Novel Enzymatic Approach to the Synthesis of Flavonoid Glycosides and Their Esters

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Abstract: Flavonoids such as (+)catechin can be efficiently solubilised in supersaturated solutions prepared with donor glycosides, e.g., *p*-nitrophenyl glycosides, di- and higher oligosaccharides, and poly(ethylene glycol) dimethyl ether in sufficiently high concentration for their efficient enzymatic glycosylation. Under these conditions several glycosidases readily accept (+)catechin as substrate and the target glycosides were prepared in one step in up to 26% yields. The regioselectivity of the reaction depends on the enzyme and substrate combination used; three positions, 5, 7, and 4′, in the flavonoid can be glycosylated. The resulting and similar flavonoid glycosides were further modified by regioselective acylation with vinyl esters of arylpropenoic acids using lipases as biocatalyst. The efficiency of acylation was found to diminish in the order of vinyl cinnamate > vinyl ferulate > vinyl coumarate. This work demonstrates the feasibility of assembling complex flavonoid glycoside esters in just two steps by sequential use of commercially available glycosidases and lipases. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **71**: 235–243, 2000/2001.

Keywords: flavonoid; flavonoid glycosides; catechin; glycosides; glycosidase; glycosylation; supersaturated solutions; lipase; transesterification

INTRODUCTION

Enzymes are valuable tools for the preparation of complex carbohydrates and their derivatives due to the ability of biocatalysts to generate the required type and configuration of glycosidic linkage with no requirement for elaborate protection/deprotection strategies (see reviews, Davis, 2000; Fernandez-Mayoralas, 1997; Gambert and Thiem, 1997; Murata and Usui, 2000; Nilsson, 1996; Wang et al., 1995; Wong, 1995; Van Rantwijik et al., 1999, and references therein). Generally, the main advantage of using enzymes such as glycosidases and glycosyltransferases is that it is often possible to significantly simplify the synthetic protocol by reducing the overall number of steps required.

Recently, we described an efficient new system for the synthesis of anomerically pure ethoxylated glycosides and disaccharides using supersaturated substrate solutions as reaction media (Millqvist-Fureby et al., 1998a,b,c). Further investigation of this system revealed that a number of commercially available enzymes were catalytically competent under these reaction conditions and that the secondary hydroxyl groups of glycoside acceptors (e.g., 6-*O*-acetyl glycosides) could be glycosylated to obtain disaccharides in preparative yields of up to 28% (MacManus and Vulfson, 2000). The objective of the present work was to establish whether other complex natural substrates can be efficiently glycosylated in supersaturated solutions and to assess the scope and limitations of this methodology. In particular, we were interested in developing a simple approach to the preparation of flavonoid glycosides and their esters, as these compounds are currently receiving much attention as "nutraceutical" components of the diet. In particular, flavonoid and isoflavons were reported to reduce the risk of prostate and breast cancer, osteoporosis, and to improve short-term memory in the elderly (Adlercreutz et al., 1995; DiCarlo et al., 1999; Lee, 1999; Morton et al., 2000; Sangwan et al., 1998; Tijburg et al., 1997).

More than 1,400 flavonoid glycosides and their derivatives, mainly isolated from various natural sources, have been described (Barron and Ibrahim, 1996). In nature the (iso)flavonoid core and the glycosidic component are assembled separately and the glycoside is then produced by the action of specific glycosyl transferases. The resulting (iso)flavon glycosides are often further acylated on the primary position of sugar moieties by acyl-CoA dependent synthases to give the corresponding esters of acetic, malonic, or arylpropenoic acids such as caffeic, cinnamic, *p*-coumaric, or ferulic acid. The function of glycosylation and esterification is yet unknown and it is presumed that the derivatisation pattern governs the interaction of (iso)flavons with particular cell types and/or tissues. Clearly, the availability of a relatively simple synthetic approach to the preparation of these complex molecules would much facilitate the investigation of their biological properties. (+)Catechin was selected as a suitable model compound for the purpose of this investigation because it is structurally representative and is available in high purity at a reasonable cost. Also, the resulting glycosylation products were of in-

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terest due to the diverse biological activities of catechin and its derivatives, i.e., antitumor, antimutagenic, and antihypercholestemic action (Kada et al., 1985; Oguni et al., 1988).

MATERIALS AND METHODS

Chemicals

Cellulase (EC 3.2.1.4, from *Aspergillus niger*, 0.39 units/mg solid), α-amylase (EC 3.2.1.1, Type II-A: from Bacillus species, 1,840 units/mg solid), α-D-glucosidase (EC 3.2.1.20, from *Bacillus stearothermophilus*, 151 units/mg protein), *p*-Nitrophenyl-β-D-fucopyranoside (fucoside-β-D-*O*PNP), maltose, naringin hydrate, rutin hydrate, and all other chemicals, silica gel (70–230 mesh, 60Å), and organic solvents used in this work were obtained from Aldrich Chemical Co. (Dorset, UK). Novozyme 435 and Lipozyme IM were obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). Dextrin 10 (from maize starch) and (+)catechin hydrate were purchased from Fluka (Dorset, UK). Quercitrin, isoquercitrin, and luteolin were supplied by Indofine Chemical Co. (NJ, USA). Vinyl cinnamate was purchased from Lancaster Synthesis (Lancashire, UK).

Preparation of Supersaturated Solutions and Enzymatic Transglycosylation

In a typical small-scale experiment, substrates, i.e., (+)catechin and a glycon donor were weighed into a 2 mL screw-capped glass vial and citrate buffer (50 mM, pH 5.2) and poly(ethylene glycol) dimethyl ether (PEG-$(OCH_3)_2$) were added as specified below and in the legends to the tables. The mixture was heated until the components dissolved (approx. to 95°C) and the vial was then transferred to an incubator at 37°C. The reaction was started by the addition of enzyme, dissolved in the same citrate buffer. Aliquots of the reaction mixture were withdrawn at relevant time intervals analysed by TLC (developing solvent: ethyl acetate / acetic acid / water 3:1:1, v/v/v).

