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Glycosyltransferase-catalysed cascade reactions for biocatalytic synthesis of flavonoid glucosides

PhD thesis

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Abstract

Due to their powerful antioxidant, anti-inflammatory and anti-bacterial properties flavonoids, a large class of secondary plant metabolites, are considered as highly beneficial ingredients in functional food and pharmaceuticals. However, similar to various other natural products their bioavailability is restricted by poor water solubility which can be overcome by glycosylation. In order to maintain expedient bioactivities the attachment of sugars needs to be regio- and stereoselective. In contrast to alternative chemical and enzymatic approaches glycosylations by Leloir glycosyltransferases (GTs) can provide the required specificity. These enzymes use an activated sugar donor substrate, typically a NDP-glucose, for transfer of the glycosyl residue onto a specific position of an acceptor molecule. Unfortunately the need of costly activated sugar donors strongly limits their synthetic applicability. Therefore this thesis aimed to establish a generally applicable regeneration system for uridine 5'-diphosphate (UDP)-glucose, the most common sugar donor. Sucrose synthase (SuSy) catalyses the reversible transfer of glucose from sucrose to UDP and thereby enables *in situ* regeneration of UDP-glucose in GT-SuSy cascade reactions. As model reactions we mainly studied regioselective 2′-*O*- and 3′-*C*-glucosylation of the dihydrochalcone phloretin by *Pc*OGT and *Os*CGT, respectively. The resulting *O*-glucoside phlorizin is the major phenolic compound in apple trees while the *C*-glucoside nothofagin is a prominent antioxidant from rooibos tea. Furthermore the selective synthesis of the 2′-*O*- (davidioside) and 4′-*O*-glucoside (confusoside) of davidigenin was achieved for the first time. By coupling glycosylations to UDP-glucose recycling by SuSy in one pot yield and rate enhancements were accomplished in addition to cost efficient sugar donor supply. Reaction engineering mainly focussed on the particularly desired synthesis of *C*-glucosidic nothofagin. *C*-glycosides are rare in nature and challenging to synthesise but due to their resistance to acidic and enzymatic hydrolysis particularly attractive pharmaceutical targets. Aside of sugar donor supply low solubility and substrate inhibition of acceptor molecules pose a major limitation in flavonoid glycosylation. Addition of DMSO as cosolvent and stepwise addition of phloretin in a fed-batch mode largely eased this constraint in nothofagin synthesis. Further improvement of phloretin solubility was achieved by substitution of DMSO with β-cyclodextrin which also abolished the frequently observed enzyme inactivation in presence of organic solvents. Therefore not only the need for substrate and enzyme feed became obsolete but also overall catalytic efficiency was largely increased. Finally nothofagin was also prepared from phlorizin in a novel *O*- to *C*-glucosidic bond rearrangement approach. Reversible *O*- and irreversible *C*-glucosylation were therefore coupled in one pot. Similar to the SuSy-GT coupled conversions also in this glycosyltransferase cascade reaction UDP-glucose was *in situ* formed from catalytic amounts of UDP.

Zusammenfassung

Flavonoide sind eine bedeutende Gruppe sekundärer Pflanzenstoffe, die durch ihre stark antioxidativen, entzündungshemmenden und antibakteriellen Eigenschaften wesentliche Inhaltsstoffe von funktionellen Lebensmitteln und Arzneimitteln sind. Die häufig durch schlechte Wasserlöslichkeit eingeschränkte biologische Verfügbarkeit kann durch Glykosylierungen überwunden werden. Um die erwünschten Bioaktivitäten zu erreichen, müssen Glykosylierungen regio- und stereoselektiv erfolgen. Im Gegensatz zu chemischen und anderen enzymatischen Methoden kann dies durch Leloir-Glykosyltransferasen (GTs) erzielt werden. Für den Transfer von Zuckern an spezifische Positionen von Akzeptormolekülen benötigt man typischerweise durch Nukleosiddiphosphate aktivierte Zuckerdonoren. Die hohen Kosten dieser Donoren stellen jedoch eine starke Limitierung für synthetische Anwendungen dar. Ziel der Arbeit ist ein allgemein anwendbares Regenerationssystem für Uridin-5'-Diphosphat (UDP)-Glukose, den am weitesten verbreiteten Zuckerdonor, zu etablieren. Sucrose Synthase (SuSy) katalysiert den reversiblen Glukosetransfer von Sucrose auf UDP wodurch *in situ* Regenerierung von UDP-Glukose in GT-SuSy Kaskadenreaktionen ermöglicht wird. Als Modellreaktionen wurden hauptsächlich die regioselektive 2′-*O*- und 3′-*C*-Glukosylierung des Dihydrochalkons Phloretin durch *Pc*OGT beziehungsweise *Os*CGT untersucht. Das dabei gebildete *O*-Glukosid Phlorizin ist die verbreitetste phenolische Verbindung in Apfelbäumen während das *C*-Glukosid Nothofagin ein bedeutendes Antioxidans in Rooibos Tee ist. Zusätzlich wurden erstmalig das 2′-*O*- (Davidiosid) und 4′-*O*-Glukosid (Confusosid) von Davidigenin selektiv synthetisiert. Durch Kopplung von Glykosylierungen mit SuSy zur UDP-Glukose Regenerierung konnten neben kosteneffizienter Zuckerdonor Bereitstellung auch signifikant erhöhte Ausbeuten und Umsetzungsraten erreicht werden. Das Hauptaugenmerk lag hierbei auf der Reaktionsoptimierung für die besonders gefragte Synthese des *C*-Glukosids Nothofagin. Durch ihre außergewöhnliche Hydrolysebeständigkeit besteht ein besonderes Interesse an *C*-glukosidischen Pharmazeutika. Jedoch sind diese Verbindungen in der Natur nicht weit verbreitet und auch schwer zu synthetisieren. Neben der Verfügbarkeit der Zuckerdonoren sind die geringe Löslichkeit und potentielle Substratinhibierung der Akzeptoren weitere Hürden in der enzymatischen Synthese von Flavonoid-Glykosiden. Durch Beimengung von DMSO in Kombination mit schrittweiser Zugabe von Phloretin konnten diese Limitierungen in der Synthese von Nothofagin größtenteils beseitigt werden. Durch Austausch von DMSO durch β-Cyclodextrin wurde eine weitere Verbesserung der Löslichkeit von Phloretin erreicht sowie die häufig auftretende Enzyminaktivierung durch organische Lösungsmittel eliminiert. In Folge dessen wurden nicht nur Substrat und Enzymzugabe während der Reaktion überflüssig, sondern auch die katalytische Effizienz konnte merklich gesteigert werden. Letztendlich wurde Nothofagin auch aus Phlorizin in einer neuartigen *O*- zu *C*-glukosidischen Umlagerung, durch Verknüpfung reversibler *O*- mit irreversibler *C*-Glykosylierung, synthetisiert. In dieser Kaskadenreaktion wurde wie in den SuSy-Reaktionen UDP-Glukose *in situ* aus katalytischen Mengen an UDP gebildet.

Contents

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COMMUNICATIONS

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Leloir Glycosyltransferases and Natural Product Glycosylation: Biocatalytic Synthesis of the *C*-Glucoside Nothofagin, a Major Antioxidant of Redbush Herbal Tea

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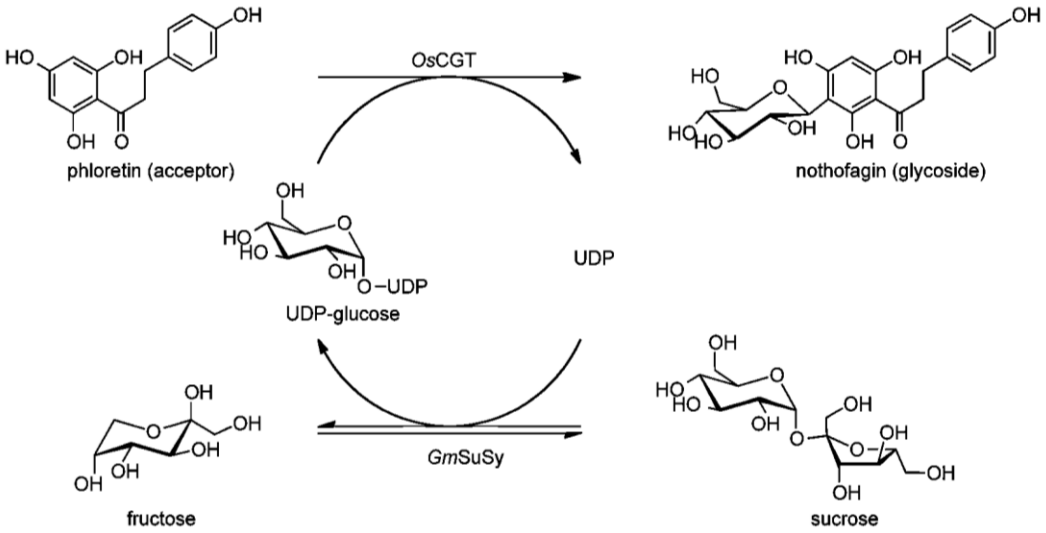
Abstract: Nothofagin is a major antioxidant of redbush herbal tea and represents a class of bioactive flavonoid-like *C*-glycosidic natural products. We developed an efficient enzymatic synthesis of nothofagin based on a one-pot coupled glycosyltransferase-catalyzed transformation that involves perfectly selective 3′-*C*-β-D-glucosylation of naturally abundant phloretin and applies sucrose as expedient glucosyl donor. *C*-Glucosyltransferase from *Oryza sativa* (rice) was used for phloretin *C*-glucosylation from uridine 5′-diphosphate (UDP)-glucose, which was supplied continuously *in situ* through conversion of sucrose and UDP catalyzed by sucrose synthase from *Glycine max* (soybean). In an evaluation of thermodynamic, kinetic, and stability parameters of the coupled enzymatic reactions, poor water solubility of the phloretin acceptor substrate was revealed as a major bottleneck of conversion efficiency. Using periodic feed of phloretin controlled by reaction progress, nothofagin concentrations (45 mM; 20 g L^{-1}) were obtained that vastly exceed the phloretin solubility limit (5–10 mM). The intermediate UDP-glucose was produced from catalytic amounts of UDP (1.0 mM) and was thus recycled 45 times in the process. Benchmarked against comparable glycosyltransferase-catalyzed transformations (e.g., on quercetin), the synthesis of nothofagin has achieved intensification in glycosidic product formation by up to three orders of magnitude (μM→mM range). It thus makes a strong case for the application of Leloir glycosyltransferases in biocatalytic syntheses of glycosylated natural products as fine chemicals.

Keywords: carbohydrates; *C*-glycosides; glycosyltransferases; natural products; UDP-glucose recycling

Many bioactive natural products contain sugar molecule(s) as part of their structure.[1] Their physiological activity, selectivity and pharmacological properties are often derived from the sugar component(s).[2] Therefore, glycosylation is often central to a natural product's efficacy in the particular application considered. Aside from therapeutic uses,[3] glycosylated natural products have raised interest as functional food additives and cosmetic ingredients.[4] Glycosylation pattern engineering is regarded as a highly promising way of functional diversification of natural products.[2b,3] This might contribute to the creation of new bioactive substances and drug leads.

In nature, the selective modification of target compounds with sugars is catalyzed by glycosyltransferases (EC 2.4).[5] These enzymes use an activated donor substrate, typically a nucleoside diphosphate (NDP)-sugar, for transfer of the glycosyl residue onto a specific position of an acceptor molecule. Glycosyltransferases display splendid regioselectivity and stereochemical control in the transformations catalyzed,[6] and they are therefore widely recognized as highly valuable glycosylation catalysts.[7] However,



Scheme 1. Synthesis of nothofagin is achieved by enzymatic *C*-glucosylation of phloretin from sucrose *via* UDP-glucose. *Os*CGT and *Gm*SuSy are telescoped in one pot, and the reaction proceeds in the presence of catalytic amounts of UDP. Fructose is the only by-product formed. The overall equilibrium lies far on the *C*-glycoside product side, driven by the reaction of *Os*CGT and the presence of sucrose in excess.

synthetic applications of glycosyltransferases have so far been quite restricted due to complexities of the enzymes (e.g., low specific enzyme activity and stability)[8] and the supply of donor and acceptor substrates for the enzymatic reactions.[9]

The majority of natural product glycosylations involve *O*-glycosidic bonds. Glycosylations at carbon, by contrast, are relatively rare and to date only a small number of natural *C*-glycosyltransferases have been reported.[10] The *C*-glycosidic linkage displays outstanding resistance to chemical or enzyme-catalyzed hydrolysis, surpassing that of the corresponding *O*-glycosidic linkage by a large amount.[11] *C*-glycosides have therefore attracted considerable attention for functional substitution of physiologically active *O*-glycosidic compounds having low *in vivo* lifetimes.[12]

Aryl glucosides derived from flavonoid-like aglycones (Scheme 1) present a very interesting class of plant natural products that may involve either a *C*- or an *O*-glycosidic linkage.[13] They show a highly significant profile of biological activities that typically include strong antioxidant and radical scavenger functions, but also comprise antiviral and cytotoxic effects.[14] Because product isolation directly from the plant is often impractical, compounds must also be prepared by bottom-up synthesis. Despite notable recent advancements,[15] chemical methodologies involve multiple steps and are therefore generally neither atom-efficient nor high-yielding. We demonstrate in this study that single-step glycosyltransferase-catalyzed transformation *in vitro* presents a powerful tool for aryl *C*-glycoside synthesis. We show that when offered the dihydrochalcone phloretin as acceptor, *C*-glycosyltransferase from rice (*Oryza sativa*; *Os*CGT) reacts with uridine 5′-diphosphate (UDP)-glucose to give the 3′-*C*-aryl β-D-glucoside nothofagin (Scheme 1) as a single transfer product. Nothofagin is a natural substance found in redbush herbal tea and represents a structural class of bioactive aryl *C*-glycosides.[16] For efficient nothofagin synthesis, we coupled the *C*-glucosyltransferase reaction to enzymatic *in situ* supply of the glucosyl donor substrate (Scheme 1): UDP-glucose is produced from sucrose and UDP using recombinant sucrose synthase from soybean (*Glycine max*; *Gm*SuSy). While the applied internal UDP-glucose regeneration is known in principle and has been applied to enzymatic reactions involving different glycosyl donor substrates,[17] a critical test of its performance capability in the synthesis of natural product glucosides such as nothofagin[17e–h] remains outstanding. We show the application of comprehensive step-by-step reaction engineering to overcome complexities inherent to this and similarly coupled glycosyltransferase systems and report an efficient, high-yielding biocatalytic production of nothofagin.

We first examined reactions of *Os*CGT and *Gm*SuSy separately and determined their kinetic and thermodynamic characteristics. Enzymes were obtained from *Escherichia coli* expression cultures and purified to apparent homogeneity by Strep-tag affinity chromatography, as described in the Supporting Information (Methods, Figure S1). Their activities were determined using enzymatic or HPLC-based assays (Supporting Information, Methods). The reaction of *Os*CGT was monitored with an HPLC assay capable of distinguishing between nothofagin and potential alternative products resulting from *O*-glucosyl transfer at the 2′ or 4′ position of the acceptor (Supporting In-

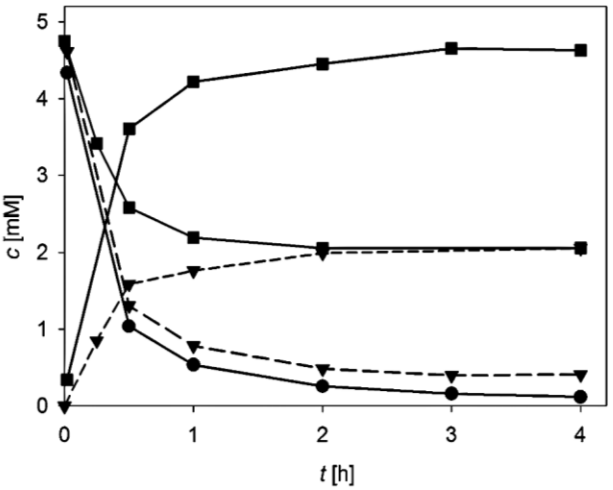


Figure 1. Time course analysis for individual enzymatic reactions catalyzed by *Os*CGT and *Gm*SuSy at pH 7.5 and 30 °C. Nothofagin synthesis by *Os*CGT (black symbols): 80 mU mL^{-1}, 5 mM phloretin (triangle, dashed line), 4.75 mM UDP-glucose (circle, solid line), nothofagin (square, solid line). Reactions of *Gm*SuSy (grey symbols): 50 mU mL^{-1}, 5 mM of each substrate, UDP-glucose in sucrose synthesis (squares, solid line) and cleavage (triangles, dashed line).

