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SELECTIVE CHEMICAL MODIFICATIONS OF POLYMYXIN B

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Abstract: Polymyxin B (**1**) monohydrochloride was converted to the tetra-BOC derivatives **1b** and **1c** by reaction with di-*tert*-butyl dicarbonate. The structures of these protected intermediates were established utilizing a degradative sequence that afforded **3** and **5**. A method for the deprotection 2,4-dinitrophenylamines to the free amine, utilizing a strongly basic ion-exchange resin, was developed for use in the degradative sequence. The tetra-BOC derivatives **1b** and **1c** were used to prepare several Polymyxin B derivatives **6–27** at the DAB_1 and DAB_9-γ-amine. The antibacterial activity of these selectively functionalized derivatives is reported here.

Introduction

Polymyxin B (**1**) is a clinically important member of the polymyxin group of antibiotics that are cyclic peptides highly potent against gram-negative bacteria.[1] Nephrotoxicity and neuromuscular blockade are serious side effects associated with this class of antibiotics and hence their clinical usage is primarily as topical agents.[2] Polymyxin B (**1**)[3] contains five free γ-amino groups from the α,γ-diaminobutyric acid (DAB) units and these functionalities are attractive handles that can be used for chemical modifications/derivatizations aimed at improving the therapeutic ratio of the parent compound. Several such modifications of **1** reported previously, have shown that monoacylation of **1** retained the antibacterial potency whereas di- and polyacylation caused an appreciable loss in potency.[1] However, the chemistry used in all of those studies was uncontrolled and did not establish which of the amino groups of **1** were derivatized. We report here, methodology that enabled us to prepare selectively several γ-amine derivatives of **1** at DAB_1 and DAB_9, and the antibacterial activity of these derivatives.

Chemistry

Our approach for the monofunctionalization of **1**, was based on the protection of the most reactive/basic amino group by protonation with strong acids while introducing a protective group on the remaining four amino groups in the molecule. Preliminary experiments had shown that the penta-acetate salt of **1** reacted with di-*tert*-butyl dicarbonate to form the penta-BOC derivative **1a** thereby demonstrating that protonation with weak acids did not protect the amino groups from reacting with the reagent; the hydrochloride salt of **1** on the other hand was stable to this reagent. These observations were applied towards the preparation of tetra-BOC protected polymyxin B (Scheme 1). Thus, polymyxin B monohydrochloride,[4] prepared as a homogeneous solution by dissolving the free base **1** in methanol containing AcOH and HCl (1 equiv) was reacted with di-*tert*-butyl dicarbonate to afford the penta-BOC derivative **1a** (23%), and the tetra-BOC derivatives **1b** (36%) and **1c** (15%).

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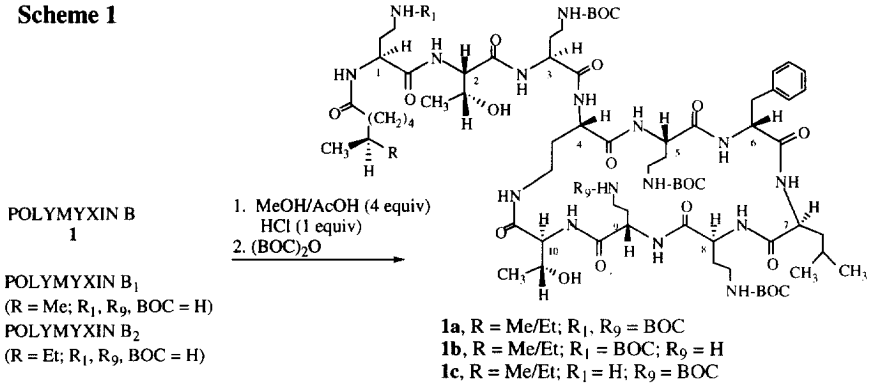
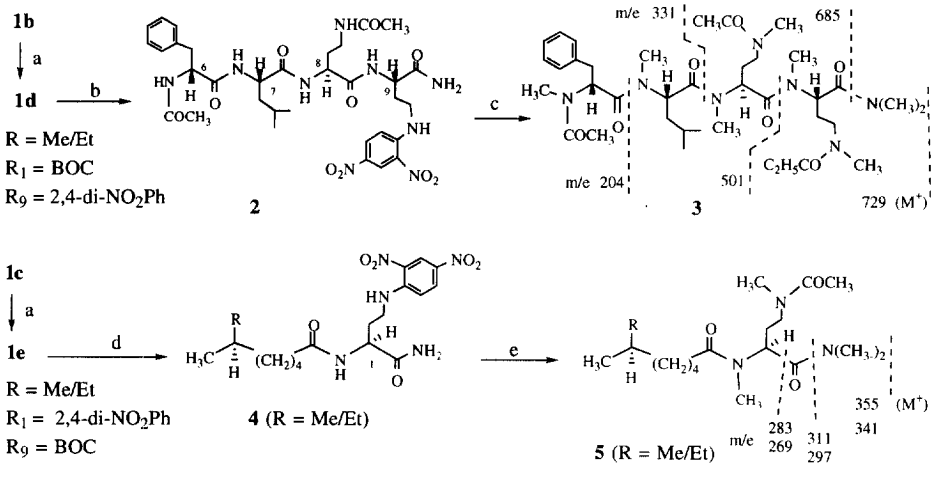
Scheme 1