To determine the solubility of substrates in the reaction media, saturated solutions were prepared by equilibrating the compounds at 37°C overnight. A sample was taken and after centrifugation the concentration of (+)catechin and fucoside-β-D-*O*PNP in the supernatant was determined spectrophotometrically.

Preparative Synthesis of (+)Catechin Glycosides

Synthesis of (+)Catechin β-D-fucopyranoside (see Fig. 1A)

Fucoside-β-D-*O*PNP (0.20 g, 0.70 mmol), (+)catechin hydrate (0.60 g, 2.07 mmol), and poly(ethylene glycol) dimethyl ether (Mn 500, 0.4 g) were mixed with 50 mM citrate buffer, pH 6.0 (1.9 mL), and heated to 95°C to dissolve the reactants. The reaction mixture was transferred to a shaker-incubator (125 rpm) at 37°C. After 20 min, cellulase (0.20 g in 0.1 mL buffer) was added to start the reaction. The progress of the reaction was monitored by TLC (developing solvent: ethyl acetate / acetic acid / water 3:1:1 (v/v/v), $Rf_{fucoside} = 0.60$). After 3 days the reaction was

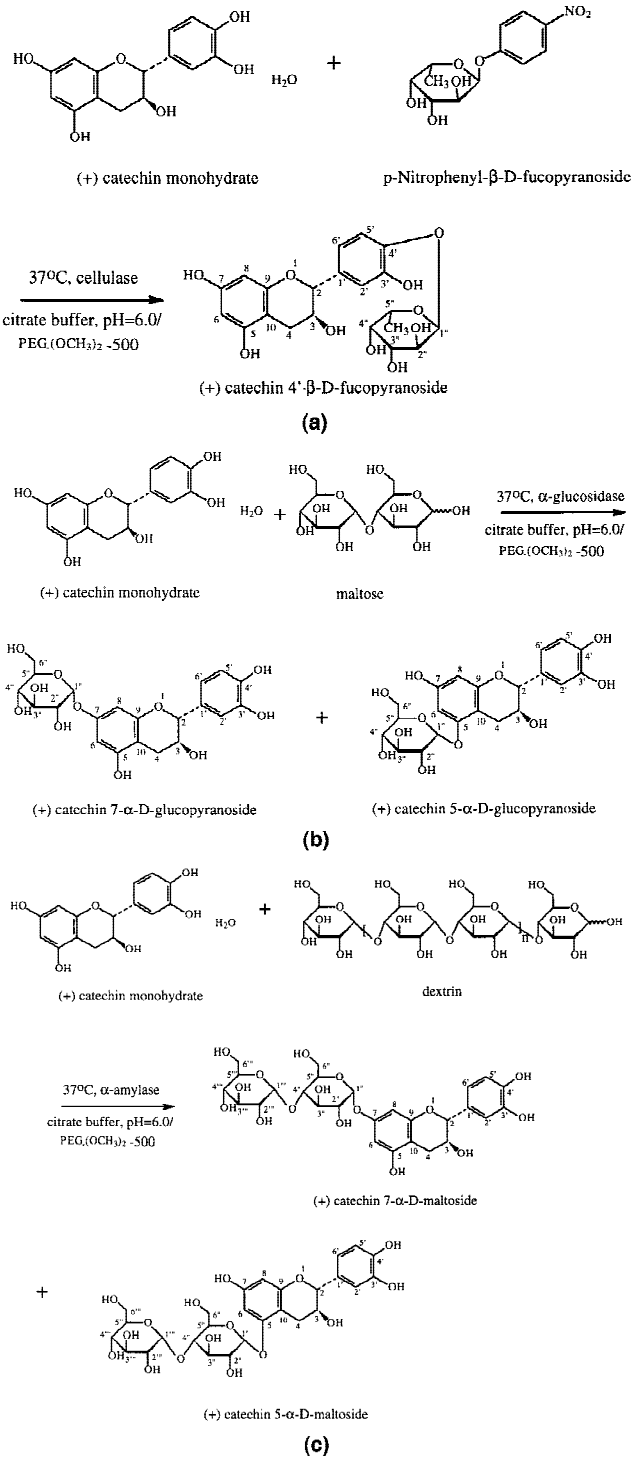

Figure 1. Glycosidase-catalysedsynthesis of (+)catechin β-D-fucopyranoside (**A**), (+)catechin α-D-glucopyranoside (**B**), and (+)catechin α-D-maltoside (**C**).

stopped and water was evaporated under vacuum at 50–60°C using a standard Büchi rotor evaporator for about an hour. The product was isolated on a silica gel column (20 × 1.5 cm) eluting with chloroform first, then using gradient elution with chloroform/methanol from 2:1 to 1:2 (v/v). The product catechin 4′-*O*-β-D-fucopyranoside (0.08 g, 0.18 mmol) was obtained in 26% yield, based on fucoside-β-D-*O*PNP.

^{13}C-NMR (methanol-d_4) δ: 16.8 (-CH_3), 28.5 (C-4), 68.8 (C-3), 72.1 (C-2″), 72.4 (C-5″), 72.9 (C-4″), 74.7 (C-3″), 82.5 (C-2), 95.5 (C-6), 96.3 (C-8), 100.7 (C-10), 104.9 (C-1″), 116.0 (C-2′), 118.7 (C-5′), 119.8 (C-6′), 136.4 (C-1′), 146.5 (C-3′), 148.6 (C-4′), 156.8 (C-9), 157.6 (C-5), 157.9 (C-7).

Synthesis of (+)Catechin α-D-glucopyranosides (see Fig. 1B)

D-maltose monohydrate (2.00 g, 5.55 mmol), (+)catechin hydrate (6.00 g, 20.67 mmol), and poly(ethylene glycol) dimethyl ether (Mn 500, 4.0 g) were mixed with 50 mM citrate buffer, pH 6.0 (19.8 mL), and heated to 95°C to dissolve the reactants. The reaction mixture was transferred to a shaker-incubator (125 rpm) at 37°C. After 20 min, α-D-glucosidase (100 U in 0.2 mL citric buffer) was added. The progress of the reaction was monitored by TLC as described above ($Rf_{glucoside}$ = 0.48). After 24 h, the water was evaporated under vacuum 50–60°C using Büchi rotor evaporator for about 1 h and the product was isolated on a silica gel column (25 × 2 cm) eluting with chloroform first, then using gradient elution with chloroform/methanol from 1:1 to 1:5 (v/v). The product (+)catechin maltoside (0.50 g, 1.11 mmol) was obtained in 20% yield, based on maltose. The NMR data showed that the product was a mixture of (+)catechin 7-*O*-α-D-glucopyranoside and (+)catechin 5-*O*-α-D-glucopyranoside, which were present in approximately equal quantities. The separation of the two products was carried out by repeated preparative TLC (developing solvent: ethyl acetate / acetic acid / water 3:1:1 (v/v/v).

