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R(−)PANTOYLLACTONE-β-D-GLUCOPYRANOSIDE: CHARACTERIZATION OF A METABOLITE FROM RICE SEEDLINGS

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Abstract—A new derivative of pantoic acid, *R*(−) pantoyllactone-β-D-glucopyranoside, has been isolated from rice seedlings and its structure determined. β-Glucosidase hydrolysed it to D-glucose and *R*(−)pantoyllactone. Alkaline hydrolysis converted it to the salt of 2-*R*(−)pantoic acid β-D-glucopyranoside. It accumulated in rice shoots but not in roots.

INTRODUCTION

In an effort to increase the amount of biological information extractable from ^{1}H NMR spectra of tissue extracts by assignment of unknown signals, our attention was drawn to two such signals present in shoot extracts of aetiolated rice, *Oryza sativa* L., seedlings [1]. Both signals were absent from the root extracts, suggesting some relevance for the parent compound. The compound was found to be *R*(−)pantoyllactone-β-D-glucopyranoside, the condensation product of D(−)pantoyllactone (PL) with β-D-glucose, henceforth pantoyllactone glucoside (PLG), a new member in the family of pantoic acid derivatives. Glucosylated derivatives of pantothenic acid, the most important member of the family, are known [2]. To our knowledge this is the first glucosylated derivative of PL to be isolated from a natural source. Its chemical synthesis has been outlined [3] but the pure compound was not isolated and characterized.

This paper reports on PLG isolation from rice seedlings, on its structure determination and transformation into pantoic acid glucoside (PAG).

RESULTS AND DISCUSSION

Proton NMR spectra of $HClO_4$ shoot extracts of aerobically grown rice seedlings (Fig. 1A) show two singlet signals at 1.27 and 1.12 ppm, of the same intensity, most probably CH_3 signals according to their chemical shift. The parent compound was unknown. The chemical shift of both signals was unaffected by changes of pH between 4 and 8 (not shown) indicating a molecule devoid of ionizable functions. The putative presence of two methyl residues prompted the exploration of a chromatographic separation based on hydrophobic interactions. Indeed, the isolation of the highly pure compound (Fig. 2) was achieved by the use of a XAD-2 column. Typically 8 μmol of the substance were obtained from 10 g of fresh wt material with a 90% recovery.

In most but not all preparations an additional substance appeared, which was characterized by two signals 0.005 ppm apart centred at 0.944 ppm at pH 7.0. It constituted 1–5% of the main product but it could increase up to 15% when $HClO_4$ extracts were stored for several weeks at −20° before separation. This substance could be selectively removed by a QAE-Sephadex column.

The purified compound was studied by mass spectrometry. The 70 eV EI mass spectrum showed a weak peak at *m/z* 293 $[M + H]^+$ reasonably due to a self-chemical ionization process. The methanol–CI spectrum showed *m/z*: 293 $[M + H]^+$ (10) (accurate mass 293, 1237 fitting with a $C_{12}H_{21}O_8$ composition); 163 $[M + H - ROH]^+$ (19); 131 $[ROH + H]^+$ (100); 129 $[ROH - H]^+$ (27). The above results indicate a molecule of composition $C_{12}H_{20}O_8$ and M_r 292 resulting from the condensation of a hexose $C_6H_{12}O_6$ with pantoyllactone (ROH) $C_6H_{10}O_3$.

The structure of PLG was established by means of two-dimensional NMR homo- and hetero-nuclear correlation techniques (Tables 1 and 2). The ^{1}H NMR spectrum of PLG in DMSO-d_6 shows the presence of a doublet at 4.32 ppm (J = 7.8 Hz), connected to a carbon which resonates at 102.4 ppm. These values are characteristic of an anomeric proton with a β-glucosidic

