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Enzymatic Synthesis of 1-*O*-Phenylcarboxyl-β-D-Glucose Esters

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A procedure for the facile preparation of various 1-*O*-phenylcarboxyl-β-D-glucose esters has been developed. Although such compounds are widely distributed in higher plants, only fragmentary knowledge exists about their synthesis and properties. The method reported here utilizes a UDP-glucose-specific glucosyltransferase from oak leaves. Under the catalysis of this enzyme, several differently ring-substituted 1-*O*-benzoylglucoses were synthesized. The reaction products were purified by reversed-phase HPLC and characterized by uv, ir, and ^{1}H NMR spectroscopy. The described procedure appears most suitable for small-scale preparations sufficient for many biochemical investigations, and particularly for the synthesis of radioactively labeled esters.

INTRODUCTION

It is well documented that 1-*O*-glucose esters of hydroxylated benzoic and cinnamic acids are almost ubiquitously occurring constituents of higher plants (*1–4*). In accordance with the first report of Corner and Swain (*5*) it is now evident that they are generally synthesized from the free acid and uridine-5′-diphosphoglucose (UDPG; references cited in *6*). Only recently has it been recognized that these esters function also as metabolically active intermediates; they were found to serve as acyl donors in transesterification reactions with choline (*7, 8*), malate (*9, 10*), quinate (*11–13*), *myo*-inositol (*14*), or tartaric acid (*15*) as alcoholic moieties. Surprisingly, they can act also as both acyl donor and acceptor in one reaction, yielding diacyl derivatives of glucose (*16–19*) or quinate (*20*).

In the course of our investigations on the biosynthesis of gallotannins, we required various 1-*O*-acylglucoses, particularly the benzoyl derivatives, as substrates for enzyme specificity studies. These compounds are usually prepared either by chemical synthesis or by extraction from natural sources. Both methods have certain disadvantages, e.g., the necessity of introducing protecting groups into the reactants or the difficulty of obtaining suitable plant materials with sufficiently high concentrations of the desired ester. As an attractive alternative, we

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decided therefore to attempt an enzymatic synthesis using a β-glucogallin (1-*O*-galloyl-β-D-glucose) forming glucosyltransferase that had been recently detected by us (*21*) and was shown to be active also with several related aromatic acids (*6*). This approach was thought to provide a convenient tool to prepare the comparatively low quantities required for biochemical studies and, as a particular advantage, it should allow the synthesis of radioactively labeled acylglucoses of high specific activity. Moreover, we became aware that the data published to date on this class of compounds are rather fragmentary. Considering the growing importance of these esters within the area of plant secondary metabolism, it appeared thus appropriate to attempt also their fuller characterization within the scope of this project.

Under this premise, and as reported below, seven different 1-*O*-benzoylglucose esters were enzymatically prepared and characterized by uv, ir, and NMR spectroscopy. Among these, anisoyl-β-D-glucose, to our knowledge, is described for the first time. The chemical synthesis and some properties have been published for the 1-*O*-glucose derivatives of benzoate (*22, 23*), *p*-hydroxybenzoate (*24*), vanillate (*25*), gallate (*26*), anthranilate (*27*), and gentisate (*28*). In addition, the glucose esters of benzoate (*29*), *p*-hydroxybenzoate (*30*), and veratrate (*31*) have been isolated from biological sources.

EXPERIMENTAL

HPLC Procedures

The rate of glucose ester formation in preparative incubation mixtures was measured by isocratic reversed-phase HPLC on Lichrosorb RP-18 columns (Merck CGC glass cartridges), particle size 5 μm, 180 × 3 mm i.d., at a flow rate of 1 ml/min. Due to largely differing polarities of the acid components, specifically adapted solvents had to be used to achieve clear resolution: protocatechuic (10% methanol), *p*-hydroxybenzoic (21%), vanillic (23%), syringic (25%), benzoic (27%), veratric (31%), anisic (32%); the aqueous component was 0.05% H_3PO_4.

Purity of synthesized glucose esters was checked by reversed-phase HPLC with the acetonitrile-H_3PO_4 gradient given by Haslam and co-workers (*32, 33*) (for details, see Fig. 2). Under these conditions, the following retention times were observed for the ester and the less polar parent acid, respectively: protocatechuic 14.1, 15.3 min; *p*-hydroxybenzoic 17.0, 20.0; vanillic 18.9, 23.0; syringic 20.6, 24.5; benzoic 22.5, 30.8; veratric 25.4, 32.5; anisic 27.1, 36.6.

