Bu-2470, A NEW PEPTIDE ANTIBIOTIC COMPLEX

II. STRUCTURE DETERMINATION OF Bu-2470 A, B_1, B_{2a} AND B_{2b}

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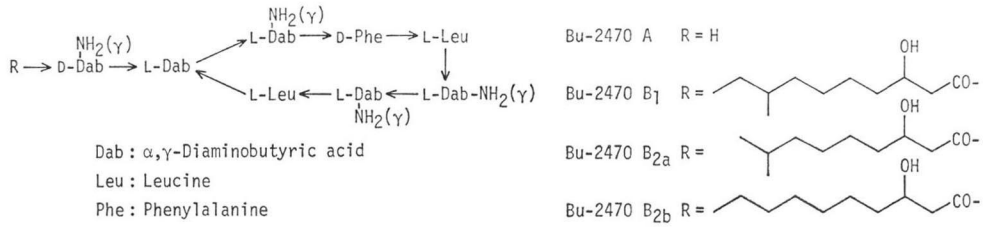
The structures of Bu-2470 A, B_1, B_{2a}, and B_{2b} have been determined. Bu-2470 A is a simple octapeptide having no fatty acid moiety, while Bu-2470 B_1, B_{2a} and B_{2b} are octapeptides that have been acylated with a β-hydroxy C_{11} or C_{10} fatty acid. The octapeptide structure of Bu-2470 components was found identical with that of octapeptin C_1, hence generic names of octapeptin C_0, C_2, C_3 and C_4 are proposed for Bu-2470 A, B_1, B_{2a} and B_{2b}, respectively.

Bu-2470 is a new peptide antibiotic complex produced by a strain of *Bacillus circulans*. The producing organism, fermentation, isolation and properties of Bu-2470 A, B_1 and B_2 have been reported[1]. This paper describes the structure determination of Bu-2470 A, B_1, B_{2a} and B_{2b}.



Constituent Amino Acids of Bu-2470 A

The amino acid analysis of the acid hydrolysate of Bu-2470 A indicated the presence of phenylalanine (Phe), leucine (Leu) and α,γ-diaminobutyric acid (Dab) with a molar ratio of 1 : 2 : 5. The specific rotation values (in 5 N HCl) of the isolated amino acids are $+16°$ for Leu, $+5°$ for Phe and $+11°$ for Dab, indicating that Bu-2470 A consists of two L-Leu, one D-Phe, and one D- and four L-Dab[2]. No fatty acid was detected in the hydrolysate.

Bu-2470 A was treated with 2,4-dinitrofluorobenzene in sodium bicarbonate solution to afford the penta-*N*-2,4-dinitrophenyl (DNP) derivative of Bu-2470 A. Acid hydrolysis of the DNP derivative in 6 N HCl yielded α,γ-bis-DNP-Dab, γ-DNP-Dab, Dab, Leu and Phe. For identification purpose, an authentic sample of bis-DNP-Dab was synthesized by a published method[3], and γ-DNP-Dab was obtained from the acid hydrolysate of DNP-colistin[4]. The release of free Dab and bis-DNP-Dab from the DNP-Bu-2470 A suggested a cyclic peptide structure with a branched chain, one Dab being at the junction of the peptide ring and the other Dab at the *N*-terminus of the branched chain.

Thus, the amino acid composition, including number and chirarity, of Bu-2470 A and the location of two Dab constituents described above are the same as those reported for octapeptin C_1[5].

Fatty Acid Moieties of Bu-2470 B_1 and B_2

Bu-2470 B_1 and B_2 were heated with 6 N HCl for 16 hours. The amino acid analysis of the hydrolysate showed that the amino acid constituents of Bu-2470 B_1 and B_2 are same as those of Bu-2470 A. In addition, the presence of a lipophilic acidic substance was indicated in the hydrolysate of Bu-2470 B_1 and B_2.