(+)Catechin 7-O-α-D-glucopyranoside. ^{13}C-NMR (methanol-d_4) δ: 28.4 (C-4), 61.9 (C-6″), 68.5 (C-3), 71.2 (C-4″), 73.1 (C-2″), 74.2 (C-3″), 74.7 (C-5″), 82.7 (C-2), 97.2 (C-6), 98.0 (C-8), 99.0 (C-1″), 103.7 (C-10), 115.3 (C-2′), 116.2 (C-5′), 120.0 (C-6′), 132.1 (C-1′), 146.1 (C-3′, 4′), 156.8 (C-9), 157.6 (C-5), 157.9 (C-7).

(+)Catechin 5-O-α-D-glucopyranoside. ^{13}C-NMR (methanol-d_4) δ: 27.8 (C-4), 61.9 (C-6″), 68.5 (C-3), 71.2 (C-4″), 73.2 (C-2″), 74.4 (C-3″), 74.7 (C-5″), 82.5 (C-2), 96.8 (C-8), 98.0 (C-6), 98.6 (C-1″), 103.0 (C-10), 115.1 (C-2′), 116.2 (C-5′), 119.7(C-6′), 132.1 (C-1′), 146.1 (C-3′, 4′), 156.7 (C-9), 157.1 (C-7), 158.2 (C-5).

Synthesis of (+)Catechin α-D-maltoside (see Fig. 1C)

Dextrin 10 (2.00 g), (+)catechin hydrate (6.00 g, 20.67 mmol), and poly(ethylene glycol) dimethyl ether (Mn 500, 4.0 g) were mixed with 50 mM citrate buffer, pH 6.0 (19.8 mL), and heated to 95°C to dissolve the reactants. The reaction mixture was transferred to a shaker-incubator (125 rpm) at 37°C. After 20 min, α-amylase (100 mg in 0.2 mL buffer) was added to start the reaction. The progress of the reaction was monitored by TLC as described above ($Rf_{maltoside}$ = 0.31). After 24 h the water was evaporated under vacuum at 50–60°C using Büchi rotor evaporator for about 1 h and the product was isolated on a silica gel column (25 × 2 cm) eluting with chloroform first, then using gradient elution with chloroform/methanol from 1:1 to 1:10 (v/v). The desired product (+)catechin maltoside (0.10 g, mmol) was obtained in 5% (w/w) yield, based on dextrin. The NMR analysis showed that the product was a mixture of (+)catechin 7-O-α-D-maltoside and (+)catechin 5-O-α-D-maltoside, which were present in approximately equal quantities.

(+)Catechin 7-O-α-D-maltoside. ^{13}C-NMR (methanol-d_4) δ: 28.4 (C-4), 61.7 (C-6″), 62.7 (C-6‴), 68.5 (C-3), 71.5 (C-4″), 72.9 (C-4‴), 73.0 (C-2″), 73.1 (C-2‴), 74.2 (C-3″), 74.8 (C-5″, 5‴), 75.1 (C-3‴), 82.8 (C-2), 97.2 (C-6), 98.0 (C-8), 98.9 (C-1″), 103.1 (C-1‴), 103.7 (C-10), 115.2 (C-2′), 116.3 (C-5′), 120.0 (C-6′), 132.1 (C-1′), 146.2 (C-3′, 4′), 156.9 (C-9), 157.5 (C-5), 157.9 (C-7).

(+)Catechin 5-O-α-D-maltoside. ^{13}C-NMR (methanol-d_4) δ: 28.0 (C-4), 61.7 (C-6″), 62.7 (C-6‴), 68.5 (C-3), 71.5 (C-4″), 72.9 (C-4‴), 73.0 (C-2″), 73.1 (C-2‴), 74.2 (C-3″), 74.8 (C-5″, 5‴), 75.1 (C-3‴), 82.7 (C-2), 96.6 (C-8), 97.8 (C-6), 98.5 (C-1″), 102.9 (C-1‴), 103.1 (C-10), 115.0 (C-2′), 116.1 (C-5′), 119.8 (C-6′), 132.1 (C-1′), 146.2 (C-3′, 4′), 156.6 (C-9), 157.2 (C-7), 158.1 (C-5).

Synthesis of Vinyl Coumarate

The vinyl ester substrates were prepared by vinyl exchange reaction of vinyl acetate and the corresponding aromatic acid. Thus, in a three-necked flask were placed *p*-coumaric acid (1.64 g, 0.01 mol), vinyl acetate (15 mL, 0.16 mol), mercury acetate (66 mg, 4% w/w), and THF (10 mL). The reaction mixture was stirred under nitrogen for 30 min, then sulphuric acid was added (2 μL, 0.04 mmol), and the reaction mixture was heated to 40°C with stirring. After 12 h the reaction was terminated by addition of an excess of sodium acetate. After removal of the solvent and the excess of vinyl acetate, the residue obtained was subjected to column chromatography on silica gel using a gradient elution with petroleum ether/ethyl acetate from 250:1 to 180:40. Vinyl coumarate (1.20 g, 6.3 mmol), a white crystalline solid was obtained in 63% yield.