Preparation and Assay of Glucosyltransferase

About 2-month-old oak leaves (80 g) were homogenized and the cell-free extract was purified by treatment with ion-exchange resin and fractionation with $(NH_4)_2SO_4$ as described earlier (*6*); the previously reported step with $CaPO_4$-gel proved unnecessary and was omitted. The resulting concentrated enzyme solution was desalted by gel filtration on Sephadex G-25 (PD-10 columns, Pharmacia). Enzyme activities and protein concentrations were determined as in Ref. (*6*).

Synthesis and Purification of Glucose Esters

In each experiment, reaction mixtures (total volume 10 ml) containing 1.4 mmol Tris–HCl buffer, pH 6.5, 0.45 mmol acid (adjusted to pH 6.5), 0.40 mmol UDPG and the enzyme obtained from 80 g leaf material were incubated at 44°C. After reaching maximal conversion as determined by isocratic HPLC (*vide supra*), the reaction was terminated by acidification with 1 N HCl (pH 1–2) and the precipitated protein was removed by centrifugation. The supernatant was extracted with ether (3 times) and the aqueous phase was clarified by two filtration steps (1, Whatman GF/C glass fibers; 2, Schleicher & Schüll RC—60 membrane filters, pore size 1 μm). The filtrate was purified in several portions by preparative HPLC (Merck Lichrosorb RP-18, 5 μm, column 30 × 2 cm, flow rate 15 ml/min). Polar contaminants were washed out with 5% methanol in 0.05% acetic acid, followed by elution of the glucose ester with 25% methanol. This fraction was concentrated by rotary evaporation, lyophilized, and dried *in vacuo* over P_2O_5 and KOH at 65°C.

Characterization of Glucose Esters

Melting points (uncorrected) were determined under the microscope with a Kofler heat bench. For uv and ir measurements see Table 2.

^{1}H NMR spectra were recorded at 200 MHz in DMSO-d_6 as internal standard and interpreted as follows:

1-O-p-Hydroxybenzoyl-β-D-glucose. cf. Fig. 3.

1-O-Anisoyl-β-D-glucose. δ 3.85 (s, OCH_3), 4.60, 5.05, 5.20, 5.40 (4 H, O*H*-2,3,4,6), 5.50 (d, ^3J = 7.5 Hz, H-1), 7.10 (d, arH-3,6), 7.95 (d, arH-2,6).

1-O-Protocatechuoyl-β-D-glucose. δ 5.50 (d, ^3J = 7.5 Hz, H-1), 6.80 (d, arH-5), 7.35 (d, arH-6), 7.40 (s, arH-2), 9.55 (s, arOH).

1-O-Vanilloyl-β-D-glucose. δ 3.80 (s, OCH_3), 4.60, 5.05, 5.20, 5.40, (4 H, O*H*-2,3,4,6), 5.50 (d, ^3J = 7.5 Hz, H-1), 6.90 (d, ^3J = 9 Hz, arH-5), 7.48 (d, ^4J = 2 Hz, arH-1), 7.52 (dd, arH-6).

1-O-Veratroyl-β-D-glucose. δ 3.80 (s, OCH_3), 3.85 (s, OCH_3), 4.60, 5.10, 5.20, 5.40, (4 H, O*H*-2,3,4,6), 5.50 (d, ^3J = 7.0 Hz, H-1), 7.10 (d, ^3J = 8.5 Hz, arH-5), 7.50 (d, ^4J = 2 Hz, arH-2), 7.65 (dd, arH-6).

1-O-Syringoyl-β-D-glucose. δ 3.80 (s, OCH_3), 5.50 (d, ^3J = 7.5 Hz, H-1), 7.25 (s, arH-2,6).

RESULTS AND DISCUSSION

Substrate Specificity of the Glucosyltransferase

During our studies on the biosynthesis of β-glucogallin it was found that the glucosyltransferase catalyzing the formation of this ester exhibited appreciable activity also in the presence of certain other cinnamic and benzoic acids (*6*). As a prerequisite for the intended preparation of such esters we therefore investigated the affinity of this enzyme toward an extended number of potential substrates.

