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Enzymatic Synthesis of α-Glucosides of Resveratrol with Surfactant Activity

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Abstract: We report the synthesis of a series of α-glucosyl derivatives of resveratrol (3,5,4′-trihydroxystilbene) by a transglycosylation reaction catalyzed by the enzyme cyclodextrin glucanotransferase (CGTase) using starch as glucosyl donor. Several reaction parameters (temperature, solvent composition, enzyme concentration and starch/resveratrol ratio) were optimized. The yield of α-glucosylated products reached 50% in 24 h. The structures of the derivatives were determined by a combination of amyloglucosidase-hydrolysis tests, MS and 2D-NMR. Three families of products were obtained: glucosylated at 3-OH, at 4′-OH and at both 3-OH and 4′-OH. The bonds between glucoses were basically α(1→4). Interestingly, the water solubilities of the α-glucosylated derivatives were at least 65- and 5-fold higher than those of resveratrol and the natural β-glucosylated derivative (piceid), respectively. In contrast with piceid, the synthesized α-glucosylated compounds exhibited surfactant activity, with critical micelle concentration (CMC) values in the range 0.5–3.6 mM. Although the incorporation of a glucosyl moiety caused a loss of antioxidant activity (more pronounced in the position 3-OH compared with 4′-OH), the fact that the glycosides need to be converted into the aglycones before they are absorbed minimizes such an effect. In contrast, the modification of physicochemical properties such as solubility and partition coefficient by glycosylation could exert a positive influence on the bioavailability of resveratrol.

Keywords: antioxidants; cyclodextrin glucanotransferases (CGTases); glycosylation; piceid; resveratrol; surfactants

Introduction

Resveratrol (**1**) is a phytoalexin synthesized by plants as a defence mechanism in certain cases (fungal attacks, injury, UV exposure, etc.).[1] It is composed of a stilbene core with three phenolic groups in positions 3, 5 and 4′. As occurs with flavonoids,[2] resveratrol is commonly present in plants bound to sugars as β-glycosides. Thus, several derivatives of resveratrol (Figure 1) have been identified in the roots of *Poligonum cuspidatum* such as piceid **2** (3-*O*-β-D-glucosyl-resveratrol), resveratroloside **3** (4′-*O*-β-D-glucosyl-resveratrol), piceatannol **4** (3,5,3′,4′-tetra-*O*-hydroxystilbene) and its glucosylated derivative **5** (4′-*O*-β-D-glucosyl-piceatannol). Piceid is the major polyphenol found in the root of *P. cuspidatum*[3] and the major resveratrol derivative in grapes.[4]

Resveratrol possesses a variety of antioxidant,[5] anti-inflammatory,[6] estrogenic,[7] anticancer,[8] cardioprotective,[9] neuroprotective[10] and immunomodulatory[11] bioactivities. Its ability to activate various deacetylase enzymes (sirtuins) has been recently discovered and could be responsible for the mentioned properties[12], and in particular of a delaying effect on aging.[13]

Glycosylation of resveratrol may cause different effects on its bioavailability. On the one hand, the sugar moiety of polyphenol glycosides plays a major role in their absorption.[14] Polyphenols are hydrophobic scaffolds exhibiting poor absorption, resulting in a very

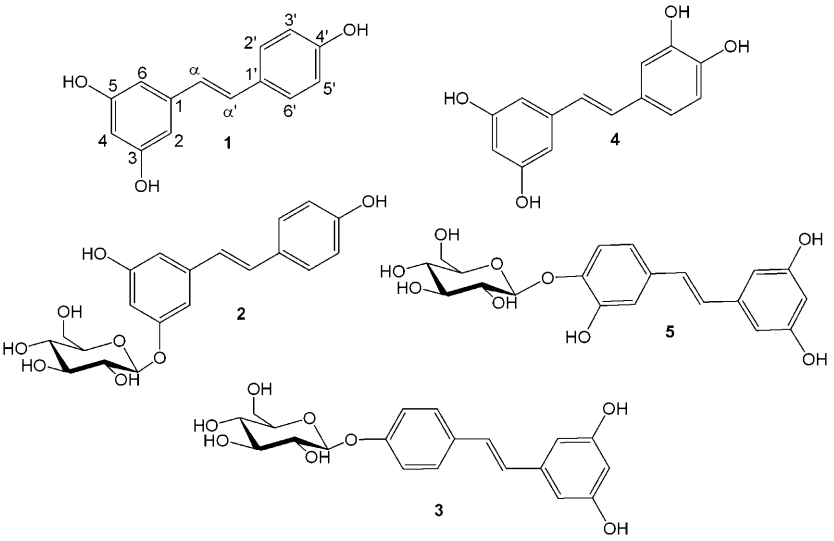

Figure 1. Polyphenols found in the root of *Polygonum cuspidatum*.

low concentration in the circulatory streams.[15] The modification of physicochemical properties such as solubility and partition coefficient by glycosylation seems to exert a positive influence on the entry of polyphenols into enterocytes.[16] For example, 3-*O*-glycosides of the flavonoid quercetin exhibited unexpectedly improved bioavailability.[17] On the other hand, it is well reported that glucosylated polyphenols are first deglycosylated at the intestinal wall before diffusing into the cells. After absorption, polyphenols aglycones are conjugated with glucuronic acid or sulphate in the intestinal enterocytes and in the liver.[2] Glycosylation may also exert other benefits for the application of bioactive compounds, including formulation, protection from oxidation by masking phenolic groups, and improvement of bioavailability of drugs that need to pass through the blood-brain barrier.[18]

The stereo- and regioselectivity of enzymes is considered a valuable alternative to chemical synthesis for the preparation of structurally well-defined glycoconjugates.[19] For the *in vitro* glycosylation of natural products, glycosyltransferases and glycosidases have been successfully employed. The only previous reports on the biocatalytic glycosylation of resveratrol were performed using whole cells of *Bacillus cereus* yielding the piceid **2**[20] and *Streptococcus mutans* that formed the alpha-derivative 3-*O*-α-D-glucosyl-resveratrol.[21]

In the present work, we report the enzymatic synthesis of various α-glucosyl-resveratrol derivatives by a transglycosylation reaction catalyzed by cyclodextrin glucanotransferases (CGTases, EC 2.4.1.19). The antioxidant and surfactant properties of the novel compounds were evaluated.

