ODS, 10 μm, 250×40 mm I.D. with isocratic elution. Isoleucine-polymyxin B_1 (Ile-B_1) was characterized by fatty acid and amino acid analysis, although the exact position of the amino acid replacement was not elucidated [9].

In this report, the isolation of pure single polymyxin B components is described. ^{1}H and ^{13}C NMR was used to characterize the components. Molecular masses were confirmed by mass spectrometry.

2. Experimental

2.1. Sample, solvents and reagents

Acetonitrile HPLC Grade S was from Rathburn (Walkerburn, UK). Anhydrous sodium sulphate was from Merck (Darmstadt, Germany). Trifluoroacetic acid was from Acros Organics (Geel, Belgium). Phosphoric acid solution (6.8%, v/v, dilution) was prepared from 85% m/m phosphoric acid (Acros Organics). Sulphuric acid (98%, m/m) and ammonia solution (25%, m/m) were from BDH (Poole, UK). Dimethylpropane sulfonic acid, sodium salt (DSSA) was from Merck. Polymyxin B sulphate was from Ludeco (Brussels, Belgium). Water was distilled twice.

2.2. Apparatus

The preparative work was carried out on a Varian Model 5000 liquid chromatography system comprising a reciprocating pump, a UV detector set at 215 nm and a Varian 4270 integrator. A Valco injector was equipped with a 500 μl loop. A preparative column 250×12.5 mm I.D., packed with PLRP-S, 8 μm, 1000 Å (Polymer Labs., Church Stretton, UK) was maintained at 60°C by means of a water bath heated by a Julabo EM thermostat (Julabo, Seelbach, Germany). The apparatus used for evaporating the fractions comprised a Rotavapor (Büchi Labortechnik, Switzerland), a Laboport vacuum pump (KNF Neuberger, Germany) and a circulating Digital Temperature Controller (PolyScience, IL, USA) filled with ethylene glycol–water (1:1) as the cooling agent at −20°C. YMC-Pack Pro C_{18}, 5 μm (YMC, Wilmington, NC, USA), 250×4.6 mm I.D. stationary phase was used for analytical work, and YMC-Pack Pro C_{18}, 5 μm, 250×2.0 mm I.D. for LC–MS analysis. For analytical work, the LC system consisted of an L-6200 intelligent pump, a Model 655A-40 autosampler set to inject 100 μl, a Model L-4000 variable-wavelength UV detector set at 215 nm (Merck–Hitachi, Darmstadt, Germany) and an electronic integrator HP 3396 Series II (Hewlett-Packard, Avondale, PA, USA). MS was performed with an LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray interface operated in the positive-ion mode. NMR spectra were determined using a 200 MHz Varian Gemini apparatus or a Varian Unity 500 spectrometer (Palo Alto, CA, USA).

3. Results and discussion

3.1. Development of preparative separation

During method development, the selectivity was investigated using different preparative LC methods. Preliminary studies carried out on an open column as well as medium-pressure liquid chromatography on silica gel resulted in poor resolution as the polar polymyxin B components were highly retained by the polar silanol groups of the silica stationary phase. Preparative reversed-phase liquid chromatography was therefore used.

In earlier work in this laboratory, polymyxin B was separated analytically into more than 10 components on a poly(styrene–divinylbenzene) (PLRP-S), 1000 Å, 8 μm, 250×4.6 mm I.D. column with a mobile phase of acetonitrile–sodium sulphate (0.7%, m/v)–phosphoric acid solution (6.8%, v/v, dilution)–water (18:50:5:27, v/v) at a flow-rate of 1.0 ml/min [10]. This analytical system was transferred to a preparative LC system using a large bore PLRP-S, 1000 Å, 8 μm, 250×12.5 mm I.D. column maintained at 60°C and replacing phosphoric acid with trifluoroacetic acid. This volatile acid facilitated

the evaporation of the collected fractions. The mobile phase comprised acetonitrile–sodium sulphate (0.7%, m/v; pH adjusted to 2.5 with trifluoroacetic acid)–water (18:50:32) at a flow-rate of 4.0 ml/min. Detection was by UV at 215 nm. Polymyxin B sulphate was dissolved in water to give a concentration of 100 mg/ml solution. This solution (500 μl) was injected repeatedly onto the preparative column, and the fractions from each peak were collected manually and combined. After evaporation to dryness, the isolated fractions were desalted by precipitating the free bases from aqueous solution by adding ammonium hydroxide up to pH 9. After filtration, the salt-free base was transformed back to polymyxin B sulphate in solution using sulphuric acid and then evaporated to dryness. Further purification was achieved by re-chromatography under the same preparative LC conditions used in the first chromatography. A total of 10 fractions were collected, but after the re-chromatography and purification processes, only seven products were obtained.