TABLE 1

Specificity of the Glucosyltransferase toward Various Aromatic Acids

Substrate	Substitution pattern	V_{max} (pmol sec^{-1})	K_m (mM)	V_{max}/K_m
C_6C_1-acids				
Vanillic	4-OH, 3-OMe	5.2	1.6	3.3
Protocatechuic	3-OH, 4-OH	4.5	1.4	3.2
Syringic	4-OH, 3-OMe, 5-OMe	4.6	1.9	2.4
Veratric	3-OMe, 4-OMe	4.6	2.0	2.3
p-Hydroxybenzoic	4-OH	4.5	2.0	2.3
Isovanillic	3-OH, 4-OMe	3.1	1.6	1.9
Gallic	3-OH, 4-OH, 5-OH	2.9	1.6	1.8
Anisic	4-OMe	4.2	3.2	1.3
Benzoic	—	3.4	6.7	0.5
3,4,5-Trimethoxybenzoic	3-OMe, 4-OMe, 5-OMe	1.1	2.5	0.4
Salicylic	2-OH	0.3	3.0	0.1
C_6C_2-acids				
Phenylacetic	—	0.2	4.4	0.05
C_6C_3-acids				
Isoferulic	3-OH, 4-OMe	2.5	1.2	2.1
Caffeic	3-OH, 4-OH	2.7	1.4	1.9
p-Methoxycinnamic	4-OMe	2.4	1.3	1.8
Sinapic	4-OH, 3-OMe, 5-OMe	0.8	0.8	1.0
p-Coumaric	4-OH	2.3	2.6	0.9
3,4,5-Trimethoxycinnamic	3-OMe, 4-OMe, 5-OMe	0.4	0.6	0.7
Ferulic	4-OH, 3-OMe	1.5	2.1	0.7
o-Coumaric	2-OH	1.6	2.9	0.6
m-Coumaric	3-OH	1.3	2.9	0.4
Cinnamic	—	1.1	9.1	0.1
Phenylpropionic	—	~0	—	—

This was done by determining the maximal velocity of the enzymatic reaction and the corresponding K_m values. The quotient of these two parameters provides a reliable measure for the substrate specificity of enzymes. It is evident from the data compiled in Table 1 that benzoic acids are better converted than cinnamic acids; within each group a certain preference for di- and trisubstituted acids can be observed. In addition, the previously (*6*) proposed systematic name of the enzyme, UDPG: vanillate 1-*O*-glucosyltransferase according to the best substrate, has been corroborated by this detailed study.

As a consequence, we chose several highly to moderately active benzoic acids for further investigations; the general reaction for the formation of such esters is depicted in Fig. 1. It is obvious, however, that also a number of cinnamic acids would represent suitable substrates.

Synthesis and Purification of Glucose Esters

For practical reasons, and in contrast to a previous communication (*6*), it was decided to work with enzyme preparations that had only been purified and con-

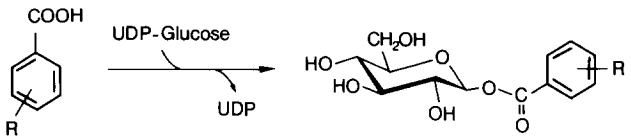

FIG. 1. Reaction catalyzed by 1-*O*-β-D-glucosyltransferase.

centrated by ammonium sulfate fractionation (cf. experimental section). The thus resulting low purification factor is compensated, however, by several advantages: preparation of the enzyme solution requires only a few hours, losses of enzymatic activity are minimal, and favorably high concentrations of both enzyme and substrates can easily be maintained in the reaction mixtures.

It was further necessary to adapt the previously developed analytical enzyme assay to the new preparative purpose. Raising the substrate concentrations to a final value of 40–45 mM and the incubation temperature to 44°C gave optimal results.

Other problems were encountered with respect to the incubation time required for maximal ester synthesis. Because of varying enzyme activities, together with the above-reported large differences of the reactivities of the acid substrates (cf. Table 1), it was impossible to standardize this parameter. Moreover, upon prolonged incubation even decreasing product concentrations were observed occasionally, a fact that must be attributed to a contaminating esterase activity. Monitoring the progress of the reaction by the previously reported, very time-consuming chromatographic method (*6*) was impossible. The problem was solved by the application of reversed-phase HPLC under isocratic conditions. Due to the pronounced polarity differences of the individual acid substrates, methanol concentrations in the eluant varying between 10% (protocatechuoylglucose) and 32% (anisoylglucose) had to be used in this procedure (for details, see Experimental section).

