NOVEL GLYCOSIDASE INHIBITORS, NOJIRIMYCIN B AND D-MANNONIC-δ-LACTAM

ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL PROPERTY

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A new aminosugar named nojirimycin B (1) has been isolated as its bisulfite adduct from the culture broth of *Streptomyces lavendulae* SF-425, together with nojirimycin. Microbiological oxidation of **1** with *Gluconobacter suboxydans* IAM 1829 gave a δ-lactam (**2**). The structures of **1** and **2** were determined to be 5-amino-5-deoxy-D-mannopyranose and D-mannonic-δ-lactam, respectively, on the basis of ^{1}H NMR spectroscopy and X-ray structural analysis. Both **1** and **2** exhibited powerful inhibitory activity against rat epididymal α-mannosidase and apricot β-glucosidase.

Microbial glycosidase inhibitors have been the subject of extensive investigations[1], of which nojirimycin was the first inhibitor isolated[2]. This was followed by isolations of acarbose[1,3], 1-deoxynojirimycin[4,5], valienamine[6], oligostatin[7], valiolamine[8], *etc.* These inhibitors are all sugar analogs containing nitrogen in their molecules.

In this paper, we report a new nitrogen containing sugar analog, nojirimycin B* and its oxidation product, D-mannonic-δ-lactam, both of which showed potent inhibitory activity against glycosidases.

Preparation of Nojirimycin B and D-Mannonic-δ-lactam

Nojirimycin B (**1**) was co-produced with nojirimycin by *Streptomyces lavendulae* SF-425. Both antibiotics were isolated from the fermentation broth by the use of Amberlite IR-120 resin and Dowex 1X2 resin. Separation of two antibiotics was achieved by fractional crystallization of bisulfite adducts, and nojirimycin B bisulfite adduct (**1a**) was crystallized after most of nojirimycin bisulfite adduct were recovered.

A free base of **1** was prepared by treating **1a** with barium hydroxide. On paper chromatography developed with a mixed solvent of BuOH - pyridine - H_2O, 6: 4: 3, **1** free base showed Rf value of 0.36, while **1a** and nojirimycin showed Rfs of 0.13 and 0.25, respectively. However, the preparation of **1** contained a small amount of an unknown substance with Rf 0.27, which was probably derived from spontaneous decomposition of **1** and was difficult to remove.

The microorganisms capable of forming gluconic acid from glucose, *i.e. Gluconobacter suboxydans* IAM 1829, *Pseudomonas ovalis* IFO 12051, *Rhizopus delemar* IAM 6015 and *Aspergillus niger* IAM

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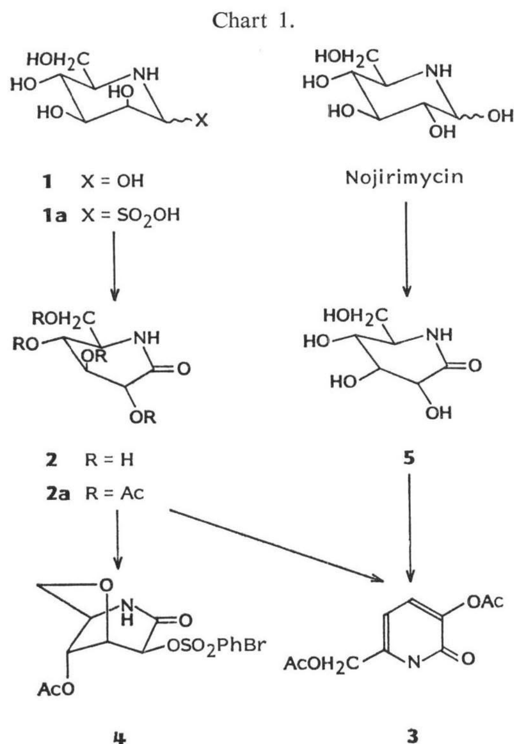
209, showed the ability to catalyze the oxidative conversion of **1** to **2**.

G. suboxydans IAM-1829 was selected as a microbial catalyst for the preparative purpose. Incubation of 2% aqueous solution of **1** and the washed cells at 28°C for 5 hours, followed by column chromatography over carbon afforded crystals of **2** in 49% yield.