Table 1. Characterization of glycosyltransferases and their reactions.[a]

Parameter	*Gm*SuSy		*Os*CGT
	Synthesis	Cleavage	
$K_{M\ sucrose}$ [mM]	–	25.5 ± 3.3[b]	–
$K_{M\ UDP}$ [mM]	–	0.13 ± 0.02[b]	–
$K_{M\ fructose}$ [mM]	3.0 ± 0.4[b]	–	
$K_{M\ UDP-glucose}$ [mM]	0.14 ± 0.03[b]	–	0.024 ± 0.004
$K_{M\ phloretin}$ [mM]	–	–	0.009 ± 0.003
k_{cat} [s^{-1}]	7.5 ± 0.4	9.3 ± 0.3	4.4 ± 0.3
Spec. act. [U mg^{-1}]	4.8 ± 0.2	5.9 ± 0.2	5.1 ± 0.3
K_{eq}		0.49 ± 0.01[c,e]	> 400[d,e]
$t_{1/2}$ [h]		18.8 ± 0.9[f]	13.8 ± 1.2[g]

[a] 30 °C, 50 mM HEPES pH 7.5, 20% (v/v) DMSO.
[b] 30 °C, 20 mM HEPES, pH 7.5.
[c] Cleavage direction (conversion of sucrose and UDP).
[d] Glycosylation direction (conversion of phloretin and UDP-glucose).
[e] Calculated from data in Figure 1.
[f] 30 °C, 50 mM HEPES pH 7.5, 20% (v/v) DMSO, 100 mM sucrose.
[g] 30 °C, 50 mM HEPES pH 7.5, 20% (v/v) DMSO, 5 mM phloretin.

formation, Figure S2).[18] *Os*CGT displayed absolute selectivity (within an error limit of ≤0.5%) for 3′-*C*-glycosylation of phloretin. We determined pH-activity dependencies for *Gm*SuSy (sucrose cleavage and synthesis) and *Os*CGT (nothofagin synthesis) at 30 °C. The resulting pH profiles revealed suitable overlap of the enzyme activities in the pH range 6.5–8.0 (Supporting Information, Figure S3). The results shown in Figure 1 indicate that *C*-glucosylation of phloretin at pH 7.5 resulted in high conversion of substrates (≥ 95%). Moreover, the enzymatic reverse reaction with nothofagin and UDP was not detectable under these conditions (Supporting Information, Figure S4). We concluded, therefore, that synthesis of nothofagin by *Os*CGT proceeds without critical thermodynamic limitations. The equilibrium for sucrose conversion is pH-dependent, and a low pH of 6 or smaller is known to favor the formation of UDP-glucose.[19] Figure 1 shows that at pH 7.5, the equilibrium constant (K_{eq}) for conversion of sucrose and UDP had a value of 0.49. However, thermodynamic constraints on the supply of UDP-glucose at elevated pH can be eliminated effectively using sucrose in excess. We therefore performed our conversion studies at pH 7.5 and 30 °C where both glycosyltransferases showed useful activity and stability (Table 1) and quantitative transformation of sucrose into nothofagin was feasible.

The low water solubility of non-carbohydrate acceptor substrates is an important issue for carrying out natural product glycosylations *in vitro*. In the case of the barely water-soluble phloretin, use of an organic cosolvent was essential to enhance the acceptor substrate availability in *C*-glycosylations catalyzed by *Os*CGT. Whereas both ethanol and DMSO up to 20% by volume caused only minor interference with *Os*CGT activity, *Gm*SuSy displayed a low cosolvent tolerance and its activity was almost completely (≥ 85%) lost in the presence of 15% ethanol. DMSO was less strongly affecting the activity of *Gm*SuSy and around 65% of the specific enzyme activity in purely aqueous buffer were retained in 20% DMSO. Furthermore, *Os*CGT stability and phloretin solubility were superior in DMSO as compared to ethanol. All conversion experiments were therefore performed in 20% DMSO, and the maximum concentration of dissolved phloretin was around 10 mM under these conditions.

Kinetic characterization of *Gm*SuSy (sucrose conversion) and *Os*CGT (nothofagin synthesis) was done at pH 7.5 and results are summarized in Table 1 along with the relevant enzyme stability and reaction thermodynamic parameters under these conditions. Both enzymes showed useful specific activities (≥ 5 units mg^{-1} protein) and were sufficiently stable under the reaction conditions with half-lives of around 19 (*Gm*SuSy) and 14 h (*Os*CGT). The Michaelis–Menten constant (K_M) of *Gm*SuSy for sucrose exceeds the corresponding K_M for UDP by two orders of magnitude. K_M values of *Os*CGT are also much lower than the sucrose K_M. Therefore, this implies that relatively high sucrose concentrations should be used in the coupled reaction to partly saturate and thus make optimum use of the *Gm*SuSy activity pres-

ent. The kinetic requirements of SuSy are therefore in good accordance with the notion of using an excess of sucrose to drive the overall conversion. We noticed that the K_M for UDP was 26-fold higher in our recombinant preparation of *Gm*SuSy as compared to the enzyme isolated from the native source.[20] This large difference in apparent UDP binding affinity might be due to effects of post-translational modification (e.g., covalent phosphorylation) that have been described for sucrose synthases in plants[21] and that may not occur in *E. coli*. The K_M for UDP-glucose was also strongly elevated (12-fold) in recombinant as compared to native *Gm*SuSy while, interestingly, the K_M values for sucrose and fructose were not affected in the recombinant enzyme. Differences between recombinant and native *Gm*SuSy were not further pursued in this study.

We performed synthesis experiments in which the initial concentration of sucrose (5–500 mM; 0.5 mM UDP) or UDP (0.005–1 mM; 100 mM sucrose) was varied while enzymatic activity (10 mU mL^{-1} *Os*CGT/*Gm*SuSy) and phloretin concentration (5 mM) were constant. The nothofagin production rate (r_P) was measured, and results are depicted in Figure 2. Dependence of r_P on the sucrose concentration was hyperbolic, with a half-saturation constant (27 mM) comparable to the K_M of *Gm*SuSy for sucrose. Therefore, it appears to primarily reflect saturation behavior of *Gm*SuSy, as noted above (Table 1). The dependence of r_P on the UDP concentration was likewise hyperbolic, with a half-saturation level (51 μM) in between the K_M values for UDP and UDP-glucose. Figure 2B) indicates that the applied "nothofagin synthase" activity, derived from the combined activities of *Os*CGT and *Gm*SuSy, was therefore utilized best at a UDP concentration of 0.5 mM or higher. Using a phloretin concentration of 5 mM, this limited the maximum number of UDP-glucose regeneration cycles (RC_{max}) to 10 (=5/0.5). It would certainly be possible to further increase this RC_{max} value by decreasing the UDP concentration relative to the phloretin concentration, but this would probably have to occur at the expense of a significant loss in r_P. It is interesting that at the lowest UDP concentration used in Figure 2 (5 μM), the observed r_P was still 10% of its maximum value at saturation with UDP.

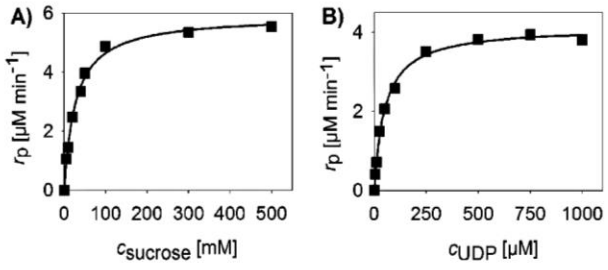


Figure 2. The nothofagin production rate (r_p) in a coupled-enzyme reaction (10 mU mL^{-1} *Os*CGT/*Gm*SuSy, 5 mM phloretin) depends on variation of **A**) the sucrose concentration (0.5 mM UDP) and **B**) the UDP (100 mM sucrose) concentration. Note: because sucrose was not fully saturating in **B**) the achieved r_p at high UDP is slightly lower than in **A**).

Aside from cost-efficient supply of the UDP-glucose donor substrate and favorable thermodynamic effects resulting from the use of high sucrose concentrations, a glucosyltransferase reaction might benefit from its coupling to the SuSy reaction kinetically. Pronounced end-product inhibition by micromolar concentrations of UDP is quite common among flavonoid *O*-glucosyltransferases[17e,g,h] and imposes severe restrictions on the direct synthetic use of these enzymes which could be decreased by continuous removal of the UDP released.[17e,22] We tested the influence of UDP inhibition on nothofagin production by comparing *Os*CGT (50 mU mL^{-1}) conversion of 5 mM phloretin (6 mM UDP-glucose, 100 mM sucrose) in absence and presence of *Gm*SuSy (50 mU mL^{-1}) (Figure 3A). Although in both reactions quantitative conversion (>99.5%) was achieved r_P showed a stronger decrease above ~75% conversion (2 h) without *Gm*SuSy and final conversions (>99%) were only reached after more than 10 h compared to less than 6 h in the presence of *Gm*SuSy. This corresponds to an approximately two-fold gain in space-time yield in nothofagin production (mM product formed/time consumed) resulting from the coupling of *Os*CGT and *Gm*SuSy reactions. UDP-glucose depletion could be excluded as an explanation for the reduction of r_P in the absence of *Gm*SuSy due to excess of UDP-glu-

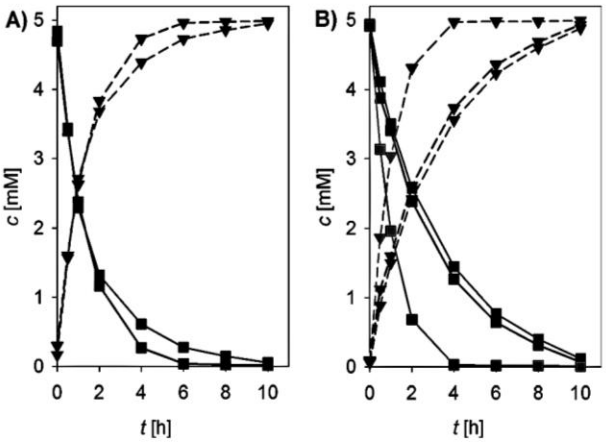


Figure 3. Conversions of 5 mM phloretin (square) to nothofagin (triangle) by *Os*CGT (100 mM sucrose): **A**) Using 50 mU ml^{-1} *Os*CGT and 6 mM UDP-glucose in the absence (grey) and presence (black) of 50 mU mL^{-1} *Gm*SuSy; **B**) variation of *Os*CGT and *Gm*SuSy activity in coupled conversions (0.5 mM UDP): 50 mU mL^{-1} *Os*CGT/*Gm*SuSy (dark grey); 50 mU mL^{-1} *Os*CGT and 250 mU mL^{-1} *Gm*SuSy (black); 250 mU mL^{-1} *Os*CGT and 50 mU mL^{-1} *Gm*SuSy (light grey, black edge).

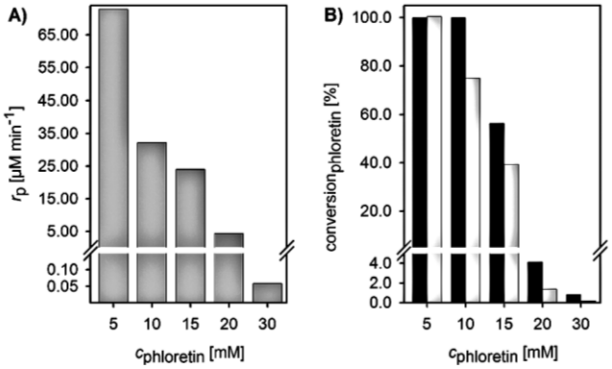


Figure 4. Batch conversions at different phloretin concentrations (100 mM sucrose, 0.5 mM UDP, 190 mU mL^{-1} *Os*CGT, 120 mU mL^{-1} *Gm*SuSy); **A**) Nothofagin production rate decreases at elevated phloretin concentrations; **B**) final conversion of soluble (black) and total applied (grey) phloretin after 24 h is limited at high phloretin concentrations by substrate inhibition and solubility.

cose (1 mM, 40-fold K_M) which was monitored throughout the conversion. Although end product inhibition at low millimolar UDP concentrations was less critical for *Os*CGT than for *O*-glycosyltransferases, *in situ* removal of UDP remains an essential feature for general application of SuSy as UDP-glucose recycling system for high level glycoside production.

Conversion rates were slightly lower when 5 mM UDP-glucose were replaced with 0.5 mM UDP in a coupled glycosyltransferase conversion (50 mU mL^{-1} *Os*CGT/*Gm*SuSy) (Figure 3B; Supporting Information, Figure S5) and complete conversion was only reached after 10 h. Also a 5-fold excess of *Gm*SuSy (250 mU mL^{-1}) over *Os*CGT (50 mU mL^{-1}) did not improve nothofagin production significantly. On the other hand a five-fold excess of *Os*CGT (250 mU mL^{-1}) over *Gm*SuSy (50 mU mL^{-1}) drastically increased the conversion resulting in complete conversion after only 4 h. Thereby *C*-glucosylation was identified as a rate-limiting step at the applied conditions. UDP-glucose levels of roughly 0.1 mM throughout all three conversions coincide with the finding that the UDP-glucose supply was not critical in the coupled conversions. Furthermore, it is worth remarking that the produced nothofagin was stable in all conversions, suggesting that the *C*-glucoside synthesis is conveniently performed under thermodynamic control and in the apparent absence of chemical or enzyme-catalyzed side reactions.

Considering that solubility of phloretin was markedly enhanced (≥5-fold) upon its *C*-glycosylation, we raised the initial concentration of phloretin in various steps to 30 mM, thereby exceeding the solubility limit of the acceptor substrate by at least 3-fold. We figured that insoluble phloretin might still be useful for the continuous *in situ* supply of acceptor substrate when gradual transformation of the dissolved phloretin occurred in the enzymatic reaction (100 mM sucrose, 0.5 mM UDP, 190 mU mL^{-1} *Os*CGT, 120 mU mL^{-1} *Gm*SuSy). Figure 4 shows that nothofagin production could not be upheld under conditions of insoluble acceptor being present (≥10 mM) and also the final product concentration after 24 h was strongly decreased in clear dependence on the phloretin concentration. Using 10 mM phloretin, which was initially dissolved completely, we noticed precipitation of the acceptor substrate over time, limiting the maximum amount of nothofagin obtainable in the reaction under these conditions despite complete conversion of all soluble phloretin (Figure 4B). Furthermore, initial rate studies of *Os*CGT revealed substrate inhibition (K_i ~5 mM) which clearly effected conversion at phloretin concentrations above 1 mM.