The thus obtained reaction mixtures were depleted from unreacted acid by extraction with ether which greatly facilitated their subsequent purification by preparative HPLC. For this latter purpose, aliquots of the aqueous phase were directly applied onto a RP-18 column. The polar contaminants (particularly UDPG and its hydrolysis products) were washed out with 5% aq. methanol. By raising the methanol concentration to 25%, the desired ester was eluted, followed by traces of residual acid. In first orientating experiments, the still unknown retention time of the ester had to be determined. This was done by chromatography of small samples of labeled ester obtained by enzymatic synthesis with [glucosyl-^{14}C] UDPG as substrate.

The ester-containing fractions were pooled, concentrated by rotary evaporation, lyophilized, and dried *in vacuo* over P_2O_5 at 65°C. Progress of the entire purification procedure was monitored by analytical HPLC; a typical example is depicted in Fig. 2. By this means, purities of ca. 99% were determined for the synthesized esters. Yields of the final products were 7–10%, i.e., about 7–13 mg of pure glucose ester could be obtained from each batch.

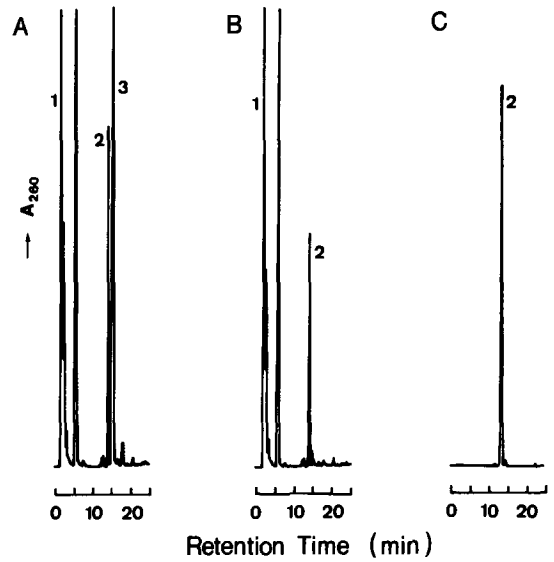

FIG. 2. HPLC-analysis of 1-*O*-protocatechuoyl-β-D-glucose. (A) Crude reaction mixture at the end of the incubation period. (B) Reaction mixture after extraction with diethyl ether. (C) Purified ester after preparative HPLC. (1) UDPG; (2) 1-*O*-protocatechuoyl-β-D-glucose; (3) protocatechuic acid. Chromatography conditions (*32, 33*): Lichrosorb RP-18 column (Merck CGC glass cartridge), particle size 5 μm, 180 × 3 mm i.d.; solvent A = acetonitrile, solvent B = 0.05% H_3PO_4; gradient: 0–5 min 0% A, 5–50 min 0–30% A; flow rate 1 ml/min.

By the same technique, but using smaller reaction mixtures, we have conveniently synthesized also β-[galloyl-^{14}C]glucogallin with a specific radioactivity of 37 kBq (1 μCi)/μmol by incubating UDPG and [^{14}C]gallic acid.

Characterization of Glucose Esters

The melting points of most of the esters synthesized by us were found to lie between 128° and 189°C, thus being considerably below the few published data on such compounds (cf. *24, 25, 30, 31*). This must clearly be attributed to the fact that the procedure described here yields amorphous products. Only 1-*O*-*p*-hydroxybenzoylglucose was obtained as a crystalline solid; in this case we determined a m.p. of 223°C which is in good accordance to literature values (*24, 30*).

No very conclusive data resulted also from elementary analyses. Recalculation of the values obtained from C,H-determinations lead to the conclusion that the different products contained water in ratios of 0.5, 1, or 1.5 mol/mol ester, respectively. This situation, however, is often encountered with such polyhydroxylated compounds, even when they had been obtained from anhydrous solvents (cf., e.g., *32*).

