NEW 5′-NUCLEOTIDASE INHIBITORS, MELANOCIDIN A AND MELANOCIDIN B

II. PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE ELUCIDATION

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New 5′-nucleotidase inhibitors named melanocidins A and B were isolated from a fermentation broth of *Nocardioides* sp. The molecular weights estimated by gel filtration were over 1,000,000. By the combination of chemical and physico-chemical analyses, we revealed their chemical structures.

As reported in the preceding paper[1], new 5′-nucleotidase inhibitors named melanocidins A and B were isolated from the fermentation broth of *Nocardioides* sp. These compounds show a unique property of inhibiting 5′-nucleotidase and are acidic polysaccharides. The determination of their chemical structures are reported in this paper.

Physico-chemical Properties

Gel filtration of melanocidins A and B[1], carried out on a column of Sepharose 4B, showed only

Fig. 1. Electrophoresis of melanocidin A.

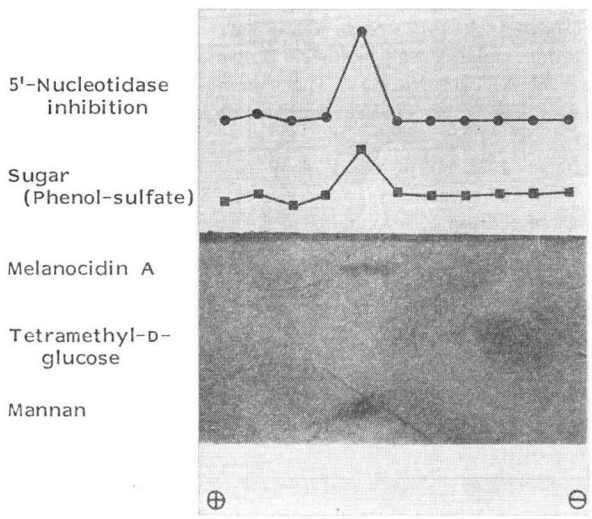


Fig. 2. Electrophoresis of melanocidin B.

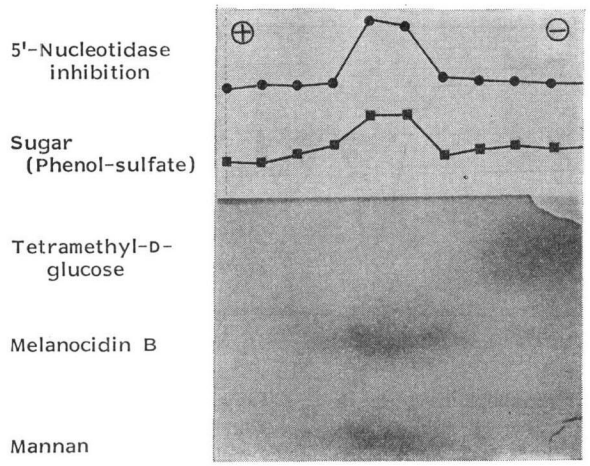
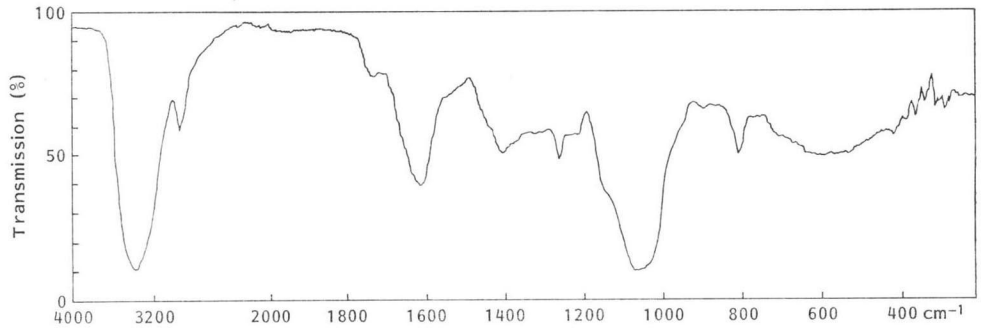


Table 1. Physico-chemical properties of melanocidins A and B.

