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A CONVENIENT METHOD FOR THE CLEAVAGE OF THE D-MANNOSYL-L-GULOSE DISACCHARIDE FROM BLEOMYCIN-A_2

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ABSTRACT

In order to elucidate the biological role of the sugar residue of the antitumor drug bleomycin, this was deglycosylated by β-elimination under mild alkaline conditions, and by solvolysis with hydrogen fluoride. The latter procedure proved to be better because it led to the complete deglycosylation without modification of the peptide, thus allowing further biological investigations of this component.

INTRODUCTION

Deglycobleomycin (**1**), the aglycon of the glycopeptide antitumor drug bleomycin (**2**), was first isolated in small amounts from culture filtrates during biosynthetic studies[1]. This compound is a useful tool for determining the nature of bleomycin–Fe(II) complex, which remains a controversial problem[2–6], and for the elucidation of the role of the sugar component in cellular processes. The preparation of this compound was subsequently reported, either by chemical hydrolysis[7] or by total synthesis[8,9]. The latter method is long and tedious, and was undertaken to allow the total synthesis of **2** or structurally simpler congeners.

The liberation of the sugar part may be obtained by β-elimination under mild alkaline conditions, but this treatment resulted in the formation of a dehydrohistidine residue[7]. Under strong acidic conditions, the peptide bonds are easily hydrolyzed, and oxidative degradation of the sugar part by classical agents, such as palladium or lead tetraacetate, also led to a concomitant hydrolysis of the peptide bonds. The previously described method[7] to obtain pure **1** used mild acid hydrolysis. However, this technique resulted in a poor yield of the desired aglycon owing to the presence of several peptide fragments and unreacted starting material in the hydrolyzate, which required a delicate separation by column chromatography. We report herein a preparation of **1** by the use of hydrogen fluoride which gave a high yield and high degree of purity as compared to the product of alkali treatment. The structure of the aglycon was confirmed by analysis of the amino acid residues and by n.m.r. spectroscopy

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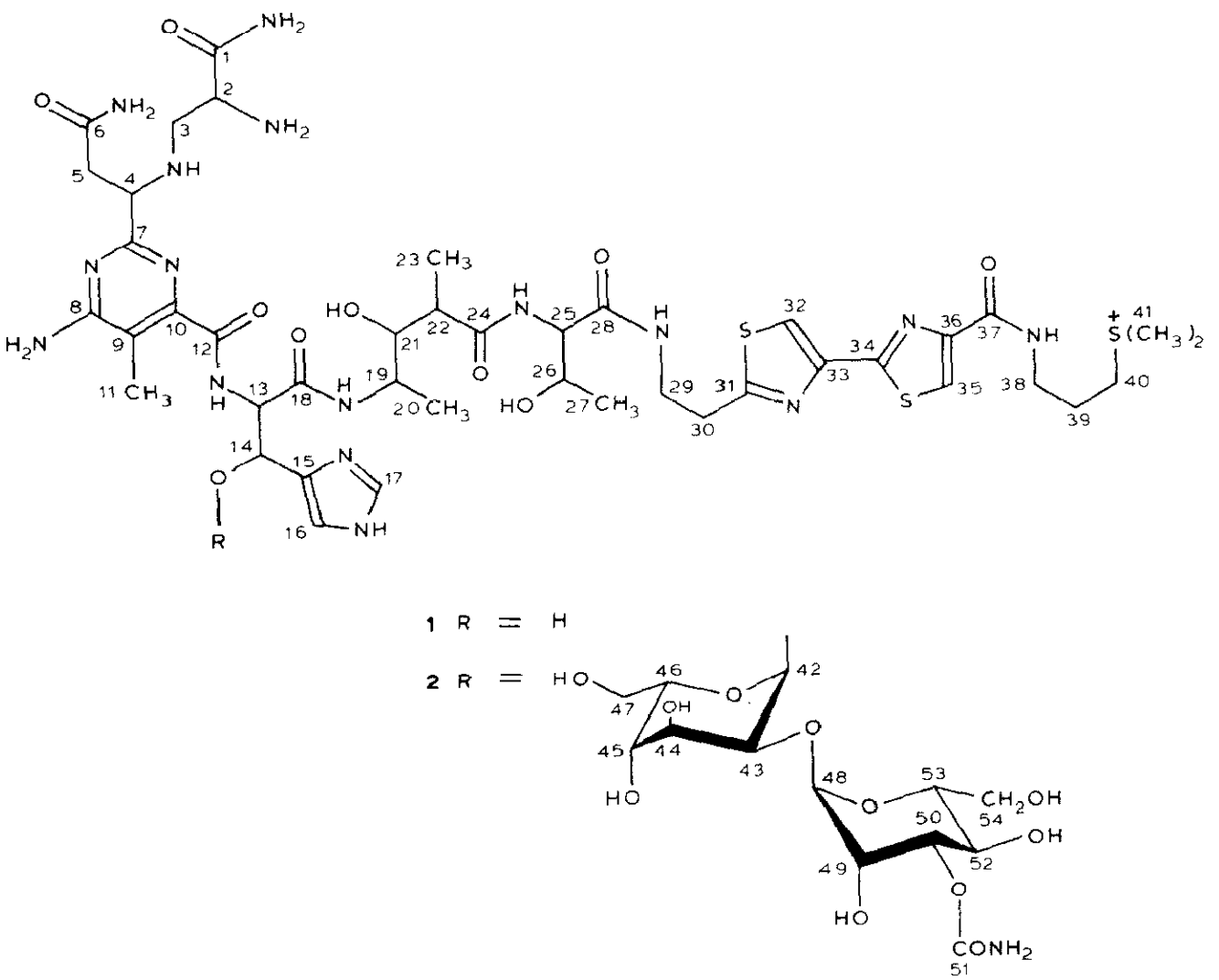