The glucose esters were further characterized by spectrophotometry. The prominent ir-bands listed in Table 2 are in full accord with the proposed structures (3500–3200 cm^{-1}: νOH; 1710: νAr-CO-O; 850: γC_{ar}-H out-of-plane vibration, lacking in the case of galloyl and syringoylglucose). Concerning the uv-data, it is

TABLE 2

Spectrophotometric Characteristics of Benzoic Acids and Their 1-*O*-β-D-Glucose Derivatives

Compound	Free acid	Glucose ester		
	λ_{max}[a] (nm)	λ_{max}[a] (nm)	ε[b] (cm^2 μmol^{-1})	ir[c] (cm^{-1})
Benzoic	224	231 (275sh)	11.8	3580–3180, 1720, 710[e]
p-Hydroxybenzoic	245	260 (300sh)	10.1	3500–3220, 1705, 840
Anisic	246	262	13.8	3570–3210, 1710, 850
Protocatechuic	252, 288	265, 300	10.0, 7.3	3510–3110, 1680, 820
Vanillic	250, 284	266, 297	8.9, 6.1	3540–3200, 1705, 870
Veratric	250, 284	265, 294	8.8, 4.4	3540–3230, 1720, 820
Gallic[d]	260	278 (330sh)	10.9	3550–3000, 1705
Syringic	262	282 (330sh)	8.0	3520–3180, 1700

[a] Recorded in 0.1 M potassium phosphate buffer, pH 7.0; sh = shoulder.

[b] The given extinction coefficients ε refer to the absorbance in 1 mM solution.

[c] Recorded in KBr.

[d] The glucose derivative was synthesized chemically (cf. *21*).

[e] γC_{ar}-H out-of-plane deformation vibration and/or ring deformation (Δ), characteristic of unsubstituted benzoic acid esters.

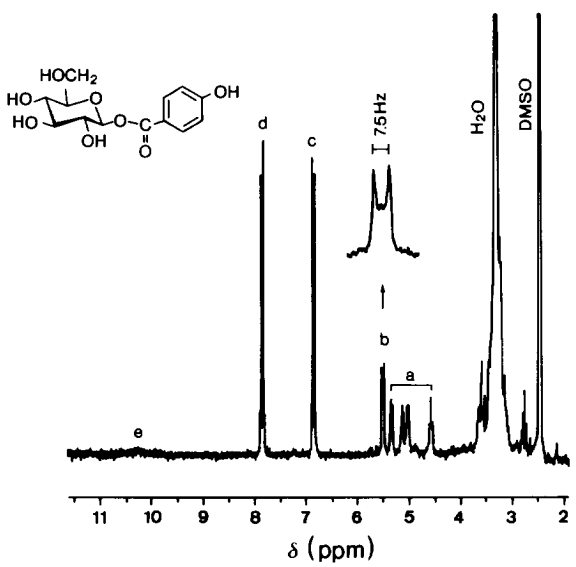

FIG. 3. ^1H NMR spectrum of 1-*O*-*p*-hydroxybenzoyl-β-D-glucose in DMSO-d_6. (a) δ 4.60, 5.05, 5.15, 5.35: broadened doublets, 4 H from OH-groups of glucose; these signals are eliminated by D_2O exchange; (b) δ 5.50: doublet, H-1 of glucose, $^3J_{1\text{-H},2\text{-H}}$ = 7.5 Hz shows β-configuration at the anomeric C-1; (c) δ 6.85: doublet, aryl H-3, H-5; (d) δ 7.85: doublet, aryl H-2, H-6; (e) δ 10.35: broadened singlet, aryl OH (eliminated by D_2O exchange).

evident that the λ_{max} values for the esters show, as expected, a moderate bathochromic shift as compared to the free acid. Similar observations have been made with cinnamoylglucose esters (*34*). On the other hand, values suggesting a slight hypsochromy have been reported for various benzoylglucoses in an earlier publication (*1*) and it must be concluded now that these latter data have been changed. As sufficient amounts of ester were available to us we have also determined their to date unknown molar extinction coefficients ε for the first time which were found to be of the same order as the well-documented values for the parent acids. Knowledge of these spectral characteristics will certainly facilitate the future qualitative and quantitative estimation of these compounds.

Definitive proof of the expected structure of the synthesized 1-*O*-acyl-β-D-glucose esters has been gained by ^{1}H NMR spectroscopy. The data from these studies are given in the experimental section; one characteristic example, including interpretation of the signals, is depicted in Fig. 3.

CONCLUSIONS

The reported enzymatic procedure adds an attractive alternative to existing conventional methods for the preparation of aromatic 1-*O*-acyl-β-D-glucose esters. No protective groups are required, and the proven specificity of the glucosyltransferase directs the acyl moiety exclusively without any precautions into the desired 1-position of glucose. These characteristics provide also a convenient tool for the facile synthesis of labeled esters of high specific radioactivity that will otherwise be accessible only by large-scale and multistep chemical methods.

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