Vinyl coumarate. ^{1}H-NMR (chloroform-d) δ: 4.69 (1H, dd, J = 1.6 and J = 6.3 Hz, O-CH = C$\underline{H}_2$), 5.03 (1H, dd, J = 1.6 and J = 14.0 Hz, O-CH = C$\underline{H}_2$), 6.35 (1H, d, J = 15.8Hz, CO-CH = C$\underline{H}$), 6.55 (1H, bs, OH), 6.92 (2H, d, J = 8.6 Hz, Ar), 7.45 (1H, dd, J = 6.3 and J = 14.2 Hz, O-C$\underline{H}$ = CH_2), 7.48 (2H, d, J = 8.6Hz, Ar), 7.78 (1H, d, J = 15.8 Hz, CO-C$\underline{H}$ = CH).

^{13}C-NMR (chloroform-d) δ: 97.9 (O-CH = C$\underline{H}_2$), 111.4

(CO-CH=CH), 126.5 (2*CH, coumarate Ar), 130.4 (C, coumarate Ar), 132.8 (2*CH, coumarate Ar), 141.8 (O-CH=CH_2), 146.9 (CO-CH=CH), 158.6 (C, coumarate Ar), 164.9 (C=O).

Synthesis of Vinyl Ferulate

Using the procedure described above and starting with ferulic acid (1.94 g, 10 mmol), vinyl ferulate (1.4 g, 6.1 mmol) was synthesised in 61% of yield as a white solid.

Vinyl ferulate. ^{1}H-NMR (chloroform-d) δ: 3.92 (3H, s, OCH_3), 4.62 (1H, dd, J = 1.4 and J = 6.3 Hz, O-CH=CH_2), 4.96 (1H, dd, J = 1.4 and J = 14.2 Hz, CO-CH=CH_2), 6.02 (1H, bs, OH), 6.30 (1H, d, J = 15.8Hz, CO-CH=CH), 6.90-7.45 (m, 3H, ferulate Ar), 7.42 (1H, dd, J = 14.2 and J = 6.3 Hz, O-CH=CH_2), 7.72 (1H, d, J = 15.8Hz, CO-CH=CH). ^{13}C-NMR (chloroform-d) δ: 55.8 (OCH_3), 97.4 (O-CH=CH_2), 109.4 (CH, ferulate Ar), 113.7 (-CO-CH=CH), 114.8 (CH, ferulate Ar), 123.4 (CH, ferulate Ar), 126.6 (C, ferulate Ar), 141.2 (O-CH=CH_2), 146.8 (-CO-CH=CH), 148.4(C, ferulate Ar), 149.8 (C, ferulate Ar), 164.2 (C=O).

Enzymatic Esterification

Preparative Synthesis of 6″-O-((+)catechin 7-O-α-D-glucopyranoside) Cinnamate

(+)Catechin 7-*O*-α-D-glucopyranoside (100 mg, 0.22 mmol) and vinyl cinnamate (153 mg, 0.88 mmol) were dissolved in *tert*-butanol/pyridine (9:1, v/v, 5 mL) and Novozyme 435 (100 mg) was added. The mixture was then shaken at 125 rpm in an incubator at 37°C. The reaction was monitored by TLC (ethyl acetate / methanol / water, 20/3/1, v/v/v) ($Rf_{glucoside}$ = 0.3, $Rf_{product}$ = 0.43). After 5 days the reaction was stopped by adding 10 mL methanol and the enzyme was filtered and the solvent was evaporated. Purification was carried out by chromatography on silica gel eluted with a gradient of chloroform/methanol from 10:1 to 2:1 (v/v). The product was obtained in 70% yield.

6″-O-((+)catechin 7-O-α-D-glucopyranoside) cinnamate. ^{13}C-NMR (methanol-d_4) δ: 28.5 (C-4), 64.8 (C-6″), 68.6 (C-3), 71.9 (C-4″), 73.3 (C-2″), 73.9 (C-3″), 75.0 (C-5″), 82.8 (C-2), 97.1 (C-6), 98.1 (C-8), 99.1 (C-1″), 103.9 (C-10), 115.3 (C-2′), 116.1 (C-5′), 118.6 (CO-CH=CH), 120.2 (C-6′), 125.5, 129.1, 130.0 (5 * CH, cinnamate Ar), 131.3 (C-1′), 135.9 (C, cinnamate Ar), 146.2 (C-3′, 4′), 146.5 (CO-CH=CH), 156.7 (C-9), 156.9 (C-5), 157.9 (C-7), 170.4(C=O).

Other flavonoid glycoside esters were prepared as follows. In a vial (20 mL) were successively added flavonoid glycoside (0.5 mmol), vinyl cinnamate, ferulate or coumarate (2 mmol, 4 equiv.), Novozyme 435 (1.5 equiv. in weight relative to flavonoid glycoside) and 10 mL of solvent (typically 9:1 acetonitrile or *tert*-butanol and pyridine). The vial was closed and the reaction mixture was put in a shaker-incubator (125 rpm) at 37°C. The reaction was monitored by TLC (ethyl acetate / methanol / water, 9/0.06/0.4, v/v/v). When no more product formation was evident the reaction was stopped by filtration. The products were purified as described above.

Naringin 6″-O-ferulate. Using the procedure described above and starting with naringin (290 mg, 0.5 mmol) and vinyl ferulate (440 mg, 2 mmol, 4 equiv.), naringin-6″-*O*-ferulate was prepared in 25% yield (92 mg, 0.12 mmol).

^{13}C-NMR (DMSO-d_6) δ: 18.2 (C-6‴), 42.2 (C-3), 55.8 (-O-CH_3), 63.3 (C-6″), 68.5 (C-5‴), 70.5 (C-4″), 70.1 and 70.6 (C-2‴and C-3‴), 71.9 (C-5″), 73.8 (C-4‴), 76.2 and 76.3 (C-2″), 77.0(C-3″), 78.7 and 78.9 (C-2), 95.4 and 96.4 (C-6 and C-8), 97.3 and 97.4 (C-1″), 100.7 (C-1‴), 103.5 (C-10), 110.2 (CH, ferulate Ar), 114.3 (CO-CH=CH), 115.6 (CH, ferulate Ar), 115.8 (C-3′ and C-5′), 123.4 (CH, ferulate Ar), 125.7 (C-1′), 128.6 and 128.7 (C-2′ and C-6′), 145.5 (C, ferulate Ar), 148.1 (CO-CH=CH), 149.6 (C, ferulate Ar), 158.0 (C-4′), 163.0 (C, ferulate Ar), 163.2 (C-9), 164.6 (C-5), 168.7 (C-7), 170.45 (C=O), 197.4 and 197.5 (C-4).