1 R = H

2 R =

RESULTS AND DISCUSSION

Exposure of **2** to anhydrous hydrogen fluoride cleaved the glycosidic linkage within 1 h at 0°, while leaving peptide bounds unaffected. The diagram (Fig. 1) of l.c. fractionation of the formed **1** shows the high yield of hydrogen fluoride cleavage (86%).

The 400-MHz ^{1}H-n.m.r. spectrum analysis of **1** clearly showed the disaccharide cleavage, by comparison with the spectrum of **2**. The signal at δ 5.25, assumed to correspond to C-1 of the L-gulosyl residue is missing, as well as the signal at δ 5.02, assigned to C-1 of 3-*O*-carbamoyl-D-mannosyl residue. The signal between δ 4.0 and 4.7, due to overlapping of the sugar and peptide signals, was better resolved in the absence of the disaccharide residue (Fig. 2). ^{13}C-N.m.r. spectroscopy confirmed the structural changes after hydrogen fluoride treatment. The well-resolved two signals at δ 98.1 and 98.7, assigned to C-1 of the gulosyl and mannosyl residues, respectively, are missing from the ^{13}C-n.m.r. spectrum of **1**, as well as the carbamoyl carbon signal at δ 158.5. Moreover, signals between δ 60.9 and 75.0, attributable to the other carbon atoms of both sugars, are missing (Fig. 3; Table I).

Sugar analysis of **2** indicated the expected mannose and gulose in a 1:1 ratio, and no sugar for **1**.