To nevertheless increase the end concentration of nothofagin in the enzymatic reaction, we changed the operation mode from batch to fed-batch, adding fresh phloretin to a concentration of 5 or 10 mM once the acceptor substrate had been depleted. Up to 8 rounds of phloretin addition were made, using a highly concentrated stock solution of 500 mM phloretin in pure DMSO to minimize the resulting volume change. Table 2 presents a summary of conditions and results and Figure 5 shows a reaction time course where in each round fresh enzyme was supplied together with 5 mM acceptor to the reaction. Feeding the phloretin acceptor was generally quite effective in enhancing nothofagin production. However, the phloretin conversion rate decreased strongly in dependence on the total amount of acceptor added to the reaction, so that without enzyme feed, the maximum concentration of nothofagin was just around 20 mM (Table 2).

Table 2. Nothofagin synthesis using controlled feed of phloretin and enzyme.[a]

$\Delta c_{phloretin}$[b] [mM]	5	5	10	10
Enzyme feed	no	yes	no	yes
$c_{phloretin}$[c] [mM]	20	45	40	60
Vol. act[c] [mU mL^{-1}]	100	550	100	600
t [h]	27	135	42	120
$c_{nothofagin}$[d] [mM]	14.6	44.1	19.7	46.6
Conversion[e] [%]	88	98	63	90
Precipitation [mM][f]	3.5	<0.1	8.9	8.1
ttn[g] (*Gm*SuSy/*Os*CGT) [1·10^3]	18/16	10/9	25/21	10/8

[a] 300 mM sucrose, 1 mM UDP, 30 °C, 50 mM HEPES pH 7.5, 20% DMSO.
[b] Amount of phloretin added per feeding.
[c] Total amount of phloretin/enzyme activity added.
[d] Final nothofagin concentration in solution.
[e] Based on final nothofagin and phloretin concentrations in solution.
[f] Difference total fed phloretin and final soluble nothofagin and phloretin.
[g] Total turnover number (mM nothofagin/mM total enzyme added).

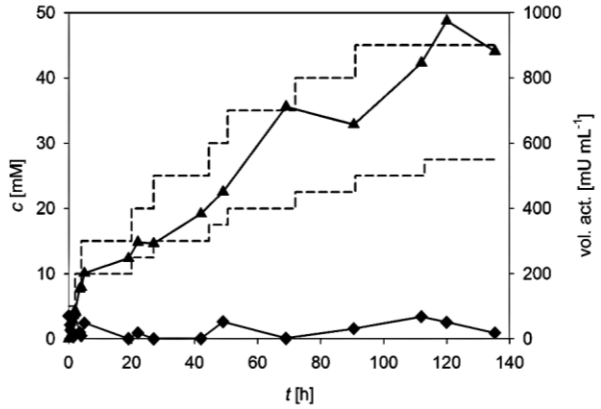


Figure 5. Controlled feeding of phloretin is useful to enhance the nothofagin concentration in the biocatalytic synthesis. *Reaction conditions:* 100 mU mL^{-1} *Os*CGT/*Gm*SuSy, 5 mM phloretin, 300 mM sucrose, 1 mM UDP. After acceptor substrate depletion, 5 mM phloretin and 50 mU mL^{-1} *Os*CGT/*Gm*SuSy were added. Symbols: phloretin added (black dashed), *Os*CGT/*Gm*SuSy added (grey dashed), nothofagin (black), phloretin (grey)

Co-addition of enzyme alleviated restrictions on the product concentration (substrate conversion), which probably resulted from the combined effect of true enzyme activity loss and product inhibition. *Gm*SuSy is inhibited by D-fructose with a reported K_i of 9 mM.[20] This could also explain why lower total turnover numbers (ttn) were obtained with enzyme feed (~10,000) than without (~20,000). We also noticed the requirement to carefully control the phloretin feed to keep the acceptor concentration well below its solubility limit during the reaction. Figure S6 (Supporting Information) depicts *in situ* precipitation of phloretin under conditions where the acceptor feeding rate was not matched to the enzymatic consumption rate. Phloretin precipitation was clearly reflected in the mass balance in solution (Table 2).

Applying a suitable stepwise feeding of phloretin (5 or 10 mM) that included supplementation with fresh enzyme, it was possible to accumulate nothofagin in a concentration of around 45 mM, equivalent to 20 g L^{-1} (Figure 5, Table 2) which corresponded to RC_{max} values of approximately 45. It has to be noted that only with addition of enzyme and a low acceptor feed (5 mM) (Figure 5) could precipitation be avoided and almost quantitative conversion (~98%) was achieved. Nothofagin was isolated from reaction mixtures in a single step using preparative reversed phase C-18 HPLC with water to methanol gradient elution. Typically more than 80% of the initially applied phloretin (≥25 mg) could be recovered as highly pure nothofagin (Supporting information, Figure S8C). The isolation procedure is simple and not limited in scale.

Glycosyltransferases currently underachieve to a large extent their often-quoted high potential as synthetically usable glycosylation catalysts.[9] Notable exceptions in the field of complex oligosaccharides notwithstanding,[17a,d,22] glycosyltransferase transformations have been realized almost exclusively at the analytical or minute preparative scale.[23] Glycosylations of similar poorly water-soluble natural product core structures such as the flavonoid quercetin have furnished hardly more than micromolar concentrations of the desired glycosidic compound.[17g,h,24] On comparison with the literature, therefore, the herein described enzymatic process of glucosylation of phloretin from sucrose stands out due to intensification, by up to three orders of magnitude, in terms of the product concentration that it has achieved for a coupled glycosyltransferase-catalyzed conversion. Furthermore, with the notable exception of the recently reported application of *Os*CGT for the production of nothofagin (~200 μM) and 2-hydroxynaringenin *C*-glucoside using engineered *S. cerevisiae* strains for whole cell conversions[23c] this is the first synthetic use of a *C*-glucosyltransferase. The here reported nothofagin process features efficient assembly of isolated *C*-glucosyltransferase in one pot with an adaptable module for UDP-glucose supply from sucrose, which serves as a highly expedient glucosyl donor for the overall conversion. We show that systematic analysis of thermodynamic conditions, kinetic properties of the glycosyltransferases and their stabilities is the key for identifying and thus eliminating critical constraints on the multi-component reaction system. Integration of biochemical optimization with reaction engineering was essential to overcome the restriction of acceptor substrate solubility. The number of UDP-glucose regeneration cycles was brought into a range (around 50) where one begins to truly capitalize on the coupling with the SuSy reaction. Reported RC_{max} values in literature are by far too small[17e 17g,17h] to justify enzymatic recycling of UDP-glucose. However, costs of the donor substrate are expected to prohibit the direct use of UDP-glucose for synthesis. Considering that unprocessed redbush tea contains nothofagin to just about 4.31 g kg^{-1} freeze-dried matter,[25] the high-yielding enzymatic synthesis developed herein is expected to remove compound availability as a critical bottleneck of the various medical and food-related applications of this aryl-*C*-glucoside.[14b,16,26] This study therefore makes a strong and so far missing case for the application of glycosyltransferases in biocatalytic synthesis of glycosylated natural products as fine chemicals.

Experimental Section

Coupled Enzymatic Conversions

Unless otherwise mentioned, standard reaction mixtures contained 5 mM phloretin, 100 mM sucrose, 0.5 mM UDP,

13 mM $MgCl_2$, 50 mM KCl, 0.13% (w/v) BSA, and 20% (v/v) DMSO in 50 mM HEPES buffer pH 7.5. Reactions were started by the addition of the indicated amounts of *Os*CGT and *Gm*SuSy. Enzymatic reactions were performed in 1.5 mL Eppendorf tubes at 30 °C using a thermomixer comfort for temperature control and agitation at 400 rpm. Samples were mixed with an equal volume of acetonitrile to stop the reaction. Precipitated protein was removed by centrifugation (13,200 rpm). The supernatant was analyzed using a reversed phase C-18 HPLC-assay.[18] All compounds of the reaction were analyzed. Reported conversions are confirmed by closed mass balance.

A detailed description of all experimental procedures can be found in the Supporting Information, which comprises: cloning of *Gm*SuSy; expression and purification of *Os*CGT and *Gm*SuSy; enzyme assays; details of the biocatalytic transformations performed; product isolation and identification; and analytical methods used. Any associated references are also given.

Acknowledgements

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Supporting Information

SUPPORTING INFORMATION

Leloir glycosyltransferases and natural product glycosylation: biocatalytic synthesis of the *C*-glucoside nothofagin, a major antioxidant of redbush herbal tea

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Table of contents:

1 Methods

1.1 Chemicals and reagents

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich in the highest purity available. Phloretin (98% purity) was obtained from AK Scientific. Enzymes for DNA manipulation and GeneJET™ Plasmid Miniprep Kit were from Fermentas. *Strep*-Tactin® Sepharose® and desthiobiotin were from IBA. BCA assay kit was purchased from Thermo Scientific.

1.2 Preparation of purified *Os*CGT and *Gm*SuSy

Construction of expression strains

The *Os*CGT gene (GenBank: FM179712) was received as a kind gift from the group of Prof. Robert Edwards (Centre for Bioactive Chemistry, Durham University, UK). It was provided in a pET-STRP3 vector which is a custom made derivative of pET-24d that enables protein expression with N-terminally fused *Strep*-tag II.[1, 2] The codon optimized gene of *Gm*SuSy (GenBank: AF030231) was synthesized with flanking *Nde*I and *Xho*I restriction sites and cloned in the plasmid pUC57 by GenScript. The gene was cut out using *Nde*I and *Xho*I restriction enzymes and inserted into the respective sites of the pET-STRP3 vector for expression as fusion protein with N-terminal *Strep*-Tag II.
Expression strains were created by transformation of electro-competent *E. coli* BL21-Gold (DE3) cells. The correct sequences were verified by sequencing the complete genes.

Protein expression and purification

The described *E. coli* strains were cultivated in 1 L baffled shaking flasks containing 250 mL Luria Bertani (LB) medium with 50 µg mL^{-1} kanamycin on a rotary shaker at 37°C and 120 rpm. Protein expression was induced at an optical density at 600 nm between 0.8 and 1.0 by addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the expression was carried out overnight at 25 °C. The cells were harvested by 30 min centrifugation at 4°C and 5000 rpm. After resuspension in water they were stored at -70°C until disruption by repeated passage through a cooled French press at 100 bar. Cell debris was removed by centrifugation for 45 min at 4°C and 13200 rpm.
The *Strep*-tag II fusion proteins were purified from the cell extract by affinity chromatography on *Strep*-Tactin® Sepharose® columns according to instructions of the manufacturer IBA. The columns had a volume of 3 mL and were operated by gravity flow. Equilibration of the column was done with 3 column volumes (CVs) of washing buffer W (100 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM EDTA). The cell extract was diluted twofold with buffer W and filtrated through a 1.2 µm cellulose-acetate syringe filter before loading on the column. After washing with 5 CVs of buffer W the proteins were eluted with 3 CVs buffer E (100 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin) whereas the first 0.5 CVs were discarded and the rest was pooled. The column was regenerated using 15 CVs of buffer R (100 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM hydroxy-azophenyl-benzoic acid) and equilibrated with 10 CVs of buffer W. Between the purification of different enzymes the columns were washed with 8 M guanidine-HCl to rule out cross-contaminations. Eluted enzymes were

concentrated and buffer exchanged to 50 mM HEPES buffer pH 7.5 using centrifugal concentrators with a Molecular Weight Cut Off of 10 kDa. Enzymes were aliquoted and small aliquots were thawed for experiments as required and did not undergo multiple freeze-thaw cycles.

Protein concentration and purity

Protein concentrations were determined using the BCA method with bovine serum albumin (BSA) as standard. Purities were estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses and Coomassie Blue staining.

1.3 Determination of enzymatic activities and conversions

HPLC-based determination of phloretin and nothofagin (*Os*CGT activity)

A HPLC-assay was used for determination of dihydrochalcone concentrations (phloretin and nothofagin) and it was also applied as main assay for measurement of nothofagin production rates in *Os*CGT conversions. Reactions were performed in 1.5 mL reaction tubes at 30°C in a thermomixer at 400 rpm. Concentrations of UDP-glucose and phloretin as well as the exact buffer conditions are listed at the respective experiment. Reactions were started with the addition of *Os*CGT and stopped by mixing an aliquot of 100 µL with 100 µL acetonitrile. At least four samples (typically every 20 min) were taken during the linear initial rate to determine activities. Precipitated protein was removed by centrifugation for 20 min at room temperature and 13200 rpm. Depending on the concentrations 5 or 10 µL of the supernatant were used for analysis on an Agilent 1200 HPLC equipped with a Chromolith® Performance RP-18e endcapped column (100-4.6 mm) from Merck. The column was thermostatically controlled at 35°C and the separation was monitored by UV detection at 288 nm. Separation of phloretin and its glycosides was achieved by following method using water with 0.1 % TFA (trifluoroacetic acid) as solvent A and acetonitrile with 0.1% TFA as solvent B, respectively. A 7.5 min long linear gradient from 20 to 47.5% B (1 mL min^{-1}) was used for product separation. It was followed by 0.05 min of a linear gradient from 47.5 to 100 % B (1 mL min^{-1}) and 1.45 min of isocratic flow at 100% B (1.5 mL min^{-1}) to wash off hydrophobic compounds. After a 0.05 min linear gradient from 100 to 20 % B (1.5 mL min^{-1}) an isocratic flow of 2.45 min at 20% B (1.5 mL min^{-1}) was applied to equilibrate the column.

One Unit of *Os*CGT used in batch or fed-batch conversions for nothofagin production was defined as the amount of enzyme producing 1 µmol nothofagin per minute under following conditions: 0.6 mM UDP-glucose, 5 mM phloretin, 50 mM HEPES, pH 7.5, 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% (v/v) DMSO.

HPLC-based determination of UDP and UDP-glucose

Determination of UDP and UDP-glucose concentrations was done by HPLC using the sample preparation protocol with acetonitrile addition described for dihydrochalcone measurements. 10 µL sample were applied on an Agilent 1200 HPLC equipped with a Chromolith® Performance RP-18e endcapped column which was thermostatically controlled at 30°C. Separation was monitored by UV detection at 254 nm. Using 20 mM potassium phosphate buffer pH 6.8 with 2 mM tetrabutylammonium hydrogen sulfate as solvent A and acetonitrile as solvent B following gradient was applied with

constant flow rate of 2 mL min^{-1}: 3 min of a linear gradient from 0 to 2% B were followed by a 7 min long linear gradient from 2 to 25% B to separate UDP-glucose from UDP. During 2 min of isocratic flow at 25% B hydrophobic compounds were washed off and after a 1 min long gradient from 25 to 0%, 2 min of isocratic flow at 0% B were applied to equilibrate the column.

Photometric assay for *Os*CGT and *Gm*SuSy sucrose synthesis activity

Activity of *Os*CGT as well as *Gm*SuSy activity in sucrose synthesis direction were also determined with a photometric assay using a modified spectrophotometric method.[3] It is based on coupling of UDP formation through pyruvate kinase (PK) and lactic dehydrogenase (LD) to equimolar NADH (ε = 6220 M^{-1} cm^{-1}) depletion which can be followed photometrically at 340 nm. The assay was performed in a discontinuous way whereas the reaction conditions for *Os*CGT measurements were as described for the HPLC based assay. Concentrations of UDP-glucose and fructose for *Gm*SuSy activity measurements are listed together with the used buffer in the corresponding sections. Samples of 150 µL were stopped by heating to 95°C for 5 min. After removing precipitated protein by centrifugation at room temperature and 13200 rpm for 20 min, 100 µL of the supernatant were mixed in a Half Micro Cuvette with 400 µL of measuring solution. It consisted of 0.42 mM phosphoenolpyruvate and 0.18 mM NADH in a 50 mM HEPES buffer pH 7 containing 13 mM $MnCl_2$, 50 mM KCl and 0.13 % (w/v) BSA. The absorbance at 340 nm was determined using a Beckman Coulter DU 800 UV/VIS spectrophotometer before addition of 0.5 µL of a solution containing the coupling enzymes (PK: 682 U mL^{-1}; LD: 990 U mL^{-1} from rabbit muscle, Sigma-Aldrich). After incubation of the sealed cuvette at 30°C for 45 min the absorbance at 340 nm was measured again and the concentration of UDP was calculated by the difference in absorbance before and after enzyme addition.
One Unit of *Gm*SuSy used in batch or fed-batch conversions for nothofagin production was defined as the amount of enzyme producing 1 µmol NAD^+ per minute under following conditions: 0.2 mM UDP, 300 mM sucrose, 50 mM HEPES, pH 7.5, 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% (v/v) DMSO.

