A NEW 5'-NUCLEOTIDASE INHIBITOR, NUCLEOTICIDIN

II. PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE ELUCIDATION

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A novel 5'-nucleotidase inhibitor, named nucleoticidin, was isolated from a fermentation broth of *Pseudomonas* sp. The molecular weight was estimated by gel filtration to be over 1,000,000. Nucleoticidin is composed of D-glucose and D-mannose at a molar ratio of 1.7 to 1.0. Combined analyses using chemical and physico-chemical methods, such as gas liquid chromatography and mass fragmentography, revealed that nucleoticidin has a structural unit with mannosyl residues at the terminal of a (1→4) linked D-glucosyl main chain with β-configuration.

As reported in the preceding paper[1], a novel 5'-nucleotidase inhibitor, nucleoticidin, was isolated from the fermentation broth of *Pseudomonas* sp. YM-3229G. It has an unique property of inhibiting 5'-nucleotidase. Therefore, it is interesting to know the chemical structure-inhibitory activity relationship of this compound. For this purpose, it is essential to elucidate the chemical structure. This paper reports the results.

Physico-chemical Properties

When gel filtration of nucleoticidin isolated by the procedure described in the previous paper[1] was carried out on a column of Sepharose 4B, only one peak was observed at a molecular weight of over 1,000,000. In addition, glass paper electrophoresis gave only one spot, as detected by the phenol-sulfate[2] and the *p*-anisidine-sulfate[3] methods, which coincided with the inhibitory activity against 5'-nucleotidase (Fig. 1).

The physico-chemical properties are summarized in Table 1. Phenol-sulfate and anthrone gave positive reactions, but Fehling and Elson-Morgan reactions were negative. This indicates that nucleoticidin is a neutral polysaccharide substance. The UV spectrum and the IR absorption spectrum shown in Fig. 2 confirm this indication. Although chemical analysis by phenol-sulfate method yielded 78% of carbohydrate using D-glucose as a standard, GC analysis of the acid hydrolysate indicated 97% of carbohydrate. This suggests that the molecule mainly consists of carbohydrates.

Structure Elucidation

Sugar composition of nucleoticidin was determined by TLC and GC. Acid hydrolysate with 2 N sulfuric acid contained D-glucose and D-mannose at a molar ratio of 1.7 to 1.0, as estimated by GC.

Fig. 1. Electrophoresis of nucleoticidin.

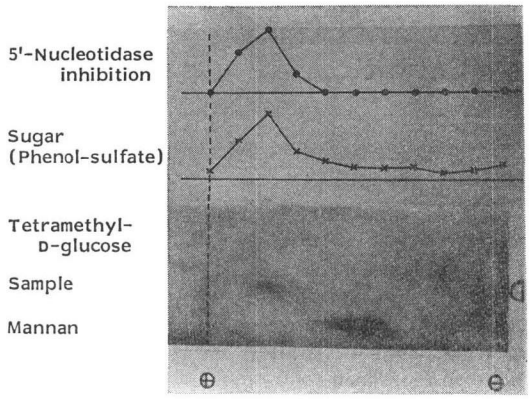


Table 1. Physico-chemical properties of nucleoticidin.

Appearance	White powder
MP	>300°C
Optical rotation	$[\alpha]_{589}^{20}$ −140° (*c* 0.50, 0.1 N NaOH)
Elemental analysis (%)	C 40.17, H 6.20, Ash 3.44
UV (H_2O)	End absorption
Color reaction (+)	Phenol-sulfate, anthrone
(−)	Fehling, Elson-Morgan, ninhydrin
Solubility: Soluble	H_2O, DMSO
Insoluble	Other common organic solvents

Fig. 2. IR spectrum of nucleoticidin in KBr disk.