Results and Discussion

Screening and Optimization of Transglycosylation

We screened different enzymes with transglycosylation activity (Table 1) in order to conjugate resveratrol with a glycosyl moiety. Due to the low solubility of resveratrol in water (approx. 30 mg per litre), it was necessary to add a cosolvent. Dimethyl sulfoxide (DMSO) showed the best properties in terms of substrates solubility and reaction yield (data not shown), in accordance with previous studies on the enzymatic glycosylation of flavonoids.[22] Compared with other cosolvents, mixtures buffer/DMSO were very efficient to solubilize resveratrol and the glycosyl donor to a significant extent. The initial composition of the reaction mixture was resveratrol (10 mg), glycosyl donor (50 mg), enzyme (65 μL) in 1 mL final volume. The reaction medium was a 50/50 v/v mixture of DMSO

Table 1. Screened enzymes for the glycosylation of resveratrol.

Enzyme	Donor	Transferred group
α-glucosidase (*Aspergillus niger*)	maltose	glucosyl-
dextransucrase (*Leuconostoc mesenteroides*)	sucrose	glucosyl-
CGTase *(Thermoanaerobacter)*	starch	glucosyl-
CGTase (*Bacillus macerans*)	starch	glucosyl-
β-fructofuranosidase (*Aspergillus aculeatus*)	sucrose	fructosyl-
β-galactosidase (*Bacillus circulans*)	lactose	galactosyl-
β-galactosidase (*Aspergillus oryzae*)	lactose	galactosyl-

and 0.2 M sodium acetate (pH 5.6). Reactions were incubated at 40 °C and analyzed by HPLC.

Under the above conditions, most enzymes did not catalyze the formation of any product, except for CGTases with starch as glucosyl donor. A similar conclusion was recently reported by Markosyan et al. exploring the glycosylation of benzoquinazolines with different glycosidic enzymes.[23] Glycosylation of phenolic compounds using simple carbohydrates as donors has been described only in very few cases. However, glucosylation of several flavonoids such as catechin, hesperidin, naringin, rutin and luteolin has been achieved with glucosyltransferases from *Streptococcus* strains[24], α-glucosidases[25] and dextransucrases[22]; in particular, CGTase is an excellent choice to glucosylate phenolic compounds due to its broad acceptor specificity.[26]

The transglucosylation activity of CGTase is known to be very dependent on the enzyme source. In our work, the CGTase from *Thermoanaerobacter* sp. (Toruzyme 3.0 L) gave a higher yield than that from *Bacillus macerans* (CGTase Amano). In contrast with the CGTase from *Bacillus macerans* (the HPLC chromatogram showed four different compounds with increasing concentration, indicating the formation of the so-called analogous series, Figure 2), the CGTase from *Thermoanaerobacter* sp. did not present the typical chromatographic profile of a mixture of derivatives with increasing glucosylation degree,[27] suggesting a more complex reaction scheme. However, the yield with *Thermoanaerobacter* CGTase was extremely small, not surpassing 2% in 24 h.

In order to improve the yield of glucosylated products, several parameters were optimized (temperature, percentage of cosolvent, enzyme concentration and w/w ratio starch/resveratrol). An increase of the reaction temperature was favourable on yield until 60 °C.

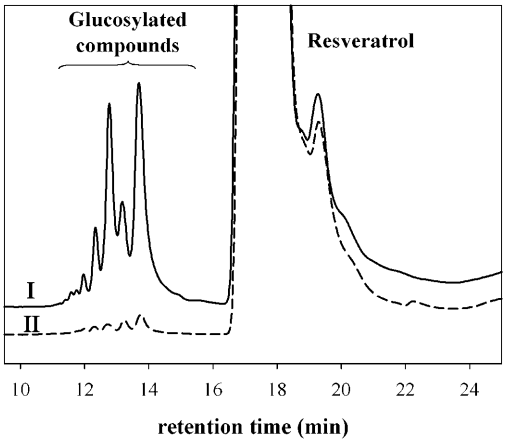

Figure 2. HPLC chromatograms (96 h of reaction) showing the formation of resveratrol glucosyl derivatives catalyzed by CGTase from: (**I**) *Thermoanaerobacter* sp. and (**II**) *Bacillus macerans.*