Determination of Structure of 2

The IR spectrum of **2** exhibited characteristic amide bands at 1640 and 1550 cm^{-1}, and resembled with that of D-gluconic-δ-lactam (**5**). Treatment of **2** with acetic anhydride and pyridine at room temperature gave tetraacetate (**2a**), mp 95°C, which showed four acetyl signals in ^1H NMR and a (MH^+) ion peak at m/z 346. On the other hand, when treated **2** at 90~100°C, 3-acetoxy-6-acetoxymethyl-2-(1*H*)-pyridone (**3**) was formed. Compound **3**, which was formed also from **5** by similar treatment, was crystallized from chloroform - ethanol, mp 135 ~ 136°C. The structure of **3** was determined from UV (λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 228 (315), 302 (330)) and ^1H NMR ($CDCl_3$, δ 2.00 ($COCH_3$), 4.75 (CH_2COCH_3), 5.92, 6.83 (two ring protons)). These results suggested that **2** was a tetrahydroxypiperidin-2-one.

Chart 1.



The stereochemistry of **2** was elucidated by X-ray crystallographic analysis as follows. *p*-Bromophenylsulfonylation of **2** with sulfonyl chloride in pyridine at 60°C, followed by acetylation gave monoacetyl-mono-*p*-bromophenylsulfonate (**4**), mp 185°C. A single crystal was prepared from chloroform - carbon tetrachloride, and its crystal data are shown in Table 1.

Fig. 1. IR spectrum of nojirimycin B bisulfite adduct (**1a**) in KBr.

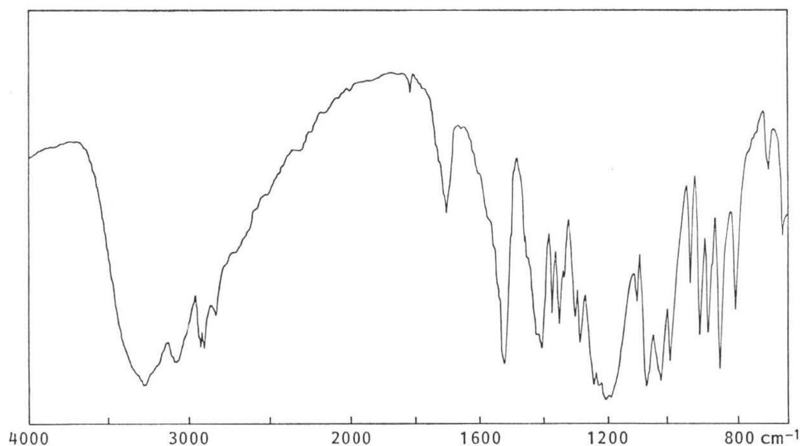


Table 1. Crystal data of D-mannonic-δ-lactam (**2**) and 4-*O*-acetyl-3,6-anhydro-2-*O*-*p*-bromophenylsulfonyl-D-mannonic-δ-lactam (**4**).

		2	4
Crystal system		Orthorhombic	Monoclinic
Space group		$P2_12_12_1$ (z=4)	C_2 (z=4)
Lattice constant	a	10.649Å	21.864Å
	b	7.916	8.260
	c	8.505	9.134
	α	90°	90°
	β	90°	85.2°
	γ	90°	90°
Lattice volume		717.2Å^3	1644.5Å^3
Density		1.64	1.70

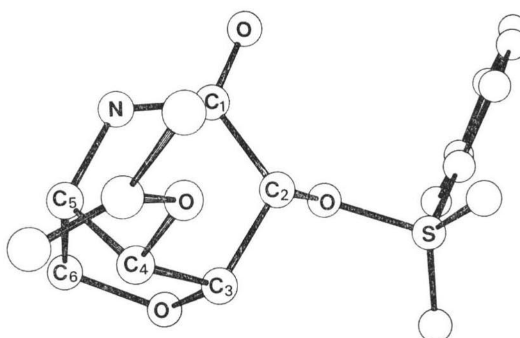


Fig. 2. Perspective drawing of the molecule of 4-*O*-acetyl-3,6-anhydro-2-*O*-*p*-bromophenylsulfonyl-D-mannonic-δ-lactam (**4**).