Naringin 6″-O-coumarate. Using the procedure described above and starting with naringin (290 mg, 0.5 mmol) and vinyl coumarate (380 mg, 2 mmol, 4 equiv.), naringin-6″-*O*-coumarate was prepared in 30% yield (106 mg, 0.15 mmol).

^{13}C-NMR (methanol-d_4) δ: 18.2 (C-6‴), 43.9 (C-3), 64.6 (C-6″), 70.0 (C-5‴), 72.0 (C-4″), 72.1 (C-2‴ and C-3‴), 73.9 (C-4‴), 75.4 (C-5″), 78.8 (C-3″), 78.9 and 79.0 (C-2″), 80.4 (C-2), 96.9 (C-8), 97.8 (C-6), 99.0 and 99.1 (C-1″), 102.5 (C-1‴), 104.5 (C-10), 114.8 (CO-CH=CH), 116.3 (C-3′ and C-5′), 116.8 (2 * CH, coumarate Ar), 127.8 (C-1′), 129.0 and 129.2 (C-2′ and C-6′), 130.7 (C, coumarate Ar), 131.1 and 131.2 (CH, coumarate Ar), 146.9 (CO-CH=CH), 158.9 (C-4′), 161.2 (C, coumarate Ar), 164.5 (C-9), 164.9 (C-5), 166.2 (C-7), 169.0 (C=O), 198.6 (C-4).

Naringin 6″-O-cinnamate. Using the procedure described above and starting with naringin (290 mg, 0.5 mmol) and vinyl cinnamate (348 mg, 2 mmol, 4 equiv.), naringin-6″-*O*-cinnamate was prepared in 30% yield (104 mg, 0.15 mmol).

^{13}C-NMR (DMSO-d_6) δ: 18.4 (C-6‴), 49.0 (C-3), 63.7 (C-6″), 68.7 (C-5‴), 70.2 (C-3‴), 70.7 (C-2‴), 70.8 (C-4‴), 72.5 (C-4″), 73.9 (C-5″), 76.3 and 76.5 (C-2″), 77.2 (C-3″), 78.9 and 79.1 (C-2), 95.6 (C-8), 96.5 (C-6), 97.3 and 97.4 (C-1″), 100.9 (C-1‴), 103.7 (C-10), 115.5 (C-3′ and C-5′), 118.1 (CO-CH=CH), 128.6 (2 * CH, cinnamate Ar), 128.8 (C-2′ and C-6′), 129.3 (2 * CH, cinnamate Ar), 128.9 (C-1′), 130.9 (C, cinnamate Ar), 134.3 (C, cinnamate Ar), 145.1 (CO-CH=CH), 158.1 (C-4′), 163.1 (C-9), 164.8 (C-5), 164.9 (C-7), 166.4 (C=O), 197.5 and 197.7 (C-4).

Isoquercitrin-6′-O-coumarate. Using the procedure described above and starting with isoquercitrin (116 mg, 0.25 mmol) and vinyl coumarate (174 mg, 1 mmol, 4 equiv.), naringin-6″-*O*-coumarate was prepared in 32% yield (49 mg, 0.08 mmol.)

^{13}C-NMR (DMSO-d_6) δ: 63.2 (C-6″), 69.9 (C-4″), 74.0 (C-2″), 74.4 (C-5″), 76.3 (C-3″), 93.6 (C-8), 98.8 (C-6),

100.7 (C-1″), 103.9 (C-10), 113.7 (CO-CH=CH), 115.4 (C-2′), 115.8 (2 CH, coumarate Ar), 116.2 (C-5′), 121.1 (C-5′), 121.6 (C-1′), 125.4 (C, coumarate Ar), 130.2 (2 * CH, coumarate Ar), 133.1 (C-3), 144.2 (CO-CH=CH), 144.9 (C-3′), 148.6 (C-4′), 156.4 (C-9), 159.6 (C-2), 159.8 (C, coumarate Ar), 161.2 (C-5), 164.2 (C-7), 168.1 (C=O), 177.4 (C-4).

Isoquercitrin-6′-O-cinnamate. Using the procedure described above and starting with isoquercitrin (116 mg, 0.25 mmol) and vinyl cinnamate (174 mg, 1 mmol, 4 equiv.), naringin-6″-*O*-cinnamate was prepared in 45% yield (67 mg, 0.11 mmol.). NMR ^{1}H and ^{13}C were identical to those previously published (Danieli et al., 1993).

Isoquercitrin-6′-O-ferulate. Using the procedure described above and starting with isoquercitrin (116 mg, 0.25 mmol) and vinyl ferulate (220 mg, 1 mmol, 4 equiv.), naringin-6″-*O*-ferulate was prepared in 15% yield (24 mg, 0.04 mmol). NMR ^{1}H and ^{13}C were identical to those previously published (Danieli et al., 1993).

Luteolin 7-O-(6″-cinnamoyl)-β-D-glucopyranoside. Using the procedure described above and starting with lutelolin 7-*O*-β-D-glucopyranoside (100 mg, 0.22 mmol) and vinyl cinnamate (153 mg, 1 mmol, 4 equiv.), Luteolin 7-*O*-(6″-cinnamoyl)-β-D-glucopyranoside was prepared in 30% yield (38 mg, 0.07 mmol). NMR ^{1}H and ^{13}C were identical to those previously published (Mizuno et al., 1987).

Analytical Methods

NMR spectra were recorded on a Jeol JNM EX 270 Fourier transform spectrometer using methanol-d_4, chloroform-d, or DMSO-d_6 as solvent. Proton spectra were recorded at 270.0 MHz and ^{13}C spectra at 67.8 MHz, both using solvent field lock. TLC was performed on silica gel *60 F_{254}* (Merck, Darmstadt, Germany) precoated on aluminium sheets. Products were visualised using orcinol-ferric chloride spray reagent or phosphomolybdic acid reagent.