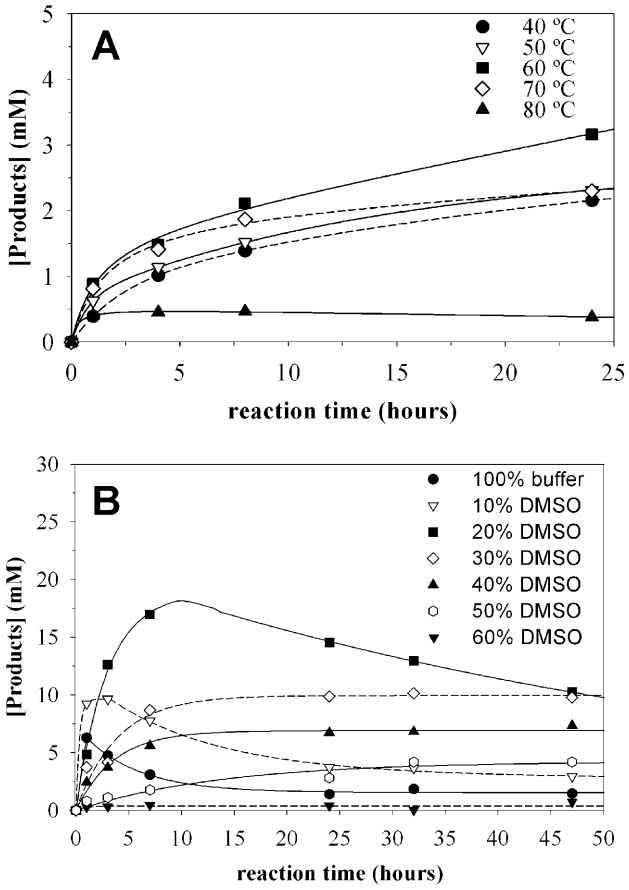

Figure 3. Effect of temperature (**A**) and DMSO concentration (**B**) on resveratrol glucosylation. The total concentration of glucosylated products is depicted in y axis.

Then, the activity decayed drastically, with negligible conversion at 80 °C (Figure 3 **A**).

We tested several concentrations of cosolvent (Figure 3 **B**) concluding that 20% (v/v) DMSO was the optimum in terms of total yield of glucosylated products. This solvent composition represented a compromise between substrate solubility and enzyme efficiency. Blackwood and Bucke reported that CGTases exhibit good performance in the presence of small amounts of polar organic solvents.[28]

After the optimization of the different parameters, the best conditions were as follows: 10 mg mL^{-1} resveratrol (44 mM), 60 mg mL^{-1} starch, 0.2 M sodium acetate (pH 5.6)/DMSO 80/20 (v/v), 560 units CGTase per gram resveratrol (measured in the formation of β-cyclodextrin, see Experimental Section). Under the above conditions, 50% of the initial resveratrol was converted into glucosylated products in 24 h. The yield was significantly higher than that described by Shim et al. with *Streptococcus mutans* cells; they reported 18% of 3-*O*-α-D-glucosyl-resveratrol in 48 h[21], but using only 1 mg mL^{-1} (4.4 mM) resveratrol.

Scaling-Up and Enzymatic Hydrolysis of the Transglycosylation Products

We scaled-up the reaction with the CGTase from *Thermoanaerobacter* sp. in order to isolate and characterize the different glucosides. The reaction was scaled to 20 mL as described in the Experimental Section, and the products purified by semipreparative HPLC. Figure 4 shows the typical chromatogram obtained. Monoglucosides (**6a**, **6b**), diglucosides (**7a**–**7d**), triglucosides (**8a**–**8d**) and tetraglucosides (**9a**, **9***) were further identified using mass spectrometry (ESI-TOF, see Supporting Information). The isolated yields are given in Table 2.

In previous reports on CGTase-catalyzed transglycosylations, the bonds between glucosyl groups were in the majorly α(1→4).[27,29,30] In order to confirm the presence of such bonds in our polyglucosylated products, an enzymatic hydrolysis method based on amyloglucosidase specificity was performed.[31,32] We incubated a mixture of glucosylated resveratrol derivatives (**6b**, **7c**, **8d**) with a pure amyloglucosidase [previously we checked that the enzyme was able to hydrolyze only α(1→4) bonds such as those contained in maltotriose, and was not contaminated with other activities]. As illustrated in Figure 5, the HPLC analysis showed that, after 22.5 h incubation with amyloglucosidase, di- and triglucoside peaks (**7c**, **8b**) disappeared substantially, with a concomitant increase of the monoglucoside derivative and the appearance of free resveratrol. These results indicated that most bonds between glucoses were α(1→4).

Considering that the glycosides need to be converted into the aglycones before they are absorbed into the blood circulation,[14] the above results suggest that amylolytic enzymes in the intestinal tract will be able to hydrolyze α(1→4) bonds between glucoses and,

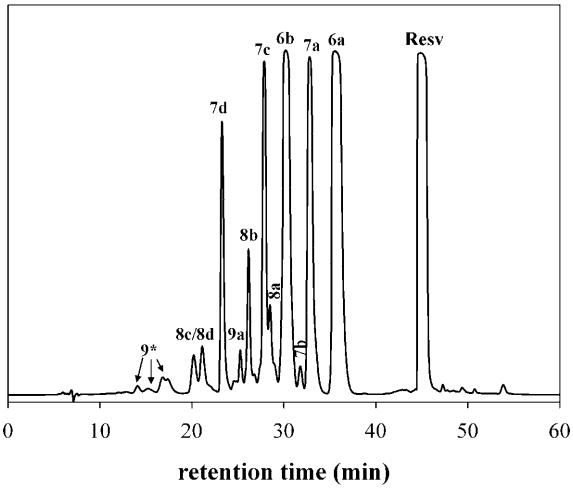

Figure 4. Semipreparative HPLC chromatogram showing the different products obtained at 15 h with the CGTase from *Thermoanaerobacter* sp.

