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Preparative Biosynthesis of Natural Glucosides and Fluorogenic Substrates for β-Glucosidases followed by *in vivo* ^{13}C NMR with High Density Plant Cell Cultures

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Abstract: The natural glucosides, picein (**2**), o-coumaric acid glucoside (**4**), and cinnamic acid glucoside (**5**) could be prepared by preparative biosynthesis in yields of 91, 65 and 69% resp., using high density plant cell suspension cultures. The cell suspensions also glucosylated synthetic phenolic compounds at remarkable rates. Fluorescein was glucosylated in yields > 90% and the product (**6**) could be obtained in high purity (> 98%). *In vivo* ^{13}C NMR spectroscopy at the natural abundance of ^{13}C has been successfully applied to follow the formation of such glucosides.

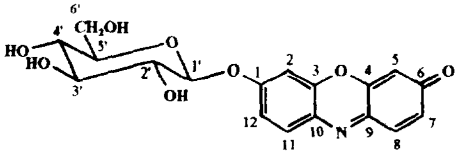
INTRODUCTION

Recently we have demonstrated that cultivated cells of the plant *Rauwolfia serpentina* Benth. ex Kurz have an excellent glucosylating activity. Many phenolics are *in vitro* transformed into the corresponding glucosides by crude enzyme extracts[1]. In agreement with these findings this cell culture system also converts phenolic compounds *in vivo* into their glucosides sometimes with exceptionally high transformation rates as reported for hydroquinone[2].

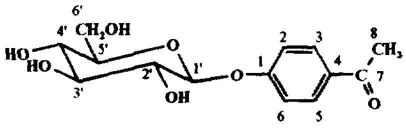
We have now carried out experiments to glucosylate *in vivo* on the one hand a number of natural phenolic compounds leading to relatively rare glucosides and on the other hand synthetic phenolics like fluorescein and resorufin in order to prove that this method can be applied for preparative syntheses on the gram scale.

Synthetic fluorogenic substrates for enzymes are commonly used to measure the activity of hydrolytic proteins, e.g. glycosidases. In the case of the synthesis of appropriate fluorogenic substrates for glucosidases, the Koenigs-Knorr reaction[3] is the method usually applied. A typical example is the chemical synthesis of resorufin-O-β-D-glucopyranoside (**1**), which has been previously described[4,5]. Although plant cultures have often been used for biotransformation[6,7], and for special cases at semi-technical scale[7], they have not been applied to prepare such synthetic fluorogenic glucosides from their aglycones.

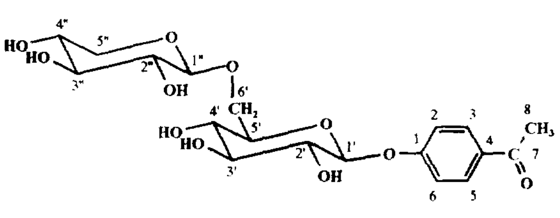
The gram scale preparation of glucosides from a range of natural and synthetic phenolics by high density cell cultures and the application of *in vivo* NMR to follow such transformations is described in this paper.



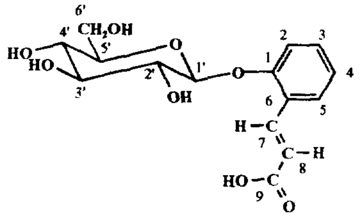
Resorufin-O-ß-D-glucopyranoside (**1**)



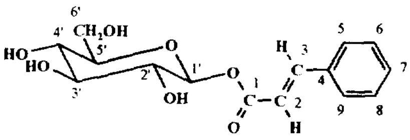
Picein (**2**)



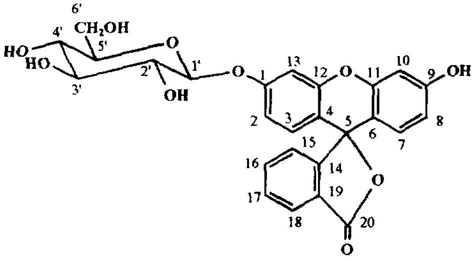
p-Acetyl-phenyl-primeveroside (**3**)



o-Coumaric acid-O-ß-D-glucopyranoside (**4**)



trans-Cinnamoyl-O-ß-D-glucopyranoside (**5**)



Fluorescein-O-ß-D-glucopyranoside (**6**)