Photometric assay for *Gm*SuSy sucrose cleavage activity

The measurement of linear initial rates for sucrose cleavage by *Gm*SuSy were performed by coupling the production of UDP-glucose to NADH formation using human UDP-α-D-glucose 6-dehydrogenase (hUGDH).[4] The oxidation of one molecule UDP-glucose to UDP-α-D-glucuronic acid is accompanied with the reduction of two NAD^+ molecules to NADH (ε = 6220 $M^{-1}cm^{-1}$) which is monitored photometrically at 340 nm. Concentrations of UDP and sucrose as well as the exact buffer conditions are listed at the respective experiment. The reaction volume was typically 1 mL and conversions were performed in 1.5 mL reaction tubes at 30°C in a thermomixer at 400 rpm. The conversion was started by adding *Gm*SuSy. At least 4 samples (typically every 10 min) of 150 µL were withdrawn during the linear initial rate of the reaction. The reactions were stopped by heating the aliquots to 95°C for 5 min. Precipitated protein was removed by 20 min of centrifugation at room temperature and 13200 rpm. 100 µL of the supernatant were mixed with 400 µL of measuring solution containing 2.5 mM NAD^+, 0.05 % Triton™ X-100 and 100 mM HEPES pH 8.0 in a Half Micro Cuvette. The absorbance at 340

nm was determined using a Beckman Coulter DU 800 UV/VIS spectrophotometer before addition of 1.5 mU of hUGDH. After incubation of the sealed cuvette at 30°C for 45 min the absorbance was measured again and the concentration of UDP-glucose was calculated by the difference in absorbance before and after hUGDH addition.

1.4 Characterization of single enzymes

Determination of pH-profiles

The standard protocol of photometric *Os*CGT activity assay was modified by using a buffer mixture of 12.5 mM HEPES, 12.5 mM Tris and 25 mM CAPS of the respective pH. Reaction buffers were prepared from pH 6.0 to 11.0 in steps of 0.5 pH units. The HEPES concentration of the measuring solution was increased from 50 to 100 mM and it contained 16.25 mM $MgCl_2$ instead of 13 mM $MnCl_2$. The actual pH of the reaction mixture was determined as the average of pH measurements at the beginning and at the end of the observed time span.

Measurements of linear initial rates for sucrose cleavage by *Gm*SuSy were performed at 30°C by using the standard protocol of the photometric *Gm*SuSy activity assay. The reactions mixtures contained 100 mM sucrose, 2 mM UDP and 20 mM of buffer. 20 mM MES (pH 4.5 - 7.0), 20 mM HEPES (pH 7.0 - 8.5) and 20 mM CHES (pH 8.5 - 10.5) buffers were prepared in steps of 0.5 pH units. The actual pH of the reaction mixture was determined as the average of pH measurements at the beginning and at the end of the observed time span.

Linear initial rates for sucrose synthesis by *Gm*SuSy were measured with the standard photometric assay at 30°C using 15 mM fructose and 2 mM UDP-glucose in the buffers described for sucrose cleavage. Average pH during conversion was again calculated as average from samples withdrawn at beginning and end of the reaction.

Determination of equilibrium constants (K_{eq})

Equilibrium constant of *Os*CGT was determined by running standard glycosylation reaction (80 mU mL^{-1} *Os*CGT, 5 mM phloretin, 4.75 mM UDP-glucose) until no further conversion was observed. A standard buffer (50 mM HEPES, pH 7.5, 50 mM KCl, 13 mM $MgCl_2$, 0.13% (w/v) BSA, 20% (v/v) DMSO) was used. From all samples phloretin and nothofagin as well as UDP and UDP-glucose concentrations were determined on HPLC using the appropriate protocols. However, quantification of UDP failed and therefore only nothofagin, phloretin and UDP-glucose concentrations could be used for K_{eq} determination.

To test if there is any reverse activity detectable a conversion of 5 mM phloretin and 5 mM UDP-glucose (100 mU mL^{-1} *Os*CGT) was completed as described and subsequently another 5 mM UDP were added to test for deglycosylation of nothofagin. Therefore samples were taken as described over 12 hours and phloretin and nothofagin concentrations were determined per HPLC. Furthermore nothofagin and UDP were used directly as substrate in a reverse reaction using 200 mU mL^{-1} *Os*CGT (1 mM nothofagin, 1 mM UDP, 50 mM HEPES, pH 7.5, 13 mM $MgCl_2$, 50 mM KCl, 0.13% (w/v) BSA, 5% (v/v) ethanol). Nothofagin was extracted from an almost quantitative conversion of phloretin by

*Os*CGT and contained therefore still small amounts of phloretin. Samples were taken over 23 hours and the analyzed by HPLC for phloretin production.

Sucrose cleavage (5 mM sucrose, 5 mM UDP) and synthesis (5 mM fructose and 5 mM UDP-glucose) were catalyzed by 50 mU mL^{-1} *Gm*SuSy using otherwise the same conditions as in *Os*CGT conversions. UDP-glucose concentrations were measured in all samples per HPLC. Comparable to *Os*CGT experiments UDP could not be quantified and concentrations of UDP, sucrose and fructose had to be calculated from starting concentrations and final UDP-glucose level. For K_{eq} calculations data from sucrose cleavage and synthesis was combined.

Determination of half saturation constants

Half saturation and phloretin inhibition constant of *Os*CGT were determined by measurement of linear initial rates at 30°C in 50 mM HEPES, pH 7.5 containing 50 mM KCl, 13 mM $MgCl_2$, 0.13% (w/v) BSA and 20% (v/v) DMSO using the HPLC-assay. For each substrate 10 different concentrations in a suitable range were used. Phloretin was varied from 0.01 - 10 mM (4 mU mL^{-1} *Os*CGT, 2 mM UDP-glucose) and UDP-glucose was varied from 0.005 - 5 mM (4 mU mL^{-1} *Os*CGT, 1 mM phloretin).

For *Gm*SuSy half saturation constants were determined in sucrose cleavage and synthesis direction. All conversions were made with 2 mU mL^{-1} *Gm*SuSy at 30°C with 20 mM HEPES, pH 7.5 as buffer. Nine different concentrations of each substrate were used and all activities were measured using the appropriate photometric assays. Sucrose was varied from 5 - 300 mM (2 mM UDP), UDP from 0.0025 - 5 mM (400 mM sucrose), fructose from 0.05 - 30 mM (2 mM UDP-glucose) and UDP-glucose from 0.01 - 5 mM (40 mM fructose).

Influence of cosolvents on *Os*CGT and *Gm*SuSy activity

To test the effect of cosolvents on *Os*CGT and *Gm*SuSy (sucrose cleavage) activity initial rate measurements were done at different concentrations of ethanol or DMSO. Reference measurements for *Gm*SuSy (2 mU mL^{-1}, 0.2 mM UDP, 100 mM sucrose, 50 mM HEPES pH 6.5, 10 mM $MgCl_2$) were made without any organic solvent and those for *Os*CGT (0.2 mU mL^{-1}, 0.6 mM UDP-glucose, 0.1 mM phloretin, 50 mM HEPES pH 7, 13 mM $MnCl_2$, 50 mM KCl, 0.13% (w/v) BSA) were done at 5% of the respective solvent. *Gm*SuSy and *Os*CGT activity were measured using described photometric assays.

Determination of enzyme stability

Enzymes were incubated for certain times (0-24 h) in the final reaction mix at 30°C and 400 rpm on a thermomixer but without UDP (*Gm*SuSy) or UDP-glucose (*Os*CGT), respectively. Reactions were started by addition of UDP or UDP-glucose and the loss of activity in dependence of incubation time was used to fit first order inactivation kinetics for calculation of half-lifes. In case of *Os*CGT glycosylation activity was measured by HPLC (15 mU mL^{-1}, 5 mM phloretin, 1 mM UDP-glucose) and sucrose cleavage of *Gm*SuSy was determined photometrically (2 mU mL^{-1}, 100 mM sucrose, 1 mM UDP). The buffer for *Gm*SuSy was 50 mM HEPES, pH 7.5 with 13 mM $MgCl_2$, and 20 % (v/v) DMSO and that for *Os*CGT additionally contained 50 mM KCl and 0.13 % (w/v) BSA.

1.5 *Os*CGT conversions coupled with *Gm*SuSy

Batch conversions of *Os*CGT coupled with *Gm*SuSy

Standard reaction mixtures contained 5 mM phloretin, 100 mM sucrose, 0.5 mM UDP, 13 mM $MgCl_2$, 50 mM KCl, 0.13 % (w/v) BSA, 20 % (v/v) DMSO and 50 mM HEPES buffer pH 7.5. Enzymatic conversions were initiated by the addition of the respective amount of *Os*CGT and *Gm*SuSy. Reactions were performed in 1.5 mL reaction tubes at 30°C in a thermomixer at 400 rpm. Samples were analyzed with the HPLC-based *Os*CGT assay to determine the concentrations of phloretin and nothofagin.
For optimizing the conversion conditions the concentrations of the substrate were varied separately: UDP (0.005 - 1 mM; 10 mU mL^{-1} *Os*CGT, 10 mU mL^{-1} *Gm*SuSy), sucrose (5 - 500 mM; 10 mU mL^{-1} *Os*CGT, 10 mU mL^{-1} *Gm*SuSy) and phloretin (5 - 30 mM; 190 mU mL^{-1} *Os*CGT, 120 mU mL^{-1} *Gm*SuSy). On variation of sucrose and UDP only initial nothofagin production rates were analyzed during the first two hours (3-4 samples) but on variation of phloretin complete conversion curves over 24 hours were recorded.
For comparison of phloretin conversion by *Os*CGT with and without coupling to UDP-glucose recycling with *Gm*SuSy instead of 0.5 mM UDP 6 mM UDP-glucose were used. Both enzymes were applied at 50 mU mL^{-1}.

Fed-batch conversions of *Os*CGT coupled with *Gm*SuSy

Phloretin concentrations of the batch conversion were modified for the fed-batch experiments. The reaction solution contained 300 mM sucrose, 1 mM UDP, 13 mM $MgCl_2$, 50 mM KCl, 0.13 % (w/v) BSA, 20 % (v/v) DMSO in 50 mM HEPES buffer (pH 7.5). Separate reactions with 5 and 10 mM phloretin, respectively were made in a total volume of 2 mL. Reactions were started by enzyme addition (100 mU mL^{-1} *Os*CGT/*Gm*SuSy) and incubated at 30°C and 400 rpm on a thermomixer. Directly after taking samples phloretin and nothofagin concentrations were measured on HPLC and upon depletion of phloretin, new substrate was added from a stock of 500 mM phloretin in DMSO. The increase of substrate concentration was thereby equivalent to the initial concentrations of 5 and 10 mM, respectively. Separate reactions were made with and without feeding of fresh enzyme (50 mU mL^{-1}) at the same time as the substrate feed.

1.6 Product isolation and identification by HPLC and NMR

The product of phloretin conversions by *Os*CGT has previously been identified as nothofagin using ^{1}H and ^{13}C NMR.[5] Therefore 1 mM phloretin was converted to nothofagin (2 mM UDP-glucose in 50 mM HEPES pH 7, 13 mM $MnCl_2$, 50 mM KCl, 0.13% BSA and 5% (v/v) ethanol) and the resulting product was almost quantitatively extracted from the reaction solution by repeated extraction with ethyl acetate. After removing the solvent under reduced pressure the product was dissolved in $MeOD_4$ for NMR analysis. HPLC retention time of the product of coupled conversion of *Os*CGT with *Gm*SuSy was compared with nothofagin for product verification.
Nothofagin was purified by preparative reversed phase C-18 HPLC. Proteins were removed from phloretin conversions by centrifugal concentrators with a Molecular Weight Cut Off of 10 kDa. The HPLC system from Knauer Technologies (smartline pump 1000 V7603, smartline DAD UV detector

2600, manual 6 port injection valve) was equipped with a C18-reversed phase VP 125/21 Nucleodur 100-5 C18ec column. Water containing 0.1% TFA was used as solvent A and methanol as solvent B. Separation of nothofagin from other compounds was achieved by a 20 min long gradient from 20 to 100% B followed by 6 min of isocratic flow at 100% B. Separation was monitored by UV-detection at 288 nm. Fractions containing nothofagin were pooled, methanol was evaporated under reduced pressure and water was removed by freeze drying.

2 Results

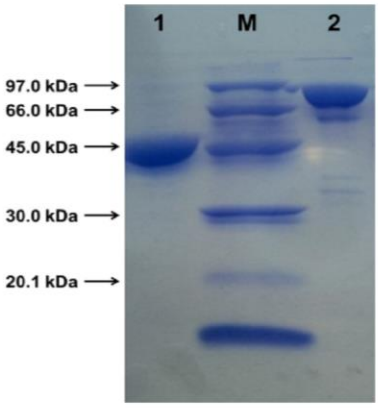

Figure S1: SDS-PAGE of enzymes purified by *Strep-tag* affinity chromatography: Lane 1: *Os*CGT (51.3 kDa); Lane 2: *Gm*SuSy (94.1 kDa); Lane M: Low Molecular Weight standard (GE Healthcare)

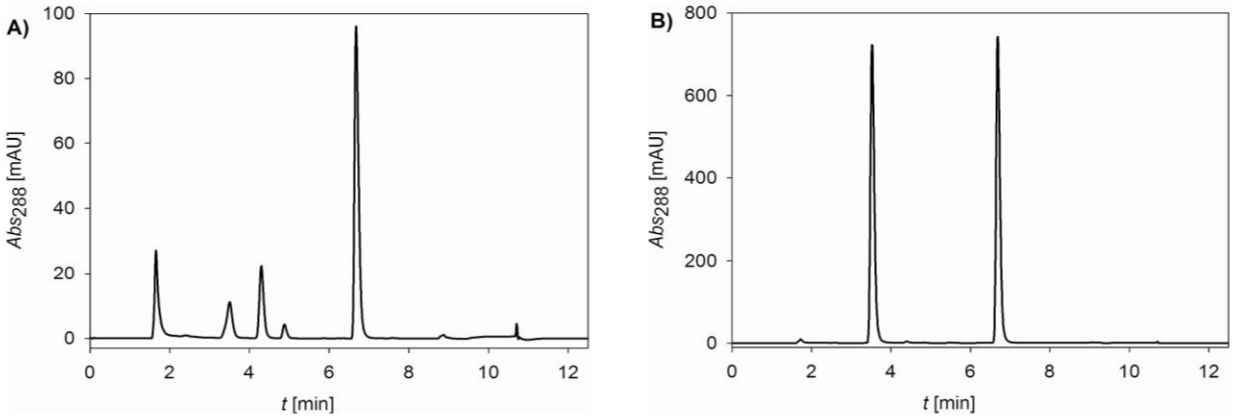

Figure S2: Separation of phloretin from its glycosides using RP-HPLC: A) mixture containing non-binding compounds like UDP(-glucose) (1.7 min), 3'-*C*-glycoside nothofagin (3.5 min), 2'-*O*-glycoside phlorizin (4.3 min), 4'-*O*-glycoside (4.9 min) and phloretin (6.7 min); B) partial phloretin conversion using *Os*CGT and *Gm*SuSy contains only 3'-*C*-glycoside nothofagin (3.5 min), phloretin (6.7 min) and small amounts of UDP(-glucose)

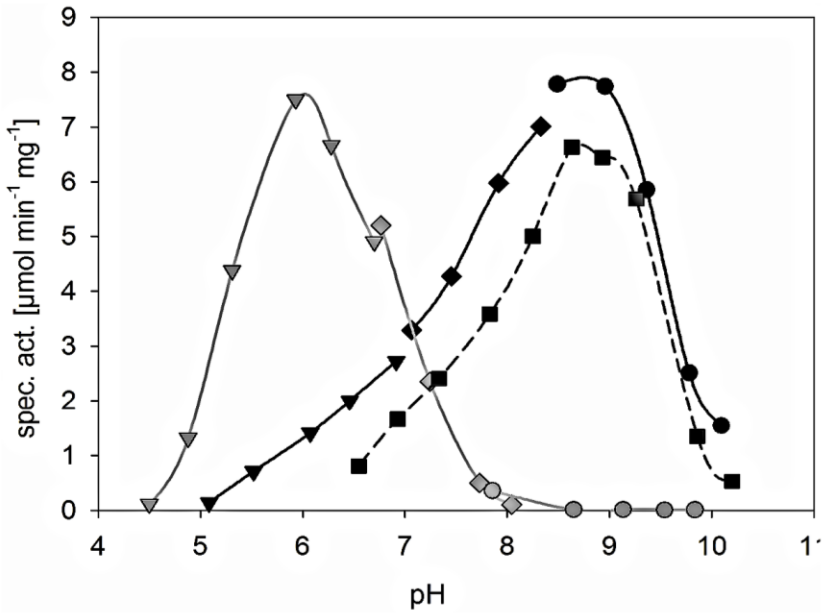

Figure S3. pH profiles for nothofagin synthesis by *Os*CGT (dark grey, dashed line), sucrose cleavage (light grey) and sucrose synthesis (black) by *Gm*SuSy using a mixture of HEPES, Tris and CAPS (square), MES (triangle), HEPES (diamond) or CHES (circle) as buffer.