Amino acid analysis after hydrogen fluoride cleavage gave the same nin-

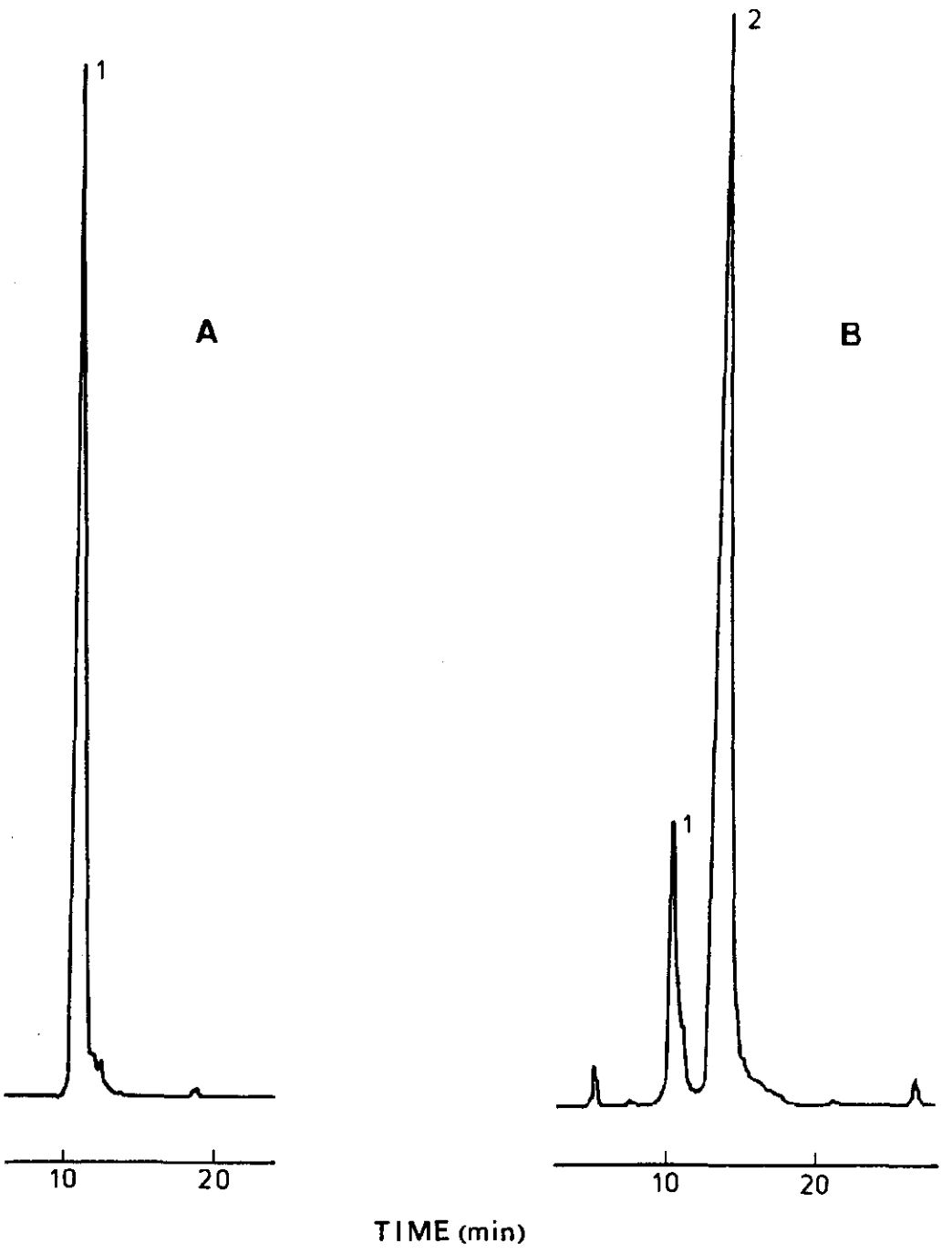

Fig. 1. L.c. chromatograms of **2** before (A), and after hydrogen fluoride treatment (B). Peak 2 corresponds to **1**.

hydrin-positive products for **1** and **2**, *i.e.*, L-threonine, 2-amino-2-[6-amino-(4-carboxy-5-methyl-pyrimidin-2-yl)]propionic acid, 4-amino-3-hydroxy-2-methyl-pentanoic acid, L-*erythro*-β-hydroxyhistidine, and β-aminoalanine (Fig. 4). Under the present conditions, other amino acids related to the bithiazole part were not detected.

The main finding of this study is that hydrogen fluoride cleavage keeps β-hydroxyhistidine intact, whereas alkali degradation induces a β-elimination and formation of dehydrohistidine. Moreover, hydrolysis of the peptide part with formation of dehydroamino acid may occur under the latter conditions. This was confirmed by the formation of DL-histidine, after hydrogenation with palladium-on-carbon of the β-elimination products, followed by acid hydrolysis. When the same treatment was applied to the hydrogen fluoride cleavage product, no histidine peak was detected by amino acid analysis. An excess of anisole is necessary to protect