RESULTS AND DISCUSSION

Formation of Natural Glucosides

One of the naturally occurring phenolic glucosides we first synthesized by feeding the appropriate aglycone to high density plant cell cultures was p-hydroxy-acetophenone-O-β-D-glucopyranoside (**2**), also named picein. It has been isolated from *Picea abies* needles, in which it is a typical glucoside[8], but also has been identified in some Salicaceae species[9], *Arctostaphylos uva-ursi*[10] and *Picrorhiza kurrooa*[11]. Recently, **2** became an interesting compound, because it is apparently important as a marker substance for tree damage. Severely injured needles of Norway spruce trees showed higher amounts of **2** (7-16fold) than healthy spruce needles[12] and the ratio of **2** to its aglycone was affected by SO_2-containing emissions[13]. Furthermore **2** was used as a component in desulfurizing agents[14] and in etching masks for semiconductor materials[15]. The chemical preparation of **2** is possible by the method of Glaser and Wulwek in yields of 75%[16]. The *Rauwolfia* cells employed here efficiently converted the aglycone into **2**. By *in vivo* ^{13}C NMR experiments it could be demonstrated that the cells glucosylate p-hydroxy-acetophenone quantitatively. As illustrated in figure 1, the time course of the conversion can be followed using ^{13}C signals of C-2/C-6 and C-3/C-5, respectively or the intensity of the anomeric carbon C-1'.

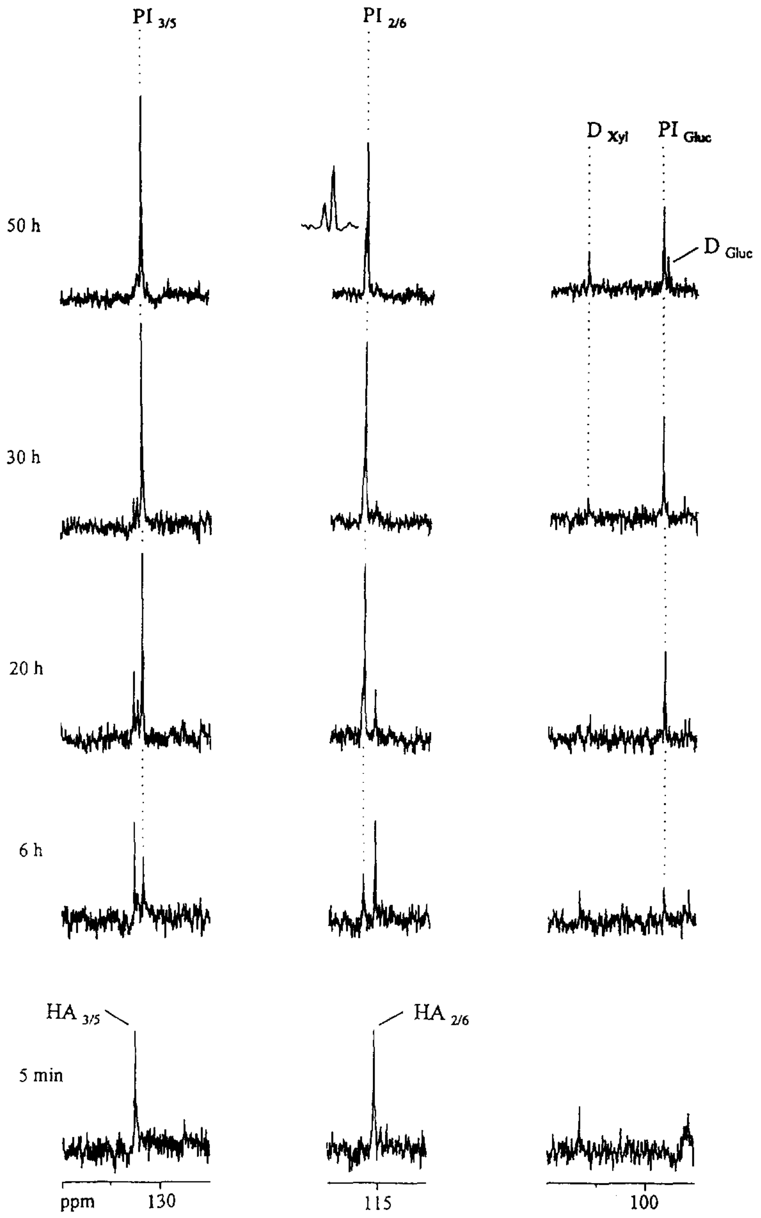


Figure 1: Biotransformation of p-hydroxy-acetophenone to picein (2) and p-acetyl-phenyl-primeveroside (3) shown by 100.6 MHz *in vivo* ^{13}C NMR spectroscopy of cells from suspension cultures of *Rauwolfia serpentina* at the natural abundance of ^{13}C.
Spectra recorded sequentially starting 5 min (time zero), 6 h, 20 h, 30 h and 50 h after addition of 3 mmol p-hydroxy-acetophenone to a cell suspension of about 300 g wet cells cultivated for four days in LS-medium (450 ml total volume). All samples contained about 1.4 ml D_2O. Each spectrum represents 5600 accumulations (measuring time 2 h) with a pulse repetition of 1.3 s. Digital resolution is 0.76 Hz/data point. Resolution enhanced spectral part was obtained by Gaussian-multiplication (GB: 0.15, LB: -6 Hz).
Abbreviations used: HA $_{2/6}$, HA $_{3/5}$: aromatic carbons of p-hydroxy-acetophenone; PI $_{2/6}$, PI $_{3/5}$: aromatic C-atoms of 2; PI $_{Gluc}$: C-1 of the glucose unit of 2; D $_{Gluc}$, D $_{Xyl}$: C-1 of the glucose unit and C-1 of the xylose unit of 3.