Table 2. Structure assignation and yield of the main products obtained in the CGTase-catalyzed glucosylation of resveratrol.[a]

Compound	Mass	Structure	Type	Name	Isolated yield [mg][b]
6a	MonoGlc		I	3-*O*-α-D-glucosyl-resveratrol	28.4
6b			II	4'-*O*-a-D-glucosyl-resveratrol	20.5
7a	DiGlc		I	3-*O*-α-D-maltosyl-resveratrol	12.0
7b			I	3-*O*-[a-D-glucosyl-(1?2)-a-D-glucosyl]-resveratrol	n.d.
7c			II	4'-*O*-a-D-maltosyl-resveratrol	10.5
7d			IV	3,4'-di-*O*-a-D-glucosyl-resveratrol	4.1
8a	TriGlc		I	3-*O*-α-D-maltotriosyl-resveratrol	n.d
8b			II	4'-*O*-a-D-maltotriosyl-resveratrol	6.1
8c/8d			IV	3-*O*-α-D-maltosyl-,4′-*O*-α-D-glucosyl-resveratrol	n.d
			IV	3-*O*-α-D-glucosyl,4′-*O*-α-D-maltosyl-resveratrol	n.d
9a	TetraGlc		II	4′-*O*-α-D-maltotetraosyl-resveratrol	n.d
9*			IV	*structure not determined*	n.d

[a] *Conditions:* 200 mg resveratrol, 1.4 g starch, 2.4 mL Toruzyme, 4 mL DMSO, 13.6 mL 0.2 M sodium acetate (pH 5.6), 60 °C, 15 h.

[b] n.d. = not determined: recovered yield was less than 1 mg.

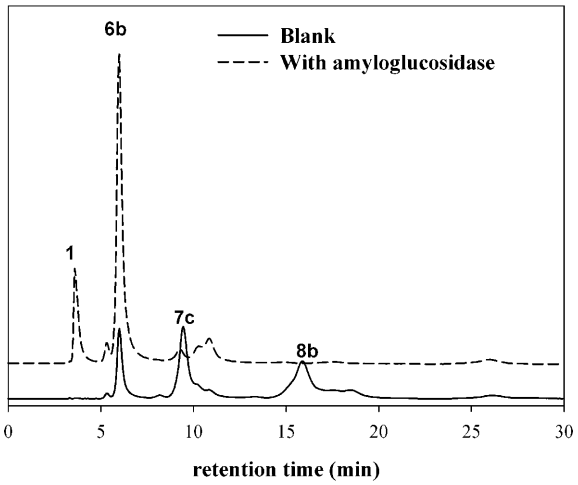

Figure 5. HPLC analysis showing the enzymatic hydrolysis of glucosylated resveratrol derivatives by the action of amyloglucosidase after 22.5 h.

more slowly, the bond between resveratrol and glucose, yielding the free resveratrol and thus presumably favouring its absorption.

The chromatogram in Figure 5 also indicated the presence of several diglucosides not hydrolyzed by amyloglucosidase. These compounds could contain two glucosyl moieties in different resveratrol phenolic positions, or other bonds between glucoses could be involved. In this context, we assayed the amyloglucosidase Spirizyme from Novozymes, which is also able to hydrolyze kojibiose (2-*O*-α-D-glucopiranosyl-D-glucose) apart from maltose and maltodextrins. When the mixture of glucosylated resveratrol derivatives was treated with Spirizyme (data not shown), we observed the disappearance of other diglucoside peaks, suggesting that the minor derivatives could contain α-(1→2) bonds between glucose moieties.

Product Characterization by NMR

Five different patterns of glucosylated derivatives could be initially expected (Figure 6): Type I, 3-*O*-glucosyl-resveratrol; Type II, 4′-*O*-glucosyl-resveratrol; Type III, 3,5-*O*-glucosyl-resveratrol; Type IV, 3,4′-*O*-glucosyl-resveratrol and Type V, 3,5,4′-*O*-glucosyl-resveratrol. The complete elucidation of the structure of the isolated compounds was carried out by 2D-NMR. A combination of homonuclear (COSY, TOCSY, NOESY, ROESY) and heteronuclear sequences (HSQC, HMBC) was used, assisted by 1D-selective NOE or TOCSY-type experiments.

None of the elucidated structures presented either Type III or Type V patterns. This was due, probably, to the steric hindrance between two glucosyl moieties in the spatially close 3- and 5-positions. The structures of the isolated products are summarized in Table 2.

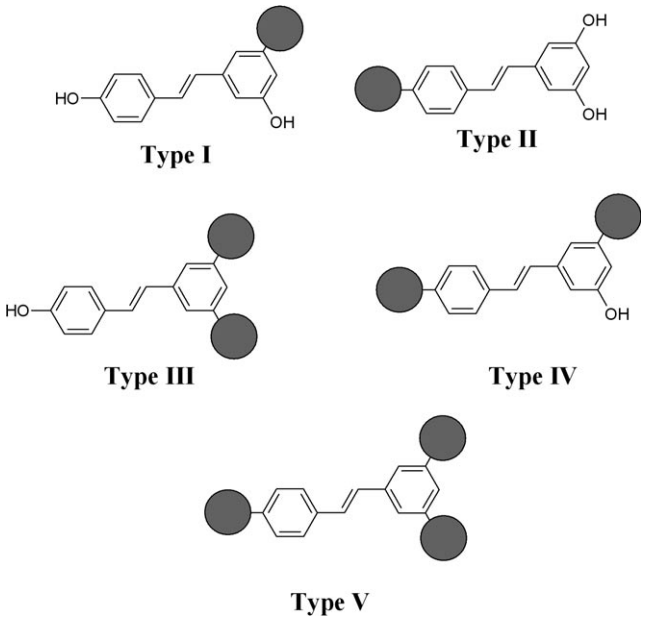

Figure 6. Possible substitution patterns in resveratrol glucosylation.

The triglucosides **8c**/**8d** could not be univocally identified. The tetraglucosides **9*** (see Figure 4) showed an important heterogeneity, so it was not possible to determine the position of the glucosyl groups, except in the case of **9a**, which was characterized as a maltotetraosyl derivative at the 4′-OH position (see Supporting Information for NMR analysis).

The diglucoside **7b** was difficult to isolate in pure form as its concentration in the reaction was very low, so the assignation of an α(1→2) bond between the two glucoses was based on amyloglucosidase-catalyzed hydrolysis assays.