Thus, Bu-2470 B_1 was briefly hydrolyzed with 6 N HCl for 30 minutes and the hydrolyzate was extracted with diethyl ether. Evaporation of the etherial extract afforded a fatty acid which was identified as 3-hydroxy-8-methyldecanoic acid by the GC-MS analysis of its methyl ester. In a similar manner, the hydrolysate of Bu-2470 B_2 yielded two fatty acids, which were identified as 3-hydroxy-8-methylnonanoic acid and 3-hydroxydecanoic acid. Authentic samples of these fatty acids were obtained from the acid hydrolysate of Bu-1880[6] and antibiotic EM-49 complex[7]. Bu-2470 B_2 was therefore shown to be comprised of two subcomponents having different fatty acid moieties. The two subcomponents were designated as Bu-2470 B_{2a} (which contains 3-hydroxy-8-methylnonanoic acid) and Bu-2470 B_{2b} (which contains 3-hydroxydecanoic acid).

Enzymatic Deacylation of Bu-2470 B and Octapeptins

KIMURA and HIRAKI reported that cells of *Pseudomonas* sp. strain M-6-3 possessed an enzymatic activity that deacylates polymyxins and the enzyme was named polymyxin acylase[5]. A strain of *P. aeruginosa* K-102 (our culture collection number: Pa-74) was found to produce the same type of enzyme capable of deacylating colistin and polymyxin. Although it has been reported to the contrary[2,5], the polymyxin acylase was found to be capable of deacylating the octapeptin group of antibiotics.

Bu-2470 B was suspended in pH 7 phosphate buffer and mixed with a preparation of polymyxin acylase. The suspension was incubated at 37°C with shaking for 3 days. The enzymatic reaction mixture was filtered and extracted with 1-butanol at pH 2 to remove unchanged Bu-2470 B. The aqueous layer was then made alkaline (pH 9.5) and extracted again with 1-butanol. The second butanol extract was evaporated and the residue was purified by chromatography (Diaion HP-20) to afford a bioactive fragment which was identified as Bu-2470 A. Enzymatic deacylation of Bu-2470 B_1 and B_2 gave the same product, Bu-2470 A. Thus, Bu-2470 A, B_1 and B_2 have been shown to have the same octapeptide structure. Component A was non-acylated while Bu-2470 B_1 and B_2 were acylated with different fatty acid moieties.

EM-49 complex[9] and octapeptin C_1 (antibiotic 333-25)[10] were also treated with the polymyxin acylase at 37°C for 5 days affording their deacylation products. Table 1 shows HPLC data for the deacyl derivatives of Bu-2470 B, octapeptin C_1 and octapeptins A and B. These results indicated that deacyl Bu-2470 B and deacyloctapeptin C_1 were identical with Bu-2470 A but apparently different from deacyloctapeptin A and deacyloctapeptin B. The identity of deacyl Bu-2470 B and deacyloctapeptin C_1 with Bu-2470 A was further verified by their IR and NMR spectra.

Table 1. HPLC of Bu-2470 A and deacyl derivatives of Bu-2470 B, octapeptin C_1 and octapeptins A and B.

	HPLC (Rt in minutes)
Bu-2470 A	25.9
Deacyl Bu-2470 B	25.9
Deacyloctapeptin C_1	25.9
Deacyloctapeptin A	14.9
Deacyloctapeptin B	19.7

Column: Finepak SIL C_{18}.
Mobile phase: CH_3CN - 0.005 M tartrate buffer (pH 3.0), 19: 81 containing 0.05 M Na_2SO_4 and 0.005 M 1-butanesulfonic acid.

Thus, the peptide structure for each of the Bu-2470 components is identical with that of octapeptin C_1 and the structures which follow are

assigned for Bu-2470 A, B_1, B_{2a} and B_{2b}.

