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Inhibition of Intestinal α-Glucosidase Activity and Postprandial Hyperglycemia by Moranoline and Its N-Alkyl Derivatives

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Moranoline (1-deoxynojirimycin) isolated from mulberry root bark (Mori Cortex), is a potent intestinal α-glucosidase inhibitor. The IC_{50} values for sucrase and maltase in various animals ranged around 10^{-7}M. Postprandial hyperglycemia in sucrose-, starch-, or maltose-loaded rats was significantly supressed by simultaneous administration of moranoline in doses over 6 mg/kg. In contrast to the potent inhibition of intestinal α-glucosidase, the inhibition of β-glucosidase, glucoamylase, and α-amylase was weak.

Among the N-substituted alkyl derivatives of moranoline, the methyl and ethyl derivatives had more potent hypoglycemic activity than moranoline in sucrose- or starch-loaded rat models. Nojirimycin, or 2,5-dihydroxymethyl 3,4-dihydroxypyrrolidine (DMDP), which structurally resembles moranoline, only weakly inhibited α-glucosidase but strongly inhibited β-glucosidase.

Postprandial hyperglycemia in diabetic patients can be avoided only with strict dietary control. Non-enzymatic glycosylation of various plasma and tissue proteins proceeds actively during periods of hyperglycemia. The dysfunction of these glycosylated proteins seems to be the cause of many of the lesions which develop gradually in patients with diabetes mellitus.[1]

A new approach to prevent postprandial hyperglycemia may emerge from the therapeutic use of α-amylase or α-glycosidase inhibitors.[2] In the course of screening for inhibitors of intestinal α-glucosidase, 1-deoxynojimycin, designated "moranoline," has been isolated from Mori Cortex (root bark of the mulberry tree),[3,4] leaves of *Jacobinia suberecta*,[5] and a culture filtrate of *Streptomyces* species.[6] This compound was obtained from Nojirimycin[7] by catalytic degradation.[8] Soon after the first finding of moranoline in nature by Murai *et al.*,[3] two other research groups, Frommer *et al.* and Murao and Miyata, isolated this compound independently from the culture filtrate of a *Bacillus* species,[9,10] and a *Streptomyces Species*,[11] respectively.

This paper describes the inhibition by moranoline[12] and its N-alkyl derivatives[13] of glucosidase activity and of the postprandial rise of blood glucose.

MATERIALS AND METHODS

Chemicals. Moranoline (1-deoxynojirimycin, M.W. 163.2, Fig. 1) was isolated from Mori Cortex.[3,4] N-substituted alkyl derivatives of moranoline were synthesized in the research laboratories of Nippon Shinyaku Co., Ltd.[13] Nojirimycin and nojirimycin bisulfate were synthesized in the same laboratories by the method of Inouye *et al.*[8] 2,5-Dihydroxymethyl 3,4-dihydroxypyrrolidine (DMDP) was isolated from *Derris elliptica*.[14]

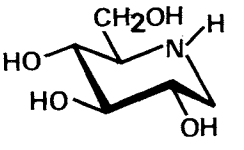

FIG. 1. Moranoline.
1-Deoxynojirimycin.

Synthesis of N-substituted alkyl derivatives of moranoline. While a number of procedures are available for synthesizing the contemplated compounds of this study, the commonest procedure is alkylation of the corresponding N-norcompound, moranoline.

This alkylation, in turn, may be done by various alternative procedures, for example by reacting moranoline with an alkyl halide, by N-acylating moranoline and then reducing the N-acyl derivative to the N-alkyl compound, and by reductive alkylation using a carbonyl compound. It is also advantageous to start with moranoline having suitably protected hydroxyl groups such as benzyl, benzoyl, or acetyl, in which case the N-alkylation reaction is followed by removal of the protective groups.

The following examples are intended to further describe the method by which the compounds of this study are produced.