Kinetics of Resveratrol Glucosylation

The kinetics of transglucosylation was studied with the two CGTases. Reactions were followed during 50 h, and the 8 main glucosylated derivatives were quantified by HPLC (Table 3). The maximum yield was obtained at 5 h with the CGTase from *Thermoanaerobacter* sp., whereas in the case of CGTase from *B. macerans* the highest production was achieved at 26 h.

The presence of a mixture of glucosylated compounds may have interest in terms of absorption of resveratrol at the intestinal wall when they are orally administered. Recently Biasutto et al. observed that when glucosyl groups were incorporated to resveratrol *via* a succinate linker, the absorption was delayed and the curve showing the concentration of resveratrol derivatives in blood *vs.* time was shifted to longer times in comparison with resveratrol.[15] They suggest-

Table 3. Concentration of the 8 main products obtained in the CGTase-catalyzed glucosylation of resveratrol, determined by HPLC.[a]

Compound	Concentration (mM)[b]	Concentration (mM)[c]
6a	6.6	1.4
6b	4.0	0.6
7a	3.8	0.9
7c	2.4	0.4
7d	0.5	0.2
8a	0.7	0.3
8b	1.3	0.4
9a	1.1	0.1

[a] *Conditions:* 10 mg mL^{-1} resveratrol (44 mM), 60 g/L starch, 0.2 M sodium acetate (pH 5.6)/DMSO 80/20 (v/v), 5.6 units CGTase per ml.
[b] Using CGTase from *Thermoanaerobacter* sp. in 5 h.
[c] Using CGTase from *Bacillus macerans* in 26 h.

ed the administration of a mix of the aglycone (resveratrol) and its glycosylated derivative to produce a long-lasting increase in circulating levels of the polyphenols and its metabolites.

In our case, the presence of derivatives with different degrees of glycosylation may even extend the half-life of resveratrol and its metabolites in the circulatory streams. This has been attributed to the fact that the time needed for complete hydrolysis of the different glycosylated compounds in the intestinal tract, which is a previous requirement before polyphenol absorption, may vary substantially depending on the glycosylation pattern.

Trolox Equivalent Antioxidant Capability (TEAC)

Although it is expected that the synthetic compounds are completely deglycosylated before absorption,[15] we studied their antioxidant activity (TEAC assay) in order to analyze the role of different phenolic groups on the properties of resveratrol.

Because resveratrol is a lipophilic compound that has a very low solubility in water, the TEAC assay was developed in ethanolic solutions. The antioxidant activities of resveratrol and seven of its glucosylated derivatives were measured. The antioxidant mechanism of these compounds is associated with the presence of hydroxy groups that are able to donate a hydrogen to another compound. The results of the assay are represented in Figure 7 **A** for those compounds glucosylated totally or partly at 3-OH, and Figure 7 **B** for those glucosylated exclusively at 4′-OH.

The incorporation of a glucosyl moiety to the position 3 (Figure 7 **A**) caused a higher loss of antioxidant activity than in position 4′ (Figure 7 **B**), which seems to imply that phenolic 3-OH is more important for the antioxidant activity than the corresponding 4′-OH. This fact is in concordance with our previous results studying a series of resveratrol esters.[33] Furthermore, the antioxidant activity decreased more substantially when increasing the number of glucosyl moieties. The TEAC values, calculated from the slopes of linear regressions of Figure 7, are summarized in Table 4. The derivative with the higher TEAC value (**6b**) has an antioxidant activity of only 69% compared with resveratrol.

Surface Tension and and Critical Micelle Concentration (CMC)

It was expected that the incorporation of one or various glucosyl moieties to resveratrol **1** could impart surfactant properties to such a hydrophobic molecule. We measured the surfactant properties of several α-glucosylated derivatives (the monoglucosides **6a** and **6b**, and the diglucosides **7a**, **7c** and **7d**) as well as the natural β-glucosylated piceid **2**. Surprisingly, piceid did not exhibit surfactant properties (Figure 8), which could be attributed to the spatial configuration of the β-anomer.

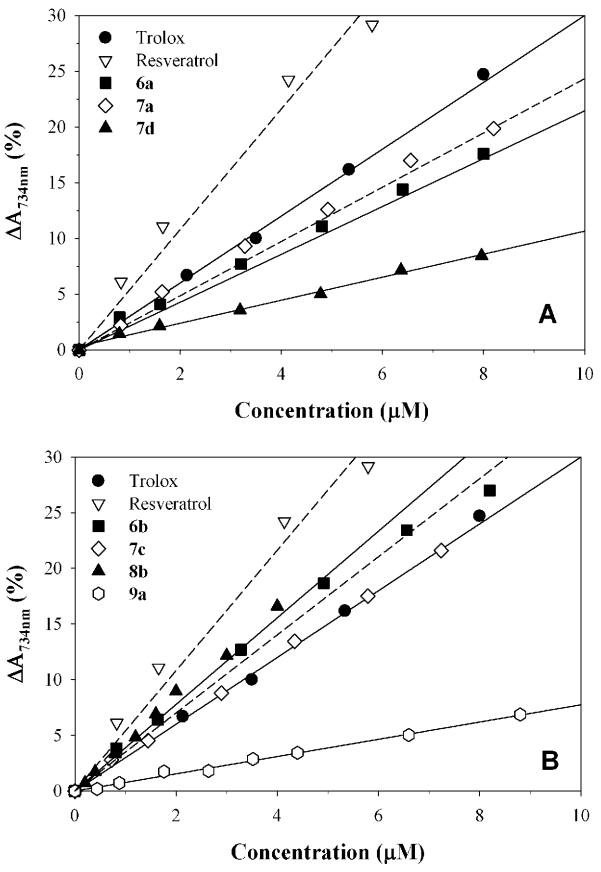

Figure 7. Effect of different resveratrol derivatives on ABTS$^{\bullet+}$ reduction: (**A**) glucosylated only at 3-OH, or both at 3-OH and 4′-OH; (**B**) glucosylated exclusively at 4′-OH.