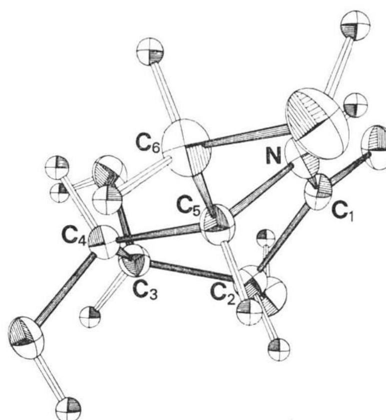


Fig. 3. Perspective drawing of the molecule of D-mannonic-δ-lactam (**2**).

Fig. 2 illustrates the framework of **4** drawn by ORTEP[9], indicating the 4-*O*-acetyl-3,6-anhydro-2-*O*-*p*-bromophenylsulfonyl-D-mannonic-δ-lactam structure. Thus, the configurations at C(2), C(3) and C(5) of **2** were determined. The absolute configuration also was determined to be D-sugar series by the use of anomalous diffractions involving bromine atom. However, the configuration at C(3) that involved 3,5-anhydro ring formation was remained unsettled.

By tracing the sulfonylation reaction by the use of TLC and ^{1}H NMR spectroscopy, it was found that the initial product formed was the 2,6-di-*O*-*p*-bromophenylsulfonate, which was converted into the 3,6-anhydro derivative (**4**), even under ice-cooling condition. Assuming that the 3,6-anhydro bridge was formed by intramolecular nucleophilic attack of C(3) hydroxyl group to C(6) that bore the leaving sulfonyloxy group, the configuration at C(3) in **2** could be assigned to L. This was confirmed by the direct X-ray analysis of **2**.

A single crystal of **2** was prepared from aqueous ethanol, and its crystal data are shown in Table 1. The framework of **2** determined by X-ray analysis is shown in Fig. 3. The relative configurations of hydroxyl groups at C(2), C(3), C(4) and C(5) are in accord with those of **4**, thus confirming the stereochemistry at C(3). Four ring atoms of C(1), C(3), C(4) and NH forms almost a plane, but two bonds involving C(1)-N and C(3)-C(4) are twisted 6.8°. Two other ring atoms of C(2) and C(5) are projected one side above the plane. This means that **2** forms a twist-boat conformation.

Table 2 shows chemical shifts and coupling constants (*J*) of **2**, **2a** and **4**. The *J* values of **2** in D_2O and **2a** in deuteriochloroform were consistent with those calculated from the dihedral angles determined by X-ray according to Karplus equation: H-C(2)-C(3)-H, 65.5°, H-C(3)-C(4)-H, 104.5°; H-C(4)-C(5)-H, 156.6°. This indicated a similar twist-boat form in solution. Ready formation of the 3,6-anhydro bridge was thus favored by the inverted form of the twist-boat found in crystals and solution, whereby C(3) hydroxyl group and C(6) carbon are close to each other. On the other hand,

Table 2. Chemical shifts (δ) and coupling constants (J) of nojirimycin B bisulfite adduct (**1a**), D-mannonic-δ-lactam (**2**), its tetraacetate (**2a**) and 4-*O*-acetyl-3,6-anhydro-2-*O*-*p*-bromophenylsulfonyl-D-mannonic-δ-lactam (**4**).

	1a D_2O	**2** D_2O	**2a** $CDCl_3$	**4** $CDCl_3$ - DMSO-d_6 (9:1)
δ H_1	4.98			
H_2	5.10	4.40	5.64	5.05
H_3	4.21	4.10	5.46	4.54
H_4	4.51	4.91	5.08	5.20
H_5	3.82	3.45	3.80	
H_6	*ca* 4.5	3.73	4.31, 4.15	4.00
$COCH_3$			2.12, 2.14	2.16
$J_{1,2}$	1.3			
$J_{2,3}$	2.5	3.5	3.4	
$J_{3,4}$	10.0	5.0	4.0	2.1
$J_{4,5}$	10.0	5.0	4.0	6.5
$J_{5,6}$	4.5	5.0	5.1	
$J_{5,6'}$	3.5	10.5	7.0	

J values of **4** differed from those of **2** and **2a**, being consistent with the distorted pyranose ring indicated by X-ray analysis.