		Melanocidin A	Melanocidin B
Appearance		White powder	White powder
MP		>300°C	>300°C
$[\alpha]_{589}^{20}$		+40° (*c* 0.5, H_2O)	+2° (*c* 1.0, H_2O)
Anal (%)		C 39.89, H 5.88	C 37.80, H 5.53
UV (H_2O)		End absorption	End absorption
Color reaction	(+)	Phenol-sulfate, anthrone, carbazole-sulfate	Phenol-sulfate, anthrone, carbazole-sulfate
	(−)	Fehling, Elson-Morgan, ninhydrin	Fehling, Elson-Morgan, ninhydrin
Solubility	Soluble	H_2O, DMSO	H_2O, DMSO
	Insoluble	Other common organic solvents	Other common organic solvents

Fig. 3. IR absorption spectrum of melanocidin A in KBr disk.



one peak for both compounds at a molecular weight of over 1,000,000. In addition, glass paper electrophoresis gave only one spot as detected by phenol-sulfate[2] and *p*-anisidine-sulfate[3] methods, which coincided with the inhibitory activity against 5'-nucleotidase (Figs. 1 and 2).

The physico-chemical properties are summarized in Table 1. Phenol-sulfate, anthrone and carbazole-sulfate gave positive reactions, but Fehling and Elson-Morgan reactions were negative, indicating that both melanocidins A and B are acidic polysaccharides. IR absorption spectra shown in Figs. 3 and 4, and UV absorption spectra confirm this indication.

Fig. 4. IR absorption spectrum of melanocidin B in KBr disk.

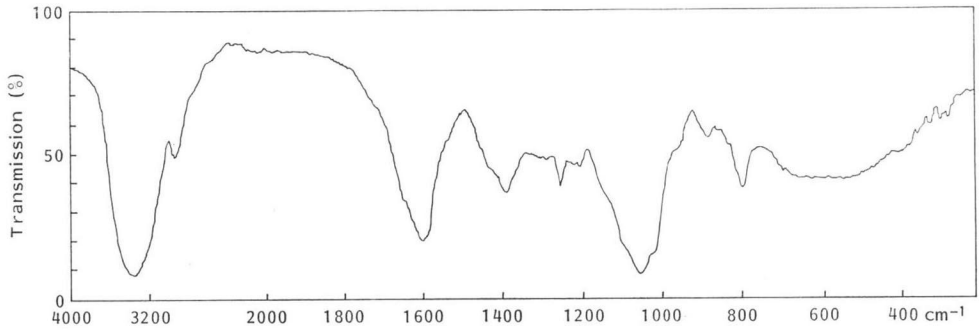


Table 2. Composition of melanocidins A and B.

	Composition		Molar ratio[c]
	Gal A[a]* (%)	Total hexose[b] (%)	Glu*: Gal*
Melanocidin A	25	52	1 : 1.4
Melanocidin B	21	70	0 : 1.0

[a] Determined by the carbazole-sulfate method using galacturonic acid as a standard.
[b] Determined by the phenol-sulfate method using galactose as a standard.
[c] Determined by GC as the trimethylsilyl derivatives.
* Abbreviation: Gal A, galacturonic acid; Glu, glucose; Gal, galactose.

Table 3. Methylation analysis of melanocidin B and acetic anhydride-triethylamine degraded melanocidin B.

Methylated sugar (as alditol acetate)	Molar ratio	
	A[a]	B[b]
2,3,4,6-Tetra-*O*-methyl-D-galactose	—	0.8
2,4,6-Tri-*O*-methyl-D-galactose	1.9	1.1
2,6-Di-*O*-methyl-D-galactose	1.0	1.0

[a] Alditol acetates obtained from permethylated melanocidin B.
[b] Alditol acetates obtained by the methylation of the acetic anhydride-triethylamine degraded products.

Structural Elucidation

Sugar composition of melanocidins A and B were determined by TLC and GC, and carbazole-sulfate[4] and phenol-sulfate[2] methods. Table 2 shows that melanocidin A consists of galactose, glucose and galacturonic acid while melanocidin B is composed of galactose and galacturonic acid. The structure of melanocidin B was determined first. Melanocidin B and the uronic acid-degraded[5] melanocidin B were permethylated[6,7], and the fully methylated products were subjected to hydrolysis, reduction and acetylation. The identities and relative concentrations of the sugars from the hydrolysate were determined by GC-MS[8,9] and by a more sensitive technique of single-ion mass fragmentography[10]. The data from the methylation analysis are presented in Table 3. These results show that galacturonic acid is located at the terminal position of the branched chain, that it connects with galactose through 1 to 3 bond, and that two of three galactose residues are 3-substituted and the other is 3,4-di-substituted (branching point in the chain).