The newly developed method used for the isolation of polymyxin components is a highly efficient preparative method resulting in milligram amounts of single components in contrast to the earlier methods which only yielded microgram amounts. From a 10 g polymyxin B sulphate injected mass an approximate yield obtained for B_1 was 3 g; B_2, 2 g; B_3, 0.2 g; B_4, 0.3 g; B_5, 30 mg; B_6, 0.15 g and Ile-B_1, 50 mg. An analytical LC method developed previously [11] was used for determination of the chromatographic purity. Solutions (0.5 mg/ml) of the purified compounds were injected (100 μl) for separation on YMC-Pack Pro C_{18}, 5 μm stationary phase with acetonitrile–sodium sulphate (0.7%, m/v)–phosphoric acid solution (6.8%, v/v, dilution)–water (22.25:50:5:22.75, v/v) at a flow-rate of 1.0 ml/min. The chromatographic purity, calculated by normalization, was 95% for B_1, 95% for B_2, 93% for B_3, 96% for B_4, 86% for B_5, 96% for B_6 and 96% for Ile-B_1. Fig. 1 shows the analytical profile of a commercial polymyxin B sample. Peaks with areas below 0.5% after normalization are not numbered on the chromatogram.

3.2. Structure determination

The structures of isolated single polymyxin B components were determined by spectrophotometric methods. The amounts isolated allowed the deduction of the complex polymyxin structures by NMR and mass spectrometry, thus rendering superfluous classical amino acid and fatty acid analysis. ^1H and ^{13}C NMR spectra were determined with a 200 MHz Varian Gemini apparatus in ^2H_2O with DSSA as external standard. For polymyxin B_5, which was in very small amount, a Varian Unity 500 spectrometer was used. Methyl, methylene and methine carbons were differentiated by ^{13}C NMR using the full DEPT (distortionless enhancement through polarization transfer) technique, as implemented in the Varian VNMR Software version 5.3b.

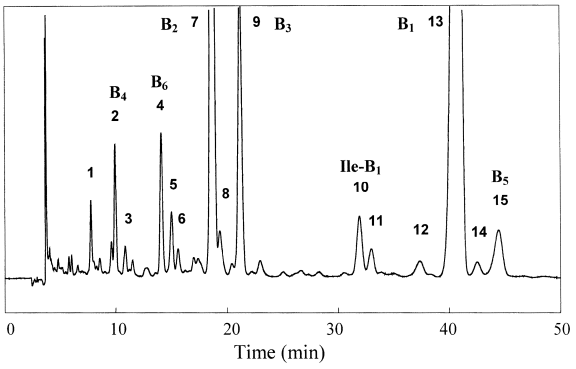


Fig. 1. Analytical profile of a 50 μg polymyxin B sulphate commercial sample. Column: YMC-Pack Pro C_{18}, 5 μm, 250× 4.6 mm I.D. maintained at 30°C. Mobile phase: acetonitrile–sodium sulphate (0.7%, m/v)–phosphoric acid solution (6.8%, v/v)–water (22.25:50:5:22.75) at a flow-rate of 1.0 ml/min. Detection: UV at 215 nm. 2, Polymyxin B_4; 4, polymyxin B_6; 7, polymyxin B_2; 9, polymyxin B_3; 10, isoleucine-polymyxin B_1; 13, polymyxin B_1; 15, polymyxin B_5; other peaks are of unknown identity.

^1H NMR spectra of polymyxin B components revealed the presence of an aromatic group in the molecules. The structures of the examined compounds were further derived from ^{13}C NMR. Using the full DEPT technique, which divides the peaks into sub-spectra enabling clear interpretation, the carbon atoms incorporated in the different groups were assigned. Besides isoleucine polymyxin B_1 (Ile-B_1), all the polymyxin B components examined differed only in the fatty acid moiety at the end terminus. Table 1 lists a summary of the assignment of ^{13}C signals for different C atoms of the fatty acid moiety. The presence of a hydroxyl group on the fatty acid moiety of polymyxin B_6 was deduced from the downfield shifted CH carbon at 71.8 ppm. MS analysis confirmed that B_6 has a higher molecular

Table 1
^{13}C NMR chemical shift assignments for the fatty acid C atoms of polymyxin B components