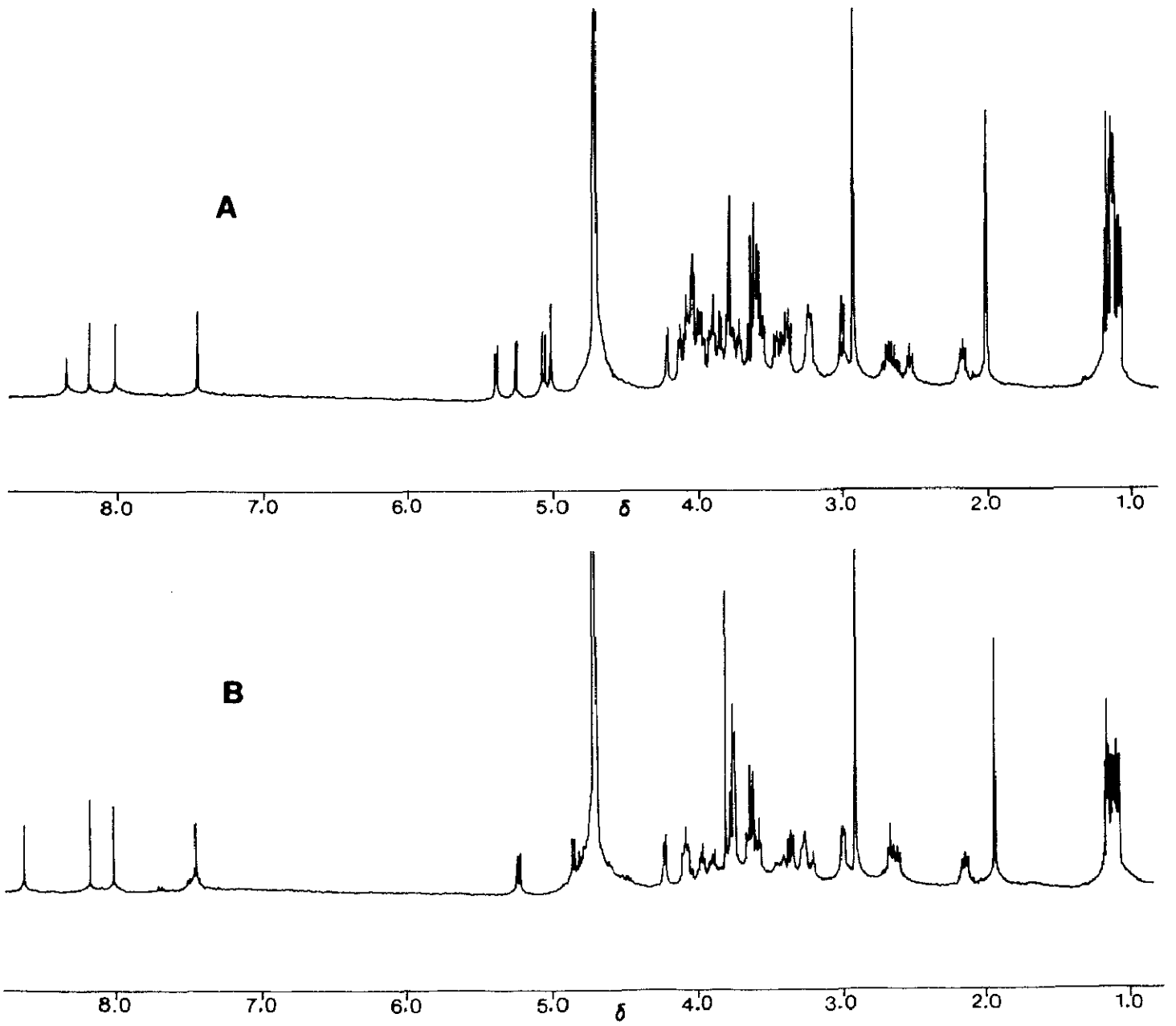

Fig. 2. ^{1}H-N.m.r. spectrum of **2** (A) and **1** (B).

heterocyclic aromatic rings from alkylating by glycosyl fluorides produced by hydrogen fluoride solvolysis, the reagent acting as a Friedel–Crafts catalyst[17], and it is not possible to study sugar recovery in the presence of anisole.

The material prepared by this method was tested for DNA cleavage. It is clear that **1** is able to cleave DNA at higher concentrations than **2** (Fig. 5). Similar results[6] were obtained with material prepared by another method. Thus, this deglycosylation is a technique of choice to recover **1** in a high degree of purity, under very convenient experimental conditions, and is, therefore, applicable to other glycopeptide antitumor antibiotics.