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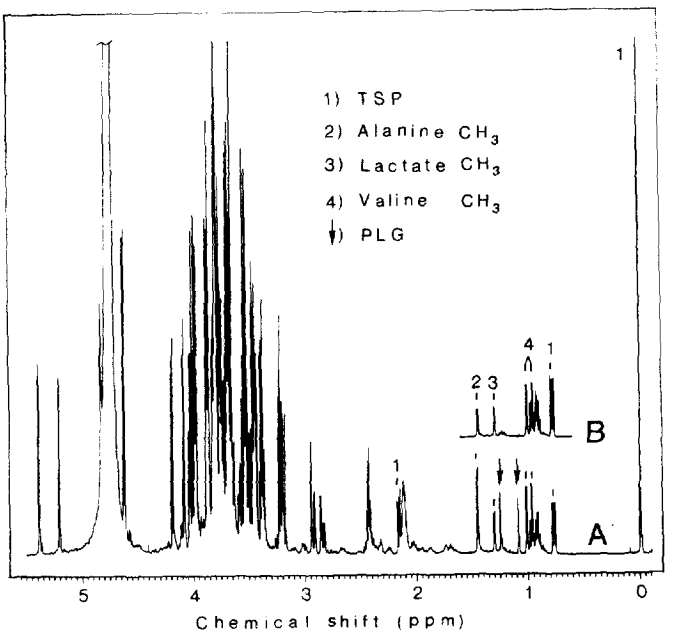

Fig. 1. ^{1}H NMR spectra of $HClO_4$ extracts of tissues from 5-day-old aetiolated aerobic rice seedlings. (A) shoot; (B) root. Arrows indicate the CH_3 signals of PLG. The extract from 1 g of fresh wt tissue was dissolved in 1 ml of D_2O.

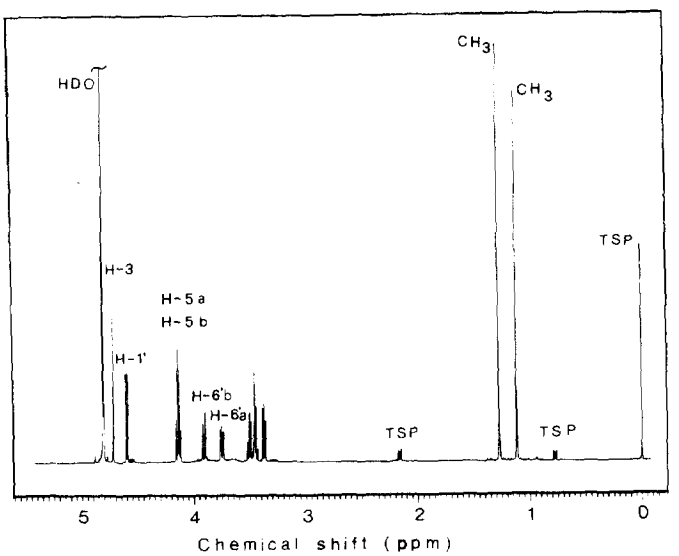

Fig. 2. ^{1}H NMR spectra of a 10 mM solution of pure PLG in D_2O. See Fig. 3 and Table 1 for the numbering of protons.

linkage. The two-dimensional COSY-DQF (correlation spectroscopy-double quantum filtered) experiment and the high values of the H–H coupling constants (Table 1) clearly established the presence of a glucose moiety. The ^{1}H signal at 3.93 ppm in DMSO-d_6 is a deceptively simple AB system, because in water (D_2O) solution it splits in two doublets with a geminal coupling constant of 9.0 Hz (Table 1) and has been attributed to a $-OCH_2-$group. Heteronuclear long-range connectivities were measured by means of a two-dimensional HMBC (heteronuclear multiple bond correlation) experiment (Table 2). All these correlations prove the presence of the five-membered lactone ring, formed by pantoic acid, with a β(1'-3) glycosidic linkage. The structure of the compound is shown in Fig. 3.