Discussion

The class name of "octapeptin" has been proposed[11] for a group of octapeptide antibiotics acylated with a fatty acid residue. EM-49 α and β are now designated as octapeptins $A_1 \sim A_3$, which contain no phenylalanine (Phe) in the peptide structure. EM-49 γ and δ, having one L-Phe, are renamed octapeptins $B_1 \sim B_3$. Octapeptin C_1 is a synonym for antibiotic 333-25, which contain D-Phe as a structural element. The peptide structure of Bu-2470 components is the same as that of octapeptin C_1. Since Bu-2470 B_1, B_{2a} and B_{2b} differ from octapeptin C_1 only in the fatty acid moiety, they are designated as octapeptin C_2, C_3 and C_4, respectively. Bu-2470 A has a non-acylated octapeptide structure, which is the first such occurrence in the octapeptin group of antibiotics. It is proposed to extend the original nomenclature system to include non-acylated octapeptide antibiotics by placing subscript number "0" after the letter. Thus, Bu-2470 A can be called octapeptin C_0.

Experimental

Hydrolysis of Bu-2470 A

A solution of Bu-2470 A (300 mg) in 15 ml of 6 N HCl was heated at 105°C for 15 hours in a sealed tube. The reaction mixture was concentrated *in vacuo* to dryness. The residue was dissolved in 1 ml of water and chromatographed on a column of Dowex 50WX4 (12×250 mm). The column was developed with increasing concentration of hydrochloric acid (0.1 N~1.0 N), the elution being monitored by ninhydrin test and TLC (system A-107*). The appropriate fractions were collected and concentrated *in vacuo* to yield the following 3 amino acids:

	Leu	Phe	Dab		Leu	Phe	Dab
Eluted with	0.3 N HCl	0.5 N HCl	1 N HCl	TLC (silica gel plate)			
				System A-107*	0.47	0.59	0.02
Yield	78 mg	45 mg	237 mg	System A-108*	0.35	0.37	0.07
				$[\alpha]_D^{28.5}$ (*c* 1.0, 5 N HCl)	+15°	+5°	+11°
				Chirality assigned	L	D	1 D+4 L

* TLC system; A-107: Phenol - H_2O (4: 1), A-108: 1-BuOH - AcOH - H_2O (12: 3: 5).

Preparation and Acid Hydrolysis of Penta-(2,4-dinitrophenyl)-Bu-2470 A

A solution of dinitrofluorobenzene (236 mg) in 2 ml of ethanol was added to an aqueous solution (4 ml) of Bu-2470 A (100 mg) and $NaHCO_3$ (134 mg), and the mixture was stirred for 1.5 hours at 20°C in a dark room. Yellow solid precipitated was collected by filtration, washed with water and benzene, and then dried *in vacuo*. The crude product was purified by silica gel chromatography. The column (10×200 mm) was eluted with $CHCl_3$ - MeOH (93: 7) and the elution was monitored by the characteristic yellow color and TLC. The appropriate fractions were collected and evaporated *in vacuo* to give 111 mg of the DNP derivative of Bu-2470 A. λ_{max} in 1.0% dioxane - MeOH: 247 nm ($E_{1cm}^{1\%}$ 472). TLC (SD-105*): Rf 0.43.

A sample of DNP-Bu-2470 A (5 mg) was heated at 105°C with 0.5 ml of 6 N HCl in a sealed tube for 15 hours. The reaction mixture was concentrated *in vacuo* and lyophilized. The yellow solid thus obtained was analyzed by TLC and the following five compounds were identified:

	TLC (Rf) System PL-111*	Detection		TLC (Rf) System PL-111*	Detection
α,γ-bis-DNP-Dab	0.63	Yellow color	Leu	0.38	Ninhydrin
γ-DNP-Dab	0.45	〃	Dab	0.06	〃
Phe	0.41	Ninhydrin			

* TLC system; SD-105: $CHCl_3$ - MeOH (9: 1), PL-111: $CHCl_3$ - C_2H_5OH - 14% NH_4OH (4: 7: 2).