Table 4. TEAC values of resveratrol and its α-glucosides.

Compound	TEAC
1	1.70
6a	0.75
6b	1.17
7a	0.84
7c	1.00
7d	0.36
8b	0.98
9a	0.26

The 3-OH monoglucoside **6a** showed a typical surfactant behaviour, characterized by a linear decrease of the surface tension *vs.* logarithm of concentration until reaching the CMC. In the case of **6b**, we observed two inflexion points. The first one is commonly due to the micelle preaggregation (denominated critical aggregation concentration or CAC[34]); the second corresponds to its CMC, which was quite similar to the value of compound **6a**. The diglucosides **7a** and **7c** also showed surfactant activity (plots not shown). In the case of **7d**, the CMC was calculated (0.49 mM) but the surface tension (γ) decreased only to values close to 60 mN/m. This molecule can be related to the so-called bolamphiphilic surfactants[35,36] due to the presence of two glucoses in opposite sides of the stilbene ring (structural Type IV). Comparing the three diglucosides of resveratrol, **7a** caused a higher decrease of the surface tension ($\leq$48.8 mN/m) than **7c** and **7d** ($\leq$66.7 and 59.6 mN/m, respectively). The main results of this study are summarized in Table 5.

It is noteworthy that the solubilities in water of the synthesized derivatives (α-glucosylated) and the natural compounds (β-glucosylated) differ significantly. The solubility of resveratrol in water was approx. 0.03 g/L (30 ppm), whereas that of piceid was 0.37 g/L. However, the solubilities of the novel α-glucosides were higher than 2 g/L in all cases, which represents at least a 65-fold increase with respect to resveratrol.

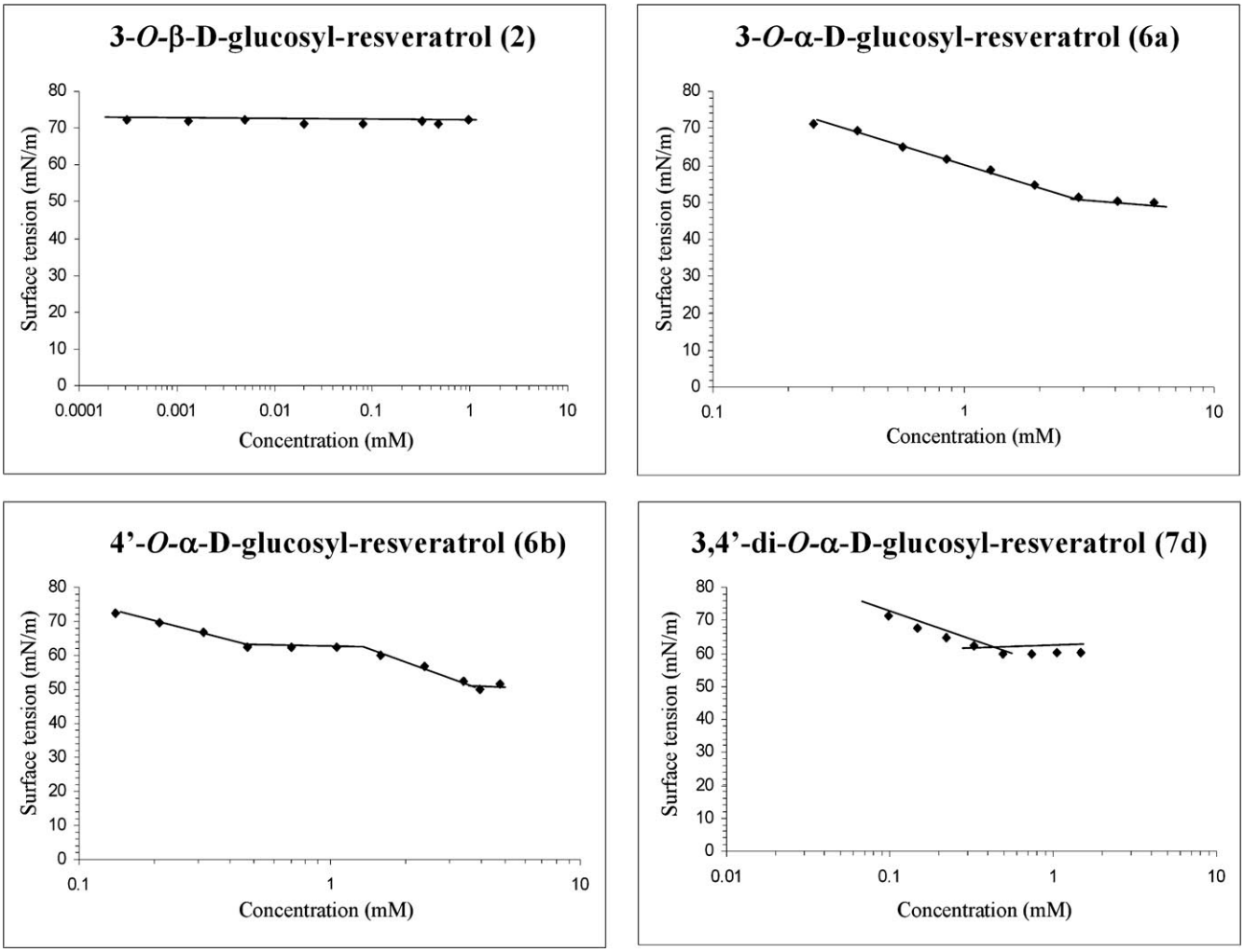

Figure 8. Variation of surface tension *vs.* concentration for different glucosylated resveratrol derivatives.