To confirm the β-configuration of the glycosidic bond and further characterize the molecule, PLG was subjected to the action of α- and β-glucosidases. The

Table 1. ^1H and ^{13}C chemical shifts and coupling constants of PLG and of PAG Na salt (all chemical shifts are referenced to TSP)

Atom numbering	PLG			PAG-Na
	^1H* (ppm)	^1H† (ppm)	^{13}C‡ (ppm)	^1H (ppm)
CH_3	1.02	1.12	19.3	0.942
CH_3	1.18	1.27	22.8	0.947
2			174.7	
3	4.60	4.72	78.8	3.74
4			40.6	
5a	3.93	4.12§	75.0	—§
5b		4.15§		—§
1′	4.32 (7.8)	4.60	102.4	4.38
2′	3.03 (9.0; 7.8)	3.37	73.5	3.38
3′	3.17 (9.0; 8.6)	3.51	76.7	—§
4′	3.07 (9.6; 8.6)	3.44	70.1	—§
5′	3.12 (9.6; 5.8; 2.2)	3.47	77.4	—§
6′a	3.67 (11.7; 2.2)		61.3	3.71
6′b	3.46 (11.7; 5.8)			3.83

*In DMSO-d_6 solution at 45°. The coupling constants (Hz) are reported in parentheses.
†In D_2O solution at 25°.
‡The assignments were performed by inverse ^1H ^{13}C correlation experiments in DMSO solution.
§Not assigned.

Table 2. Two-dimensional ^1H–^{13}C long-range correlations from HMBC experiment of PLG in DMSO-d_6 at 45°*

	H-3	H-5	CH_3	CH_3	H-1′
C-2	++	+			
C-3		+	++	++	+
C-4	+	+	++	++	
C-5			++	++	
C-1′	+				
CH_3a	+	±		++	
CH_3b	+	++	++		

*Cross-peaks: ++, strong intensity; +, medium intensity; ±, weak intensity.

^1H NMR signals of the geminal CH_3 groups of PLG at 1.27 ppm and 1.12 ppm undergo a shift at 1.19 ppm and 1.07 ppm, respectively, when the PLG is hydrolysed to glucose and free PL [4]. Accordingly, the glucosidases were added to the sample of PLG inside the NMR tube and the changes of the intensity of the CH_3 signals of PLG and PL were recorded as function of time. Figure 4 clearly confirms the β-configuration of the glycosidic bond as established by the NMR spectroscopy structural studies. Following hydrolysis with β-glucosidase the resulting PL was extracted with diethyl ether with an overall recovery of 95% of the PL present in PLG. Maximal molar ellipticity of isolated PL was $[\Theta]_{219}$-16.000, against $[\Theta]_{219}$-17.300 of authentic PL, indicated 95% natural *R* configuration of PL in PLG [5]. The less

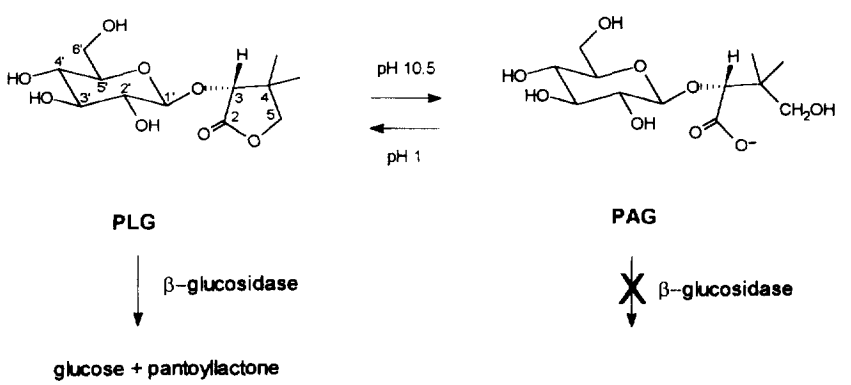

Fig. 3. Structures of *R*(−)pantoyllactone-β-D-glucopiranoside (PLG) and of 2-*R*(−)pantoate-β-D-glucopiranoside (PAG). pH dependent interconversion of the two metabolites and response to β-glucosidase are shown.