RESULTS AND DISCUSSION

The major difficulty in performing enzymatic glycosylation of flavonoids is their extremely low solubility in water. Indeed, according to the Merck index catechin is only slightly soluble in cold water, and our own measurements gave a value of about 10 mg/mL at 37°C (see Table I). It is not surprising, therefore, that glycosidase-catalysed synthesis of flavonoid glycosides has not been described in the literature, although there are several communications on the use of particular transferases, e.g., sucrose phosphorylase from *Leuconostoc mesenteroides* (Kitao et al., 1993) and "glucose synthase" from a soil bacterium (Funayama et al., 1994) for glycosylation of certain natural polyphenols. In our own experiments, too, we observed no product formation in the reaction between *p*-nitrophenyl glycosides and (+)catechin under conventional reaction conditions, i.e., saturation with catechin using a range of commercially available glycosidases.

Table I. Composition of supersaturated solutions containing (+) catechin and maltose.

(+)Catechin (g)	Maltose (g)	PEG-$(OCH_3)_2$-500 (g)	Stability
0.01*	—	—	Not applicable
0.04*	0.3	0.05	Not applicable
0.1	0.2	—	Stable after 7 days
0.2	0.1	—	Precipitation after 2 h
0.2	0.2	—	Precipitation after 18 h
0.2	0.3	—	Precipitation after 18 h
0.3	0.1	—	Precipitation after 0.5 h
0.3	0.2	—	Precipitation after 2 h
0.3	0.3	—	Precipitation after 18 h
0.3	0.1	0.05	Stable after 7 days
0.4	0.1	0.05	Precipitation after 6 h
0.4	0.2	0.05	Precipitation after 20 h
0.4	0.3	0.05	Stable after 7 days
0.4	0.1	0.10	Stable after 7 days
0.4	0.2	0.10	Stable after 7 days
0.4	0.3	0.10	Stable after 7 days
0.5	0.3	0.05	Precipitation after 0.5 h
0.5	0.3	0.10	Precipitation after 0.5 h
0.5	0.3	0.15	Phase separation

The samples (excluding the two shaded lines) were prepared by mixing (+)catechin, maltose, and PEG-$(OCH_3)_2$ with 1 mL citrate buffer (pH 6.0), heating the mixture to 95°C to dissolve the components, and then cooling it and incubating at 37°C.

*The two "shaded" samples were prepared by adding the excess of catechin (line 1) and (+)catechin and maltose (line 2) to the buffer (1 mL) and equilibrated at 37°C. The samples were then centrifuged and the equilibrium concentration of (+)catechin was determined spectrophotometrically.

Thus, we proceeded to investigate whether one could improve the "availability" of catechin as substrate in this biotransformation by forming a supersaturated solution with the flavonoid and a suitable glycon donor. It should be noted that in order to achieve reasonable yields in transglycosylation reactions, glycon acceptors are usually employed in considerable excess over the donor glycoside and a relatively high, in absolute terms, concentration of the acceptor is required for efficient glycosylation of the secondary hydroxyl groups (Fernandez-Mayoralas, 1997; Nilsson, 1996). As before, poly(ethylene) glycols (PEGs) derivatives were used to deter crystallisation in supersaturated solutions (Milqvist-Fureby et al., 1998a) but because PEGs were likely to act as competing glycon acceptors, poly(ethylene glycol) dimethyl ether (PEG-$(OCH_3)_2$; Mn 500) was employed in these experiments.

Tables I and II show the results of the experiments carried out with varying concentrations of PEG-$(OCH_3)_2$, catechin, and maltose or Fucoside-β-D-*O*PNP, respectively. It is evident from the data presented that the solubility of catechin in the reaction mixture can indeed be increased substantially by forming supersaturated solutions with the donor of glycon and PEG-$(OCH_3)_2$. Thus, catechin was soluble at only about 10 mg/mL in buffer and 40 mg/mL in 30% maltose – 5% PEG-$(OCH_3)_2$ solution, respectively, after overnight shaking at 37°C (see the shaded lines in Table I). However, when as much as 400 mg of catechin was mixed with 300

Table II. Composition of supersaturated solutions containing (+)catechin and Fucoside-β-D-*O*PNP

(+)Catechin (g)	Fucoside-β-D-*O*PNP (g)	PEG-$(OCH_3)_2$-500 (g)	Stability
0.01*	—	—	Not applicable
—	0.004[a]	—	Not applicable
0.30	0.10	0.05	Precipitation after 0.5 h
0.30	0.20	0.05	Precipitation after 0.5 h
0.30	0.10	0.10	Stable after 7 days
0.40	0.20	0.10	Precipitation after 18 h
0.40	0.20	0.15	Stable after 7 days
0.50	0.20	0.15	Phase separation

The samples (excluding the two shaded lines) were prepared by mixing (+)catechin, Fucoside-β-D-*O*PNP, and/or PEG-$(OCH_3)_2$ with 1 mL citrate buffer (pH 6.0), heating the mixture to 95°C to dissolve the components, and then cooling it and incubating at 37°C.

*The two "shaded" samples were prepared by adding the excess of (+)catechin (line 1) and Fucoside-β-D-*O*PNP (line 2) to the buffer (1 mL) and equilibrated at 37°C. The samples were then centrifuged and the equilibrium concentration of (+)catechin and Fucoside-β-D-*O*PNP was determined spectrophotometrically.

mg of maltose and 50 mg of PEG-$(OCH_3)_2$ in 1 mL of water and the mixture was heated to 95°C and then cooled to 37°C to form a supersaturated solution, no precipitation of catechin was observed for several days.

This observation clearly shows that catechin can be efficiently solubilised under the conditions of supersaturation and, crucially, the resulting solutions were sufficiently stable to attempt enzymatic transglycosylations. Similar results were obtained with other substrates, e.g., Fucoside-β-D-*O*PNP (Table II) and *p*-nitrophenyl-α-D-glucopyranoside or other flavonoids, e.g., quercitin and morin (not shown). In the former case, prolonged incubation at 37°C led to solubilization of about 10 mg of catechin and 4 mg Fucoside-β-D-*O*PNP in 1 mL of buffer when these compounds were tested separately and in the absence of added PEG-$(OCH_3)_2$. However, when the reactants were combined at much higher concentrations (400 mg of catechin, 200 mg of Fucoside-β-D-*O*PNP, and 150 mg of PEG-$(OCH_3)_2$ in 1 mL of buffer), the resulting supersaturated solution remained stable at 37°C and no crystallisation was observed for days.