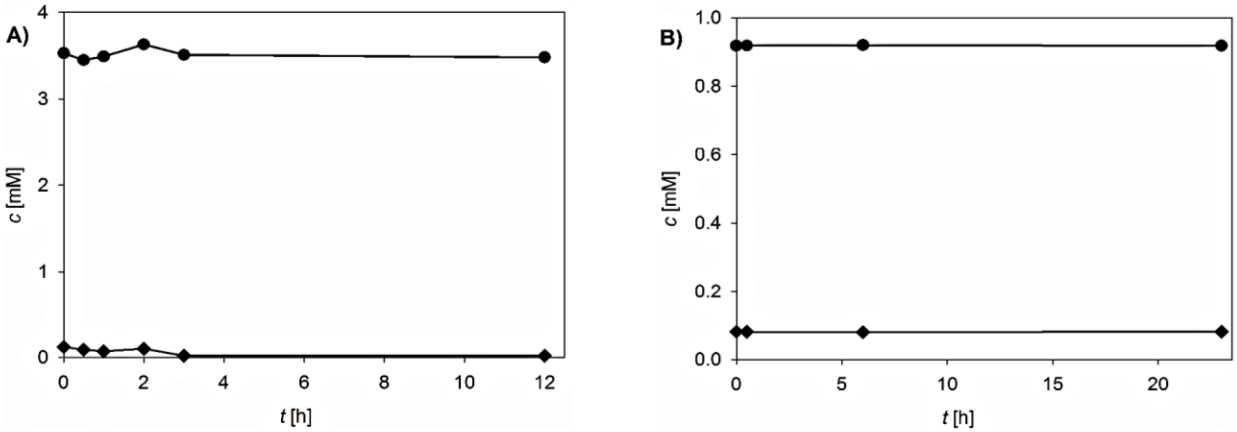

Figure S4: Deglycosylation of nothofagin (black) to phloretin (grey) by *Os*CGT could not be observed: A) 5 mM phloretin and 5 mM UDP-glucose were almost completely converted by 100 mU mL^{-1} *Os*CGT (not shown). When adding further 5 mM UDP (dilution) no deglycosylation of nothofagin was observed; B) Incubation of ~1 mM nothofagin containing some phloretin with 1 mM UDP (200 mU mL^{-1} *Os*CGT) did also not result in any further phloretin production.

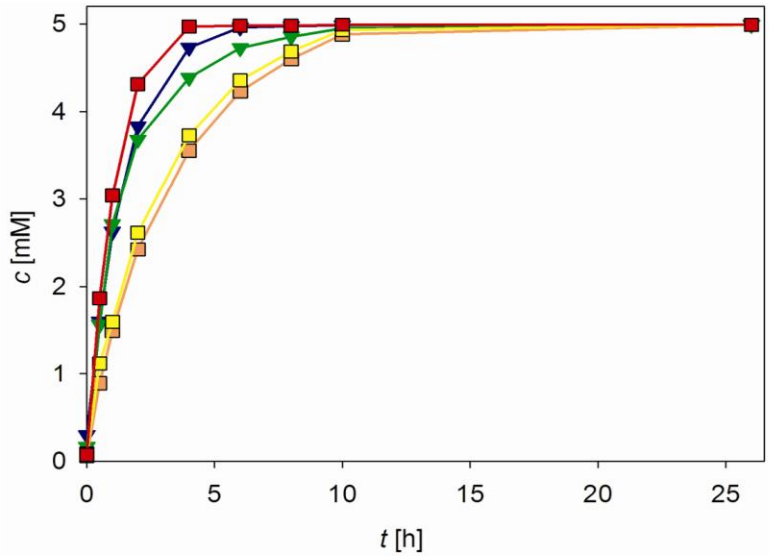

Figure S5. Nothofagin production in *Os*CGT catalyzed conversions of 5 mM phloretin (100 mM sucrose) at various conditions: 50 mU mL^{-1} *Os*CGT, 6 mM UDP-glucose (green); 50 mU mL^{-1} *Os*CGT/*Gm*SuSy, 6 mM UDP-glucose (blue); 50 mU mL^{-1} *Os*CGT/*Gm*SuSy, 0.5 mM UDP (orange); 50 mU mL^{-1} *Os*CGT, 250 mU mL^{-1} *Gm*SuSy, 0.5 mM UDP (yellow); 250 mU mL^{-1} *Os*CGT, 50 mU mL^{-1} *Gm*SuSy, 0.5 mM UDP (red)

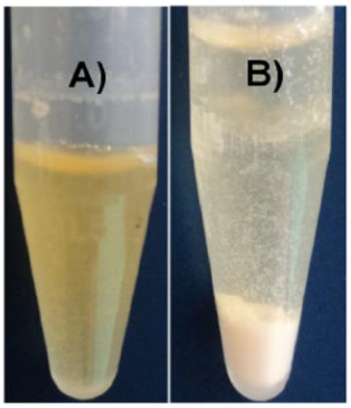

Figure S6: Increased solubility of nothofagin compared to phloretin: A) In a fed-batch conversion of *Os*CGT and *Gm*SuSy with feeding of 5 mM phloretin and enzyme in total 45 mM phloretin were applied and fully converted to nothofagin causing only enzyme to precipitate; B) In an identical solution without enzyme most of the applied 45 mM phloretin was precipitated.

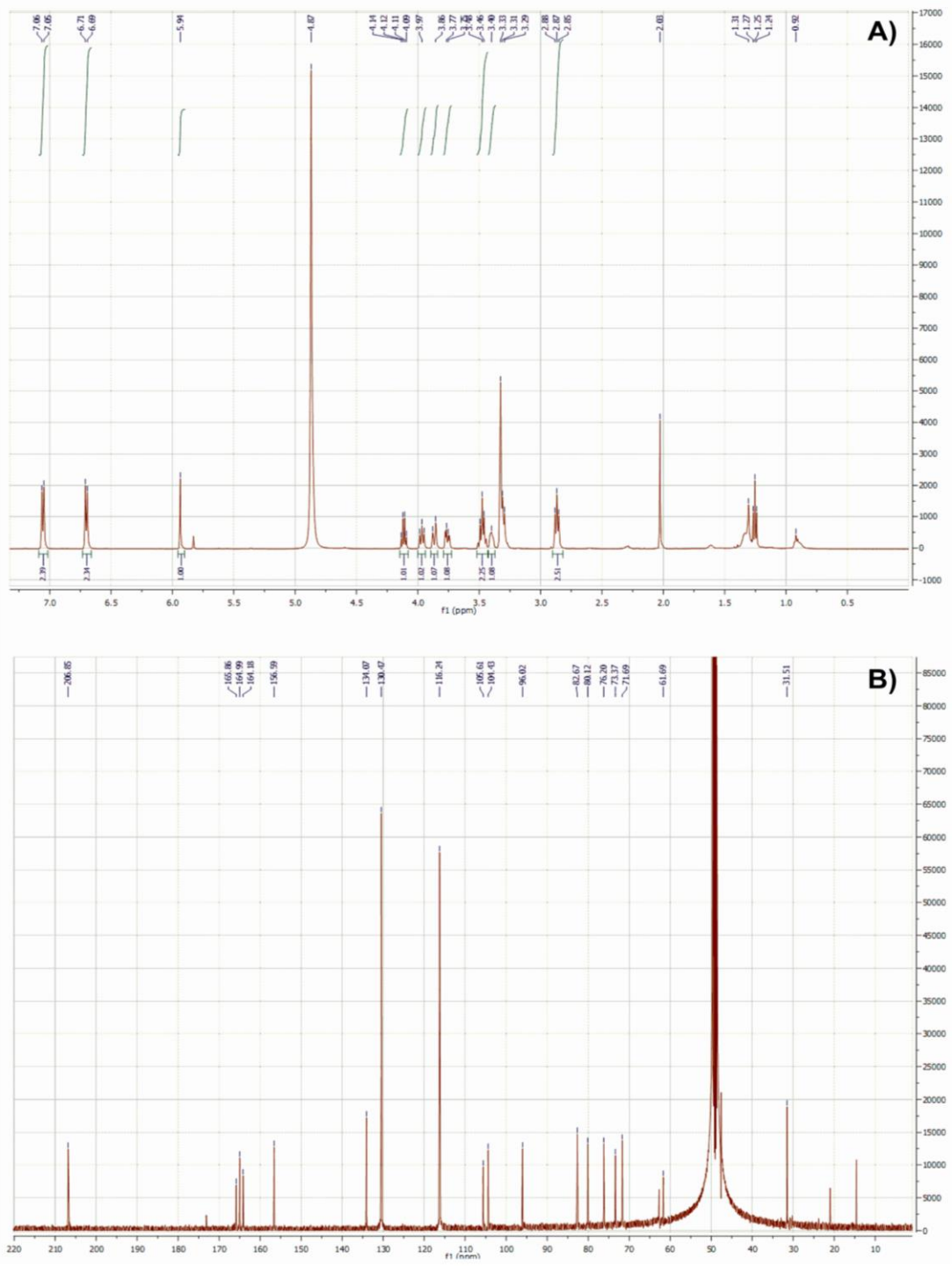

Figure S7: ^{1}H A) and ^{13}C-NMR B) of nothofagin after extraction with ethyl acetate from enzymatic conversion of phloretin by *Os*CGT.

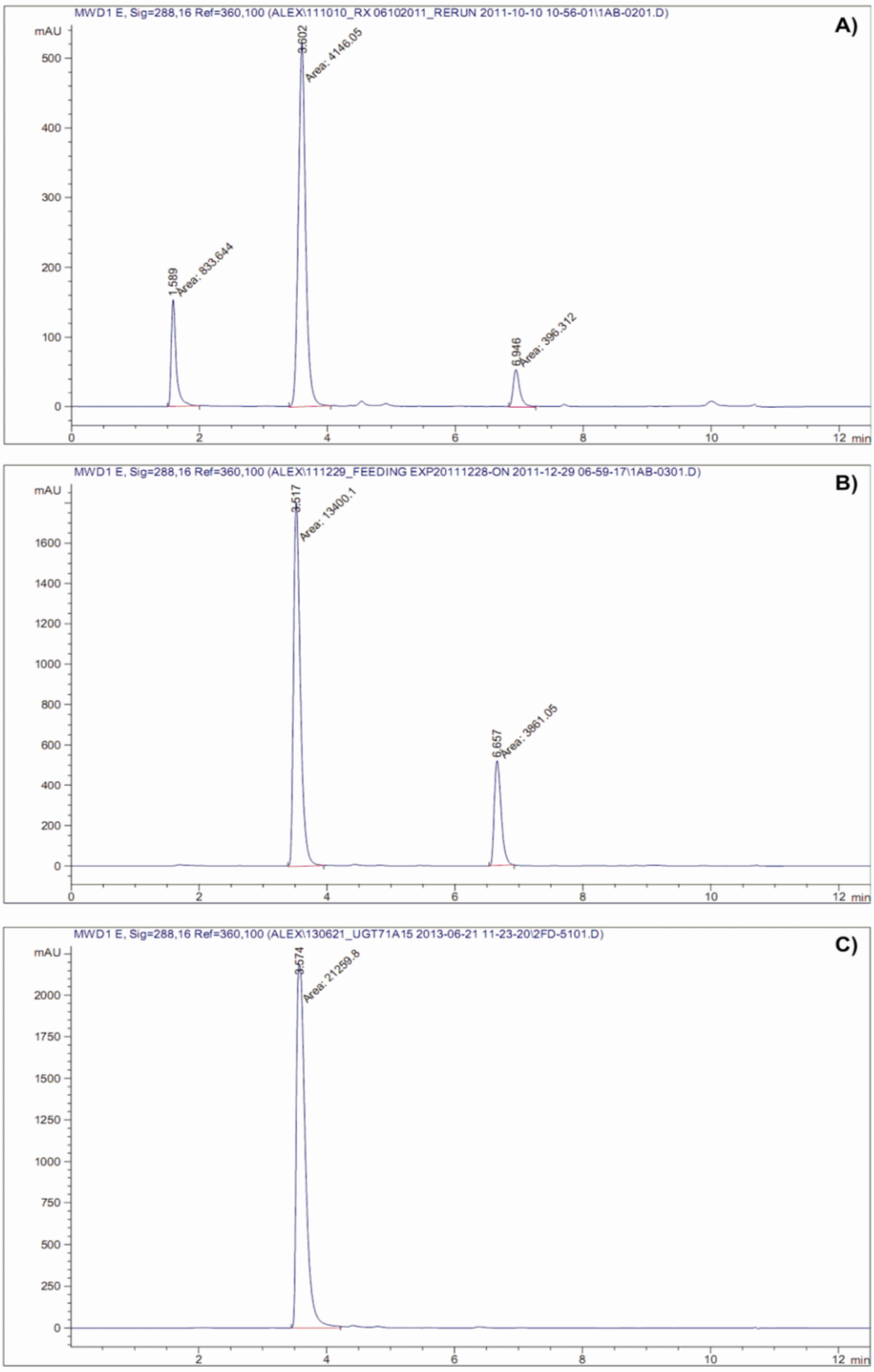

Figure S8: HPLC chromatograms (UV detection at 288 nm) showing nothofagin peak at ~3.5 min either from authentic standard A), after partial phloretin conversion in a coupled reaction of *Os*CGT and *Gm*SuSy B) and after purification by reversed phase HPLC C).