N-Methyl derivative; in a mixture of 7 ml of aqueous formalin and 15 ml of formic acid, moranoline (mp 204~205°C; $[\alpha]_D^{24}+45°$ (H_2O)) (1.5 g) is heated under reflux for 20 hr. The reaction mixture is then concentrated to dryness under reduced pressure, the residue is dissolved in water, and the insolubles are removed. The filtrate thus obtained is passed over an ion exchange resin column (Dowex 50 W × 4) and, after the column is rinsed with water, the absorbed material is eluted with 0.28% aqueous ammonia. The eluate is concentrated to dryness under reduced pressure and the crystalline residue is recrystallized from ethanol, whereby the contemplated compound is obtained, mp 142~143°C, $[\alpha]_D^{24}+15.5°$ (H_2O), yield 1.2 g. Treatment with an equimolar amount of *p*-toluenesulfonic acid gives the corresponding *p*-toluenesulfonate which, in turn, is recrystallized from methanol, mp 198~199°C, $[\alpha]_D^{24}+12.2°$ (methanol).

N-Propyl derivative; 1.0 g of benzyl derivative of moranoline (mp 44~47°C) is dissolved in 50 ml of DMF, then 3.0 g of anhydrous potassium carbonate and 2.0 g of propyl bromide are added. The mixture is stirred with heating at 60±3°C for 12 hr, after which it is diluted with water and extracted with benzene. The extract is dissolved in a mixture of 10 ml of 47% hydrobromic acid and 7 ml of acetic acid, then heated at 100°C for one hour. After cooling, 100 ml of methanol is added into the reaction mixture, and the solution is hydrogenated over 300 mg of 5% palladium-charcoal. After this catalytic reduction has been completed, the catalyst is filtered off and the filtrate is concentrated to dryness under reduced pressure. The residue is dissolved in water and the solution is passed over an ion exchange resin column (Dowex 50 W × 4). The effluent is distilled under reduced pressure to recover 205 mg of the contemplated compound as a colorless oil. This product is converted to the *p*-toluenesulfonate which, in turn, is recrystallized from isopropyl alcohol, mp 208~211°C, $[\alpha]_D^{24}+0.4°$ (methanol).

N-Isobutyl derivative; 1.0 g of the hydrochloride of benzyl derivative of moranoline (mp 185~189°C) is dissolved in 20 ml of pyridine. Following the addition of 1.1 g of isobutyryl chloride, the solution is reacted at room temperature for 24 hr. Then the reaction mixture is concentrated to dryness under reduced pressure, the residue is extracted with ether, and the ethereal layer is washed with diluted alkali and acid. The ether is then distilled off, whereupon 1.1 g of the N-isobutyryl derivative is obtained as a colorless oil. IR spectrum: ν_{max} (film) 1670 cm^{-1}. This isobutyryl derivative is, without further purification, reduced in 20 ml of tetrahydrofuran using 500 mg of lithium aluminum hydride, with heating and stirring for 2 hr and the reaction mixture is treated in the usual manner. The resultant colorless oil is dissolved in a mixture of 10 ml of 47% hydrobromic acid and 10 ml of acetic acid and heated at 100°C for one hour. Then the mixture is diluted with 100 ml of methanol followed by the catalytic reduction over 300 mg of 5% palladium-charcoal.

Thereafter, the reaction mixture is treated as was the N-propyl derivative and the resultant oil is converted to the corresponding *p*-toluenesulfonate which, in turn, is recrystallized from isopropanol, mp 192~194°C, $[\alpha]_D^{25}$ −8.3° (methanol), yield 0.46 g.

Preparation and assay of intestinal α-glucosidases. The following male animals were used for sources of enzymes: ddY mice, 10 weeks old (Shizuoka Laboratory Animal Center); KK mice, 3 months old (Nippon Shinyaku); Sprague Dawley (SD) rats, 11 weeks old (Shizuoka Laboratory Animal Center); SD rats with streptozotocin-induced diabetes, 9 weeks old (STZ rats, injected intraperitoneally with 40 mg/kg of streptozotocin at 5 weeks old after overnight starvation); JW/NIBS rabbits, 6 months old (Rabbiton Institute Inc.); one beagle dog, 14 months old (Laboratory Research Enterprise); one rhesus monkey, 13 years old (Nippon Shinyaku).