Next, the configuration of melanocidin B was clarified by ^{13}C NMR analysis (Fig. 5). Signals at δ 104.0, 103.9, 100.5 and 96.9 ppm were assigned to four anomeric carbons, and a signal at δ 176.6 ppm to the carboxyl group of a D-galacturonic acid residue. Anomeric carbon atoms of pyranoses[11] with α configuration are reported to show resonances in the δ 95~103 ppm region, and those with β configuration at δ 103~108 ppm. Therefore, it is concluded that there are two α- and two β-pyranosidic residues in one repeating unit. These assignments are also consistent with the low $[\alpha]_{589}^{20}$ value [+2°

Fig. 5. ^{13}C NMR spectrum of melanocidin B.

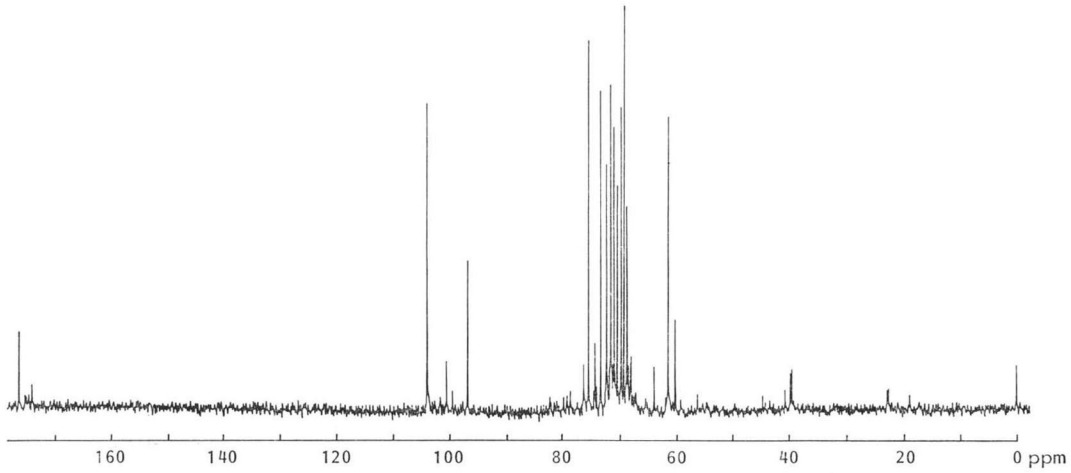
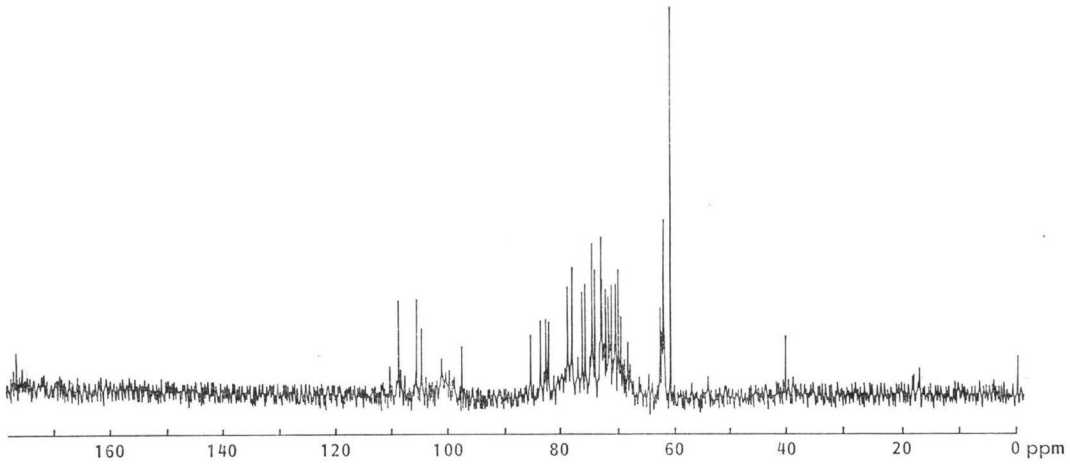
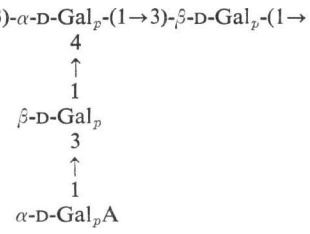


Fig. 6. ^{13}C NMR spectrum of melanocidin A.