Although immediately after feeding of 410 mg of p-hydroxy-acetophenone to 300 g wet cells (9 g dwt) in a total volume of 450 ml only the signals of the starting material could be observed at 115.2 and 131.3 ppm, after 6 h the aromatic carbons of **2** (115.8 and 130.8 ppm) showed up as well as the anomeric C-1' at 99.1 ppm. After 20 h the total conversion of the aglycone was about 80% and 30 h after feeding of the cells, nearly complete transformation to **2** (90%) and the diglycoside, p-acetyl-phenyl-primeveroside (**3**) (6%), was measured using NMR spectroscopy.

The formation of **3** could be monitored by the signals of C-1 of glucose (98.9 ppm) and C-1 of xylose (102.9 ppm). After 50 h of incubation the total yield of the biosynthesis of **3** was about 25%. These results indicate that *in vivo* NMR can be efficiently used even at the natural abundance of ^{13}C to follow biosynthetic processes in living plant cells. Despite the *in vivo* monitoring of the formation of arbutin, ursubin and methylursubin by NMR[17,18], the formation of **2** described here is a further example demonstrating that NMR can now be routinely used to follow the dynamics of bioconversions in intact plant cells provided that high density cultures with efficient transformation rates are employed. Excellent formation of **2** (90%) could still be observed up to amounts of 2.0 g/l medium fed with p-hydroxy-acetophenone. The physical and spectroscopical data of the isolated compound **2** were in complete agreement with those described in literature[19]. Biotransformation studies of p-hydroxy-acetophenone with root cultures of *Panax ginseng* showed, in contrast to the *Rauwolfia* cells employed here, a transformation rate of only 17% in roots and medium, and **3** could not be observed[20].

In addition to **2**, the o-coumaric-O-β-D-glucoside (**4**) has been discussed in the literature as a further possible marker for tree damage[21]. Therefore we proved the capability of the suspended *Rauwolfia* cells to glucosylate o-coumaric acid. Adding this acid continuously to the cell culture (1.0 g per l nutrition medium) we observed a biotransformation rate of 65%. This transformation offers an interesting synthetic approach to this particular glucoside and it is noteworthy that the appropriate ester glucoside, which also could have been synthesized, was not found under the conditions of the here described biotransformation.

To prove further the *in vivo* transformation capability of the plant cell system, we fed trans-cinnamic acid in gram amounts to the cells. As one could assume from the high glucosylations discussed above, this transformation was also efficient. Based on HPLC analysis a conversion of about 70% into trans-cinnamoyl-O-β-D-glucoside (**5**) has been monitored after a feeding time of only 5 days. Previous work on cinnamic acid described mostly the degradation of cinnamic acid in plant cell cultures[22-24].

Glucosylation of Synthetic Compounds

Three synthetic compounds have been tested as potential substrates for their *in vivo* glucosylation, 6-bromo-2-naphthol, resorufin and fluorescein. All three phenolics were glucosylated in considerable amounts although the naphthol-derivative was a rather poor substrate being transformed into its O-β-D-glucoside only to an extent of ca. 10% (table 1). Obviously each phenolic substance respectively, whether it is a naturally occurring or synthetic compound, will be glucosylated on a preparative scale by the *Rauwolfia* cells but with greatly varying yield. Since 6-bromo-2-naphthol can be glucosylated in excellent yield chemically[25], the procedure described here is, in fact, not advantageous when compared to the Koenigs-Knorr reaction.

Table 1: Biotransformation Rates of Natural and Synthetic Phenolic Compounds with Cell Suspension Culture of *Rauwolfia serpentina*.