EXPERIMENTAL

Material. — Bleomycin-A_2 **(2)** was obtained from Roger–Bellon Laboratories. It was lyophilized for 48 h and dried overnight in a desiccator *in vacuo* at room temperature before use.

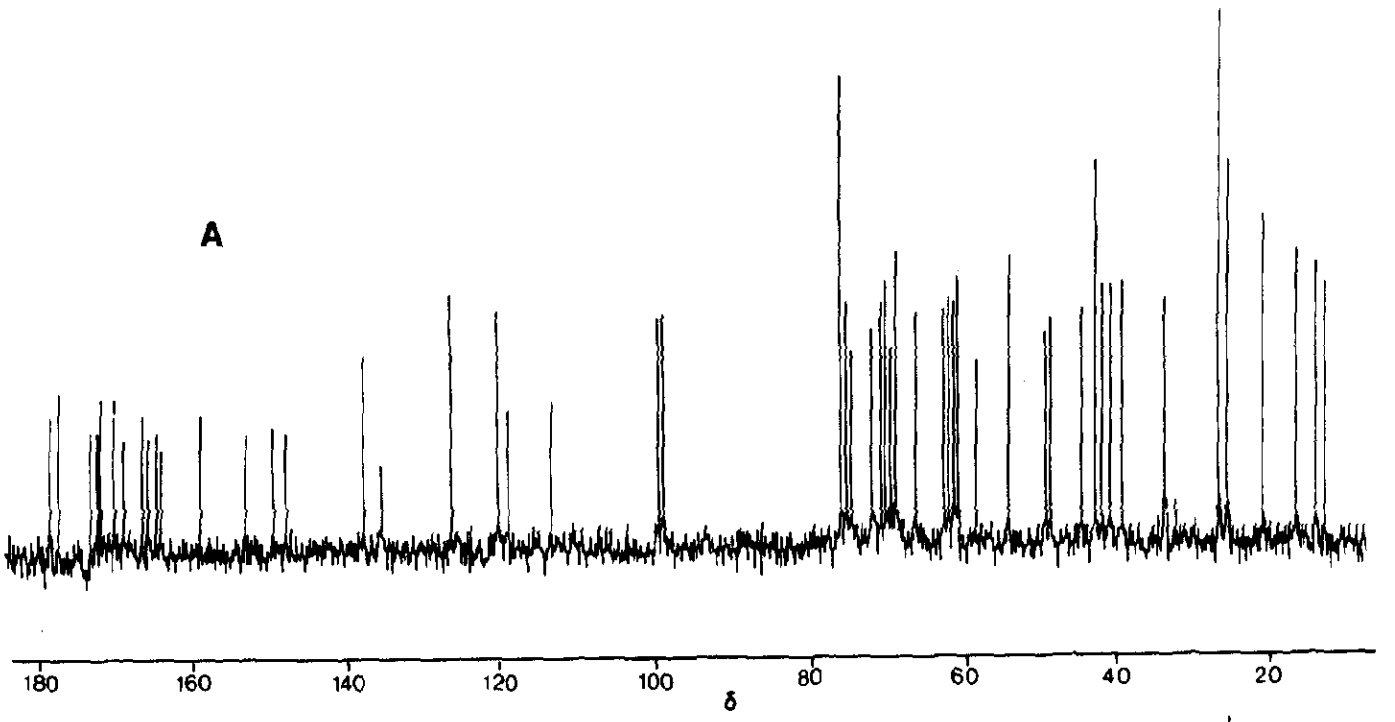

Fig. 3. ^{13}C-N.m.r. spectrum of **2** (A) and **1** (B).

^{1}H- and ^{13}C-N.m.r. spectroscopies. — The spectra were recorded for solutions (~10 mg in 0.6 mL) in D_2O at ambient temperature, with a Brucker AM 400 WB spectrometer. The ^{1}H-chemical shifts (δ) are reported relative to the signal of Me_4Si, external and the assignments were made according to the literature[11,12].