Isolation of Fatty Acids from Bu-2470 B_1 and B_2, Bu-1880 and EM-49

A solution of Bu-2470 B_1 (10 mg) in 0.5 ml of 6 N HCl was heated at 105°C for 0.5 hour in a sealed tube. The hydrolysate was added with 5 ml of water and extracted with two 5-ml portions of diethyl ether. The ethereal solution was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give an oily residue (2 mg). The oil was taken into 3 ml of ether and treated with an excess of diazomethane in ethereal solution. Evaporation of the solvent gave the methyl ester (2 mg) which was analyzed by gas chromatography (GC; Shimadzu GC-4BPT) and GC-mass spectrometry (GC-MS; Shimadzu LKB-9000). In the same manner, Bu-2470 B_2 (10 mg), Bu-1880 (10 mg) and EM-49 (10 mg) were hydrolyzed and the fatty acid moieties converted to methyl esters. The GC and GC-MS analysis of these methyl esters are shown below:

Fatty acid methyl esters isolated from	GC* (Rt in minutes)	GC-MS** (*m/z*)			Identification
		M^+	Base peak	Diagnostic peaks	
Bu-2470 B_1	5.77	216	103	199, 167, 57	Methyl 3-hydroxy-8-methyldecanoate
Bu-2470 B_2	4.28	202	103	185, 137, 109, 107	Methyl 3-hydroxy-8-methylnonanoate
	4.80	202	103	185, 127, 110	Methyl 3-hydroxy-decanoate
Bu-1880[6]	5.79	216	103	199, 167, 57	Methyl 3-hydroxy-8-methyldecanoate
EM-49 complex[7]	4.27	202	103	185, 137, 109, 107	Methyl 3-hydroxy-8-methylnonanoate
	4.79	202	103	185, 127, 110	Methyl 3-hydroxy-decanoate
	5.77	216	103	199, 167, 57	Methyl 3-hydroxy-8-methyldecanoate

* OV-17 3%, He, temperature programming 5°C/minute from 140°C.

** OV-17 1.5%, He, temperature programming 5°C/minute from 140°C.

Isolation of Deacyl Bu-2470 B, Deacyl EM-49 and Deacyloctapeptin C_1

A suspension of Bu-2470 B (100 mg) in 30 ml of 1/15 M SÖRENSEN's phosphate buffer (pH 7.0) was combined with polymyxin acylase (120 mg) and the mixture incubated at 37°C with shaking for 3 days. The enzymatic reaction mixture was centrifuged, and the clear supernatant was adjusted to pH 2.0 and extracted with two 20-ml portions of 1-butanol. Evaporation of the extract yielded 26 mg of unchanged Bu-2470 B. The aqueous layer was adjusted to pH 9.5 with 2 N NH_4OH and extracted again with two 20-ml aliquots of 1-butanol. Second butanol extracts were combined and concentrated *in vacuo* to afford the crude solid of deacyl Bu-2470 B (55 mg). The solid was dissolved in 2 ml of water, adjusted to pH 2.0 with 1 N HCl and chromatographed on a column of Diaion HP-20 (10×160 mm). The column was developed with water and the fractions were monitored by ninhydrin assay and TLC (system S-118*). The yield of deacyl Bu-2470 B was 32 mg.

By essentially the same procedure as described in the above experiment, EM-49 complex (10 mg) was treated with polymyxin acylase (12 mg) affording 5.0 mg of deacyl EM-49 along with 1.5 mg of EM-49 recovered. Similarly, deacylation of octapeptin C_1 (10 mg) with polymyxin acylase (12 mg) yielded 4.9 mg of deacyloctapeptin C_1 and 2.5 mg of unchanged octapeptin C_1.

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* TLC system S-118: $CHCl_3$ - MeOH - 28% NH_4OH (1 : 2 : 1).

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