Substrate	HPLC Gradient	HPLC Retention time [min] Substrate	HPLC Retention time [min] Product	Transformation rate [g/l medium]	Transformation rate [%]
p-Hydroxy-acetophenone *	I	6.14	2.19	1.64	91
o-Coumaric acid *	II	8.10	4.38	1.08	65
trans-Cinnamic acid *	I	10.23	2.90	1.20	69
6-Bromo-2-naphthol *	III	8.42	2.58	0.13	10
Fluorescein **	II	8.90	3.80	1.90	91
Resorufin **	I	8.90	4.20	0.49	19

* = 0.5 g/500 ml nutrition medium
** = 1.0 g/500 ml nutrition medium

As outlined in tables 1 and 2 the cell culture system was also tested for the generation of fluorogenic substrates for glucosidases like e.g. the synthesis of the O-β-D-glucopyranosides of resorufin and fluorescein. Indeed, even these synthetic phenolic compounds were transformed by the different cell suspension culture systems (table 2), although the biotransformation rates were very different. Although the rate of the synthesis of fluorescein-mono-O-β-D-glucopyranoside (**6**) ranged between 11% and 94% depending on the cell suspension culture employed, the maximum of resorufin glucosylation reached only ~ 35%.

Table 2: Biotransformation of Fluorescein and Resorufin with Different Cell Suspension Cultures

Cell culture	Transformation rate in [%] **6**	Transformation rate in [%] **1**
Rauwolfia serpentina (L.) Benth. ex Kurz	90.7	19.4
Rhazya stricta Decaisne x *Rauwolfia serpentina* Benth. ex Kurz	93.5	34.6
Catharanthus roseus (L.) G. Don	38.1	5.1
Nicotiana plumbaginifolia Viv.	32.0	19.9
Nicotiana tabacum L.	12.2	11.1
Solanum marginatum L.	16.0	n.t.
Solanum lycopersicum L.	11.4	n.t.

n.t. not tested

Of the seven tested cell suspensions those of *Rauwolfia* and its recently described hybrid cell culture (*Rauwolfia* x *Rhazya stricta*)[26-28] were the most efficient, showing biotransformation of fluorescein over 90% and optimum values for resorufin of ~ 20 and ~ 35%, respectively. It is obvious that the latter compound is more toxic to the plant cells and is therefore not glucosylated in such high yields as fluorescein. It is, however, interesting to note that the chemical synthesis recently published for **1** reported only 41% yield[5], but seems to be superior to the "biological synthesis" described here. This result is, however, not true for the transformation of fluorescein, which can be applied in a concentration of up to 2 g/l to the cells and is excellently glucosylated.

Using a three step purification procedure (fig. 2) which consisted mainly of AD-2, MLCCC and silica gel chromatography 53% of **6** was obtained with a purity of ca. 98% as determined by HPLC. Besides this product, a further product identified as the diglucoside of fluorescein was also formed. When the isolated **6** was incubated with a commercially available β-glucosidase a complete conversion was observed, indicating the high purity of the glucoside and its application as a fluorogenic substrate for β-glucosidases. Since the obtained product shows significantly greater purity than the commercially available[29] **6**, the biological formation described here and the procedure of purification of **6** provides a new synthetic approach to this fluorogenic compound.

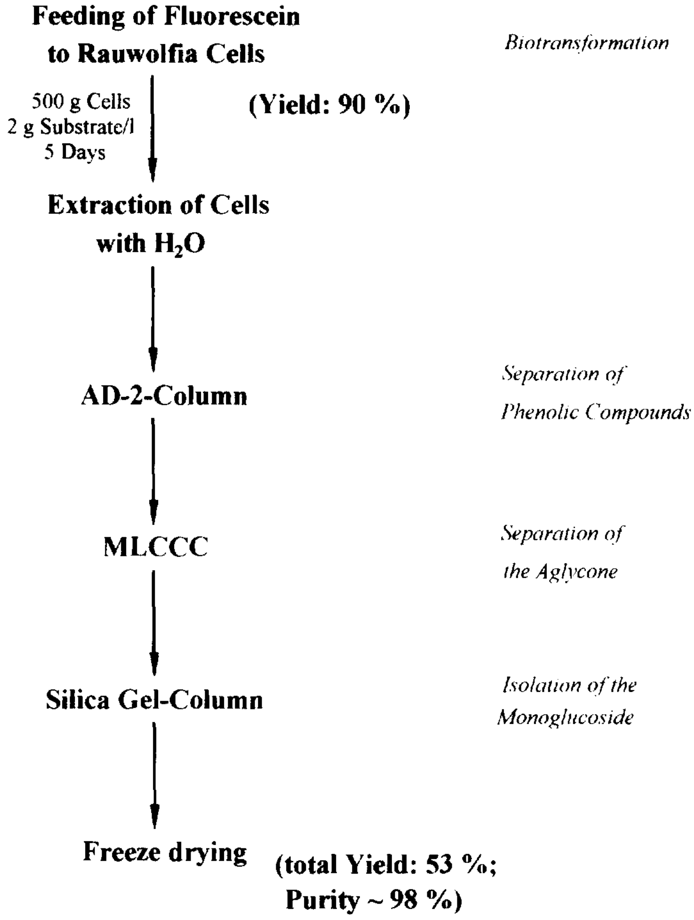


Figure 2: Isolation Scheme for Fluorescein-mono-glucoside (**6**)

In summary, high density plant cell cultures of *R. serpentina* can be successfully applied to the biological synthesis of rare O-β-D-glucosides of a number of natural and synthetic phenolic compounds. These glucosides can be prepared on a preparative scale in gram amounts. *In vivo* ^{13}C NMR at the natural abundance of ^{13}C can be used efficiently to follow and optimize these biotransformation processes as demonstrated for the formation of **2**.