Encouraged by this highly improved "solubility" of catechin under the conditions of supersaturation, we performed several transglycosylation reactions using a range of commercially available glycosidases. In every case a substantial amount of PEG-$(OCH_3)_2$ was used to maintain the solubility of catechin and the donor glycosides/saccharide in the reaction mixture. The glycosylations were carried out for up to 3 days at 37°C and the progress of the reaction was analysed by TLC. As a control, the same reactions were carried out in the absence of catechin and the comparison between the two respective reaction mixtures was used to determine the formation of any possible glycosylation products of catechin.

As a result of these preliminary experiments, three reactions with Fucoside-β-D-*O*PNP (Fig. 1A), maltose (Fig. 1B), and dextrin (Fig. 1C) as glycon donors were selected for the preparative synthesis. The rationale behind this choice was as follows: The expected glycosylation products of catechin (6-deoxy-glycoside (the fucoside), glucoside, and maltoside, respectively) would provide us with a range of representative structures. The preparative synthesis of these three catechin glycosides was carried out as described in detail in Materials and Methods and, after enzyme inactivation and drying, the reaction mixtures were subjected to column chromatography on silica gel; 26% and 20% yield of catechin-β-D-fucopyranoside and catechin-α-D-glucopyranoside (calculated on the basis of the limiting substrate) were obtained in the reaction with Fucoside-β-D-*O*PNP and maltose, respectively. When dextrin was used as substrate, 100 mg of catechin α-D-maltoside was isolated from the reaction mixture containing 2 g of dextrin, i.e., 5% yield on w/w basis.

Once obtained, the three catechin glycosides were analysed to establish the position of glycosylation. It was found that transglycosylation with maltose gave two products which were first separated by column chromatography and then identified as (+)catechin 7-*O*-α-D- glucopyranoside and (+)catechin 5-*O*-α-D- glucopyranoside (Fig. 1B) by comparison of their NMR spectra with those of the known natural products (both glycosides were isolated from commercial rhubarb by Kashiwada et al. (1986)). (+)Catechin 7-*O*-α-D- glucopyranoside was also isolated from Douglas fir (*Pseudotsuga mannziesii*) inner bark by Foo and Karchesy (1989). By analogy with the above, the transglycosylation products obtained in the reaction with dextrin were identified as (+)catechin 7-*O*-α-D-maltoside and (+)catechin 5-*O*-α-D-maltoside, while the reaction with Fucoside-β-D-*O*PNP gave (+)catechin 4′-β-D-fucopyranoside as a sole product of the biotransformation (identification has been done by comparison of the NMR spectra recorded with the known natural compound (+)catechin 4′-β-D-glucoside (Kashiwada et al., 1986)). It seems clear, therefore, that under the reaction conditions used hydroxyl groups in at least three positions (5, 7, and 4′) in flavonoids can be glycosylated in one step by employing crude, inexpensive, commercially available glycosidases.

It should be stressed that the yields in the above three reactions could have been improved by thorough optimisation of the reaction conditions and/or purification protocols used. However, this was not attempted since the primary objective of the work was to assess the validity of our synthetic approach rather than to maximise the yield of any particular product. Similarly, although preliminary results indicated that other flavonoids, e.g., quercitin, can be glycosylated under similar conditions as judged by TLC, no preparative synthesis was carried out for the same reason. Instead, we investigated the feasibility of enzymatic acylation of (+)catechin glycosides as this reaction, if successful, would give easy access to a very wide range of natural products. It should be noted in this context that protease-catalysed acylation of several flavonoid glycosides with trifluoroethyl butanoate was first described by Riva and co-workers (Danieli, et al., 1989, 1990; Kodelia et al., 1993). However, the reaction with natural phenolic acids of interest has not proceeded satisfactorily (Riva et al., 1995; see also Danieli et al., 1993; Riva et al., 1996, for a chemo-enzymatic approach to the preparation of malonyl flavonoid glycosides and their subsequent chemical conversion to the cinnamate), although the same group have subsequently performed the acetylation of several flavonoid glycosides with vinyl acetate (Danieli et al., 1997). More recently, the lipase-catalysed synthesis of some aryl propenoic acid esters of arbutin and isoquercitrin were reported by Japanese scientists (Nakjima et al., 1997, 1999; Kohji et al., 1999).

In order to investigate the efficiency of enzymatic acylation of flavonoid glycosides with natural aryl propenoic acids, vinyl esters of cinnamic, ferulic, and coumaric acids were purchased or prepared according to the general method available (Adelman, 1949; Swern and Jordan, 1963; Hopff and Osman, 1968). Initially, the acylations were carried out on a few mg scale to assess the progress of the reaction and to select particular substrate combinations for preparative scale synthesis. Such a qualitative assessment led us to the following observations: 1) among several enzymes tested, two lipases, from *Mucor miehei* (Lipozyme) and from *Candida antarctica* (Novozyme), were able to catalyse the reaction with appreciable yields, with the latter enzyme giving noticeably higher conversions; 2) two solvent mixtures, i.e., *tert*-butanol-pyridine (9:1 v/v) and acetonitrile-pyridine (9:1 v/v) appeared to be the best among numerous solvent combinations tested. Under these conditions (*tert*-butanol-pyridine, Novozyme), the acylation of (+)catechin 7-*O*-α-D-glucopyranoside with vinyl cinnamate proceeded well and after 5 days at 37°C (Fig. 2), the desired product, 6″-O-((+)catechin 7-O-α-D-glucopyranoside) cinnamate, was isolated by column chromatography on silica gel in 70% preparative yield. This is in line with the yield reported for isoquercitin glucoside cinnamate (68%) in a very recent communication by Nakajima et al. (1999), who used a similar synthetic procedure.