Determination of Structure of 1

The fact that **1** was transformed to **2** in reasonable yield by microbiological oxidation suggested the 5-amino-5-deoxy-D-mannose structure for **1**. This was confirmed by ^1H NMR analysis of **1a** shown in Table 2. Large $J_{3,4}$ and $J_{4,5}$ values are consistent with *trans*-diaxial orientation of H(3), H(4) and H(5). Moderate $J_{2,3}$ value indicated the equatorial orientation of H(2). These values were in agreement with those of mannopyranose of C_1 conformation. The anomeric H(1) appeared as only one signal, indicating the predominant presence of an epimer, but it was difficult to determine the configuration of H(1) from $J_{1,2}$ value, because of the equatorial orientation of H(2) proton.

Inhibitory Activities of 1, 1a and 2 against Three Glycosidases

Table 3 shows the inhibition constants (K_i) for **1a** and **2** against apricot emulsin and rat epididymal α-mannosidase, together with the K_m values for the substrates employed. All of the K_i values were substantially small, although the substrates had relatively favorable K_m values. The LINEWEAVER-BURK plots, from which these values were obtained, showed the inhibition to be competitive in each cases.

Table 3. K_m values for substrates and K_i values for nojirimycin bisulfite adduct (**1a**) and D-mannonic-δ-lactam (**2**) against apricot β-glucosidase and rat epididymal α-mannosidase.

Substrates were *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-α-D-mannoside.

	Apricot β-glucosidase (M)	Rat epididymal α-mannosidase (M)
K_m	4.2×10^{-3}	7.9×10^{-3}
K_i (**1a**)	5.6×10^{-6}	1.2×10^{-5}
K_i (**2**)	9.2×10^{-7}	9.5×10^{-5}

Table 4 shows the 50% inhibitory concentrations of **1**, **1a**, **2**, **5** and 1-β-D-glucosylamine[10] against the above two glycosidases and *Trichoderma viride* β-glucosidase. The inhibitory activity of **1** was potent against apricot emulsin and α-mannosidase but less active against *Trichoderma* β-glucosidase. Notably, **1a** was two- to five-fold more potent than the parent **1** against these

Table 4. Comparative inhibitory activities of nojirimycin B (**1**), its bisulfite adduct (**1a**), D-mannonic-δ-lactam (**2**), D-gluconic-δ-lactam (**5**) and 1-β-D-glucosylamine against apricot β-glucosidase, *Trichoderma* β-glucosidase and rat epididymal α-mannosidase.

Compound	Concentrations required to cause 50% inhibition		
	β-Glucosidase		α-Mannosidase
	Apricot (M)	*Trichoderma* (M)	Rat epididymal (M)
1	1.0×10^{-5}	—	5.1×10^{-5}
1a	4.8×10^{-6}	1.8×10^{-3}	2.5×10^{-5}
2	1.1×10^{-6}	$>10^{-2}$	1.2×10^{-4}
5	1.2×10^{-4}	1.0×10^{-3}	1.5×10^{-3}
1-β-D-Glucosylamine	1.0×10^{-3}	2.0×10^{-6}	—

enzymes. The lactam (**2**) was even more active than **1a** against apricot emulsin, but was less active against α-mannosidase and no activity against *Trichoderma* β-glucosidase. When compared with the corresponding gluconolactam (**5**), **2** was 10- to 100-fold more active than **5** against emulsin and α-mannosidase but less active against *Trichoderma* β-glucosidase. In contrast, 1-β-D-glucosylamine used as a reference compound showed more potent activity against *Trichoderma* β-glucosidase than apricot β-glucosidase.

Nojirimycin B (**1**) showed weak activity against *Xanthomonas oryzae* on paper disk assay (inhibition zone 23.9 mm at 500 μg/ml), but no activity against other bacteria.

Discussion

Two kinds of nitrogen-containing sugar analogs have been reported as glycosidase inhibitors. The first group has a nitrogen in the ring, and the second group a nitrogen outside of the ring. They may be regarded as isosters of the ring oxygen and the glycosidic oxygen, respectively. Nojirimycin, 1-deoxynojirimycin and D-gluconic-δ-lactam belong to the first group, whereas 1-glycosylamines[10] and probably acarbose, oligostatin, valienamine, valiolamine belong to the second group.