EXPERIMENTAL

^1H and ^{13}C spectra were recorded on Bruker ARX-400 or AM-400 spectrometer. Specific rotation was measured with a Perkin-Elmer 241 polarimeter. Mass spectra analyses were carried out using a Finnigan MAT 44S quadrupole instrument in EI-mode and a Finnigan MAT 90 (FAB, FD). Multilayer-counter-current-chromatography (MLCCC) (Zinsser) was applied for separation of the aglycone using $CHCl_3/MeOH/H_2O$ (7:13:8) as a two phase solvent system with the aqueous phase as mobile phase. Serdolit® AD-2 (0.3 - 1.0 mm) from Serva was used for adsorption chromatography, and silica gel 60 (0.063 - 1.0 mm) from Merck for column chromatography. TLC was carried out on Merck silica gel 60 F_{254}, solvent systems: [A] $EtOAc/MeOH/H_2O$ (7:2:1), [B] $CHCl_3/MeOH$ (3:1) with detection under UV light (254 and 365 nm) or by spraying with 3-methoxyphenol/H_2SO_4 reagent. HPLC analyses were performed on a Merck-Hitachi system connected to a LiChrospher® 60-column (RP-select B; 4 x 125 mm) with the solvent system $CH_3CN:H_2O$, pH 2.3 adjusted with H_3PO_4, gradient I 15:85 $\rightarrow$ 45:55, II 25:75 $\rightarrow$ 55:45, III 35:65 $\rightarrow$ 60:40, all within 10 min and with a flow rate of 1 ml/min.

In vivo NMR

After addition of 410 mg (3 mmol) of p-hydroxy-acetophenone to 300 g wet cells (9 g dwt) of the suspension culture of *Rauwolfia serpentina*, grown for 4 days in 1 l Erlenmeyer flasks containing 250 ml LS-medium[30], ca. 8 ml suspended cells were transferred after 5 min, 6 h, 20 h, 30 h and 50 h to a 10 mm NMR tube containing 1.4 ml D_2O. Cells were allowed to settle down for 15 min before measurements were started with 100 dummy scans to establish stable temperature conditions (25 °C ± 0.5). The spectra were obtained on a 400 DRX (100.6 MHz ^{13}C) instrument with standard Bruker software. For each spectrum 5600 FID's were accumulated (measuring time 2 h). Pulse angle was 30° with a pulse repetition time of 1.3 s. The sweep width of 25000 Hz and the memory size used (32 K) lead to a digital resolution of 0.76 Hz/data point. Resolution enhanced spectral parts were obtained by Gaussian-multiplication (GB: 0.15, LB: -6 Hz).

High Density Cell Suspension Culture and Addition of the Aglycones

Cell cultures were grown under typical conditions on gyratory shakers (100 rpm) under constant illuminaton (~ 600 Lux) at 24-26 °C using LS-medium. Cells were filtered aseptically after a growth period of seven days and 300 g of fresh cells were transferred to 150 ml LS-medium delivering a stage of high density cells, which were allowed to grow for four days. At this time 0.5 g of the corresponding aglycone dissolved in 200 ml LS-medium were added continuously over a period of 3 days. After a further 2 days, cells were harvested by suction filtration and extracted twice with MeOH under reflux for 30 min. The medium was frozen and stored at - 20 °C. The amounts of aglycones and glucosides in the cells or medium were determined by HPLC. For isolation of **6** on a preparative scale (> 0.5 g), 500 g fresh cells were added to 400 ml LS-medium and 2 g fluorescein was applicated to the culture after four days as an aqueous solution (13 mM) at a rate of 4 ml/min.