The electrospray ionization mass spectra of both **1b** and **1c** showed the expected molecular ion signals at *m/e* 1604, 1590 in agreement with four BOC functionalities and R = Me and Et. Scheme 2 shows the degradative sequence that was developed to establish the structures of these two tetra-BOC derivatives **1b** and **1c**. Tetra-BOC

Scheme 2[a]

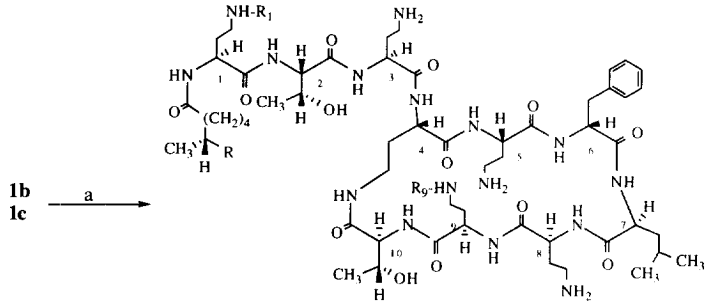


[a]Reagents: (a) i. 2,4-dinitrofluorobenzene/CH_2Cl_2 /Et_3N. (b) i. 1 N HCl-AcOH/0.5 h ii. 1 N HCl /45 °C/3 days iii. Ac_2O/Et_3N iv. CH_2N_2 /Et_2O v. NH_3 /MeOH. (c) i. Amberlite ion-exchange resin IRA-401S (OH^-)/30% H_2O-Me_2CO ii. propionic anhydride/Et_3N iii. DMSO/NaH/CH_3I. (d) i. 1 N HCl-AcOH/0.5 h ii. 1 N HCl/45 °C/3 days iii. CH_2N_2/Et_2O iv. NH_3/MeOH. (e) i. Amberlite ion-exchange resin IRA-401S (OH^-)/30% H_2O-Me_2CO/3h ii. Ac_2O/Et_3N iii. DMSO/NaH/CH_3I.

1b was reacted with 2,4-dinitrofluorobenzene to afford the 2,4-dinitrophenyl (DNP) derivative **1d** (γ-DNP-DAB_9), which was subjected to dil. HCl hydrolysis under mild conditions. The major yellow spot was isolated by chromatography of the products from this hydrolysis followed by acetylation, reaction with diazomethane and ammonolysis to afford the amide **2**. The DNP group in **1d** served as a convenient visual tag to locate the fragment

precursor of **2** formed in the hydrolytic step. However, for MS sequencing purposes, the DNP group needed to be replaced by an alternative tag. We developed a simple method[5] for the cleavage of DNP-amines and applied it to **2** and **4**. The method involved stirring a solution of **2** in aqueous acetone, with a strongly basic ion-exchange resin (Amberlite IRA-401S, OH^- form); the deprotection was complete in a few hours as evidenced by the resin beads turning yellow from sequestering the 2,4-dinitrophenol. The resulting colorless solution of the free amine was acylated with propionic anhydride (used to tag the amino group derived from the DNP deprotection), and the product was then permethylated utilizing methylsulfinyl carbanion/MeI in DMSO[6] to afford **3**. The EI mass spectrum fragmentation pattern of the permethylated product **3** (Scheme 2) was in agreement with the aminoacid sequence for the tetrapeptide and more significantly the fragment ions m/e 685/501 established the location of the DNP in **1d** and hence the free amino group in **1b** was the γ-amino-DAB_9. A similar degradative sequence was utilized for **1c**. Thus, its DNP derivative **1e** was subjected to HCl hydrolysis as described above. The only hydrolysis fragment containing the DNP group (yellow color, insoluble, ninhydrin negative) was converted to the methylester with diazomethane, followed by ammonolysis to afford the amide **4**. The DNP group was cleaved with ion-exchange resin (Amberlite IRA-401S, OH^- form), followed by acetylation, and permethylation[6] to afford **5**. The EI mass spectrum fragmentation pattern of **5** established the location of the DNP group in **1e** and hence the free amino group in **1c** was the γ-amino-DAB_1.