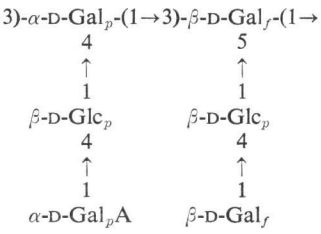


(water)] of melanocidin B. The signals for C-1 in methyl α- and β-D-galactopyranosides are reported to appear at δ 99.5 and 103.9 ppm[12], respectively. Thus, close signals at δ 104.0 and 103.9 ppm were assigned to C-1 of two 3-*O*-substituted β-D-galactopyranosyl residues, and a signal at δ 100.5 ppm was assigned to a 3,4-di-*O*-substituted α-D-galactopyranosyl residue. The remaining signal at δ 96.9 ppm is assigned to the D-galactopyranosyluronic acid residue, which is α-linked. A similar signal at high field is observed for the C-1 of an α-D-glucopyranosyluronic acid residue, as reported in the previous paper[13]. From these results, the following structure (**2**) can be proposed for one repeating unit of melanocidin B.

Structure of melanocidin A was elucidated by reference to the data on melanocidin B. Melanocidin A contained D-glucose, D-galactose and D-galacturonic acid (Table 2). The ^{13}C NMR spectrum (Fig. 6) showed signals at δ 110.7, 109.2, 105.9, 105.1, 101.6 and 98.0 ppm, suggesting the presence of four β-anomeric linkages and two α linkages. The presence of two large C-1 signals at δ 110.7 and 109.2 ppm in the ^{13}C NMR spectrum is characteristic of β-D-galactofuranosyl unit[14]. The signals at



(2)



(1)

Table 4. Methylation analysis of melanocidin A and acetic anhydride-triethylamine degraded melanocidin A.

Methylated sugar (as alditol acetate)	Molar ratio	
	A[a]	B[b]
2,3,4,6-Tetra-*O*-methyl-D-glucose	—	0.84
2,3,5,6-Tetra-*O*-methyl-D-galactose	1.0	1.0
2,3,6-Tri-*O*-methyl-D-glucose	1.9	1.1
2,6-Di-*O*-methyl-D-galactose	1.9	2.3

[a] Alditol acetates obtained from permethylated melanocidin A.

[b] Alditol acetates obtained by the methylation of the acetic anhydride-triethylamine degraded products.

δ 101.6 and 98.0 ppm are similar to the data on melanocidin B. Therefore, these signals are assigned to C-1 of a 3,4-di-*O*-substituted α-D-galactopyranosyl residue and C-1 of an α-D-galactopyranosyluronic acid residue, respectively. The remaining signals at δ 105.9 and 105.1 ppm were assigned to C-1 of β-D-glucopyranosyl residue, by comparison with the value for methyl-α- and methyl-β-D-glucopyranoside[15]. The result of methylation analysis (Table 4) shows that galacturonic acid is located at the terminal position of one branching chain in the repeating unit of the polysaccharide and that it is bound to glucose through 1 to 4 linkage. The occurrence of 2,3,5,6-tetra-*O*-methyl-D-galactose indicates that this D-galactose is furanosidic and located at the terminal of another branching chain. These results indicate that melanocidin A is highly branched, and that one repeating unit consists of six hexose residues. From these results, structure (**1**) can be proposed for one repeating unit of melanocidin A.

Experimental

General

Optical rotations were measured with a Jasco J-20K polarimeter. IR spectra were recorded as KBr disk (polysaccharides) or in carbon tetrachloride (methylated polysaccharides) with a Jasco PS-701 spectrometer. GC was performed with a Jeol JGC-20K equipped with a flame-ionization detector and fitted with a glass column (2 mm×3 m) packed with 3% OV-1 on Chromosorb W (60~80 mesh) at 150~200°C (2°C/minute). For combined GC-MS of the partially methylated alditol acetates, Jeol DX-300 was used. A glass column (3 mm×2 m) was filled with 3% ECNSS-M on Gaschrom Q (100~200 mesh) at 190°C and a nitrogen flow rate of 50 ml/minute was used. The spectra were taken at 70 eV electron energy in a mass range of 30~400. ^{13}C NMR spectra were recorded at 70°C in D_2O with a Jeol GX-400 spectrometer in the pulsed Fourier-transform (F. t.) mode with complete proton-decoupling. The chemical shifts were obtained by use of tetramethylsilane as an external standard.