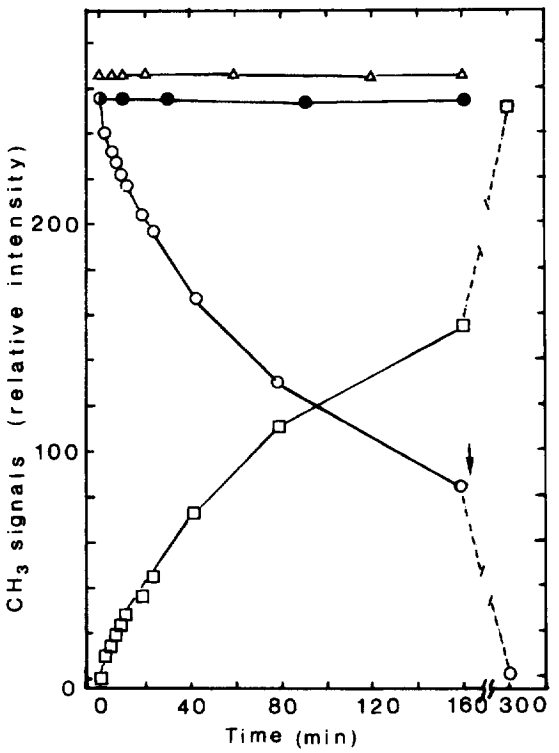

Fig. 4. Action of α- and β-glucosidases on PLG and on the Na salt of PAG. To 0.5 ml of a 10 mM solution of PLG, 10 μl of β-glucosidase were added and the reaction monitored measuring the intensities of the CH_3 signals of PLG, decreasing (○), or the intensities of the CH_3 signals of PL, increasing (□), with time. The mean intensity of the geminal CH_3 signals is reported. The arrow marks the further addition of β-glucosidase. Action of 10 μl of α-glucosidase on an identical solution of PLG (●). Action of 20 μl of β-glucosidase on 0.5 ml solution of 10 mM Na salt of PAG at pH 7.0 (△). The temperature was 25° and all solutions contained 10% D_2O for NMR lock.

than 100% *R* configuration may be due to experimental errors rather than to the presence of some *S* isomer. Maximal molar ellipticity of PLG was $[\Theta]_{21}$-10.000.

Hydrolytic opening of the lactone ring of PLG to a carboxylate anion is to be expected at a alkaline pH [6]. At pH 10.5 and 25° the reaction was complete within 3 hr. The solution was then brought to pH 7.0 with HCl, freeze-dried and the product redissolved in D_2O for ^1H NMR analysis. Opening of the lactone ring of PLG with the generation of the carboxylate anion of PAG caused changes in the ^1H NMR spectrum of the molecule, particularly sharp for the signals of the geminal CH_3 groups (Table 1).

The solution of the sodium salt of pantoic acid glucoside (Na PAG) was then brought to pH 1.0 with HCl and to 50° to induce lactonization back to PLG [7]. The lactonization was complete after 6 hr and the overall recovery in the cycle, PLG to NaPAG and back to PLG, was found to be 100% within the experimental error.

Opening of the lactone ring and introduction of a negative charge on the molecule renders the PAG Na salt completely resistant to the action of β-glucosidase (Fig. 4). PAG is clearly the metabolite appearing in low amounts in preparations of PLG and in particular when $HClO_4$ extracts were stored at −20° for long time. This last phenomenon can be attributed to a lactone-hydrolysing activity present in tissues and not completely denatured by $HClO_4$. Further investigations are needed to establish if PAG is present in the tissues or if it is an artefact of the extraction procedure.

The accumulation of PLG in rice shoots was characterized by an initial fast increase to a maximum of 24 nmol per shoot at day 5 after sowing (not shown). Thereafter a decline followed, suggesting the utilization of this substance by the growing seedling. The possibility that PLG represents a temporary store of pantoic acid for the synthesis of pantothenic acid is presently being investigated. It is to be noted that the biosynthesis of pantoic acid and related compounds is poorly understood in plants [8].

To the authors' best knowledge this is the first report of the existence of PLG in living organisms. The finding is interesting because the pantoyl residue is related to central roles of metabolism through pantothenic acid and CoA. In a study on the prebiotic origin of pantoic acid and on the possible roles of the free OH group of the pantoic acid moiety of CoA, the existence of α-hydroxy derivatives of PL, like PLG, was suggested [6].

EXPERIMENTAL

General. Rice seedlings, cultivar Arborio, were raised and $HClO_4$ extracts of tissues were prepared as previously reported [1]. Amberlite XAD-2 practical grade 20–50 mesh was used; α- and β-glucosidases were from yeast and almond, respectively.