3 References

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A two-step *O*- to *C*-glycosidic bond rearrangement using complementary glycosyltransferase activities

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A two-step *O*- to *C*-glycosidic bond rearrangement using complementary glycosyltransferase activities†

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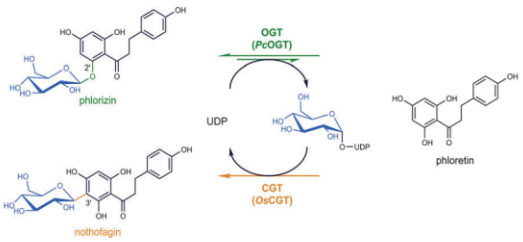
An efficient 2′-*O*- to 3′-*C*-β-D-glucosidic bond rearrangement on the dihydrochalcone phloretin to convert phlorizin into nothofagin was achieved by combining complementary *O*-glycosyltransferase (OGT) and *C*-glycosyltransferase (CGT) activities in a one-pot transformation containing catalytic amounts of uridine 5′-diphosphate (UDP). Two separate enzymes or a single engineered dual-specific O/CGT were applied. Overall (quantitative) conversion occurred in two steps *via* intermediary UDP–glucose and phloretin.



Scheme 1 Conversion of phlorizin into nothofagin using a direct *O*- to *C*-glucoside rearrangement in two catalytic steps.

Leloir glycosyltransferases (GTs) are selective catalysts of synthetically useful glycosylation reactions.[1,2] Naturally they catalyse glycosyl transfer from an activated donor, typically a nucleoside-diphosphate (NDP) sugar, onto metabolic target acceptor(s) (ESI,† Scheme S1a).[3] Recognising the synthetic scope of glycosyltransferase reactions run backwards, researchers introduced two-step exchange reactions to the field (ESI,† Scheme S1b).[4–6] Complementary glycosyltransferase activities are combined in a one-pot conversion where NDP-sugar or an acceptor substrate for the actual synthetic transformation is generated *in situ* from a reactant glycoside and NDP *via* a reverse glycosyltransferase reaction. The overall catalytic conversion is steered to achieve swapping of glycosyl residues between different acceptor substrates (aglycon exchange),[4,6–10] or to result in an alternative glycosylation of a single acceptor compound (sugar exchange).[4,7,11] Exchange processes were exploited in different glycosylations of small molecules and enabled glycoengineering of natural products *in vitro*.[12]

In this communication, we have discovered that phenolic *O*- to aromatic *C*-glycosidic bond rearrangement (Scheme 1; ESI,† Scheme S1c) is achievable by coupled glycosyltransferase reactions. The overall conversion is formally equivalent to a chemical Fries-type rearrangement;[13] however, the mechanisms are distinct. Glycosyltransferase conversions involve intermediary release of aglycon and NDP-sugar, followed by regio- and stereoselective *C*-glycosylation.

A proof of principle was obtained for conversion of phlorizin, the 2′-*O*-glucoside of the dihydrochalcone phloretin, into the corresponding 3′-*C*-glucoside nothofagin (Scheme 1). The catalytic process involved *O*-glucosyltransferase (OGT) and *C*-glucosyltransferase (CGT) activities, derived from a specificity matched pair of enzymes from pear (*Pyrus communis*; *Pc*OGT)[14] and rice (*Oyrza sativa*; *Os*CGT),[15] or from an engineered "promiscuous" *O*/*C*-glucosyltransferase (O_CGT). Only catalytic amounts of uridine 5′-diphosphate (UDP) were required in the conversion to generate intermediary UDP–glucose and phloretin *via* reverse reaction of the OGT. The overall two-step rearrangement proceeded under thermodynamic control whereby a large driving force for the second step, aromatic *C*-glycosylation, enabled nothofagin formation in quantitative yields. The phlorizin–nothofagin pair stands for a number of homologous *O*- and *C*-glycosidic natural products, in that the *O*-glycoside occurs naturally in relatively high abundance over the quite rare *C*-glycoside.[16,17] Fruit trees (*Rosaceae*) contain large amounts of phlorizin in their bark[18] whereas significant quantities of nothofagin were reported only from redbush herbal tea.[19] Direct *O*- to *C*-glycosidic bond rearrangement makes effective use of both the glycon and the aglycon moiety of the substrate. In principle, therefore, it presents an expedient and particularly atom-efficient transformation of *O*-glycosides

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† Electronic supplementary information (ESI) available: Experimental procedures used; the scheme of reverse glycosyltransferase reactions; enzyme purity; deglycosylation of phlorizin by *Pc*OGT; the effect of cosolvents and substrate concentrations on rearrangement; HPLC trace of phloretin (glycosides); potentiometric titrations; optimisation of conversion by *Os*CGT_I121D. See DOI: 10.1039/c4cc00536h

(*e.g.* flavonoid glycosides) readily available from the natural products pool. Due to their high resistance to hydrolysis, the resulting *C*-glycosides are of special interest for development of bioactive substances with enhanced *in vivo* half-lives.[16,20]

Purified preparations of *Pc*OGT and *Os*CGT were obtained from *E. coli* overexpression cultures using His-tag and Strep-tag affinity chromatography, respectively. The Ile^{121} → Asp variant (I121D) of *Os*CGT, previously shown to exhibit dual specific O_CGT activity,[21] was produced and isolated in the same way as the wild-type enzyme (ESI,† Fig. S1). All reactions were carried out with 20% (by volume) DMSO to enhance phloretin solubility. We confirmed that the yield and the selectivity of the bi-enzymatic rearrangement were not affected by DMSO (ESI,† Fig. S3). Enzymes were fully active and exhibited suitable stability under these conditions. Phloretin, phlorizin, nothofagin, and other phloretin glucosides were identified and quantified by HPLC (ESI,† Fig. S4b).[21] UDP–glucose and UDP were measured by capillary zone electrophoresis. OGT and CGT activities were determined using HPLC-based assays.

We noticed that rearrangement of phlorizin to nothofagin could become effective only when main requirements concerning enzyme specificity, reaction kinetics, and reaction thermodynamics in each step were adequately met. *Pc*OGT and *Os*CGT were known to glucosylate phloretin at position 2′-*O*[14,21] and 3′-*C*,[21,22] respectively. Interference from secondary *O*-glucoside hydrolase activity in the enzymes used could also be discounted based on earlier evidence.[21] Moreover, *Os*CGT alone was inactive as a "rearrangement enzyme" when phlorizin and UDP were offered as substrates. *C*-Glucosylation of phloretin from UDP–glucose was known to be largely irreversible (K_{eq} > 400; 30 °C, pH 7.5).[23] However, kinetic and thermodynamic characteristics of the *Pc*OGT reaction required clarification. Fig. 1a compares the pH-rate profile for deglucosylation of phlorizin to UDP to the pH-rate profile for glucosylation of phloretin from UDP–glucose. *Pc*OGT was more active in the direction of phlorizin synthesis (18.3 U mg^{-1}) than degradation (5.6 U mg^{-1}) at the respective optimum pH. Interestingly, despite the rather uniform pH effects on enzyme activity in each reaction direction (except for the slight pH range shift), we realised that the reaction equilibrium constant (K_{eq} = [phlorizin][UDP]/[UDP–glucose][phloretin]) increased dramatically in response to pH change from 6.5 to 8.8 (Fig. 1b). Attainment of reaction equilibrium was affirmed rigorously under all conditions, ruling out interference from enzyme activity loss at high or low pH. It was also confirmed that the pH was stable during the conversions. Correlation between log K_{eq} and pH was linear with a large slope of +1.6 (±0.1), implying that conversion of phlorizin and UDP involves the uptake of proton(s). Potentiometric titration of each compound present in the reaction (ESI,† Fig. S5) revealed the likely importance of protonation of UDP (pK_a ~ 5.6). The immediate ramification of results in Fig. 1b is that exploitation of the *Pc*OGT reverse reaction for supplying substrates in adequate steady-state concentrations to the *Os*CGT reaction will only be practical at pH 7.5 or lower. Half-saturation constants of *Os*CGT were determined to be 0.009 mM for phloretin and 0.024 mM for UDP–glucose,[23] defining lower limits to the respective substrate concentration for effective utilization of the *C*-glucosylation activity present.

Rearrangement of phlorizin (5 mM) into nothofagin was examined in a one-pot reaction that contained UDP (2 mM), *Pc*OGT, and *Os*CGT. Because the optimum pH for *C*-glycosylation (pH 8.5)[23] did not match pH conditions applicable to phlorizin conversion by *Pc*OGT, we tested the coupled enzyme reaction at different pH values in the range 5.9–7.8. Nothofagin was produced under all conditions used, demonstrating the system's functionality in principle. Fig. 2a compares phlorizin consumption to the corresponding formation of nothofagin and phloretin after 5 h of reaction. Interestingly, whereas the actual phlorizin conversion was not strongly affected by pH change in the applied range, the resulting product distribution, nothofagin compared to phloretin, showed pronounced pH dependence. Accumulation of phloretin at low pH indicated critical limitations due to insufficient *Os*CGT activity under these conditions. However, *C*-glucosylation was quite effective in the pH range 6.7–7.8 where only small amounts of phloretin were detected next to the main product nothofagin (2.5 mM; 50% substrate conversion).

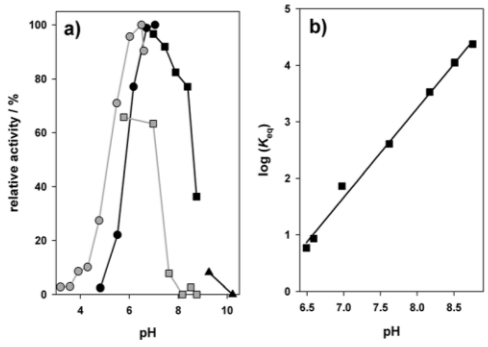

Fig. 1 pH effects on *Pc*OGT activity for phlorizin synthesis and degradation (a), and on the reaction equilibrium constant (b). (a) Relative activities of *Pc*OGT; black symbols (forward reaction: 0.1 mM phloretin, 0.6 mM UDP–glucose, 100% = 18.3 U mg^{-1}); grey symbols (reverse reaction: 1.0 mM phlorizin, 2.0 mM UDP, 100% = 5.6 U mg^{-1}). The buffers used were citrate (circles), tris (squares), and CAPS (triangles). (b) K_{eq} is for synthesis of phlorizin and UDP from phloretin and UDP–glucose.

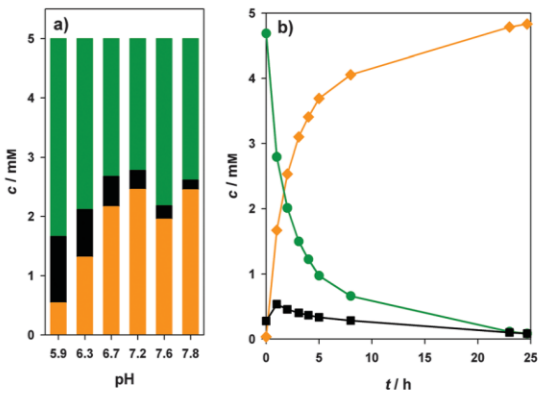

Fig. 2 Conversion of phlorizin (5 mM) *via* *O*- to *C*-glycosidic bond rearrangement in the presence of 2 mM UDP using 100 mU mL^{-1} *Pc*OGT and 50 mU mL^{-1} *Os*CGT. (a) Product distribution after reaction for 5 h at different pH conditions. (b) Reaction time course at pH 7.0. Colours show phlorizin (green), nothofagin (orange), and phloretin (black).

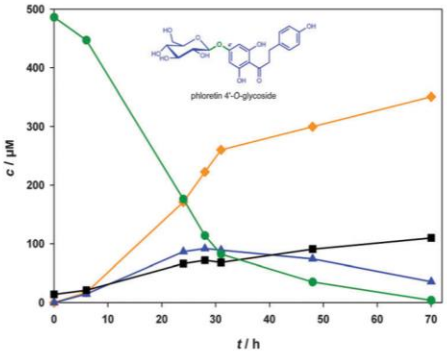

Fig. 3 Conversion of 0.5 mM phlorizin (green) by I121D mutant of *Os*CGT (2.5 mU mL^{-1}) in the presence of 5 mM UDP and 1 mM UDP–glucose. Further symbols: phloretin (black), nothofagin (orange), 4′-*O*-glucoside of phloretin (blue).

Fig. 2b shows a full time course for enzymatic reaction at pH 7.0, demonstrating that quantitative conversion of phlorizin into nothofagin was made possible under these conditions. Isolation of highly pure nothofagin is typically achieved by reversed phase C-18 HPLC in yields of more than 80%.[23] The phloretin concentration attained maximum (~0.5 mM) early during the reaction, only to drop to a very low level later on. This time dependence was fully consistent with the proposed role of phloretin as an intermediary product in an overall two-step rearrangement process. Evidence that immediately after the reaction started, nothofagin formation was clearly lagging behind the formation of phloretin (see the phloretin "burst" of ~0.3 mM at $t = 0$ h in Fig. 2b) gave additional indication of the reaction in two discrete biocatalytic steps.

Kinetic analysis (ESI,† Fig. S6) showed that the phlorizin consumption rate (r_P) was hyperbolically dependent on UDP and phlorizin concentration, resulting in apparent half-saturation constants of 1.22 ± 0.16 mM (phlorizin) and 0.074 ± 0.010 mM (UDP).

The overall rearrangement rate (r_N; nothofagin formation) paralleled r_P under all conditions, except at high phlorizin concentration (≥3 mM) where r_N dropped off strongly in relation to r_P. The behaviour of r_N is explained by substrate inhibition of *Os*CGT at high phloretin concentration.[23] However, disparity between r_P and r_N did not diminish the nothofagin yield when 10 mM phlorizin was applied (ESI,† Fig. S4a). High rearrangement rates could be maintained with as little as 0.25 mM UDP (ESI,† Fig. S6b) confirming the requirement for only catalytic amounts of UDP in the overall conversion.

We recognised the interesting possibility of performing the two-step rearrangement using only a single enzyme that exhibits OGT as well as CGT activity. The I121D mutant of *Os*CGT which catalyses glucosyl transfer to O2 and O4, next to C3 of the phloretin acceptor, was considered useful.[21] Kinetic properties of the mutant required adaptation of reaction conditions for synthesis of nothofagin from phlorizin (ESI,† Methods, Fig. S7–S9). In particular, the UDP concentration was increased to 5 mM to promote an otherwise inefficient reverse reaction from phlorizin substrate, which was applied at a lowered concentration of 0.5 mM. Additionally, UDP–glucose was supplied at 1.0 mM to drive the *C*-glucosylation.

Fig. 3 displays the time course of reaction catalysed by I121D mutant under these conditions. Turnover frequency of the mutant (~0.001 s^{-1})[21] restricted the amount of enzyme activity usable in the reaction, resulting in relatively slow conversion. However, all of the initial phlorizin was converted, and nothofagin was obtained in 70% yield. Unlike in the reaction catalysed by coupled OGT and CGT (Fig. 2b), the phloretin concentration increased steadily during the mutant-catalysed reaction, approaching a maximum value of ~0.1 mM at the time when phlorizin was depleted (Fig. 3). Therefore, this indicates that *C*-glucosylation was rate limiting for the overall rearrangement catalysed by the mutant. Interestingly, small amounts of phloretin 4′-*O*-glucoside were also produced and used up later in the conversion (Fig. 3). Therefore, this implied O2 to O4 positional rearrangement in phloretin *O*-glucosides catalysed by the mutant.

Summarising, the biocatalytic rearrangement discovered in this study is a remarkable chemical transformation that might open up new opportunities for synthesis of aromatic *C*-glycosidic natural products or natural product-like structures from (readily available) phenolic *O*-glycosidic substrates. Unlike glycosyltransferase exchange reactions in which overcoming thermodynamic restrictions presents the main issue,[4,8,24] the example of phlorizin conversion into nothofagin shows that (quantitative) *O*- to *C*-glycosidic bond rearrangement is promoted by a large driving force on the *C*-glycosylation. While the study provides a clear proof of principle, expansion of the synthetic scope of the biocatalytic rearrangement will be important. Development of new pairs of complementary enzymes through discovery work and protein engineering is required whereby identification of new CGT enzymes will be the key.