Table 5. Surfactant properties of some glucosyl derivatives of resveratrol.

	6a	6b	7d
CAC (mM)	n.d.[a]	4.72	n.d.[a]
CMC (mM)	3.33	3.59	0.49
γ_{CMC} (mN/m)[b]	50.0	50.5	59.6
C_{20} (mM)[c]	2.56	2.18	–
pC_{20}	2.59	2.66	–
Γ (mol/cm^2)[d]	$3.5\cdot10^{-10}$	$4.6\cdot10^{-10}$	$3.0\cdot10^{-10}$
A (Å^2)[e]	46.9	35.8	56.0
Solubility (g/L)	>2.5	>2.0	>0.82

[a] Not detected.
[b] Surface tension at CMC.
[c] Concentration needed to decrease the surface tension of water by 20 mN m^{-1}.
[d] Γ: surface excess concentration according to the Gibbs equation.
[e] A: area occupied per molecule adsorbed at the saturated interface.

Conclusions

A series of α-glucosides of resveratrol have been enzymatically synthesized, purified and characterized using MS and NMR. Most of them are novel compounds. The glucosides showed a remarkable surfactant activity which make it useful to include them in different delivery systems and administration forms.[16] Considering that the glycosides need to be converted into the aglycones before they are absorbed into the blood circulation, the presence of a mixture of glucosylated compounds may exert a long-lasting increase in circulating levels of the polyphenols and its metabolites, as the time needed for complete hydrolysis of the different glycosylated compounds in the intestinal tract, may vary substantially depending on the glycosylation pattern. To demonstrate that the bioavailability of resveratrol can be modified upon glucosylation, pharmacokinetics studies of the synthesized derivatives are required.

Experimental Section

Materials

Resveratrol from *Polygonum cuspidatum* was purchased from Shangai Seebio Biotechnology. Amyloglucosidase from *Aspergillus niger* (Spirizyme Fuel) and CGTase from *Thermoanaerobacter* sp. (Toruzyme 3.0L) were kindly donated by Novozymes A/S. CGTase from *Bacillus macerans* (CGTase Amano) was kindly supplied by Amano Enzyme Inc. Pure amyloglucosidase from *Aspergillus niger* (ref. 10115) was purchased from Sigma. Partially hydrolyzed starch from potato (Passelli SA2) was from Avebe (Foxhol, The Netherlands). 2,2′-Azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), kojibiose and maltodextrins were purchased form Sigma. All other reagents were of the highest available purity and used as purchased.

Determination of Enzymatic Activity

Among the compounds that can be synthesized by CGTases, β-cyclodextrin (a cyclic oligosaccharide composed of seven D-glucopyranoside units) can enclose a molecule of phenophthalein inside the structure, changing a pink solution (at basic pH) to a colourless one. An ethanolic solution of phenophthalein (3.75 mM) was diluted with 0.2 M sodium carbonate buffer (pH 9.7) to obtain a 55 μM concentration. The reaction mixture was composed of 0.2 M sodium carbonate pH 9.7 (0.45 mL), 50 g/L starch (0.5 mL) and 50 μL enzyme (or buffer). This mixture was incubated at 50 °C for 15 min. After that, an aliquot of 20 μL was added to 180 μL of phenolphthalein solution (55 μM) and the absorbance was measured at 554 nm in a microplate reader (Versamax, Molecular Devices). One enzyme unit (U) was defined as that catalyzing the formation of 1 μmol of β-cyclodextrin per min.

General Procedure for Enzymatic Reactions on an Analytical Scale

A stock resveratrol solution was prepared in DMSO, and starch was dissolved (10–100 mg mL^{-1}) in 0.2 M sodium acetate buffer (pH 5.6). Both solutions were mixed in a sealed 15-mL dark vial to a final resveratrol concentration of 44 mM (10 mg mL^{-1}), using different DMSO/buffer ratios. The mixture was kept under nitrogen at 40–80 °C with 150 rpm orbital stirring (SI50, Stuart Scientific). The enzyme was added to a final concentration of 1–10 U mL^{-1}. Aliquots (200 μL) were withdrawn at intervals, filtered using an Eppendorf tube containing a Durapore® 0.45 μm filter and the progress of the reaction analyzed by HPLC.

Preparative-Scale Enzymatic Reactions

The reaction mixture contained resveratrol (200 mg, 44 mM), starch (1.4 g), Toruzyme 3.0 L (2.4 mL, 112 U), DMSO (4 mL), 0.2 M sodium acetate buffer 13.6 mL (pH 5.6). The mixture was incubated at 60 °C under similar conditions to those described above and monitored by HPLC. After 15 h, the mixture was cooled, filtered, the solvent evaporated and the crude product chromatographied, yielding different pure compounds.

HPLC Analysis

HPLC analysis was performed using a ternary pump (model 9012, Varian) coupled to a thermostatted (25 °C) autosampler (model L-2200, VWR International). The temperature of the column was kept constant at 40 °C (MEF-01 oven, Analisis Vinicos, Spain). Detection was performed using a photodiode array detector (ProStar, Varian) in series with an evaporative light scattering detector (ELSD, model 2000ES, Alltech), and integration was carried out using the Varian Star LC workstation 6.41. For the analytical reaction, the column was a Nucleosil C18 (4.6×250 mm, 5 μm, Analisis Vinicos) and mobile phase was H_2O (containing 0.1% of acetic acid) at 0.7 mL min^{-1} for 4 min. Then, a gradient from this mobile phase to 50:50 (v/v) H_2O/methanol was per-