Isolation of 6

After feeding the cells for 5 days **6** was isolated in the following manner: Cells (550 g FW) were filtered off the nutrition medium and were immediately boiled with H_2O (3 times, 1500 ml) for 30 min. The aqueous solution was passed through a Serdolit® AD-2 column (4 x 20 cm) at a flow rate of 1.5 ml/min. The column was washed with 0.5 l water followed by elution with 1 l methanol, which was dried at 50 °C under reduced pressure and redissolved in the aqueous phase of the two phase solvent system. This solution was then pumped on to a preparative coil (2.6 mm x 80 m) of a MLCCC operating with the aqueous phase as mobile phase at a flow rate of 6 ml/min in ascending mode and fractions each of 9 ml were collected. The fractions No. 6 to 43 were pooled and concentrated in vacuum in the presence of 2 g silica gel (0.063 - 1.0 mm). The silica gel was then put on the top of a silica gel column (4.5 x 10 cm) which was eluted with 300 ml of the solvent system $CHCl_3$/MeOH (7:1) and then with $CHCl_3$/MeOH (6:1) at a rate of 4 ml/h. Fractions containing pure **6** were combined, evaporated under reduced pressure, centrifuged (7000 x g) after addition of water and finally freeze dried yielding 1.09 g (53%) pure **6**. HPLC indicated the purity of **6** as 98%.

Conversion of 6 into Fluorescein by β-Glucosidase

The purity of the obtained **6** was checked by β-glucosidase and TLC. To an aqueous solution of 0.5 mg of **6** (2 mM) 10 μg β-glucosidase (Boehringer Mannheim, from almonds, 0.5 nkat) were added at 37 °C in presence of 50 mM phosphate-citrate buffer (pH 5.5). After each 2 min, 10 μl of the solution was spotted on to a TLC plate, which was developed after drying in system [B]. Complete conversion of **6** was monitored after 40 min of incubation. For quantitation, the incubation mixture was analysed by HPLC; R_t 4.5 min for **6** and R_t 8.9 min for fluorescein was measured.

Resorufin-O-β-D-glucopyranoside **(1)**

Orange powder; R_f [A] 0.51; UV λ_{max}^{MeOH} nm: 465.2, 380.4, 197.3; EI-MS of the aglycone *m/z* (rel. int. %): 255 (3), 213 (35), 185 (38), 156 (12), 149 (19), 128 (20), 87 (58), 69 (100); ^1H NMR (400 MHz, DMSO-d_6/D_2O): δ 7.79 (d, 1H, $J_{H-8/H-7}$=8.7, H-8), 7.54 (d, 1H, $J_{H-11/H-12}$=9.2, H-11), 7.15 (d, 1H, $^4J_{H-5/H-7}$=2.6, H-5), 7.10 (dd, 1H, $J_{H-7/H-8}$=8.7, $^4J_{H-7/H-5}$=2.6, H-7), 6.79 (dd, 1H, $J_{H-12/H-11}$=9.2, $^4J_{H-12/H-2}$=2.1, H-12), 6.28 (d, 1H, $^4J_{H-2/H-12}$=2.1, H-2), 4.88 (d, 1H, $J_{H-1'/H-2'}$= 7.1, H-1'), 3.7 -3.1 (m, 6H, H_A-6', H_B-6', H-5', H-3', H-2', H-4').

Picein (p-acetyl-phenyl-O-β-D-glucopyranoside) **(2)**

R_f [A] 0.62; UV λ_{max}^{MeOH} nm: 264.0, 218.6; FD-MS *m/z* (rel. int %): 299.2 ($[M+H]^+$, 17), 298.2 (100), 135.1 (7); ^1H NMR (400 MHz, DMSO-d_6/D_2O): δ 7.91 (d, 2H, $J_{H-3/H-2}$=$J_{H-5/H-6}$=8.8, H-3, H-5), 7.10 (d, 2H, $J_{H-2/H-3}$= $J_{H-6/H-5}$=8.8, H-2, H-6), 4.99 (d, 1H, $J_{H-1'/H-2'}$=7.1, H-1'), 3.66 (d, 1H, $J_{HA-6'/HB-6'}$=11.2, H_A-6'), 3.45 (dd, 1H, $J_{HB-6'/HA-6'}$=11.2, $J_{HB-6'/H-5'}$=5.8, H_B-6'), 3.40 - 3.28 (m, 2H, H-5', H-3'), 3.24 (dd, 1H, $J_{H-2'/H-3'}$=8.1, $J_{H-2'/H-1'}$=7.1, H-2'), 3.16 (d, 1H, $J_{H-4'/H-5'}$=$J_{H-4'/H-3'}$= 7.7, H-4'), 2.51 (s, 3H, H-8); ^{13}C NMR: 197.0 (C-7), 161.0 (C-1), 130.8 (C-4), 130.3 (C-3/C-5), 115.9 (C-2/C-6), 99.6 (C-1'), 76.9 (C-5'), 76.2 (C-3'), 72.9 (C-2'), 69.5 (C-4'), 60.5 (C-6'), 26.4 (C-8).