Scheme 3[a]



1b
1c —a→

6-11, R_1= Derivatives at DAB_1, R_9 = H
12-27, R_1=H, R_9 = Derivatives at DAB_9

[a]Reagents: (a) i. RCOO-active ester or N-Protected α-aminoacid *p*-nitrophenyl ester/DMF, or reductive amination with CH_3CHO, HCHO, or OHC-COO-*tert*.Bu/$NaCNBH_3$ ii. 1 N HCl/AcOH/rt.

Compounds **1b** and **1c** were utilized to prepare DAB_9 or DAB_1 N-alkylated and N-acylated derivatives (Scheme 3). Twenty two such derivatives were prepared to study the effect of functionalization of these two amino groups on the microbiological profile. Thus, reductive alkylation (appropriate carbonyl reactant/sodium cyano borohydride) of these intermediates followed by acid catalyzed debocylation provided the mono- and di- N-alkylated derivatives **6, 7, 12–15**. The N-acyl derivatives **8–11** and **16–27** were prepared by acylation of **1b** and **1c** with the 2,4,5-trichlorophenyl ester of the appropriate acid or protected α-aminoacid followed by acid catalyzed debocylation.[7]

Results

The derivatives **6–27** were screened for their in vitro antibacterial activity. Minimum inhibitory concentration (MIC) values were determined by the macrobroth dilution method[8,9] against gram-negative and gram-positive groups of *Pseudomonas*, *E. Coli*, and *Staphylococcus*. organism strains. The results are summarized in Table 1 as geometric mean MIC values. The data in Table 1 provides the following information. Mono- and di-alkyl derivatives of the DAB_1 amino group are less active than the parent compound **1**, but are three to four-fold more potent than the corresponding DAB_9 derivatives (compounds **6**, **7** vs. **12–15**). Acyl derivatives

Table 1. In vitro Antibacterial activity of Polymyxin derivatives.[8,9]

Entry #	DAB #	Derivative	Geometric Mean MIC's (μg/mL)*		
			Pseudomonas (n = 8)	*E. Coli* (n = 8)	*Staphylococcus* (n = 6)
1	----	------	0.051	0.026	2.80
6	1	Ethyl-	0.146	0.112	2.30
7	1	Diethyl-	0.206	0.208	2.40
8	1	Acetyl-	2.80	1.30	2.80
9	1	Glycyl-	0.033	0.028	8.0
10	1	γ-CBZ-L-Lysyl-	0.175	0.206	1.122
11	1	L-Arginyl-	0.207	0.453	4.48
12	9	Ethyl-	0.413	0.492	9.50
13	9	Diethyl-	0.763	0.995	9.50
14	9	Dimethyl-	0.456	0.537	16.0
15	9	bis(2-Ethanoic acid)	0.318	1.19	22.6
16	9	Chloroacetyl-	0.085	0.454	9.50
17	9	Dichloroacetyl-	0.995	1.54	0.82
18	9	Hydroxyacetyl-	1.53	1.54	22.62
19	9	6-Me-octanoyl-	2.18	3.10	0.70
20	9	L-α-NH_2- octanoyl-	0.051	0.225	0.561
21	9	γ-CBZ-L-Lysyl-	0.087	0.146	0.445
22	9	L-Lysyl-	0.032	0.173	2.80
23	9	L-Tyrosyl-	0.073	0.175	2.20
24	9	L-Arginyl-	0.030	0.160	2.0
25	9	Nitro-L-Arginyl-	2.60	1.30	1.20
26	9	L-Methionyl -	0.416	0.77	5.65
27	9	L-Cysteinyl -	2.82	1.68	11.31

* n denotes the number of microorganism strains used in MIC determinations.