formed in 8 min, and this eluent was maintained during 9 min. Finally, a gradient from this composition to the initial H_2O was performed in 2 min. The resveratrol derivatives were quantified at 308 nm. The elution of carbohydrates was followed by the ELSD detector. For the preparative-scale reactions, the column was a Mediterranea-C18 (21.2× 250 mm, 5 μm, Teknokroma, Spain). The initial mobile phase was 70:30 (v/v) methanol/H_2O (H_2O contained 0.1% of formic acid) at 9 mL min^{-1} for 10 min. Then, a gradient from this mobile phase to 45:55 (v/v) H_2O/methanol was performed in 5 min, and this eluent was maintained during 15 min. Finally, a gradient from this composition to the initial mobile phase 70:30 (v/v) metanol/H_2O was performed in 5 min. For the amyloglucosidase-hydrolysis test the column was a Luna-NH2 (4.6×250 mm, 5 μm, Phenomenex) and mobile phase was 80:20 (v/v) acetonitrile:water at 1 mL min^{-1} during 30 min. The resveratrol derivatives were detected at 308 nm. The elution of carbohydrates was followed by the ELSD detector.

ESI-MS

Samples were analyzed on a mass spectrometer (model LCQ Deca XP plus, Thermo Instruments) that contained an ionic tramp. Samples were introduced using a needle pump at 10 μL/min.

Hydrolysis Test

In order to elucidate the structure of the new compounds an enzymatic hydrolysis method was used. Two amyloglucosidases from *Aspergillus niger* were assayed: 10115 (Sigma) and Spirizyme (Novozymes A/S) The reaction mixture contained the glucosylated derivatives (40 μL of a 5 g/L solution) and the enzyme (40 μL of a solution 2.0 U mL^{-1} in 50 mM sodium acetate buffer pH 4.5). The mixture was incubated at 40 °C with 200 rpm orbital stirring (Vortemp 56, Labnet). After 24 h, the mixture was filtered using an eppendorf tube containing a Durapore® 0.45 μm filter and the reaction analyzed by HPLC.

NMR

NMR spectra of the different compounds were recorded on a Bruker DRX 500 spectrometer using deuterated water (D_2O) or DMSO-d_6 as solvent. A temperature of 298 K was employed with concentrations around 10 mM. Chemical shifts were reported in ppm, and referenced versus the DSS signal. Vicinal proton-proton coupling constants were estimated from first order analysis of the spectra. The 2D-TOCSY experiment (60 ms mixing time) was performed using a data matrix of 256×1 K to digitalize a spectral width of 5000 Hz. 2D-NOESY (600 ms) and 2D-T-ROESY experiments (500 ms) used the standard sequences provided by the manufacturer, and the data matrixes described above. In all cases, squared cosine-bell apodization functions were applied in both dimensions. The spectral widths for the HSQC spectra were 5000 Hz and 18000 Hz for the ^{1}H- and ^{13}C-dimensions, respectively. The number of collected complex points was 1028 for the ^{1}H-dimension with a recycle delay of 2 s, and 256 time increments minimum were always recorded in the ^{13}C-dimension. The *J*-coupling evolution delay was set to 3.2 ms. A squared cosine-bell apodization function was applied in both dimensions. Prior to Fourier transform the data matrixes were zero filled up to 1024 points in the ^{13}C-dimension. The spectral widths for the HMBC spectra were 5000 Hz and 25000 Hz for the ^{1}H- and ^{13}C-dimensions, respectively. The number of collected complex points was 1028 for the ^{1}H-dimension with a recycle delay of 2 s, and 256 time increments were always recorded in the ^{13}C-dimension. The *J*-coupling evolution delay was set to 66 ms. Prior to Fourier transform the data matrixes were zero filled up to 1024 points in the ^{13}C-dimension. The number of transients in every experiment was set according to the concentration of the sample.

TEAC assay

The trolox equivalent antioxidant capability (TEAC) assay described by Re et al.[37] was employed to measure the antioxidant activity of the resveratrol derivatives, with some modifications to adapt to 96-well plates. This assay is based on the ability of antioxidants in reducing ABTS radical. Briefly, ABTS (7 mM final concentration) was added to an aqueous solution of 2.45 mM potassium persulphate and kept in the dark at room temperature for 15 h to obtain the ABTS radical, which was stable for 2 days. The $ABTS^{\bullet+}$ solution was diluted with ethanol to get an absorbance of 0.70 (±0.02) at 734 nm, and equilibrated at room temperature. In each well, 20 μL of a Trolox solution (standard) or of the antioxidants (0.5–10 μM) in ethanol were added to 230 μL of adjusted $ABTS^{\bullet+}$ solution. The decrease of absorbance of $ABTS^{\bullet+}$ solution was monitored at 734 nm during 6 min using a microplate reader (model Versamax, Molecular Devices) and the decrease of absorbance (ΔA_{734nm}) for each concentration was determined using the area under the curve. The concentration *vs.* ΔA_{734nm} curve was plotted for the different compounds and used to calculate the equivalent Trolox concentration. The TEAC value was determined as the ratio between the slopes of concentration-ΔA_{734nm} curves for the corresponding antioxidant and Trolox.

Surface Tension and CMC

The surface tension measurements of the aqueous solutions were performed in a Krüss K-12 tensiometer using the Wilhelmy plate method. Stock solutions were prepared for each compound, which were conveniently diluted to measure the surface tension. The CMC values were taken from the intersection of the linear sections obtained in the plot of surface tension *vs.* logarithm of the concentration. Besides the CMC, we calculated the surface excess concentration (Γ) according to the Gibbs equation [$\Gamma = -(d\gamma/d\log c)/2.303\,nRT$] and the area occupied per molecule adsorbed at the saturated interface (A), expressed in Å^2: $A = 10^{16}/(N_A \times \Gamma)$.

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