p-Acetyl-phenyl-primeveroside **(3)**

R_f [A] 0.45; UV λ_{max}^{MeOH} nm: 263.6, 204.8; ^1H NMR (400 MHz, DMSO-d_6/D_2O): δ 7.92 (d, 2H, $J_{H-3/H-2}$=$J_{H-5/H-6}$ =8.7, H-3, H-5), 7.15 (d, 2H, $J_{H-2/H-3}$= $J_{H-6/H-5}$=8.7, H-2, H-6), 4.95 (d, 1H, $J_{H-1'/H-2'}$=7.2, H-1'), 4.16 (d, 1H, $J_{H-1''/H-2''}$=7.5, H-1''), 3.94 (d, 1H, $J_{HA-6'/HB-6'}$=11.5, H_A-6'), 3.66 (dd, 1H, $J_{H-5e''/H-5a''}$=11.2, $J_{H-5e''/H-4''}$=5.3, H-5_e''), 3.59 (dd, 1H, $J_{HB-6'/HA-6'}$=11.5, $J_{HB-6'/H-5'}$=6.5, H_B-6'), 3.56 (dd, 1H, $J_{H-5'/H-4'}$=11.6, $J_{H-5'/H-6B'}$=6.5, H-5'), 3.33 (dd, 1H, $J_{H-3'/H-2'}$=9.8, $J_{H-3'/H-4'}$=8.7, H-3'), 3.29 (dd, 1H, $J_{H-2'/H-3'}$=9.8, $J_{H-2'/H-1'}$=7.2, H-2'), 3.25 (ddd, 1H, $J_{H-4''/H-5a''}$= 10.7, $J_{H-4''/H-3''}$=8.9, $J_{H-4''/H-5e''}$=5.3, H-4''), 3.19 (dd, 1H, $J_{H-4'/H-5'}$=9.1, $J_{H-4'/H-3'}$= 8.7, H-4'), 3.06 (dd, 1H, $J_{H-3''/H-4''}$= 8.9, $J_{H-3''/H-2''}$=8.7, H-3''), 2.97 (dd, 1H, $J_{H-2''/H-3''}$=8.7, $J_{H-2''/H-1''}$=7.5, H-2''), 2.91 (dd, 1H, $J_{H-5a''/H-5e''}$=11.2, $J_{H-5a''/H-4''}$= 10.7, H-5_a''), 2.52 (s, 3H, H-8); ^{13}C NMR: 198.2 (C-7), 162.1 (C-1), 132.1 (C-4), 131.6 (C-3/

C-5), 117.2 (C-2/C-6), 104.8 (C-1"), 100.9 (C-1'), 77.3 (C-3"), 77.2 (C-3'), 77.0 (C-5'), 74.5 (C-2"), 74.1 (C-2'), 70.5 (C-4', C-4"), 69.1 (C-6'), 66.6 (C-5"), 27.6 (C-8).

o-Coumaric acid-O-β-D-glucopyranoside ***(4)***

R$_f$ [A] 0.35; UV λ_{max}^{MeOH} nm: 307.1, 268.6, 202.4; ^{1}H NMR (400 MHz, DMSO-d_6/D_2O): δ 7.55 (d, 1H, $J_{H-8/H-7}$=16.2, H-8), 7.51 (d, 1H, $J_{H-3/H-4}$=8.2, H-3), 7.21 (dd, 1H, $J_{H-5/H-6}$=8.3, $J_{H-5/H-4}$=7.2, H-5), 7.11 (d, 1H, $J_{H-6/H-5}$=8.3, H-6), 6.96 (dd, 1H, $J_{H-4/H-3}$=8.2, $J_{H-4/H-5}$=7.2, H-4), 6.36 (d, 1H, $J_{H-7/H-8}$=16.2, H-7), 4.84 (d, 1H, $J_{H-1'/H-2'}$=7.4, H-1'), 3.66 (d, 1H, $J_{HA-6'/HB-6'}$=11.8, H$_A$-6'), 3.47 (dd, 1H, $J_{HB-6'/HA-6'}$=11.8, $J_{HB-6'/H-5'}$=4.6, H$_B$-6'), 3.41-3.36 (dd, 1H, $J_{H-5'/H-4'}$=8.9, $J_{H-5'/HB-6'}$=4.6, H-5'), 3.33 (dd, 1H, $J_{H-2'/H-3'}$=8.6, $J_{H-2'/H-1'}$=7.4, H-2'), 3.27 (dd, 1H, $J_{H-3'/H-4'}$=9.1, $J_{H-3'/H-2'}$=8.6, H-3'), 3.16 (dd, 1H, $J_{H-4'/H-3'}$=9.1, $J_{H-4'/H-5'}$=8.9, H-4').