Purification of the unknown compound. The unknown compound was identified and quantitated throughout this study by ^1H NMR spectroscopy taking advantage of its well resolved CH_3 signals. The reference capillary containing sodium trimethylsylil-propionate (TSP) 40 mM was standardized, for quantitative work, against 3 mM L-alanine [9]. The unknown compound was purified by the use of a single chromatographic column of XAD-2. Before use XAD-2 was carefully cleaned as indicated in ref. [10]. The $HClO_4$ extract, 150 ml, corresponding to 10 g of fr. wt shoot material, was charged on a column of 70 ml of bed, $h = 10$ cm and $d = 3$ cm, at a flow rate of 70 ml hr^{-1} at room temperature. The column was washed with 1.5 vol. of water and the washing continued with 2 vols of water containing 5% MeOH. Finally the compound was eluted with 2 column vol. of MeOH–H_2O (1:3).

MS analysis. MS measurements were performed with a VG-MICROMASS ZAB-2F instrument, operating at 8 KV accelerating voltage. Electron-impact (EI) and MeOH–CI mass spectra were recorded with a dual EI–CI ion source, with 70 eV (100 μA) and 50 eV (2 μA) electron-beam energies, respectively. The sample was introduced directly into the ion source, at temperatures

ranging from 140 to 180°. The accurate mass was measured under CI by peak-matching device at a resolving power of 10000, with perfluorokerosene as standard. Field desorption (FD) spectra were obtained with an EI–FD ion source operating at 100°, with 12 KV extraction potential and using an emitter, activated by the standard VG-Micromass procedure, which was dipped in a MeOH solution of the sample.

NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were obtained on Bruker AMX-600 spectrometer at 600.13 and 150.9 MHz, respectively. Measurements were made on solution in D_2O at 25° and in DMSO-d_6 at 45°. Chemical shifts were referenced to TSP signals set at 0 ppm and coupling constants are given in Hz. Heteronuclear two-dimensional ^{1}H–^{13}C correlations one-bond, HMQC (heteronuclear multiple quantum correlation) [11] and multiple-bond, HMBC [12], were carried out in the ^{1}H-detected mode with broad-band decoupling in the ^{13}C domain.

Hydrolysis of the glycosidic bond and isolation of pantoyllactone. β-Glucosidase was dissolved in 0.1 M potassium phosphate buffer pH 6.8 at 50 U ml^{-1}. α-Glucosidase, ammonium sulphate suspension 200 U ml^{-1}, was used as purchased. Hydrolysis of the glucosidic bond of PLG was monitored by ^{1}H NMR spectroscopy thanks to the upfield shifts of the CH_3 signals caused by the splitting of the molecule to free PL and glucose. For the preparation of PL from PLG for CD studies 16.8 μM of PLG dissolved in 6.5 ml of water, were reacted overnight at room temperature with 1 unit of β-glucosidase. The solution was then extracted × 3 with 3 vols of Et_2O and the extracts pooled. Ether was evaporated under reduced pressure. In order to avoid losses of PL itself, the last traces were removed with a nitrogen flow, taking care not to evaporate the remaining water, about 0.2 ml. The PL solution was brought to 1 ml and the concentration measured spectrophotometrically [13] with authentic PL as standard.

Circular dichroism (CD) studies. CD measurement were done at 25° with a Jasco J-500A instrument which was calibrated with PL as standard [5].

Hydrolysis of the lactone ring of PLG and lactonization of pantoic acid glucoside back to PLG. Hydrolysis of the lactone ring of PLG was performed at pH 10.5 and 25° with a pH-stat instrument using NaOH 0.1 M as titrant. At the end of the reaction the solution was brought to pH 7.0, freeze-dried and redissolved in D_2O for ^{1}H NMR analysis of the sodium pantoate glucoside (Na-PAG) formed. The pantoate glucoside solution was then brought to pH 1.0, and the lactonization reaction let to proceed for several hours at 50°. The pH was brought to 7.0 and the sample analysed by ^{1}H NMR spectroscopy, for control of lactonization and recoveries.

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