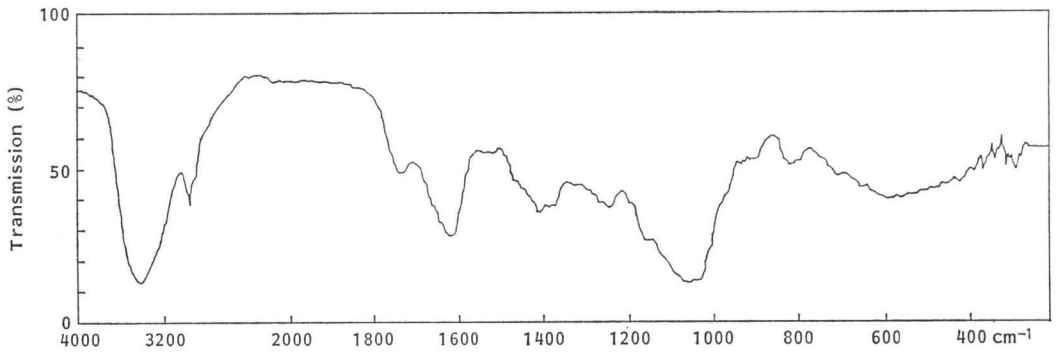


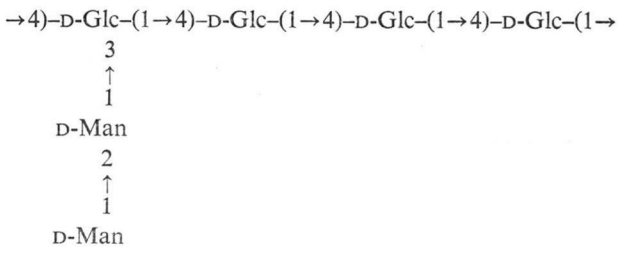
Table 2. Methylation analysis of nucleoticidin.

Methylated sugars (as alditol acetate)	RRT[a]	Molar ratio
2,3,4,6-Tetra-*O*-methyl-D-mannose	0.99	0.93
3,4,6-Tri-*O*-methyl-D-mannose	1.85	0.93
2,3,6-Tri-*O*-methyl-D-glucose	2.35	3.2
2,6-Di-*O*-methyl-D-glucose	3.52	1.0

[a] Relative retention times with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol on a 3% ECNSS-M (Kokusan Chemical Works Ltd.) column at 190°C.

The nucleoticidin was permethylated[4,5], and the fully methylated products were subjected to hydrolysis, reduction and acetylation. The identity and proportion of the sugars were determined by combined GC-MS[6,7] and by the more sensitive technique of single ion mass fragmentography[8], which revealed the presence of 2,3,4,6-tetra-*O*-methylmannose, 3,4,6-tri-*O*-methylmannose, 2,3,6-tri-*O*-methylglucose and 2,6-di-*O*-methylglucose at a molar ratio of 0.93: 0.93: 3.2: 1.0 (Table 2).

This suggests that mannosyl residues are located at the terminal position of the side chain and that the core portion consists of a (1→4) linked glucosyl main chain. In view of these results, it is concluded that nucleoticidin is composed of a structural unit of the following type:



The anomeric configuration of the sugars was estimated by cellulase digestion. When hydrolysis of nucleoticidin was monitored by measuring the appearance of reducing sugars by using the SOMOGYI-NELSON procedure[9,10], the reducing power of the medium reached a maximum at 12 hours, where the cleavage corresponded to 24% of the presumed total (1→4)-β-D-glucose bonds. This fact, together with the low specific rotation of $[\alpha]_{589}^{20}$ −140° and the drastic absorbance at 884 cm^{-1} in the IR spectrum, showed the presence of β-D-linkages but no indication of the presence of α-D-linkages.

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.

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, detection was 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 H_2O. The eluates were dialyzed against H_2O, lyophilized and assayed.

Thin-layer Chromatography (TLC)

TLC was performed on silica gel 60 plates (0.25 mm thick, Merck) or microcrystalline cellulose plates (Avicel SF, Funakoshi). The solvents used were 1-BuOH - AcOH - H_2O, 2:1:1, for silica gel plates and EtOAc - pyridine - H_2O - AcOH, 5:5:3:1, for cellulose plates. The staining reagents used were 5% methanol sulfuric acid for silica gel plates and diphenylamine - aniline[11,12] for cellulose plates.

Hydrolysis

Nucleoticidin (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 exactly neutral by the dropwise addition of 2 N NaOH. The reducing sugar was measured by the SOMOGYI-NELSON method[9,10].

Analysis of Sugar Composition

Nucleoticidin was 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 H_2O and the washings were combined with the filtrate. This pool was evaporated to a small volume and lyophilized. An aliquot of the residue was dissolved in a small volume of H_2O, and analyzed by TLC, then spots for D-glucose and D-mannose were detected. The remaining part was converted into a trimethylsilyl derivative, 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 estimated as the sum of their anomers.

Methylation Analysis

Nucleoticidin (20 mg) was permethylated by the methods of KUHN[4,5]. 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 the 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.

Degradation of Nucleoticidin with Cellulase

To a solution of nucleoticidin (20 mg) in 0.1 M sodium acetate buffer (pH 5.0, 1.5 ml) was added on excess 2 mg of cellulase (from *Aspergillus niger* Type I, Sigma). A few drops of toluene were added to prevent bacterial growth, and the mixture was incubated at 37°C. Aliquots of 0.4 ml were withdrawn at suitable intervals and the enzyme was then deactivated by heating for 10 minutes at 100°C. The reducing sugar was measured by the SOMOGYI-NELSON method[9,10].

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