trans-Cinnamoyl-O-β-D-glucopyranoside (trans-cinnamic acid-glucoside) ***(5)***

R$_f$ [A] 0.64; UV λ_{max}^{MeOH} nm: 361.8, 230.1, 223.8, 218.7, 214.3; ^{1}H NMR (400 MHz, DMSO-d_6/D_2O): δ 7.61 (d, 1H, $J_{H-3/H-2}$=15.9, H-3), 7.53 (d, 2H, $J_{H-5/H-6}$=$J_{H-9/H-8}$=8.5, H-5, H-9), 6.77 (d, 3H, $J_{H-6/H-5}$=$J_{H-8/H-9}$=$J_{H-7/H-6}$=$J_{H-7/H-8}$=8.5, H-6, H-7, H-8), 6.35 (d, 1H, $J_{H-2/H-3}$=15.9, H-2), 5.41 (d, 1H, $J_{H-1'/H-2'}$=8.0, H-1'), 3.62 (d, 1H, $J_{HA-6'/HB-6'}$=11.6, H$_A$-6'), 3.42 (dd, 1H, $J_{HB-6'/HA-6'}$=11.6, $J_{HB-6'/H-5'}$=5.4, H$_B$-6'), 3.3 - 3.0 (m, 4H, H-5', H-3', H-2', H-4').

Fluorescein-mono-O-β-D-glucopyranoside ***(6)***

Yellow Powder; R$_f$ [A] 0.57; $[\alpha]_D^{24}$= - 39.61° (c 1.0, MeOH); UV λ_{max}^{MeOH} nm: 451.5, 274.9; FAB-MS *m/z* (rel. int. %): 493.2 ([M-H]$^-$, 37), 417.4 (7), 379.3 (10), 349.3 (100), 331.1 (55), 311.2 (23), 265.2 (37); ^{1}H NMR (400 MHz, DMSO-d_6/D_2O): δ 8.01 (d, 1H, $J_{H-18/H-17}$=7.5, H-18), 7.81 (dd, 1H, $J_{H-16/H-17}$=$J_{H-16/H-15}$=7.5, H-16), 7.74 (dd, 1H, $J_{H-17/H-16}$=$J_{H-17/H-18}$=7.5, H-17), 7.29 (d, 1H, $J_{H-15/H-16}$=7.5, H-15), 7.02 (d, 1H, $^4J_{H-13/H-2}$=2.3, H-13), 6.79 (dd, 1H, $J_{H-2/H-3}$= 8.8, $^4J_{H-2/H-13}$=2.3, H-2), 6.72 (s, 1H, H-10), 6.67 (d, 1H, $J_{H-3/H-2}$= 8.8, H-3), 6.59 (s, 2H, H-7, H-8), 4.99 (d, 1H, $J_{H-1'/H-2'}$=7.4, H-1'), 3.70 (dd, 1H, $J_{HA-6'/HB-6'}$=12.2, $J_{HA-6'/H-5'}$=1.5, H$_A$-6'), 3.46 (dd, 1H, $J_{HB-6'/HA-6'}$=12.2, $J_{HB-6'/H-5'}$=5.7, H$_B$-6'), 3.40 (ddd, 1H, $J_{H-5'/H-4'}$=9.1, $J_{H-5'/HB-6'}$=5.7, $J_{H-5'/HA-6'}$=1.5, H-5'), 3.29 (dd, 1H, $J_{H-3'/H-4'}$=$J_{H-3'/H-2'}$=8.8, H-3'), 3.24 (dd, 1H, $J_{H-2'/H-3'}$=8.8, $J_{H-2'/H-1'}$=7.4, H-2'), 3.16 (dd, 1H, $J_{H-4'/H-5'}$= 9.1, $J_{H-4'/H-3'}$=8.8, H-4'); ^{13}C NMR: 168.76 (C-20), 159.6 (C-1), 158.9 (C-9), 152.5 (C-12), 151.7 (C-11), 135.1 (C-16), 130.0 (C-17), 129.1 (C-10), 128.9 (C-8), 126.0 (C-14), 124.7 (C-18), 124.0 (C-15), 113.4 (C-19), 113.2 (C-2), 112.3 (C-7), 112.2 (C-4), 109.3 (C-6), 103.3 (C-13), 102.2 (C-3), 100.0 (C-1'), 82.6 (C-5), 77.1 (C-5'), 76.4 (C-3'), 73.1 (C-2'), 69.6 (C-4'), 60.6 (C-6').

Fluorescein-di-O-β-D-glucoside

Yellow powder; R$_f$ [B] 0.33; UV λ_{max}^{MeOH} nm: 327.7, 273.2, 234.5; FAB-MS *m/z* (rel. int. %): 655.5 ([M-H]$^-$, 26), 643.6 (10), 551.5 (12), 459.4 (19), 367.3 (30), 331.3 (33), 275.3 (100).

6-Brom-2-naphthyl-O-β-D-glucopyranoside

R$_f$ (A) 0.46; UV λ_{max}^{MeOH} nm: 332.6, 274.2, 264.5.

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