Nojirimycin B (**1**), its bisulfite adduct (**1a**) and D-mannonic-δ-lactam (**2**), new members of the first group, are characterized by a D-mannose configuration. They showed potent inhibitory activity not only against rat epididymal α-mannosidase but also against apricot β-glucosidase. Notably, the lactam (**2**) is one of the most potent inhibitors against apricot emulsin so far reported.

Two interesting observations are made on the structure-activity relationships of nojirimycin B and its derivatives against apricot emulsin. First, the configuration at C(2) appears not to be important for the inhibitory activity against this enzyme, since nojirimycin B possessing the D-mannose configuration is comparable or slightly superior in activity to nojirimycin[11] possessing the D-glucose configuration.

Second, compound **2** with the twist-boat conformation showed more potent activity than did **1** with the chair conformation or **5** with the half-chair conformation[12]. This is worth noting, since this is not consistent with the half-chair ring form of inhibitors postulated as transition-state analogs[13,14].

On the other hand, **1a** and **2** showed only slight inhibitory activity against *Trichoderma* β-glucosidase, while 1-β-D-glucosylamine was more active against *Trichoderma* β-glucosidase than against apricot emulsin, indicating different modes of inhibitory action between two types of enzyme inhibitors.

The most potent inhibitor of α-mannosidase is compound **1**. Its bisulfite adduct (**1a**) and its lactam (**2**) are one-order of magnitude less active, as judged from the 50% inhibition concentration. Here, the importance of the substrate analogy including configurations at C(1) and C(2) is indicated for the inhibitory activity.

These results suggest that nojirimycin B and its lactam would be useful tools for study on the mode of action of various glycosidases.

Experimental

Melting points were determined with a Yamato capillary apparatus. Optical rotations were obtained with a Perkin-Elmer 141 polarimeter, UV spectra with a Hitachi 323 spectrometer, and IR spectra in KBr disks with a Hitachi 215 spectrometer. ^1H NMR spectra at 100 MHz were recorded on a Varian XL-100 spectrometer, and mass spectra on a Hitachi M-80 spectrometer. TLC was carried out by using silica gel plates F_{254} (E. Merck). Spots were detected by spraying with 10% sulfuric acid.

Isolation of 1

Streptomyces lavendulae SF-425 was fermented using a 300-liter fermentor according to the procedure previously reported[15]. The culture filtrate (150 liters) was applied onto a column of Amberlite IR-120 resin (H form, 15 liters). After washing with H_2O, the column was eluted with 0.5 N NH_4OH. The eluate was assayed for the inhibitory activity against apricot emulsin[16]. The bioactive fractions (30 liters) were concentrated to 2 liters, and was applied onto a column of Dowex 1X2 resin (OH form, 2 liters). The column was developed with H_2O. The bioactive fractions were combined, and concentrated to 570 ml, to which EtOH (230 ml) was added. Sulfur dioxide gas was slowly introduced under ice-cooling until nojirimycin bisulfite adduct crystallized out. The crystals (116 g) were separated, and the mother liquor and washings were combined, and concentrated to 290 ml, from which another crop (14.4 g) of nojirimycin bisulfite adduct was crystallized.

Concentration of the mother liquor and subsequent bubbling with sulfur dioxide afforded crystals of nojirimycin B bisulfite adduct (**1a**) (12.5 g). This was recrystallized from H_2O as colorless needles, mp 163~165°C (dec), $[\alpha]_D^{20}$ +4.6° (*c* 0.5, H_2O), no UV maximum in H_2O. The IR spectrum is illustrated in Fig. 1.

Anal Calcd for $C_6H_{13}NO_7S$: C 29.63, H 5.35, N 5.76, S 13.17.
Found: C 29.56, H 5.29, N 5.67, S 13.72.

Further crop (1.5 g) of **1a** was recovered from the mother liquor.

A free base of **1** was prepared from **1a** as follows. A mixture of **1a** (5.2 g) and an equivalent amount of $Ba(OH)_2$ in H_2O (150 ml) was stirred for 1 hour at room temp. The supernatant was saturated with CO_2 gas, and precipitates were removed by centrifugation. The supernatant was passed through a column of Dowex 1X2 resin (OH form, 50 ml), and the effluent was lyophilized to yield a pale yellow powder of **1** (3.4 g).