Further qualitative investigation of the reaction revealed that: 1) the reactivity of the vinyl esters decreased in the following order: cinnamate > ferrulate ≥ *p*-coumarate; and 2) the reactivity of catechin glycosides was much more dependent on the nature of the glycon than the position of glycosylation, i.e., (+)catechin-α-D-glucosides were found to be much better substrates than either the catechin fucoside or the maltosides, presumably due to the lack of primary hydroxyl group on the sugar moiety of the fucoside and relatively low solubility of the maltoside in the reaction solvents or steric hindrances, respectively.

Finally, the validity of these conclusions was further verified by investigating the acylation of a few other commercially available flavonoid glycosides under the same reaction conditions on a preparative scale (Fig. 3). In particular,

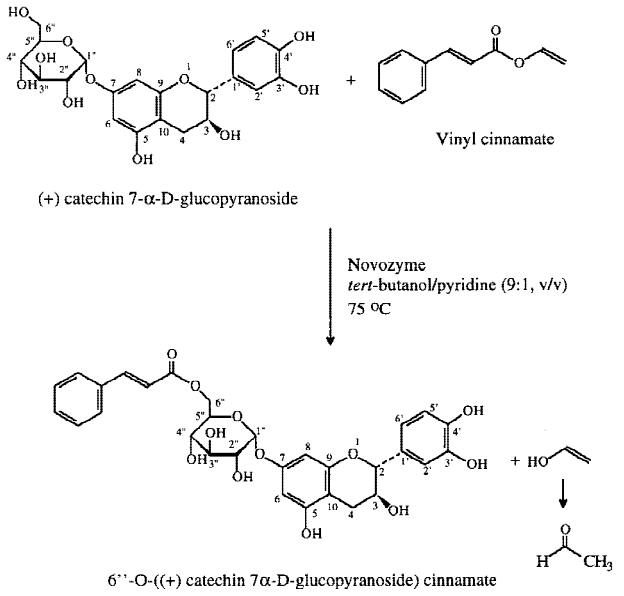

Figure 2. Lipase-catalysed synthesis of 6″-O-((+)catechin 7-O-α-D-glucopyranoside) cinnamate.

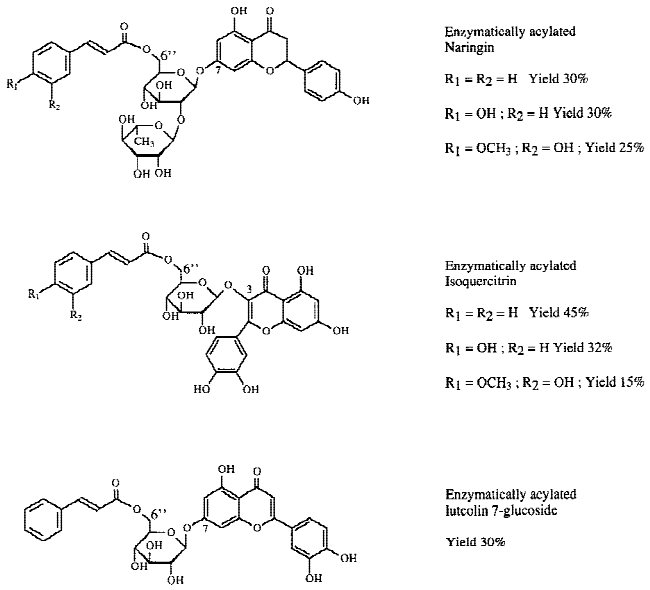

Figure 3. Structures of enzymatically synthesised flavonoid glycoside esters.

the acylation of isoquercitrin with vinyl cinnamate, ferulate, and coumarate led to the formation of the corresponding 6″-*O*-monoesters in 45, 32, and 15% preparative yields, respectively. A smaller difference was observed in the reaction with naringin, presumably due to the lower reaction rates observed with this substrate as compared to the glucosides and, hence, lesser effect of the acyl group in vinyl esters on the overall conversion. All these reactions were carried out as described above. The products were recovered by silica gel chromatography and the position of acylation was confirmed by NMR. The position of the acyl group on the flavonoid glycoside was determined by comparison of their NMR ^{1}H and ^{13}C spectra between the flavonoid glycoside and the corresponding ester. A characteristic downfield shift for the methylenic protons (6″) was observed in the acylated compounds. Similarly, the glycosyl carbon adjacent to the acyl group showed a downfield shift for the $C_{6''}$ and an upfield shift for the $C_{5''}$. From this it was deduced that flavonoid glycosides were regioselectively acylated at the $C_{6''}$ position of the sugar unit (primary hydroxyl). Neither of the two lipases catalysed acylation of 6-deoxy flavonoid glycosides, e.g., (+)catechin 4′-β-D-fucopyranoside or quercetin-3-L-rhamnopyranoside, in preparatively attractive yields, although the formation of products was noted by TLC after prolonged incubation periods.

In conclusion, it has been shown that flavonoids can be efficiently solubilised in supersaturated solutions prepared with donor glycosides, e.g., *p*-nitrophenyl glycosides, di- and higher oligosaccharides, and PEG in sufficiently high concentration for their efficient glysosylation. Under these conditions commercially available glycosidases were found to accept catechin as substrate and the target glycosides were prepared in one step in up to 26% yields. The regioselectivity of the reaction is presumed to depend on the enzyme and substrate combination used, but at least three positions, 5, 7, and 4′, in the flavonoid can be glycosylated. The resulting and similar flavonoid glycosides can be further modified by regioselective acylation with vinyl esters of arylpropenoic acids. *Candida antarctica* and, to a lesser extent, *Mucor miehei* lipases were suitable catalysts. The efficiency of acylation was found to diminish in the order of vinyl cinnamate > vinyl ferulate > vinyl coumarate. Substrates with primary hydroxyl group on the sugar moiety were acylated with relative ease, while flavonoids glycosylated with 6-deoxy sugars were found to be poor substrates. Also, the rate of acylation of flavonoids carrying a disaccharide moiety was noticeably reduced as compared to those with a monosaccharide glycon. Thus, the feasibility of a simple two-step enzymatic synthesis of these complex natural products was successfully established.

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