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Notes and references

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Two-step *O*- to *C*-glycosidic bond rearrangement using complementary glycosyltransferase activities

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Electronic Supplementary Information

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1 Methods

1.1 Chemicals and reagents

Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich (Vienna, Austria) in the highest purity available. Phlorizin dihydrate (≥98%) was purchased from Carl Roth (Karlsruhe, Germany). DNA modifying enzymes were from Thermo Scientific (Waltham, MA, US) and PCR primers were obtained from Life Technologies (Carlsbad, CA, US). Phusion® High-Fidelity DNA Polymerase was purchased from New England Biolabs (Ipswich, MA, US). *Strep*-Tactin® Sepharose® and desthiobiotin were obtained from IBA (Goettingen, Germany).

1.2 Strain construction

The *Os*CGT gene (GenBank: FM179712) was kindly provided from the group of Prof. Robert Edwards (Centre for Bioactive Chemistry, Durham University, UK) in a pET-STRP3 vector which is a custom made derivative of pET-24d that enables protein expression with an N-terminally fused *Strep*-tag II.[1-2] Introduction of the mutation I121D was described elsewhere.[3] The *Pc*OGT gene (UGT88F2; GenBank: FJ854496) was a kind gift from the group of Prof. Karl Stich (Institute of Chemical Engineering, Vienna University of Technology, Austria).[4] Flanking restriction sites for *Nde*I and *Xho*I were added and the respective internal restriction sites were removed by overlap extension PCR as described previously.[3] Subsequently the gene was inserted into *Nde*I and *Xho*I sites of a pET-28a vector for expression with N-terminally fused His-tag. Expression strains were created by transformation of electro-competent *E. coli* BL21-Gold (DE3) cells with the described plasmids.
Correct sequences of the complete genes and the regions around both cloning sites of the pET-Strep3 plasmid were verified by sequencing. Vector maps and complete DNA sequences of *Os*CGT and *Pc*OGT as well as the pET-Strep3 plasmid can be found in the Appendix.

1.3 Enzyme expression and purification

Cultivation of *E. coli* cells for protein expression was described elsewhere in detail.[3] Cells were grown at 37°C and 120 rpm in 1 L baffled shake flasks containing 300 mL LB-media (50 µg mL^{-1} kanamycin). At an optical density at 600 nm of 0.8-1.0, protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After overnight expression at 25°C cells were harvested by 30 min centrifugation at 5,000 rpm and 4°C, resuspended in water and stored at -70°C until disruption by repeated passage through a cooled French press at 100 bar. Before purification cell debris was removed by centrifugation (45 min, 13,200 rpm, 4°C) and cell extract was filtrated through a 1.2 µm cellulose-acetate filter.
*Os*CGT and *Os*CGT_I121D were purified by *Strep*-tag affinity chromatography on 3 mL gravity flow *Strep*-Tactin® Sepharose® columns at 4°C as previously described.[3] After loading cell extract the column was washed with 5 column volumes (CV) of washing buffer (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA). Enzymes were eluted with 3 CVs of elution buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin) whereas the first 0.5 CVs were discarded, and the rest was pooled.
*Pc*OGT was purified at 4°C on a 5 mL HiTrap™ Chelating FF column (GE Healthcare) loaded with Ni^{2+}. A flow rate of around 5 $mLmin^{-1}$ was applied manually using a syringe. Cell extract was loaded on the column after equilibration with 5 CVs of buffer W (20 mM Tris pH 7.4, 500 mM NaCl, 20 mM imidazole). After washing with further 5 CVs of buffer W *Pc*OGT was eluted in two steps using 4 CVs buffer E1 (20 mM Tris pH 7.4, 500 mM NaCl, 250 mM imidazole) and 5 CVs of buffer E2 (20 mM Tris pH 7.4, 500 mM NaCl, 500 mM imidazole), respectively.
Fractions containing purified proteins were concentrated and buffer was exchanged to 25 mM HEPES, pH 7.0, using centrifugal concentrators with a Molecular Weight Cut Off of 10,000. Purified enzymes were stored as small aliquots at -20°C to avoid multiple freeze-thaw cycles. Protein concentrations of purified enzymes were determined photometrically on a NanoDrop 2000 system (Thermo Scientific). Molecular weight and molar extinction coefficients were calculated using Peptide Properties Calculator. Enzyme purities were estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Coomassie Blue staining.

1.4 Quantification of dihydrochalcones – HPLC based activity assay

Quantification of phloretin and its glycosides was achieved by an HPLC based assay as described elsewhere in detail.[3] It was applied to follow all enzymatic conversions and to determine activities of *Pc*OGT and *Os*CGT (I121D). Briefly, reaction mixtures contained if not mentioned otherwise 0.6 mM UDP-glucose, 1 mM phloretin, 25 mM Tris pH 7, 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% DMSO. Conversions were started by glycosyltransferase addition. After certain incubation times at 30°C samples were withdrawn and enzymatic

conversions were stopped by mixing with an equal volume of acetonitrile. Precipitated protein was removed by centrifugation for 15 min at 13,200 rpm. Typically 10 µL of supernatant were applied on an Agilent 1200 HPLC system equipped with a Chromolith® Performance RP-18e endcapped column (100–4.6 mm) from Merck. Separation of phloretin and its glycosides was monitored by UV detection at 288 nm under thermostatic control at 35°C by following method. Solvent A: water with 0.1% TFA; Solvent B: acetonitrile with 0.1% TFA; Gradient: 7.5 min: 20-47.5% B (1 mL min^{-1}), 0.05 min: 47.5-100% B (1 mL min^{-1}), 1.45 min: 100% B (1.5 mL min^{-1}), 0.05 min: 100-20% B (1.5 mL min^{-1}), 2.45 min: 20% B (1.5 mL min^{-1})
One unit of *Os*CGT (I121D) or *Pc*OGT activity was defined as the amount of enzyme glucosylating 1 µmol phloretin per minute under the following conditions: 0.6 mM UDP-glucose, 1 mM (*Os*CGT/*Pc*OGT) or 0.1 (OsCGT_I121D) phloretin, 25 mM Tris (*Os*CGT, *Pc*OGT) or BisTris (OsCGT_I121D), pH 7, 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% DMSO at 30°C. At least 4 distinct measurements were used to calculate linear initial rates.

1.5 Quantification of UDP and UDP-glucose - capillary zone electrophoresis

Concentrations of UDP and UDP-glucose were determined by capillary zone electrophoresis. Samples were prepared as for HPLC measurements by mixing with an equal volume of acetonitrile to stop enzymatic conversion. Precipitated protein was removed by centrifugation (15 min; 13,200 rpm). Capillary zone electrophoresis was performed using a 3D capillary electrophoresis system (Hewlett Packard) equipped with a fused-silica capillary (56 cm × 50 µm) with an extended light path. The capillary was preconditioned by a 6 min flush with background electrolyte (20 mM sodium borate, pH 9.3). Samples were loaded by pressure injection (50 mbar, 10 s) and compounds were resolved at 22 kV over 22 min at 18°C. UDP and UDP-glucose were monitored on a diode array detector at 262 nm.

1.6 Characterization of *Pc*OGT reaction reversibility

To test if phloretin formation from phlorizin is a result of reverse glycosylation by *Pc*OGT, 1 mM phlorizin was incubated at 30°C in 50 mM BisTris, pH 7.0, 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% DMSO. Two mM UDP, 25 mU mL^{-1} *Pc*OGT, or both were added. Conversion of phlorizin was followed by HPLC.
To obtain pH-profiles of *Pc*OGT in glycosylation and deglycosylation direction, the standard protocol of HPLC based activity assay was modified in following manner. A set of reaction buffers was prepared from pH 3 to 10 in steps of 0.5 (25 mM citrate pH 3-7; 25 mM Tris pH 7-9.5, 25 mM CAPS pH 9.5-10). All reactions contained 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% DMSO. In glycosylation direction 0.1 mM phloretin and 0.6 mM UDP-glucose were used as substrates and reactions were started by addition of following amounts of *Pc*OGT: 60 ng mL^{-1} (pH 5-5.5); 30 ng mL^{-1} (pH 6-6.5, 9.5-10); 15 ng mL^{-1} (pH 7.0-9.0); Deglycosylation reactions (pH 3-9.5) contained 1 mM phlorizin and 2 mM UDP and were started by addition of 60 ng mL^{-1} *Pc*OGT. All reactions were incubated at 30°C and samples were withdrawn every 20 min for 1h. The actual pH of reaction mixtures was determined as the average of pH measurements at the beginning and at the end of the observed time span.
To reach equilibrium, another 8.3 µg mL^{-1} *Pc*OGT were added to reactions in deglycosylation direction after samples for initial rate measurements were withdrawn. After overnight incubation at 30°C phloretin and phlorizin concentrations were experimentally determined by HPLC. The UDP-glucose concentration was inferred from reaction stoichiometry. Equilibrium constants were calculated in glycosylation direction according to equation 1.

$$K_{eq} = \frac{c_{phlorizin} \cdot c_{UDP}}{c_{phloretin} \cdot c_{UDP-glucose}} \quad (1)$$

1.7 Potentiometric titration of phlorizin, phloretin, UDP and UDP-glucose

pK_a-values of all compounds involved in phlorizin conversion by *Pc*OGT (phlorizin, phloretin, UDP, UDP-glucose) were determined by potentiometric titration. Of all analytes, solutions of 7.5 mM were made in decarbonated water containing 20% DMSO, 13 mM $MgCl_2$ and 50 mM KCl to reproduce rearrangement conditions. Analyte volumes of 10 (UDP, UDP-glucose) or 20 mL (phlorizin, phloretin) were titrated at 30°C with 150 mM NaOH as titrant using a SenTix® Mic electrode from WTW (Weilheim, Germany) for pH monitoring.

1.8 *O*- to *C*-glucoside rearrangement by *Os*CGT and *Pc*OGT

Unless mentioned otherwise reaction mixtures contained 2 mM UDP and 5 mM phlorizin in 50 mM Tris pH 7 containing 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% DMSO. Conversions were started by addition of 100 mU mL^{-1} *Pc*OGT and 50 mU mL^{-1} *Os*CGT. All reactions were incubated at 30°C. Aliquots were withdrawn, stopped by acetonitrile addition and analysed by RP-HPLC as described.
To test the influence of cosolvents on *O*- to *C*-glucoside rearrangement conversions were carried out in presence of different concentrations (5, 10, 15 or 20%) of either ethanol or DMSO.
The effect of pH on the rearrangement was studied by replacing the standard buffer with 50 mM HEPES buffers ranging from pH 6 to 8.5 (in steps of 0.5). The actual pH of reaction mixtures was determined as the average of pH measurements at beginning and end of 5 h long conversions at 30°C.
The impact of substrate concentrations on *O*- to *C*-glycosidic bond rearrangement was studied by varying phlorizin (0.05 - 10 mM; 2 mM UDP) and UDP (0.005 - 1.2 mM; 5 mM phlorizin; 5% DMSO). Linear initial phlorizin consumption and nothofagin production rates were determined during initial 25 (phlorizin variation) or 60 min (UDP variation).

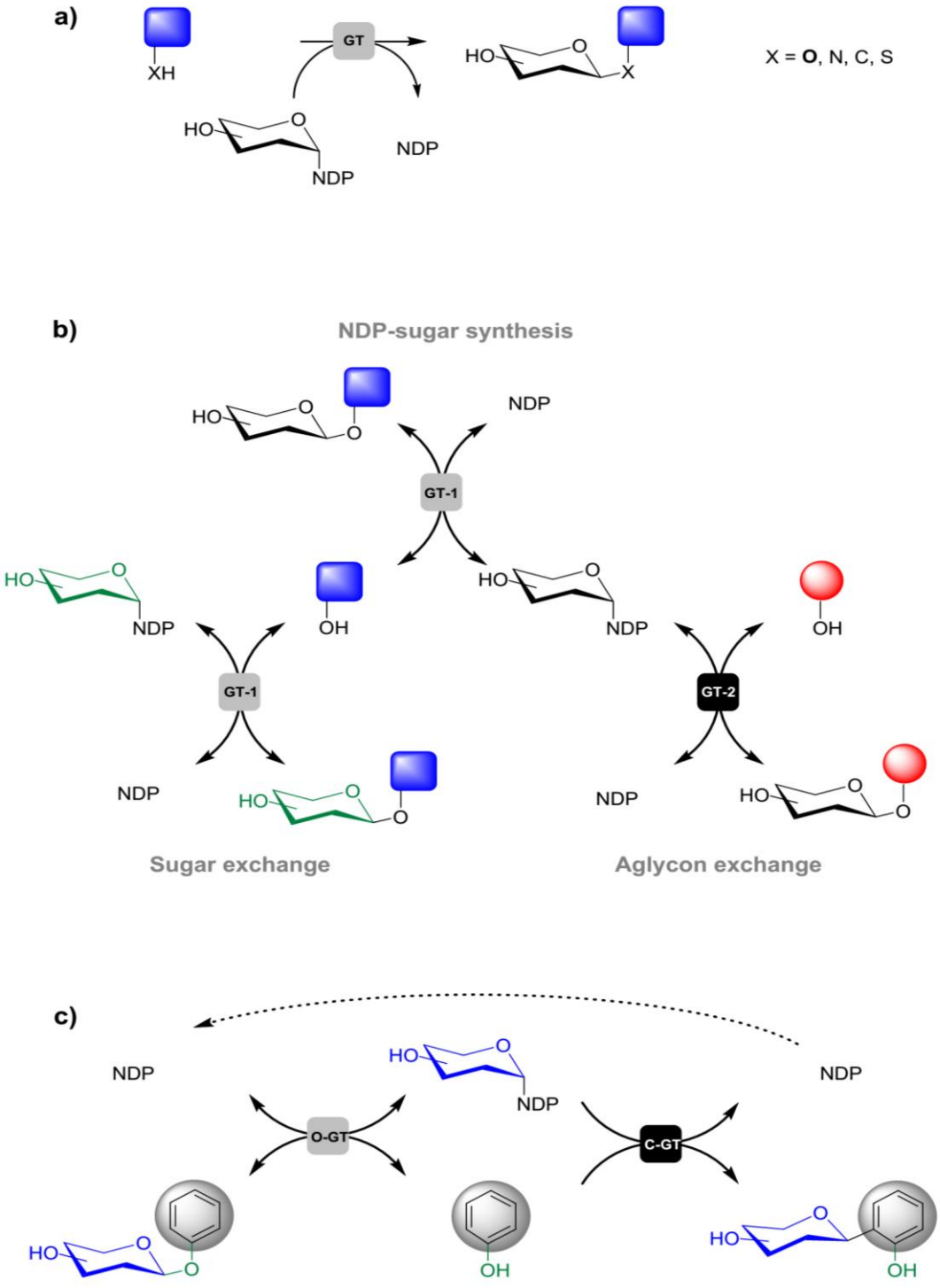
1.9 *O*- to *C*-glucoside rearrangement by *Os*CGT_I121D

Unless mentioned otherwise all conversions were made with 1.25 mU mL^{-1} *Os*CGT_I121D in 50 mM BisTris buffer, pH 7.0, containing 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% DMSO. During 24-70 h long time courses at least 7 samples were analysed by RP-HPLC as described previously.