Intestinal α-glucosidases (α-D-glucosidase, EC 3.2.1.20) were prepared by the method of Takesue[15] or of Kolínská and Kraml.[16] The small intestines of various animals were rinsed in cold saline, the mucosa was scraped off and homogenized with 5 volumes of 0.5 M NaCl–0.5 M KCl–5 mM EDTA (pH 7.0) using a Polytoron® (Kinematica). The homogenate was centrifuged at 60,000 × *g* for 60 min, the supernatant was discarded, and the sediment was washed with 10 m M potassium phosphate buffer (pH 7.4) and centrifuged again. The sediment was incubated with 10 m M potassium phosphate buffer (pH 7.0) containing 0.01% papain and cysteine for 60 min at 37°C. After papain treatment, the 105,000 × *g* (60 min) supernatant was fractionated with ammonium sulfate (45~60% saturation). The dialyzed precipitate was used for the assay.

Disaccharidase activity was measured by the method of Dahlqvist.[17] Eighty μl of inhibitor solution and 20 μl of enzyme solution from various animals (0.2~0.6 mU/μl, 1 U = 1 μmol substrate hydrolyzed/min) were added to 100 μl of 100 m M sodium phosphate buffer (pH 6.0) containing 56 m M sucrose or maltose as the substrate. The reaction mixture was incubated for 15 min at 37°C. The

reaction was stopped by the addition of 400 μl of 0.3 N barium hydroxide and 400 μl of zinc sulfate heptahydrate. Liberated glucose was measured with a glucose oxidase-peroxidase reagent (New Blood Sugar Test®, Boehringer Mannheim-Yamanouchi).

Protein was measured by the method of Lowry *et al.* with bovine serum albumin as the standard.

β-Glucosidase assay. β-Glucosidase (β-D-glucosidase, EC 3.2.1.21) activity was measured by the method of Niwa *et al.*[18] with β-glucosidase from sweet almond (Miles Laboratories): 200 μl of 0.01 M *p*-nitrophenyl-β-D-glucoside (Nakarai Chemicals), 500 μl of 0.2 M acetate buffer (pH 4.5), 100 μl of inhibitor solution, either water or methanol, and 200 μl of enzyme solution (30 μg/ml). After incubation for 15 min at 30°C the reaction was stopped by adding 2 ml of 0.1 M sodium carbonate. Liberated *p*-nitrophenol was measured by the optical density at 400 nm.

Glucoamylase assay. Starch or maltose was used as the substrate for glucoamylase (exo-1,4-α-D-glucosidase, EC 3.2.1.3) from *Rhizopus niveus* (Seikagaku Kogyo).

In the starch substrate experiment, 150 μl of 1.6% soluble starch (Nakarai Chemicals) dissolved in 0.1 M of heated phosphate buffer (pH 5.7) was mixed with 100 μl of inhibitor solution and 250 μl of enzyme solution (50 μg/ml). After incubation for 10 min at 40°C the reaction was stopped by the addition of 1 ml of 0.3 N barium hydroxide and 1 ml of 5% zinc sulfate heptahydrate. Liberated glucose was measured enzymatically as described above.

In the maltose substrate experiment, 300 μl of 33 mM maltose (Nakarai Chemicals) in 0.2 M acetate buffer (pH 4.5), 100 μl of inhibitor solution and 100 μl of enzyme solution (100 μg/ml) were mixed and incubated for 20 min at 40°C. The subsequent procedure was the same as with the starch substrate.

Amylase assay. Amylase activity was measured by Fuwa's blue value method[19] with α-amylase (EC 3.2.1.1) from *Aspergillus oryzae* (Taka amylase, Sankyo) and porcine pancreas (Sigma). Amylose solution (0.2% of Amylose B, M. W. 16,000, Nakarai Chemicals) was used as the substrate.

Carbohydrate loading test. Test compounds and either 2 g/kg of soluble starch (Kanto Chemicals), maltose (Nakarai Chemicals), or sucrose (Nakarai Chemicals) were given simultaneously by stomach tube to 5-week-old SD male rats, after overnight starvation. Blood was taken from the tail vein, and blood glucose levels were measured enzymatically (New Blood Sugar Test®, Boehringer Mannheim-Yamanouchi) and recorded as mean ± S.E. The increment of the area under the blood glucose curve (ΔAUC) was calculated by the following equation, and the dose required for 50% inhibition of ΔAUC (ED_{50}) was calculated for each test compound.

$$\Delta AUC\ (\text{min}\cdot\text{mg/dl}) = 1/2\ [(a+b)\cdot t_1 + (b+c)\cdot t_2 + \cdots]$$

$a, b, c, \cdots$, differences of blood glucose levels between initial time and the indicated time ($a=0$)

$t_1, t_2, \cdots$, differences between two consecutive times

RESULTS

Intestinal α-glucosidase inhibition by moranoline

Specific activities of partially purified intestinal α-glucosidases are shown in Table I.

TABLE I. ENZYME ACTIVITIES OF SMALL INTESTINAL α-GLUCOSIDASES

	ddY mouse	KK mouse	SD rat	STZ rat	JW rabbit	Beagle dog	Rhesus monkey
Sucrase	1.2	1.2	3.0	1.9	3.4	0.2	0.4
Maltase	1.9	2.1	11.9	5.7	8.6	0.3	0.7

Figures represent units/mg protein.

TABLE II. MOLAR CONCENTRATION OF MORANOLINE REQUIRED FOR 50% INHIBITION OF INTESTINAL α-GLUCOSIDASES

α-Glucosidase	ddY mouse	KK mouse	SD rat	STZ rat	JW rabbit	Beagle dog	Rhesus monkey
Sucrase	1.4×10^{-7}	9.6×10^{-8}	6.6×10^{-8}	1.2×10^{-7}	1.6×10^{-7}	1.2×10^{-7}	1.2×10^{-7}
Maltase	1.4×10^{-7}	7.4×10^{-8}	1.7×10^{-7}	6.3×10^{-7}	2.3×10^{-7}	1.8×10^{-7}	1.0×10^{-7}

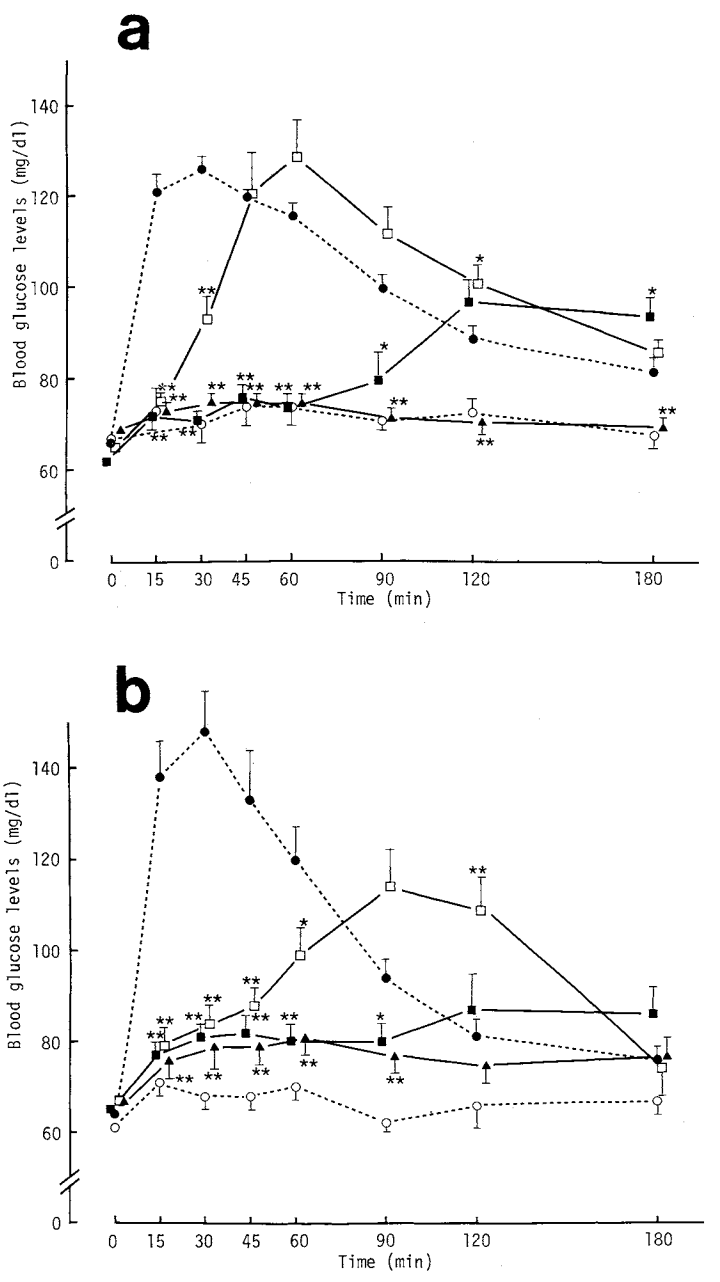

FIG. 2.

Maltase activity was higher than sucrase activity in all the species or strains tested. Rats and rabbits had higher maltase and sucrase activities than any of the other animals.

The concentrations of moranoline needed for 50% inhibition (IC_{50}) of various intestinal α-glucosidases are shown in Table II. IC_{50} values were $6.6 \sim 16 \times 10^{-8}$ M and $7.4 \sim 63 \times 10^{-8}$ M for sucrase and maltase, respectively.

Effects of moranoline on blood glucose levels in carbohydrate-loaded rats

Figure 2 shows the effects of moranoline on

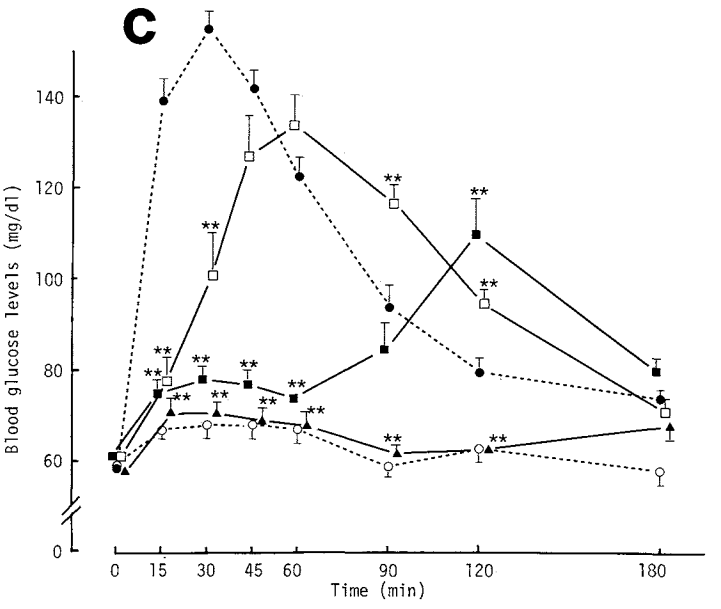

FIG. 2. Effects of Moranoline on Postprandial Blood Glucose Levels in Carbohydrate-loaded Rats

Moranoline and either 2 g/kg of sucrose, soluble starch, or maltose were given simultaneously by stomach tube to 5-week-old rats after overnight starvation. Blood was taken from the tail vein, and blood glucose levels were measured enzymatically.

Panel a, sucrose load (2 g/kg): ○---○, basal ($n=11$); ●---●, control ($n=13$); □—□, 6 mg/kg ($n=10$); ■—■, 20 mg/kg ($n=11$); ▲—▲, 60 mg/kg ($n=12$).

Panel b, starch load (2 g/kg): ○---○, basal ($n=10$); ●---●, control ($n=9$); □—□, 10 mg/kg ($n=9$); ■—■, 20 mg/kg ($n=10$); ▲—▲, 40 mg/kg ($n=10$).

Panel c, maltose load (2 g/kg): ○---○, basal ($n=9$); ●---●, control ($n=9$); □—□, 6 mg/kg ($n=8$); ■—■, 20 mg/kg ($n=8$); ▲—▲, 60 mg/kg ($n=8$).

* $p<0.05$, ** $p<0.01$ by t-test.

the blood glucose change in rats given sucrose, starch, or maltose. Moranoline, in a dose of 60 mg/kg, completely inhibited the increase of postprandial blood glucose when sucrose (2 g/kg) was given simultaneously (panel a). Twenty mg/kg of moranoline significantly reduced the blood glucose level for 90 min. At a lower dose (6 mg/kg), moranoline delayed the peak time without affecting its height. In starch-loaded rats, moranoline delayed the peak time and lowered the height of the blood glucose level in a dose-dependent manner (panel b). Panel c shows the effects of moranoline in maltose-loaded rats. A similar dose-dependent inhibition was observed.

No toxic symptom including diarrhea was observed at the doses tested here.

Glucosidase inhibitory and hypoglycemic activities of N-alkyl derivatives of moranoline

Table III shows the enzyme inhibitory and hypoglycemic activities of the various N-alkyl derivatives of moranoline (**1**) as well as of nojirimycin and DMDP. Potent inhibition of rabbit intestinal sucrase and maltase was observed with the compounds in which the carbon numbers of the alkyl chain were less than 13; among them, (**1**) and N-methylmoranoline (**2**) were the most active. The higher N-alkyl derivatives (**12**, **13**) were less active. Nojirimycin and its stable derivative, bisulfite, were comparably inhibitory to moranoline derivatives. DMDP was far less active.

Inhibition of ΔAUC for up to 180 min at a dose of 10 mg/kg of each compound was calculated when sucrose (2 g/kg) was given orally to

TABLE III. GLUCOSIDASE INHIBITORY AND HYPOGLYCEMIC ACTIVITIES OF N-ALKYL DERIVATIVES OF MORANOLINE

Compounds	R	Sucrase[a]	Maltase[a]	Sucrose load[e]		Starch load[f]		β-Glucosidase[b]	Glucoamylase[c]		α-Amylase[d]	
		IC_{50} μg/ml	IC_{50} μg/ml	Inh.[g] %	ED_{50} mg/kg	Inh.[g] %	ED_{50} mg/kg	IC_{50} μg/ml	IC_{50} starch[l]	(μg/ml) maltase[l]	Conc. mg/kg	Inh. %
1	H	0.067	0.17	23	21	36	16	272	9.5	38	8.9	50
2	CH_3	0.068	0.46	62	5.8	52	11	363	1.6	4.7	17.5	6
3	C_2H_5	0.15	0.42	80	5			395	6.9	25	1	n.d.
4	C_3H_7[m]	0.54	4.1	29				9%[h]	56	48	1	n.d.
5	$CH_2CH(CH_3)_2$[m]	1.3	4.9	9				9%[h]	200	160	1	n.d.
6	C_6H_{13}[m]	0.29	5.5	39	13			99	145	3.2	1	n.d.
7	C_8H_{17}[m]	0.23	1.6	36				86	90	52	1.75	n.d.
8	C_9H_{19}[m]	0.35	3.4	38				21%[h]	73	41	1.75	n.d.
9	$C_{10}H_{21}$[m]	0.31	3.2	26				47%[h]	68	44	1.75	n.d.
10	$C_{11}H_{23}$[m]	0.40	4.4	n.d.				28%[h]	145	165	1.75	13
11	$C_{12}H_{25}$[m]	0.81	1.8	11				31%[h]	41%[i]	39%[i]	1.75	20
12	$C_{16}H_{33}$[m]	8.0	10	22				31%[j]	11%[k]	n.d.[k]	1.75	27
13	$C_{18}H_{37}$[n]	n.d.[j]	n.d.[j]	11				29%[j]	n.d.[k]	n.d.[k]	1.75	13
Nojirimycin		0.55	0.83					2.1	143	230	5.9	31
Nojirimycin bisulfide		0.51	1.4	32	69			4.1	200	13%[i]	10	6
DMDP		31	53	24				6.4	2	7	1.75	10

[a] Rabbit intestine. [b] Sweet almond. [c] *Rhizopus niveus*. [d] *Aspergillus oryzae*.
[e] Inhibition of ΔAUC up to 180 min when sucrose (2 g/kg) was given to rats.
[f] Inhibition of ΔAUC up to 180 min when starch (2 g/kg) was given to rats.
[g] Inhibition at 10 mg/kg. [h] Inhibition at 100 μg/ml. [i] Inhibition at 200 μg/ml. [j] Inhibition at 10 μg/ml.
[k] Inhibition at 20 μg/ml. [l] Substrate. [m] Tosylate. [n] Hydrobromide salt. n.d., no detectable inhibition.

rats, and the ED_{50} was also calculated for some of the compounds. Compounds with potent sucrase inhibitory activity also tended to reduce ΔAUC greatly. Nojirimycin bisulfite and DMDP were intermediate in inhibiting ΔAUC. Inhibition of ΔAUC and ED_{50} in starch-loaded rats was also measured for (**1**) and (**2**). The order of activity of these compounds was similar to that in the sucrose-loaded model but different from that in *in vitro* maltase inhibition.

Moranoline and its N-alkyl derivatives showed only very weak β-glucosidase inhibition. In contrast, nojirimycin and nojirimycin bisulfite strongly inhibited β-glucosidase activity, as reported earlier.[18] Moranoline or its lower alkyl derivatives greatly inhibited the activity of glucoamylase from *Rhizopus niveus* when either starch or maltose was used as the substrate. Such potent inhibition of glucoamylase was very different from that by nojirimycin which showed a comparable IC_{50} to that already reported.[18] Glucoamylase inhibition of moranoline derivatives varied randomly, with IC_{50} values ranging from 1.6 to 200 μg/ml. DMDP was one of the most potent glucoamylase inhibitors examined here. Moranoline derivatives showed little or no inhibition of α-amylase activity.

DISCUSSION

Moranoline is a naturally occurring α-glucosidase inhibitor. Soon after the first isolation of moranoline from the root bark of mulberry trees,[3,4] Schmidt *et al.* isolated this compound from a culture filtrate of a *Bacillus* species and reported its inhibition of porcine intestinal sucrase, maltase, isomaltase, and glucoamylase.[10] Meanwhile, Murao and Miyata also reported glucosidase inhibition by this compound isolated from a culture filtrate of a *Streptomyces* species in various animals, plants, and microorganisms.[11] Moranoline strongly inhibited all the animal intestinal sucrase and maltase activites tested here. The IC_{50} values were around 10^{-7} M, similar to the values reported previously.[10,11,20]

To confirm their inhibitory action *in vivo*, the hypoglycemic action of these compounds was examined in sucrose-, maltose-, or starch-loaded rats. The rise of blood glucose levels in carbohydrate-loaded rats was significantly suppressed by the simultaneous administration of moranoline in a dose of 10 mg/kg. In a dose of 20 mg/kg of moranoline, the blood glucose levels were higher than those of the control at later stages (120 min or 180 min) though the peak height was lower than that of the control. This phenomenon might be explained by the reduction of the amount of moranoline in the intestine with time. Because moranoline is an absorbable compound (unpublished data), the inhibitory action of intestinal α-glucosidases may not continue for a long time. It is estimated that the hydrolysis of undigested maltose or sucrose lowers the blood glucose peak at the later stages. At a higher dose of moranoline (60 mg/kg), the blood glucose peak did not appear up to 180 min. It was shown that the α-glucosidase inhibitor, moranoline, can completely suppress postprandial hyperglycemia in starch-loaded animals without α-amylase inhibition. The results of this study prove the critical importance of intestinal α-glucosidases in starch digestion.

To obtain more potent compounds, N-alkyl derivatives of moranoline were synthesized. N-methyl (**2**) and N-ethyl (**3**) derivatives were more potent inhibitors of postprandial hyperglycemia in sucrose-loaded rats.

Structurally similar nojirimycin and DMDP had different inhibitory patterns from those of moranoline and its N-alkyl derivatives against α- or β-glucosidases or glucoamylase.

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