Electrophoresis

Electrophoresis was conducted on Whatman GF/A glass microfiber paper (20.3×25.4 cm) in 0.1 M sodium borate (pH 9.3) at 700 volt for 70 minutes, which was detected by *p*-anisidine-sulfate and by inhibitory activity against 5′-nucleotidase as follows: Each 2 cm strip containing a developed polysaccharide was cut into 2 cm segments and the polysaccharide on each segment was eluted with water. The eluates were dialyzed against water, lyophilized and assayed.

Thin-layer Chromatography (TLC)

TLC was performed on silica gel 60 plates (0.25 mm thick, Merck) or micro crystalline cellulose plates (Avicel SF, Funakoshi). The solvents used were 1-BuOH - AcOH - water, 2: 1: 1, for silica gel plates and EtOAc - pyridine - water - AcOH, 5: 5: 3: 1, for cellulose plates. The staining reagents used were 5% methanol - sulfuric acid for silica gel plates and diphenylamine - aniline[16,17] for cellulose plates.

Hydrolysis

Sample (10 mg) was hydrolyzed with 2 N H_2SO_4 (10 ml) in a boiling-water bath. Aliquots of 1 ml samples were withdrawn at suitable intervals and made just neutral by the dropwise addition of 2 N NaOH. The reducing sugar was measured by the Somogyi-Nelson method[18,19].

Analysis of Sugar Composition

Total hexose and uronic acid were estimated by phenol-sulfate[2] and carbazole-sulfate[4] methods, respectively. The two isolated polysaccharides were hydrolyzed with 0.1 N HCl for 3 hours at 100°C and the solution was evaporated to dryness. An aliquot of the hydrolysate was analyzed by TLC and spots for D-galactose, D-glucose and D-galacturonic acid were detected. The content of neutral sugars in two polysaccharides were determined by GC of the trimethylsilylated derivatives. Polysaccharides were hydrolyzed completely with 2 N H_2SO_4 in a boiling-water bath for 5 hours. The hydrolysate was neutralized with Amberlite IRA-47 (OH^-) resin. The resin was filtered off and washed three times with distilled water and the washings were combined with the filtrate. This pool was evaporated to a small volume of solution and lyophilized. The component sugars were trimethylsilylated, using trimethylsilylimidazole, and the ratios of silylated sugars were analyzed by GC using 3% OV-1 in a 2 mm×3 m glass column (temperature program 150~200°C, 2°C/minute). The silylated sugars were analyzed as the sum of their anomers. Uronic acid was also examined as follows. Polysaccharides were treated with 5% HCl - methanol at 90°C for 20 hours. The reaction mixture was neutralized by adding silver carbonate and the resulting insoluble material was removed. The solution was dried under reduced pressure. The resulting methylglucosides were trimethylsilylated, using trimethylsilylimidazole, and analyzed by GC using 3% SE-30 in a 2 mm×3 m glass column at 140~200°C (0.5°C/minute). The results of these analyses are shown in Table 2.

Acetylation of Polysaccharides[5]

Polysaccharides (100 mg) were dissolved in formamide (20 ml) and pyridine - acetic anhydride (1: 1, 10 ml) was added. The solution was kept for 24 hours at room temperature. The reaction mixture was diluted with water and dialyzed against water and then lyophilized. The acetylation was repeated once more and the IR spectrum suggested complete reaction.

Degradation of Acetylated Polysaccharides[6]

The acetylated polysaccharides (40 mg) were dissolved in acetic anhydride (16 ml) by ultrasonic treatment for 30 minutes. Triethylamine (13.5 ml) was added and the solution was kept for 2.5 hours on a boiling-water bath. The reagents were distilled off and the residue was treated with 90% aqueous acetic acid (10 ml) for 2 hours at 100°C. The mixture was cooled, dialyzed against water, and lyophilized. The recovered material was dissolved in dimethyl sulfoxide (9 ml), and 0.4 M sodium hydroxide (3 ml) was added. The solution was kept at room temperature overnight, dialyzed and lyophilized. The IR spectrum (KBr) suggested complete deacetylation. The degradation of galacturonic acid was determined by TLC.

Methylation Analysis

Polysaccharides (20 mg) were permethylated by the method of KUHN[6,7]. The completeness of permethylation was checked in the IR spectrum by the absence of absorption due to hydroxyl groups. The fully methylated samples were treated with 2 N H_2SO_4 for 8 hours at 100°C. The resulting partially methylated sugars were converted into their alditol acetates in an usual manner. The partially methylated alditol acetates were analyzed for their composition by GC-MS using 3% ECNSS-M in a 3 mm× 2 m glass column at 190°C.

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