For optimisation of substrate concentrations, 0.05, 0.5 and 5 mM phlorizin were tested in combination with 0.5 and 5 mM UDP. Evaluation of pH dependency was done using 50 mM BisTris buffers of pH 6.0, 7.0 and 8.0 for conversion of 0.5 mM phlorizin with 5 mM UDP. The effect of UDP-glucose addition (0, 0.5, 1, 5 mM) was also investigated using 0.5 mM phlorizin and 5 mM UDP. Conversion of 0.5 mM phlorizin under optimized conditions was performed with 2.5 mU mL^{-1} *Os*CGT_I121D in presence of 5 mM UDP and 1 mM UDP-glucose.

2. Results

2.1 Synthetic use of reverse glycosyltransferase reactions



Scheme S1 Natural product glycosylations through a canonical glycosyltransferase reaction (a), two-step exchange processes exploiting a suitable glycosyltransferase reaction in reverse direction (b), and *O*- to *C*-glycosidic bond rearrangement catalysed by complementary glycosyltransferase activities (c)

2.2 Determination of enzyme purity by SDS-PAGE

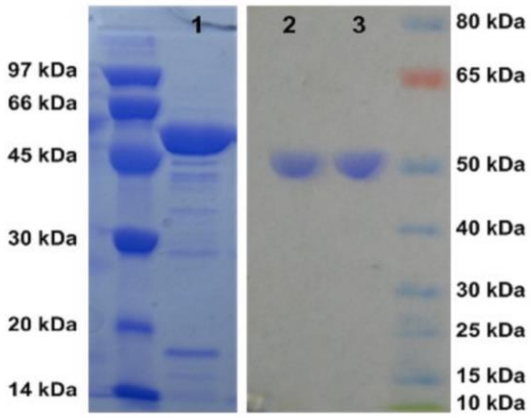


Fig. S1 SDS-PAGE of enzymes purified by affinity chromatography: Lane 1: His-tagged *Pc*OGT (55.7 kDa) purified on Ni Sepharose™; Lane 2 and 3: *Strep*-tagged *Os*CGT and *Os*CGT_I121D, respectively purified by *Strep*-tag affinity chromatography (51.3 kDa each)

2.3 Reverse glycosylation of phlorizin by *Pc*OGT

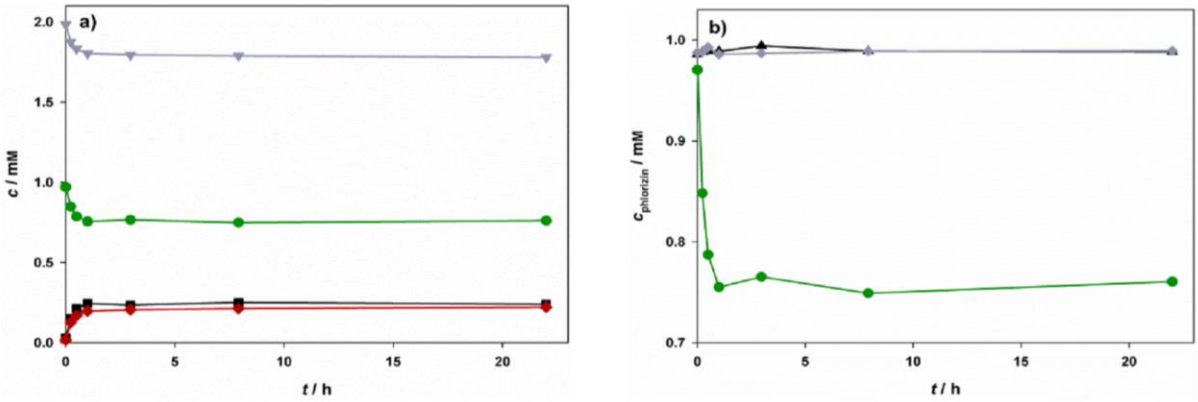


Fig. S2 Deglycosylation of phlorizin by *Pc*OGT at pH 7.0: a) 1 mM phlorizin (green) was partially deglycosylated in presence of 2 mM UDP (grey) and *Pc*OGT (25 mU mL^{-1}). Equal amounts of phloretin (black) and UDP-glucose (red) were formed. b) Phlorizin was only deglycosylated when UDP as well as *Pc*OGT were present (green). In presence of only UDP (grey) or solely *Pc*OGT (black) phlorizin was not converted. Deglycosylation of phlorizin is therefore solely achieved by enzyme-catalysed transfer of glucose to UDP and not by a potential hydrolytic side reaction.

2.4 Effect of organic cosolvents on *O*- to *C*-glucoside rearrangement

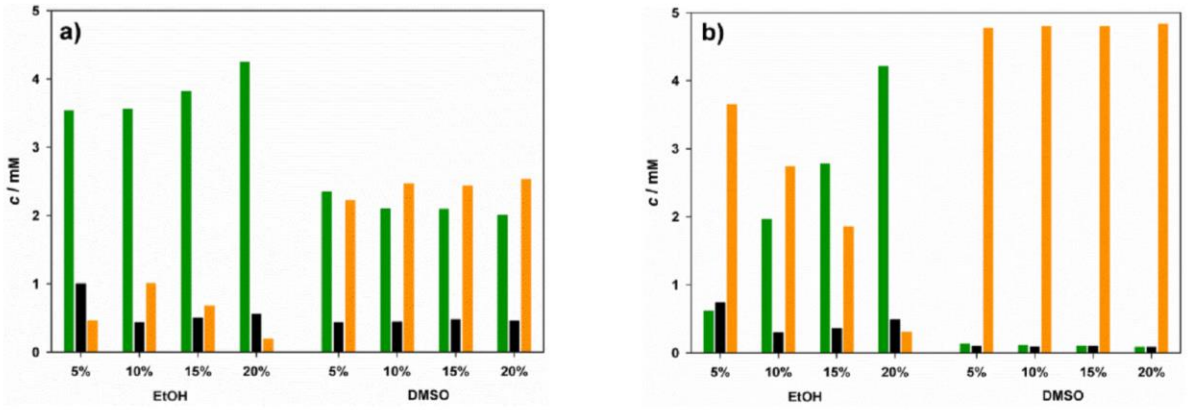


Fig. S3 Yields of *O*- to *C*-glucosidic bond rearrangement at various cosolvent conditions after (a) 2h and (b) 24h of conversion (5 mM phlorizin, 2 mM UDP, 100 mU mL^{-1} *Pc*OGT, 50 mU mL^{-1} *Os*CGT). Phlorizin (green), phloretin (black), nothofagin (orange); Any concentration of ethanol reduced conversions drastically. In contrast even the highest applied DMSO concentration of 20% showed no significant reduction of initial or final conversion.

2.5 *O*- to *C*-glucoside rearrangement in the absence of side reactions

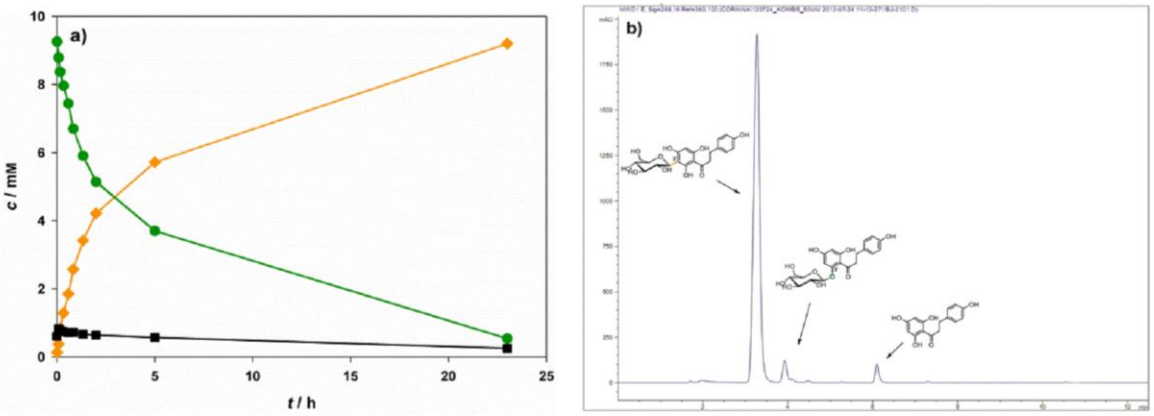


Fig. S4 10 mM phlorizin were almost completely converted to the *C*-glucoside nothofagin within 24 h (10 mM phlorizin, 2 mM UDP, 100 mU mL^{-1} *Pc*OGT, 50 mU mL^{-1} *Os*CGT). a) Time course of rearrangement: Quick initial consumption of phlorizin (green) gradually slows down after around one hour. The aglycon phloretin (black) remains relatively constant over the entire conversion. After 24 h of incubation 92% (9.2 mM) of applied phlorizin are converted to nothofagin (orange). b) HPLC analysis after 24h of conversion: The UV-trace (288 nm) shows a dominant nothofagin signal (3.3 min) with minor peaks for phlorizin (3.9 min) and phloretin (6.1 min). Absence of any further signals indicates that no side products were formed.

2.6 Potentiometric titration of phlorizin, phloretin, UDP and UDP-glucose

To investigate the cause for pH dependency of the equilibrium constant in *O*-glycosylation, potentiometric titrations of phloretin and its 4'-*O*-glucoside phlorizin (Fig. S5a) as well as UDP and UDP-glucose (Fig. S5b) were performed. Observed pK_a values were in general agreement with literature data, which allowed their assignment to functional groups (Fig. S5c).[5-6] Slight discrepancies between observed and reported pK_a values are probably caused by differences in experimental conditions (addition of DMSO, temperature, ionic strength).
The titration curve of the aglycon phloretin (Fig. S5a, black) was in good agreement with literature, the reported pK_a values being 7.0, 9.4, and 10.5.[5] Phlorizin shares with phloretin a pK_a value in the neutral pH range. This pK_a supposedly represents the first deprotonation event on one of the three phenolic hydroxyl groups on the aromatic ring involved in glycosylation. The second pK_a value of phloretin of around pH 9.3 is missing in phlorizin (green). This indicates that the pK_a represents a second deprotonation event on the same ring which is missing in phlorizin because of glucose bound to the oxygen at position 2'. A further pronounced plateau at around pH 10.8 is visible in both compounds. It most likely reflects unresolved pK_a values from a final deprotonation on the glycosylated aromatic ring and also deprotonation of the single hydroxyl groups on the second aromatic ring. The observed large increase of the equilibrium constant of *O*-glycosylation with increased pH in the range from 6.5 to 8.8 cannot be explained by deprotonation of phloretin upon glycosylation. Phloretin glycosylation causes no significant proton transfer below pH 8. At higher pH, proton release would rather occur in deglycosylation than in glycosylation direction due to increase of acidity in going from phlorizin to phloretin.
Protonation UDP and UDP-glucose can, however, explain a drastic preference of glycosylation at high pH. UDP (Fig. S5b, grey) displays a pK_a value of around 5.6, which is missing in UDP-glucose. It represents the second deprotonation of the terminal phosphate group, which is glycosylated in the case of UDP-glucose. Fig. S5b reveals that UDP is predominantly deprotonated throughout the relevant pH range (pH 6.5-8.8) where the reaction equilibrium constant exhibited pronounced pH-dependency (Fig. 1b). Sugar donor activation by a substituted phosphate-leaving group is a common feature of glycosyltransferases.[7] Therefore, directional preference for deglycosylation at low pH might be expected as a rather general feature of glycosyltransferase reactions, except in cases where the reactive group of the acceptor substrate matches the low pK_a of the sugar donor's (secondary) phosphate group. The vast majority of hydroxyl acceptor groups have comparably high pK_a and are therefore only deprotonated at high pH. Only a few studies have been performed in which pH effects on glycosyltransferase reaction equilibrium have been examined in detail. However, common trend is that reverse glycosylation is preferred at low pH (equilibrium constants and reaction rates).[8-11] Net proton release during forward glycosyl transfer from NDP-sugar was also exploited for glycosyltransferase assay development based on pH-shift.[12-13]

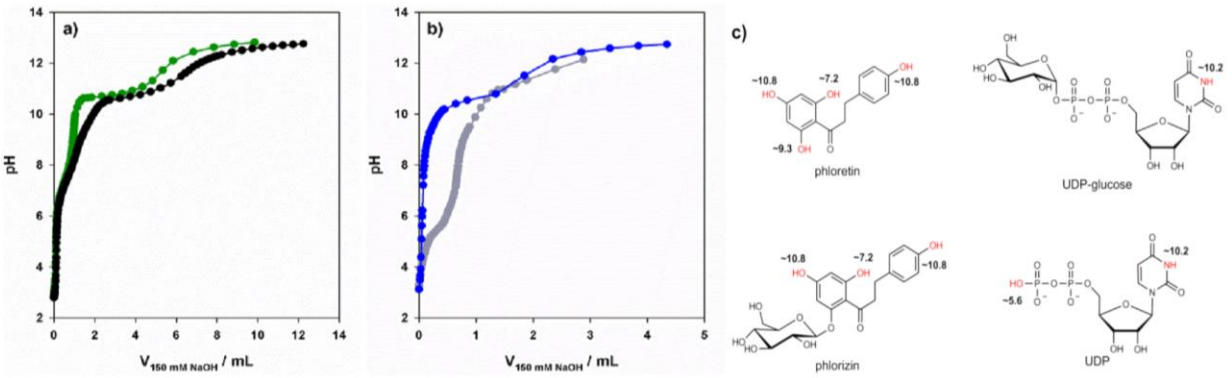


Fig. S5 Potentiometric titrations of substrates and products of *Pc*OGT-catalysed *O*-glycosylation, using 150 mM NaOH at 30°C (7.5 mM analyte in 20% DMSO, 13 mM $MgCl_2$, 50 mM KCl). a) Titration of 20 mL phlorizin (green) and phloretin (black); b) Titration of 10 mL UDP (blue) and UDP-glucose (grey); c) Structures of substrates (phloretin, UDP-glucose) and products (phlorizin, UDP) of the glycosylation reaction catalysed by *Pc*OGT. Approximate pK_a values are indicated at the respective positions.

2.7 Dependency of rearrangement rate on substrate concentrations

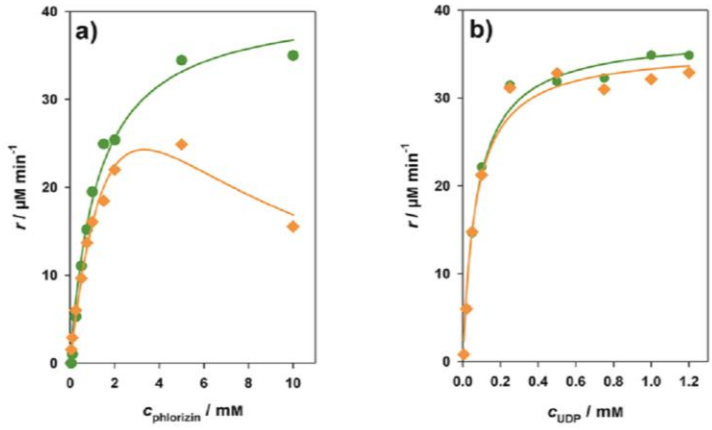


Fig. S6 Dependencies of phlorizin consumption (r_P; green) and nothofagin formation (r_N; orange) rates on the substrate concentration used. The constant substrate concentrations were 2 mM UDP (a) and 5 mM phlorizin (b). All reactions contained 100 mU mL^{-1} *Pc*OGT and 50 mU mL^{-1} *Os*CGT. Symbols show measurements and lines are fits to the data.

2.8 Optimisation of *O*- to *C*-glucoside rearrangement by *Os*CGT_I121D

Feasibility of *O*- to *C*-glucoside rearrangement by a single dual-specific *O*- and *C*-glycosyltransferase was evaluated with the promiscuous *Os*CGT mutant I121D. Initially different concentrations of UDP (0.5 and 5 mM) and phlorizin (0.05, 0.5 and 5 mM) were tested (1.25 mU mL^{-1} *Os*CGT_I121D at pH 7). