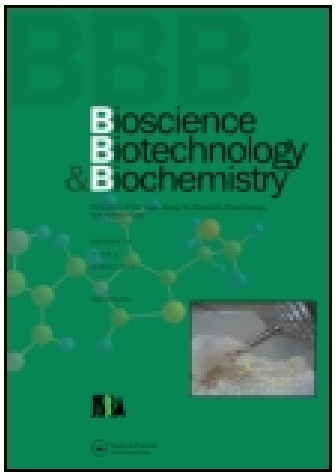

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Formation of β-Fructosyl Compounds of Pyridoxine in Growing Culture of *Aspergillus niger*†

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Two pyridoxine compounds were found to be formed in a culture filtrate of *Aspergillus niger* and *A. sydowi*, when grown in a medium containing sucrose and pyridoxine. Each of the two compounds I and II was obtained as a white powdered preparation by preparative paper chromatography, gel filtration on Toyopearl HW-40S and Sephadex G-10 columns, DEAE-cellulose column chromatography, and lyophilization. Compounds I and II were identified as 5′-*O*-(β-D-fructofuranosyl)-pyridoxine and 5′-*O*-[β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl]-pyridoxine, on the basis of the various experimental results, *viz.*, elementary analyses, UV, ^{1}H-, and ^{13}C-NMR spectra, products by hydrolysis with acid and yeast β-D-fructofuranosidase, migration on paper electrophoresis, and Gibbs reaction in the presence and absence of boric acid. Levansucrase from *Microbacterium laevaniformans* and yeast β-D-fructofuranosidase did not catalyze the β-D-fructofuranosyl transfer from sucrose to pyridoxine to give rise to β-D-fructofuranosyl-pyridoxine.

In our previous paper,[1] we reported a remarkable formation of two β-galactosylpyridoxines and a β-galactobiosylpyridoxine from lactose and pyridoxine in a growing culture of *Sporobolomyces singularis*. These three compounds were isolated in crystalline forms from the culture filtrate and identified as 4′-*O*-(β-D-galactopyranosyl)-pyridoxine, 5′-*O*-(β-D-galactopyranosyl)-pyridoxine, and 4′-*O*-[β-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl]-pyridoxine. On the other hand, 5′-*O*-(α-D-glucopyranosyl)-pyridoxine and 4′-*O*-(α-D-glucopyranosyl)-pyridoxine, new derivatives of vitamin B_6, were isolated at first by Ogata *et al.* from the culture filtrate of *Sarcina lutea* which was grown on a medium containing sucrose and pyridoxine.[2–4] Thereafter, we reported the formation of 4′-*O*-(β-D-glucopyranosyl)-pyridoxine and 5′-*O*-(β-D-glucopyranosyl)-pyridoxine from cellobiose and pyridoxine by wheat bran β-glucosidase[5] and also the accumulation of the two β-glucosylpyridoxines in plant seedlings, calluses, and cells cultured in a pyridoxine solution.[6,7] Furthermore, 5′-*O*-(β-D-glucopyranosyl)-pyridoxine[8,9] and three pyridoxine β-cellooligosaccharides[10] were found in rice bran and plant-derived foods. However, a fructosylpyridoxine has been neither chemically nor biologically synthesized. During our investigation on the formation of fructosylpyridoxine by microorganisms having oligo- and poly-fructan-forming activities, it was found that fructosyl compounds of pyridoxine were formed in the culture filtrate of *Aspergillus* spp. which were grown in the medium containing sucrose as a carbon source and pyridoxine. This paper deals with the formation of fructosyl compounds of pyridoxine, their isolation and characterization.

Materials and Methods

Materials. *Aspergillus niger* ATCC 20611 and *Microbacterium laevaniformans* ATCC 15953 were obtained from the American Type Culture Collection, Maryland, U.S.A. *Aspergillus sydowi* IAM 2544 was kindly supplied from the Institute of Applied Microbiology, Tokyo University, Tokyo. Seeds of *Asparagus officinalis*, *Lactuca sativa*, and *Allium cepa* were obtained from Yamato Plantation Co., Ltd., Tenri.

Chemicals. Pyridoxine·HCl was obtained from Nacalai tesque, Inc., Kyoto. Yeast β-D-fructofuranosidase (Boehringer Mannheim Japan, Tokyo) was purchased through commercial routes. 1-Kestose (Tokyo Kasei Kogyo Co., Ltd., Tokyo) was obtained and purified two times with ethanol. Other reagents used were of analytical grade from commercial sources.

Cultivation. *Aspergillus* spp. were grown at 30°C for 5–6 days in the dark with shaking on a culture medium (adjusted to pH 5.7) containing: sucrose, 10 g; NH_4NO_3, 0.1 g; $NaNO_3$, 0.1 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; KCl, 0.05 g; pyridoxine·HCl, 1 g as pyridoxine, and $CaCO_3$, 2 g in 100 ml of deionized water. Sucrose and $CaCO_3$ were sterilized separately, before inoculation. After 4 days of cultivation, the mycelia were removed by filtration. The filtrate was used for the isolation of pyridoxine compounds. Intact cells and cell-free levansucrase of *M. laevaniformans* were prepared as follows. The bacterium was cultivated at 30°C for 2 days with shaking on a culture medium (adjusted to pH 6.85) containing: sucrose, 2 g; meat extract, 1 g; peptone, 1 g; NaCl, 0.5 g; and yeast extract, 0.5 g in 100 ml of tap water. After cultivation, the cells were harvested by centrifugation, washed 2 times with 0.85% NaCl, till relatively free of adhering levan, and suspended in the same solution. A part of these intact cells, after centrifugation, was ground with sea sand in a chilled mortar. The mass was centrifuged at 10,000×*g* for 20 min, and the resultant opalescent yellowish supernatant was used as the crude levansucrase preparation.

Plant seedlings. Seeds were soaked in a disinfectant solution (0.25% Homai WP, Nippon Soda Co., Ltd., Tokyo) for 5 h at room temperature. After the seeds were thoroughly rinsed in running water, they were sowed in vermiculite placed on plastic plates. The seeds were germinated at 25°C in the dark. The sterilized and deionized water or a 10 mM pyridoxine·HCl solution (neutralized to pH 4.8) was sprinkled on each plastic plate every other day.

Analyses.

Assays of pyridoxine compounds. A suitable amount of culture filtrate and germinating seed extract was applied as a band on a Toyo filter paper No. 50 (40×40 cm), and developed by ascent in *n*-butanol–pyridine–water (6:4:4, v/v) (solvent A). After this was dried, purplish

† A part of this investigation was presented at the Annual Meetings of the Agricultural Chemical Society of Japan at Niigata, April, 1989.

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fluorescent bands of pyridoxine and its compounds were detected on chromatogram under a ultraviolet ray lamp (2537 Å, filter), and extracted with 0.1 M phosphate buffer, pH 6.8, for 3 h at 37°C. The amount of pyridoxine compound in each extract was measured by the diazotized *p*-aminoacetophenone method with slight modification.[3]

Enzymatic and acid hydrolysis. The reaction mixture (1 ml) containing the isolated pyridoxine compound (4 mg), 5 units of β-D-fructofuranosidase (invertase), and 0.1 M sodium phosphate buffer, pH 6.8, was incubated for 20 h at 37°C in the dark. Control experiments were done with pyridoxine (2.5 mg). The amounts of pyridoxine and sugar (fructose) released were estimated by the diazotized *p*-aminoacetophenone method[3] and the method of Nelson,[11] respectively. In acid hydrolysis, the isolated compound was heated in a boiling water bath with 0.1 N HCl for 1 h in the dark, and then concentrated to dryness *in vacuo*.The hydrolyzate was analyzed by the same techniques as described in the enzymatic hydrolysis.

Instrumental analyses. UV spectra were recorded on a Hitachi EPS-3T spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were measured on a Varian VXR-500 spectrometer at 25°C in D_2O at 500 MHz with sodium 3-(trimethylsilyl)-1-propanesulfonate as an internal standard. The spectra of acetyl compound was obtained with $CDCl_3$ as solvent and tetramethylsilane as an internal standard.

Results

Formation of new compounds of pyridoxine by several microorganisms having sucrose-metabolizing activities

Fermentation experiments were done at 30°C in the dark on a 10% sucrose medium containing 1% pyridoxine with shaking with *A. niger* ATCC 20611 and *A. sydowi* IAM 2544, which had the activity of β-(2→1)-linked fructan formation.[12-14] As shown in Table I, both strains produced two new compounds I and II which showed lower R_f values (0.68 and 0.45) than that of pyridoxine (0.80) on a paper chromatogram developed with the solvent system of *n*-butanol–pyridine–water (6:4:3, v/v) (solvent B). *A. niger* had higher forming activities of the compounds and also a lighter colored 4-day culture filtrate than those of *A. sydowi.*

On the other hand, no formation of new compounds of pyridoxine was observed in a reaction mixture containing 500 mg of sucrose, 200 mg of neutralized pyridoxine·HCl (about 1.2 mmol as pyridoxine), 5 ml of 0.2 M sodium phosphate buffer, pH 6.8, and 200 mg as dry weight of intact cells of *M. laevaniformans* in a total volume of 10 ml, after incubation at 25°C for 20 h in the dark on a shaker. Under the same reaction conditions, a remarkable production of polysaccharide (levan) took place. The formation of the pyridoxine compounds was also not detected, when examined similarly using a cell-free levansucrase preparation from the bacterium. Yeast β-D-fructofuranosidase did not transfer a β-D-fructofuranosyl residue of sucrose to pyridoxine, when incubated at 30°C for 0.5–20 h with reaction mixtures containing 100 mg of sucrose, 40 mg of neutralized pyridoxine·HCl, 1 ml of 0.1 M sodium phosphate buffer, pH 6.8, and 0.5–5 units of enzyme in a total volume of 2 ml. Thus, *A. niger* was used in the isolation of new compounds as follows.

Isolation of compounds I *and* II

To the culture filtrate (2 liter) of *A. niger* ATCC 20611, which was cultivated for 4 days on a 10% sucrose medium with 1% pyridoxine, one volume of methanol was added, and the mixture was adjusted to pH 4.8, heated for 10 min in a boiling water bath, and centrifuged. The supernatant solution was concentrated below 30°C *in vacuo*. A part of the concentrate, after adjustment of pH to 2.0, was applied on a Dowex 50W-X8 (H^+ form) column, washed successively with 0.01 N HCl and water, and eluted with 1% NH_4OH at 4°C. The eluate was observed to contain a trace of compounds (**I** and **II**) and lots of pyridoxine. The compounds were easily hydrolyzed to pyridoxine during the absorption on the column (H^+ form), although both β-glucosylpyridoxines and β-galactosylpyridoxines were stable under the same column chromatographic conditions, as described in our previous papers.[1,7] Therefore, most parts of the concentrate were applied to the 1st paper chromatography (PPC) with Toyo filter paper No. 50 in solvent B. Figure 1 showed that two purplish fluorescent compounds **I** and **II** having lower R_f values than that of pyridoxine on chromatogram could be separated from sugars by PPC. The fluorescent band of compound **I** (major one) was cut out, and extracted with acetic acid aqueous solution, pH 4.8. The extract, after concentration, was reapplied to the 2nd PPC with solvent A, to separate completely compound **I** from both compound **II** and pyridoxine. After appropriate sectioning and elution, compound **I** solution was concentrated, subjected to the 1st gel-filtration on Toyopearl HW-40S column (5.6 × 120 cm) and eluted with water. The fractions of compound I were

Table I. Formation of Pyridoxine Compounds during Fermentation

Strain	Days of cultivation	Compound I	Compound II
		(mg as PN/100 ml of culture filtrate)	
A. niger	2	86	+
ATCC 20611	4	131	33
	6	60	0
A. sydowi	2	108	+
IAM 2544	3	72	0
	5	+	0

PN, pyridoxine; +, trace.

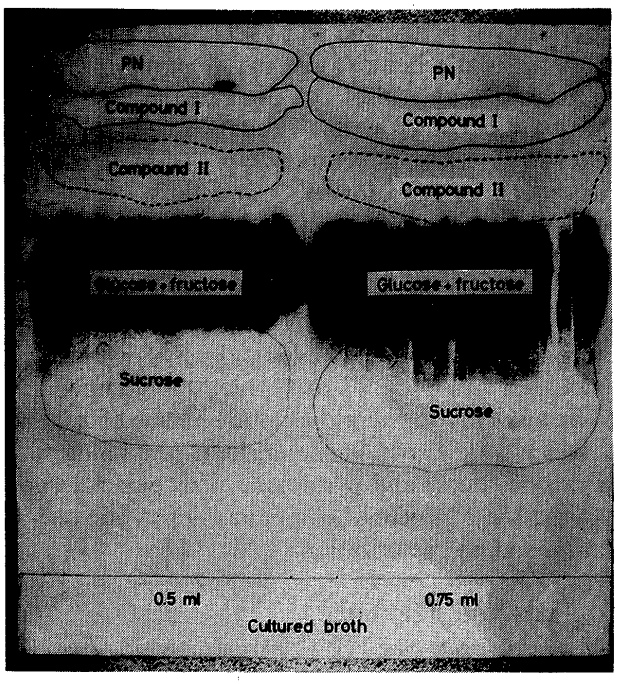
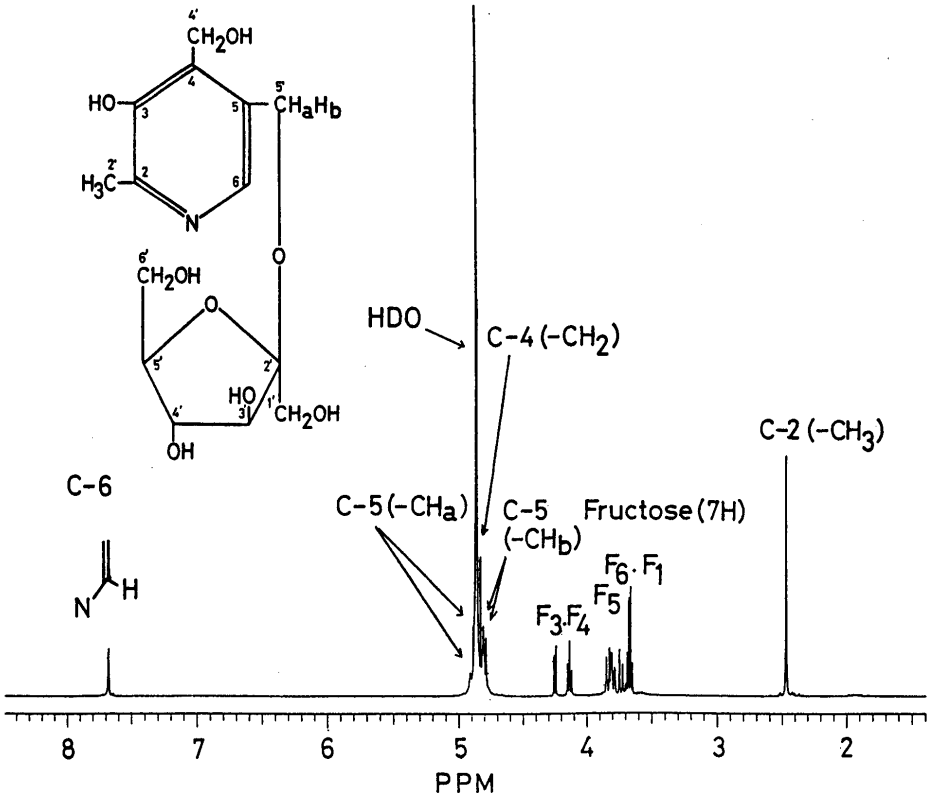


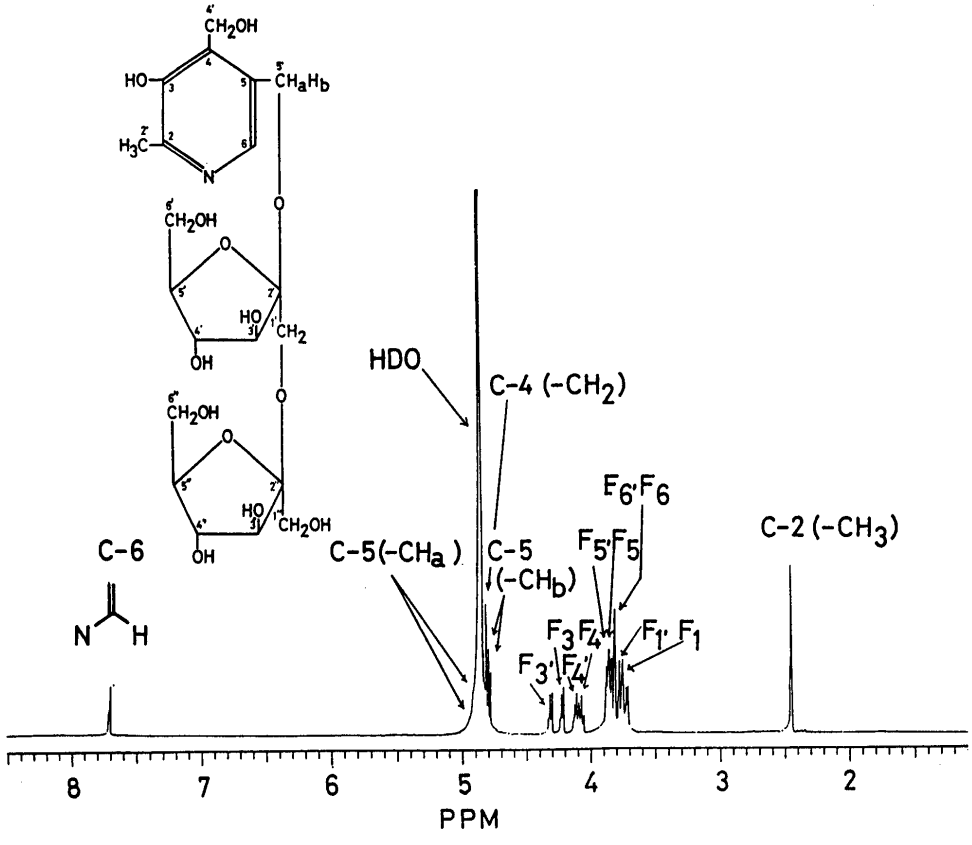
Fig. 1. A Paper Chromatogram of Products in Culture Filtrate of *A. niger* Grown on Medium containing Sucrose and Pyridoxine.

PN, pyridoxine. The silver nitrate dip method[15] was used for sugar detection on a paper chromatogram.

concentrated, decolorized with active charcoal, and lyophilized. The pale-yellowish preparation dissolved in water was put on a DEAE-cellulose (OH^-) column chromatography (5.6 × 30 cm) at 4°C. After elution with water, the desired eluent was lyophilized, and resubjected on the 2nd gel-filtration on a Sephadex G-10 column (5.6 × 90 cm), followed by elution and lyophilization. Compound I was further purified several times by precipitation with ethyl ether from the ethanol solution to give a white powdered preparation, and dried *in vacuo* on P_2O_5 (yield 1.43 g). [mp (decomp.): 191–194°C]. *Anal.* Found: C, 49.23; H, 6.10; N, 4.00%. Calcd. for $C_{14}H_{21}NO_8$: C, 50.75; H, 6.39;



Compound I



Compound II

Fig. 2. ^{1}H-NMR Spectra of Compounds I and II (D_2O).

N, 4.23%. The acetylation of compound **I** (600 mg) was done in a mixture of acetic anhydride (6 ml) and dry pyridine (6 ml), as described in our previous paper.[5] The acetylated compound **I** was purified several times from 20% ethanol solution to give a whitish powdered preparation. *Anal.* Found: C, 52.07; H, 5.59; N, 2.29%. Calcd. for $C_{26}H_{33}NO_{14}$: C, 53.51; H, 5.70, N, 2.40%.

To isolate compound **II**, the fluorescent band of compound **II** (minor one) from the concentrate of a 4-day culture filtrate was cut out, and extracted. The extract, after concentration, was applied three times on the preparative PPC with solvents B and A, and solvent A, to remove compound **I** and sugar contaminants completely. After appropriate sectioning and elution of the fluorescent band of compound **II**, compound **II** solution was concentrated and subjected to the 1st gel-filtration on a Toyopearl HW-40S column, DEAE-cellulose column chromatography, the 2nd gel-filtration on a Sephadex G-10 column, and lyophilization, successively, and then purified several times by precipitation with ethyl ether from the ethanol solution, as described in the procedures for the isolation of compound **I**. Compound **II** was obtained as a whitish powdered preparation, and dried *in vacuo* on P_2O_5 (yield 95 mg). [mp (decomp.) 206–210°C]. *Anal.* Found: C, 51.25; H, 6.03; N, 2.63%. Calcd. for $C_{20}H_{31}NO_{13}$: C, 48.68; H, 6.33. N, 2.84%.

Identification of compounds **I** *and* **II**

R_f values (0.68 and 0.45) of compounds **I** and **II** on PPC in solvent B were different from those of pyridoxine (0.80), pyridoxal (0.75), and pyridoxamine (0.31). On acid hydrolysis, compounds **I** and **II** gave pyridoxine and fructose which were confirmed by PPC and high performance liquid chromatography using a Waters carbohydrate analysis column developed with acetonitrile–water (75:25, v/v), respectively. Compounds **I** and **II** were completely hydrolyzed by yeast β-fructofuranosidase to pyridoxine and fructose in molar ratios of around 1:1 and 1:2, respectively. No reducing activity of either compound was found by the Nelson method. UV spectra of compounds **I** and **II** at 0.1 N HCl, 0.1 M sodium phosphate buffer, pH 7.0, and 0.1 N NaOH, showed the position of maxima and minima in the resemblance with those of pyridoxine. And also, compounds **I** and **II** gave the same UV spectrum (λ_{max} 294 nm) without any shoulder at 326 nm as that of pyridoxine, when saturated with boric acid at pH 6.8. In paper electrophoresis at 400 V for 2 h with 0.01 M sodium phosphate buffer, pH 7.0, both compounds migrated at a similar rate to pyridoxine toward the cathode. Compounds **I** and **II** gave positive colored reactions with diazotized *p*-aminoacetophenone and with 2,6-dichloroquinone chloroimide, but the latter reaction of both compounds was negative in the presence of boric acid. The results of the qualitative tests showed that 3- and 4′-hydroxyl groups and N-1 in both compounds were unsubstituted, and hence compounds **I** and **II** were 5′-*O*-(β-D-fructosyl)-pyridoxine and 5′-*O*-(β-fructobiosyl)-pyridoxine, respectively. Their fine structures were confirmed using ^{1}H- and ^{13}C-NMR spectra. Figure 2 shows the ^{1}H-NMR spectra of compounds **I** and **II**. The following signals of com-

Table II. ^{13}C-NMR Chemical Shifts of Compounds **I** and **II**, and Related Compounds (in D_2O)

Carbon No.	Pyridoxine	Compound I	Compound II	Carbon No.	Sucrose[a]	1-Kestose	Nystose[a]	Inulin[a]	Grass levan[a]	Methyl β-D-fructofuranoside[b]
Pyridoxine carbon				Glucopyranose carbon						
C-2	145.303	145.627	145.825	C-1	93.3	93.284	93.74	93.7	93.3	
C-2′	16.762	16.733	16.715	C-2	72.2	72.005	72.40	72.8	72.2	
C-3	160.972	160.690	161.625	C-3	73.8	73.439	73.80	74.3	73.8	
C-4	139.506	136.927	137.293	C-4	70.4	70.084	70.40	70.6	70.4	
C-4′	57.384	58.284	58.376	C-5	73.6	73.267	73.65	73.5		
C-5	136.393	133.817	134.308	C-6	61.7	60.981	61.28			
C-5′	59.816	59.806	59.854							
C-6	126.713	127.413	126.225							
Fructofuranose carbon										
C-1		61.852	61.279		62.6	61.788	62.05		62.6	60.0
C-1′			60.769			61.278	62.20	62.2	61.3	
C-1″							61.53			
C-2		104.899	104.886		104.9	104.520	104.90		104.9	104.7
C-2′			104.426			104.062	104.26	104.4	105.4	
C-2″							104.43			
C-3		77.610	77.546		77.7	77.523	77.93		77.6	77.7
C-3′			77.506			77.482	78.70	78.3		
C-3″							77.89			
C-4		75.541	75.474		75.3	74.648	75.05		76.5	75.9
C-4′			76.134			75.304	75.50	75.6		
C-4″							75.83			
C-5		82.074	82.144		82.5	82.096	82.44		81.5	82.1
C-5′			82.024			81.979	82.30	82.3	82.4	
C-5″							82.30			
C-6		63.110	63.141		63.5	63.148	63.46		64.6	63.6
C-6′			63.110			62.924	63.46	63.4		
C-6″							63.46			

[a] Reference 16. [b] Reference 17.

pounds **I** and **II** were assigned to the protons of pyridoxine moiety (δ, ppm): 2.465, 2.454 (2′-H_3), 4.796, 4.755 (5′-H_b), 4.828, 4.816 (4′-H_2), 4.900, 4.915 (5′-H_a), and 7.680, 7.680 (6-H), respectively. The additional signals at 3.5–4.3 ppm and 3.5–4.4 ppm in compounds **I** and **II**, respectively, were derived from ring protons of sugar (fructofuranose). On acetylation of compound I, both 18 proton signals of six CH_3COO groups at 2.0–2.2 ppm and a methyl signal at the 2-position of pyridoxine moiety (2.377, 3H, s) were observed. A set of signals due to a β-fructofuranosyl unit and a β-fructofuranobiosyl unit was observed in the ^{13}C-NMR spectra of compounds **I** and **II** in comparison with that of pyridoxine (Table II). Fructosylation of PN caused similar chemical shifts (2.2–2.6 ppm) of C-4 and C-5 in pyridoxine moiety of compounds **I** and **II**. Consideration of these chemical shifts alone does not permit definite conclusions concerning the fructosylation site in both compounds. In compound **II**, C-3 and C-3′, C-5 and C-5′, and C-6 and C-6′ in fructofuranose carbons resonated respectively very close to each other. In contrast, C-2′ and C-1′ were shifted upfield by about 0.5 ppm relative to the corresponding C-2 and C-1, while C-4′ was shifted downfield by 0.6 ppm. These chemical shifts of compound **II** were very similar to those of 1-kestose and nystose. The resonance at 63.141 ppm of C-6 in transferred fructose carbon in compound **II** was consistent with those (62.9–63.6 ppm) of C-6 having O-6 unsubstituted as shown by all of sucrose, 1-kestose, nystose, inulin, and methyl-β-D-fructofuranoside. Experience would indicate that any linkage to C-6 of a fructofuranose unit should normally cause a downfield displacement of the signal for C-6.[18] The linkage on C-6 of a fructofuranose unit in grass levan produces a downfield displacement of 1.0 ppm (the resonance of 64.6 ppm). These spectral data showed that the fructosylation site was present at C-1 in the fructofuranose carbon of compound **II**. Thus, compounds **I** and **II** were identified as 5′-*O*-(β-D-fructofuranosyl)-pyridoxine and 5′-*O*-[β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl]-pyridoxine, respectively.

Formation of compounds I and II in germinating seeds cultured in the presence of pyridoxine

The formation of compounds **I** and **II** from pyridoxine was examined by PPC in several plant seedlings, such as *A. officinalis*, *L. sativa*, and *A. cepa*. β-Glucosylpyridoxines were formed from pyridoxine in all the tested seedlings, as described in our previous paper.[7] But the formation of compounds **I** and **II** were scarcely detected.

Discussion

β-D-Fructofuranosidase (EC 3.2.1.26), levansucrase (EC 2.4.1.10), inulosucrase (EC 2.4.1.9) and sucrose 1^F-fructosyltransferase (EC 2.4.1.99) are well-known as enzymes catalyzing β-D-fructofuranosyl transfer from sucrose. When yeast β-D-fructofuranosidase was incubated with a 50% solution of sucrose, β-D-fructofuranosyl transfer occurred preferentially at the primary alcohol group at C-6 of fructose moiety in sucrose to give rise to 6-kestose [*O*-β-D-fructofuranosyl-(2→6)-*O*-β-D-fructofuranosyl-(2→1)-α-D-glucopyranose].[19] β-D-fructofuranosidases from *A. niger* and *A. sydowi* produced most efficiently β-D-(2→1)-linked oligofructans including 1-kestose, nystose, and 1^F-β-D-fructofuranosylnystose in a concentrated solution of sucrose.[12–14] These oligofructans occur naturally in plants such as *A. officinalis*, *A. cepa*, and *Helianthus tuberosus*. Oligo- and poly-fructans in plants were synthesized from sucrose by the combined actions of sucrose 1^F-fructosyltransferase, 1,2-β-D-fructan 1^F-fructosyltransferase (EC 2.4.1.100), and inulosucrase.[20] Levansucrases from *Aerobacter levanicum*,[21,22] *Bacillus subtilis*,[23] and *M. laevaniformans*[24] catalyzed the synthesis of levan (a β-fructofuranose polymer in which most of linkages were β-2,6) by consecutive transfers of β-D-fructofuranosyl group from sucrose.

In this work, it was found that *A. niger* and *A. sydowi* having β-(2→1)-linked oligofructan forming-activity yielded a considerable amounts of 5′-*O*-(β-D-fructofuranosyl)-pyridoxine (compound **I**) and 5′-*O*-[β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl]-pyridoxine (compound **II**) from sucrose and pyridoxine, but levansucrase from *M. laevaniformans* and yeast β-D-fructofuranosidase did not catalyze β-D-fructofuranosyl transfer from sucrose to pyridoxine. In germinating seeds of *A. officinalis*, *L. sativa*, and *A. cepa* cultured on a pyridoxine solution, β-glucosylpyridoxines were formed, but the formation of compounds I and II was scarcely detected. Examination of the formation of β-D-fructosylpyridoxines from sucrose and pyridoxine by purified enzymes of plant β-(2→1)-fructan synthesis is now being done.

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