Analytical methods. — The purity of the compounds was verified by ascending t.l.c. on precoated plates of Silica gel 60F-254, 0.25 mm (Merck) in 10:9:1 methanol–10% ammonium acetate–10% ammonia; the spots were detected by u.v. absorbance at 254 nm.

Chemically modified **1** was separated and quantitatively determined by reversed-phase, paired-ion l.c. in a Kratos analytical (New Jersey, U.S.A.) h.p.l.c. system consisting of a Spectroflow 400 solvent-delivery system, a Spectroflow 783 absorbance detector with gradient control option, and a Spectroflow 480 injector-valve module.

TABLE I

^{13}C-CHEMICAL SHIFTS (δ) OF BLM-A_2 (**2**) AND DEGLYCO-BLM-A_2 (**1**)

Carbon number	*Compound*	
	2	**1**
1	171.6	171.6
2	60.3	60.9
3	47.6	48.4
4	53.0	54.0
5	40.7	40.5
6	176.7	176.1
7	165.8	165.4
8	165.1	164.2
9	112.9	115.3
10	152.7	152.4
11	11.5	11.6
12	168.3	169.2
13	57.5	58.0
14	68.4	67.4
15	134.9	132.4
16	118.3	118.9
17	137.3	135.2
18	169.5	170.2
19	48.2	49.5
20	12.7	14.0
21	75.0	75.4
22	43.3	45.9
23	15.3	15.3
24	178.0	178.0
25	59.8	59.9
26	67.7	67.9
27	19.6	20.6
28	172.5	173.6
29	39.7	39.5
30	32.7	33.6
31	163.3	162.8
32	125.7	126.2
33	147.5	147.6
34	164.0	163.9
35	119.7	121.1
36	149.4	149.5
37	171.2	170.2
38	41.5	42.1
39	24.3	24.8
40	38.3	38.4
41	25.3	25.9
42	98.1	
43	74.2	
44	70.6	
45	69.7	
46	67.8	
47	60.9	
48	98.7	
49	68.9	
50	75.0	
51	158.5	
52	64.3	
53	73.5	
54	61.5	