of the γ-amino functionality of DAB_1 or DAB_9 which are neutral, have a reduced gram-negative and gram-positive activity as seen in compounds **8** and **16–18**; exceptions were the dichloroacetyl and 6-methyloctanoyl derivatives **17** and **19** which show a four-fold improvement in gram-positive potency. The α-amino acyl compounds **9** and **20–24** have good *Pseudomonas* potency but have lost *E. Coli* potency in contrast to the DAB_1-Gly compound **9**; the more lipophilic α-amino acyl derivatives **20** and **21** display a broader antibacterial spectrum with improved *Staph.* activity. Compounds **9**, **20**, **21**, **24**, and **27** were selected for an in vivo study[8,10] in mice in order to determine their acute iv LD_{50} and PD_{50}. The results of this study, summarized in Table 2, show that derivatization of the DAB_1 and DAB_9 amino groups leads to a slight improvement in the acute toxicity profile (LD_{50}) of the

compounds relative to **1**. In the protection study against *Pseudomonas* infection the DAB_9-L-ARG derivative **24** showed a ten-fold improvement in the PD_{50} while the other three derivatives were equal to or less effective than **1**. More notably, the ratio of the LD_{50}/PD_{50} for compound **24** shows a fifteen-fold improvement relative to **1**.

Table 2. In vivo data for selected derivatives.[8, 10]

Entry #	Derivative	LD_{50} *(iv; mpk)*	PD_{50} *(mpk)*	Ratio LD_{50} / PD_{50}
1	———	9	2	4.5
9	DAB_1-GLY	20	7	2.8
20	DAB_9- L-αNH_2-OCT	12	7	1.7
21	DAB_9- γ-CBZ-L-LYS	13	2	6.5
23	DAB_9- L-TYR	19	2.2	8.6
24	DAB_9-L-ARG	15	0.25	60.0

In summary, we report here methodology to selectively functionalize the DAB_1 or the DAB_9 amino group of Polymyxin B. The structures of the precursor tetra-BOC-polymyxin B derivatives **1b** and **1c** were established using a chemical degradation sequence which utilizes a new DNP deprotection method. In contrast with previous studies[1] on the chemical modifications of Polymyxin B, our study identifies the site of the functionalization of the parent antibiotic. Of the twenty two polymyxin B derivatives reported here, compound **24** was found to have an improved toxicity-activity profile in vivo.

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References and Notes:

1. *For reviews see:* (a) Storm, R. D.; Rosenthal, K. S.; Swanson, P. E. *Ann. Rev. Biochem.* **1977**, *46*, 723. (b) Gale, E. F.; Cundliffe, E.; Reynolds, P. E.; Richmond, M. H.; Waring, M. J. *The Molecular Basis of Antibiotic Action*, Second edition: John Wiley & Sons: New York, 1981; pp 184–195.
2. *Physicians' Drug Reference*, 52nd Edition, Medical Economics Co.,Inc., Montvale NJ, 1998, p 1067. (b) *Merck Index*, 12th Edition, Merck & Co. Inc., Rahway, NJ, 1996, p 7734.
3. Commercially available Polymyxin B which is a mixture of Polymyxins B_1/B_2 (**1**) was used in the study reported here.
4. A suspension of polymyxin B free base (9.56 g) in methanol (110 mL) was stirred while adding glacial acetic acid (1.9 g, 4 equiv) and 1 N HCl (7.94 mL, 1 equiv). Boc-dicarbonate (11.4 g, 6.6 equiv) was then added to the clear solution followed by stirring for 16 h at rt. The crude product was chromatographed on silica gel (500 mL) eluting with 7%MeOH/CH_2Cl_2 to afford (in order of elution): **1a** (3.2 g, 23%), an uncharacterized tetra-BOC product (1.1 g, 8.6%), **1b** (4.54 g, 36 %) and **1c** (1.9 g, 15 %).
5. Examples of DNP deprotection: (a) A solution of **2** (20 mg) in acetone (5 mL) and water (2.5 mL) was stirred while adding Amberlite IRA-401S (OH^-) resin in portions. The deprotection was complete when the supernatant turned colorless (2 h). The resin beads, which had turned dark orange color, were filtered out and

the clear filtrate was evaporated to afford the free amine (ninhydrin positive). (b) A solution of **4b** (75 mg) in acetone (10 mL) and water (5 mL) was stirred for 3 h with Amberlite IRA-401S (OH^-) resin (4 g) as in the previous example to afford the free amine as a colorless solid.