Carbon	B_1	B_2	B_3	B_4	B_6	Ile-B_1
CO	180.6 (1)	180.6 (1)	180.7 (1)	180.7 (1)	177.7 (1)	180.9 (1)
CH					71.8 (3)	
	36.3 (6)	29.8 (6)			36.3 (6)	36.3 (6)
CH_2	38.1 (5)	40.5 (5)	33.6 (2)	33.4 (2)	45.9 (2)	38.1 (5)
	31.6 (7)	31.2 (2)	31.2 (6)	31.2 (5)	36.4 (4)	31.5 (7)
	31.2 (2)	28.4 (4)	30.9 (5)	30.9 (4)	34.2 (5)	31.2 (2)
	28.5 (4)	28.2 (3)	30.9 (4)	27.9 (3)	31.6 (7)	28.4 (4)
	28.3 (3)		28.0 (3)	24.5 (6)		28.3 (3)
			24.6 (7)			
CH_3	21.2 (6′)	24.6 (6′)	16.1 (8)	16.0 (7)	21.2 (6′)	21.3 (6′)
	13.4 (8)	24.6 (7)			13.4 (8)	13.4 (8)

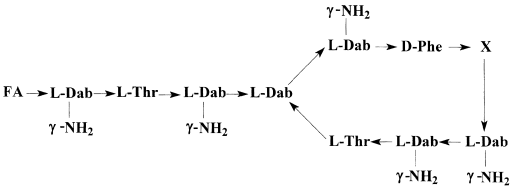
The numbers in parentheses represent the position of the carbons on the fatty acid moiety, always counted from the carbonyl group attached to the amino acid. 6′ denotes the carbon of the methyl group substituted at position 6 on the fatty acid moiety.

mass than B_1, a difference of 16. This accounted for the extra oxygen in the molecule. A 3-hydroxy-6-methyloctanoic acid (3-OH-6-MOA) structure was proposed for the fatty acid of the new polymyxin B_6, on account of the observed upfield shifts for the γ situated carbons C (1) and C (5) and of the downfield shifts for the β carbons C (2) and C (4), all the other carbon signals being almost unshifted.

The compound isolated from peak 15 (Fig. 1) yielded only a very small quantity that was just enough to measure by ^{1}H NMR, which is more sensitive than ^{13}C NMR. The presence of phenylalanine and leucine was deduced from the ^{1}H NMR analysis. MS analysis showed that it has the same mass as polymyxin B_1. A different fatty acid moiety was postulated. A straight chain nonanoic acid is proposed based mainly on the fragmentation patterns. The name polymyxin B_5 is proposed. The structures of the known (B_1, Ile-B_1, B_2, B_3 and B_4) and new (B_5 and B_6) polymyxin B components are presented in Fig. 2. The molecular mass of all examined polymyxin B components was confirmed by MS analysis. The calculated molecular masses and the measured m/z values of the singly and doubly charged peaks are shown in Fig. 2. The structures of polymyxin B components were confirmed by comparison of the fragmentation patterns of the unknown minor components with the fragmentation patterns of known polymyxin B components. The detailed interpretation of the mass spectrometry data will be described elsewhere [12].

4. Conclusion

Preparative-scale separation of polymyxin B was successfully achieved on semi-preparative PLRP-S reversed-phase with isocratic elution. The chromatographic purity was determined by analytical LC. Seven polymyxin B components were isolated and characterized using ^{1}H and ^{13}C NMR. The molecular masses were confirmed by MS. Polymyxins B_1,



Polymyxin	X	FA	Calculated mol. wt.	MS +ve mode	
				singly charged	doubly charged
B_1	L-leucine	6-MOA	1202	1203.6	602.7
Ile-B_1	L-isoleucine	6-MOA	1202	1203.5	602.6
B_2	L-leucine	6-MHA	1188	1189.6	595.5
B_3	L-leucine	OA	1188	1189.7	595.6
B_4	L-leucine	HA	1174	1175.6	588.6
B_5	L-leucine	NA*	1202	1203.7	602.6
B_6	L-leucine	3-OH-6-MOA*	1218	1219.6	610.6

*New

Fig. 2. Structures of known and new polymyxin B with the calculated molecular masses (mol. wt.) and the measured m/z values of the singly and doubly charged peaks. Dab, diaminobutyric acid; FA, fatty acid; 3-OH-6-MOA, 3-hydroxy-6-methyloctanoic acid; 6-MOA, 6-methyloctanoic acid; 6-MHA, 6-methylheptanoic acid; OA, octanoic acid; NA, nonanoic acid; HA, heptanoic acid.

Ile-B_1, B_2, B_3 and B_4 were confirmed to have previously proposed structures. Two of the components (B_5 and B_6) were isolated and characterized for the first time.

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