Preparation of 2 from 1 by Microbial Oxidation

G. suboxydans IAM 1829 was cultivated at 28°C for 42~50 hours using a medium consisting of sucrose 2%, peptone 0.5%, yeast extract 0.2%, sodium glutamate 0.2%, K_2HPO_4 0.2%, $CaCl_2$ 0.01% and $FeSO_4$ 0.001%. The washed cells (wet weight, 100 mg) thus obtained were added to 2% aqueous solution of **1** (200 mg) in 0.05 M phosphate buffer, pH 7.5. The suspension was shaken at 28°C for 5 hours. After centrifugation, the supernatant was subjected to a column chromatography over active carbon (30 ml) developed with H_2O. The effluent was assayed for inhibitory activity against apricot emulsin[16]. The active fractions were concentrated to give crystals of **2** (96 mg).

A large quantity of **2** was obtained from the crude preparation of nojirimycin containing **1** as follows. An aqueous solution of 2% crude nojirimycin (30 g) in 0.05 M phosphate buffer (pH 8.0) was incubated with the washed cells of *G. suboxydans* IAM 1829 (60 g) at 28°C for 5.5 hours in a 30-liter jar fermentor with an air flow rate of 0.5 vol/vol/minute. After centrifugation, the supernatant was successively passed through columns of Amberlite IR-120 (H form, 3 liters) and Amberlite IR-45 (OH form, 3.8 liters), and the active effluents were concentrated to crystallize **5** (18.7 g) that was derived from nojirimycin. The mother liquor was concentrated to a small volume, and applied onto a column of activated carbon (5.6×33 cm) developed with H_2O. Compound **2** with Rf 0.33 on TLC (BuOH - AcOH - H_2O, 3:1:1) was eluted after the elution of **5** with Rf 0.29. Concentration and addition of EtOH gave crystals of **2** (2.51 g), mp 169~170°C, $[\alpha]_D^{20}$ +1.6° (*c* 1.0, H_2O).

Anal Calcd for $C_6H_{11}NO_5$: C 40.67, H 6.21, N 7.91.
Found: C 40.53, H 6.28, N 7.54.

X-Ray Analysis

Three dimensional intensity data were collected using a Philips PW-1100 automatic four-circle

diffractometer with CuKα radiation. Totals of 896 and 1823 of independent reflections were collected for **2** and **4**, respectively.

The crystal structures were solved by the multi-solution method (MULTAN). The location of a bromine atom of **4** was determined by the heavy atom procedure. The positional parameters of non-hydrogen atoms were refined by the difference Fourie synthesis and the least-square method. Final refinement was performed by the block-diagonal least-square method with correction of anisotropic thermal parameters for non-hydrogen atoms and isotropic thermal parameters for hydrogen atoms. This gave R values of 0.037 and 0.106 for **2** and **4**, respectively. The positional parameters are deposited with the Cambridge Crystallographic Data Center.

Enzyme Assay

The inhibitory activity against apricot emulsin and *Trichoderma* β-glucosidase was measured as described previously[16]. *p*-Nitrophenyl β-D-glucoside was used as a substrate. Preparation of rat epididymal α-mannosidase: Five adult Donryu rats were sacrified, and 4.4 g of epididymides were obtained. The tissue was cut into small pieces and homogenized in three volumes of saline. After incubation at 38°C for 1 hour, the autolyzed homogenate was kept at −20°C overnight, thawed at room temp, and centrifuged. The resulting supernatant was stored at 5°C, and used as a crude enzyme solution. The α-mannosidase activity was retained for at least a week.

Assay for α-Mannosidase-inhibitory Activity: The crude enzyme solution diluted 30 times with 0.1 M acetate buffer (pH 5.2) was used. A reaction mixture consisted of 0.5 ml of 0.005 M *p*-nitrophenyl α-D-mannoside, 1 ml of 0.1 M acetate buffer (pH 5.2) containing inhibitor and 0.5 ml of the diluted enzyme solution. After incubation for 30 minutes at 37°C, 5 ml of 0.1 M sodium carbonate were added and *p*-nitrophenol liberated was measured colorimetrically at 400 nm.

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