6. Thomas, D. W.; Das, B. C.; Gero, S. D.; Lederer, E. *Bioch. Biophys. Res. Commun.* **1968**, *32*, 519 (b) Vilkas, E.; Lederer, E. *Tetrahedron Letters*, **1968**, *26*, 3089.
7. All new compounds gave satisfactory combustion values. Data on selected compounds: **1a**: white powder, mp 238–246 °C; *Rf* =0.84 (silica gel TLC, 10%MeOH/CH_2Cl_2); $[\alpha]_D$ -61° (*c* 4.2, MeOH); ^1H NMR (DMSO-d_6, 80 MHz) δ 0.84 (m, 12H), 1.08 (bs, 6H), 1.40 (s, 45H), 7.24 (s, 5H). **1b**: white powder, mp >260 °C; *Rf* = 0.47 (silica gel TLC, 20%MeOH/CH_2Cl_2); $[\alpha]_D$ -54° (*c* 4.1, MeOH); ^1H NMR ($CDCl_3$/CD_3OD, 400 MHz) δ 0.75 (d, 3H, *J* = 6 Hz), 0.78 (d, 3H, *J* = 6 Hz), 0.84 (t, 3H, *J* = 6 Hz), 0.88 (d, 3H, *J* = 6 Hz), 1.17 (d, 3H, *J* = 6 Hz), 1.26 (d, 3H, *J* = 6 Hz), 1.45 (s, 36H), 7.25 (m, 5H); MS(ESI) *m/e* 1604 (MH^+ for R = Et), 1590 (MH^+ for R = Me); HRMS(ESI) calcd for $C_{76}H_{130}N_{16}O_{21}Na$ 1625.9494, found 1625.9510 9 (for R = Et), calcd for $C_{75}H_{128}N_{16}O_{21}Na$ 1611.9338, found 1611.9367. **1c**: white powder; *Rf* = 0.40 (silica gel TLC, 20%MeOH/CH_2Cl_2); ^1H NMR ($CDCl_3$/CD_3OD, 400 MHz) δ 0.76, 1.19 (d, 3H, *J* = 6 Hz), 0.78 (d, 3H, *J* = 6 Hz), 0.86 (t, 3H, *J* = 6 Hz), 0.88 (d, 3H, *J* = 6 Hz), 1.20 (d, 3H, *J* = 6 Hz), 1.25 (d, 3H, *J* = 6 Hz), 1.44 (s, 36H), 7.23 (m, 5H)); MS(ESI) *m/e* 1604 (MH^+ for R = Et), 1590 (MH^+ for R = Me); HRMS(ESI) calcd for $C_{76}H_{130}N_{16}O_{21}Na$ 1625.9494, found 1625.9494 (for R = Et), calcd for $C_{75}H_{128}N_{16}O_{21}Na$ 1611.9338, found 1611.9352. **9**: $[\alpha]_D$ -150° (c, 1.6, H_2O). **14**: $[\alpha]_D$ -82° (c, 2.1, H_2O); ^1H NMR (D_2O, 80 MHz) δ 0.80 (m, 12H), 1.20 (bd, 3H), 1.28 (bd, 3H), 2.92 (s, 6H). **23**: $[\alpha]_D$ -53° (c, 2.8, MeOH). **24**: $[\alpha]_D$ -54° (c, 5.1, H_2O).
8. Waitz, A. J.; Miller, G. H.; Moss, Jr., E.; Chiu, P. J. S. *Antimicrob. Agents Chemotherapy*, **1978**, *13*, 41.
9. Antibacterial activity against several strains of gram-negative and gram-positive bacteria was determined by tube dilution in the conventional manner using 3 to 5 mL of Mueller-Hinton broth (pH 7.4). MIC values denote the concentration that caused 100% inhibition of growth of the microorganism in 24 hrs. The geometric mean of these values for each group of organisms is reported in Table 1.
10. The acute toxity determinations were carried out in male CF_1 white mice weighing 18 to 20 g each. The test compound was administered iv at dosages of 6, 9, 12, 15, or 18 mpk to seven mice in each dose group. The dose required to kill 50% of the animals was estimated by probit analysis on the basis of the number of survivors at 96 h after injection and is reported in Table 2 as the LD_{50}. Mouse protection tests were also performed in male CF_1 white mice weighing 18 to 20 g each. Sufficient inocula of *Pseudomonas aeruginosa* Stone 39 was used to cause death in control groups within 18 h of infection. One hour after intraperitoneal infection, groups of mice were given a single sc dose of the test compound at varying doses less than the LD_{50}. Mean protective doses were estimated by probit analysis based on the number of survivors at the various dosages tested at 48 h after infection and are reported in Table 2 as PD_{50} in mpk.