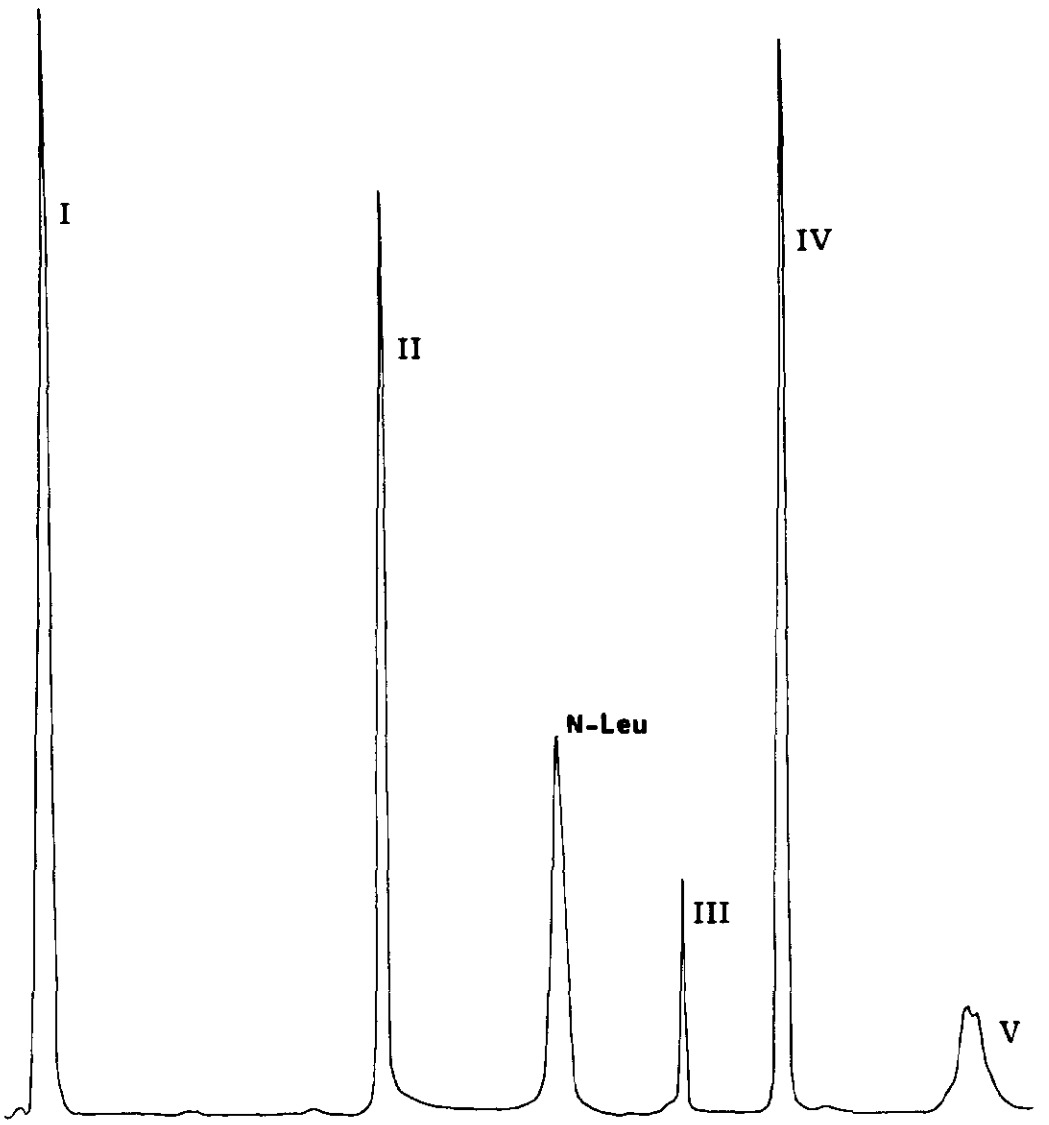

Fig. 4. Amino acid analysis of **2** and **1**: (I) L-threonine, (II) 2-amino-2-(6-amino-4-carboxy-5-methylpyrimidin-2-yl)propionic acid, (III) 4-amino-3-hydroxy-2-methylpentanoic acid, (IV) L-*erythro*-β-hydroxyhistidine, and (V) β-aminoalanine; N-Leu, *N*-leucine.

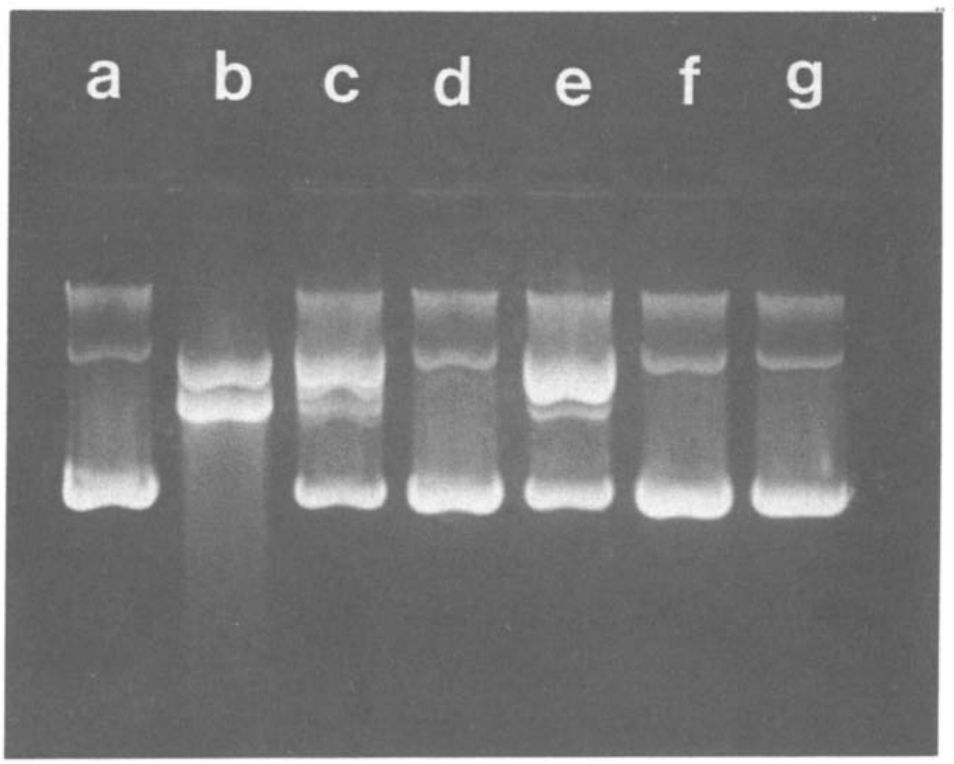

Fig. 5. Agarose-gel electrophoresis pattern: (a) Control, (b) μM BLM-A_2 (**2**), (c) 0.1μM BLM-A_2 (**2**), (d) 0.01μM BLM-A_2 (**2**), (e) μM deglyco-BLM-A_2 (**1**), (f) 0.1μM deglyco-BLM-A_2 (**2**), and (g) 0.01μM deglyco-BLM-A_2 (**1**).

A linear gradient of 30–50% methanol in water [each containing 5.0mM pentanesulfonic acid and 0.5% (v/v) glacial acetic acid, and the pH of water being adjusted to 4.3 with conc. NH_4OH] was applied for 25 min at a flow-rate of 0.8 mL/min through a 150 × 4.6 mm ultrasphere ODS (Altex) column (5 μm particle size), and detection was by u.v. absorbance at 292 nm. Compounds **1** and **2** were analyzed as the Cu(II) chelates.

Sugar analysis. — Compounds **2** and **1**, the latter obtained after alkali degradation or hydrogen fluoride solvolysis, were methanolyzed by 1.5M methanolic HCl for 24 h at 85°, and pertrimethylsilylated with Sylon HTP (Supelco, Bellafonte, PA) in the presence of *myo*-inositol as an internal standard. The *O*-trimethylsilyl methyl glycosides were analyzed by g.l.c. with a Hewlett–Packard model 5840 gas chromatograph equipped with dual-flame ionization detectors and a glass column (180 × 0.3 cm) containing 3% OV-17 on Chromosorb WHP, 80–100 mesh (Supelco). The oven temperature was programmed from 120° up to 200° at a rate of 8°/min. For determination of the free disaccharide, the preparation was directly trimethylsilylated with Sylon HTP in the presence of *myo*-inositol.

Amino acid analysis of aglycon. — The ninhydrin-positive products of hydrolysis of **1** and **2** with 6M HCl for 20 h at 105° were separated by ion-exchange chromatography and identified by comparison with commercially available (L-threonine, β-aminoaniline) and chemically synthetized compounds. L-*erythro*-β-hydroxyhistidine was synthetized as described[13], 2′-aminoethyl-2,4′-bithiazole-4-carboxylic acid was prepared according to a general procedure for the synthesis of bithiazole derivatives[14,15], and the N-terminal residue of **1** and **2** was obtained after *S*-methylation of 3-(methylthio)propylamine with methyl iodide.

Hydrogen fluoride solvolysis. — A classical HF line, designed for deprotection of synthetic peptides after chemical polymerization, was used. Thoroughly dried **2** (~40 mg) was added to anisole scavenger (1.5 mL) in a Kel-F reaction vessel cooled to 0° in an ice bath. Anhydrous HF (15 mL) was then distilled over from the reservoir, and the reaction was allowed to proceed for 1 h with continuous stirring. At the end, after complete HF removal, the sample was taken up in anhydrous ether to remove the anisole, and **1** was obtained as a solid.

Alkali treatment. — Compound **2** was maintained in 50mM NaOH for 16 h at 37°. The liberation of the disaccharide was monitored by an increase in u.v. absorption at 290 nm. The mixture was deposited on a Sephadex G-10 column (12 mm × 900 mm), and eluted with water. The second peak was collected and passed through AG 50W-X8 (H^+) cation-exchange resin to recover the disaccharide which was detected by u.v. absorption at 290 nm and an automated orcinol method[10].

DNA breakage activity of **1** *and* **2**. — To the plasmide (6 μg of pKH47 with an adenovirus insert) in 50mM Tris·HCl buffer (pH 8.0) were added β-mercaptoethanol (10mM), various concentrations of **1** or **2**, and $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ in the same final concentration as the compounds. After 30 min at room temperature, the mixture was analyzed by gel electrophoresis in 1% agarose containing 0.5 μg/mL of ethidium bromide.

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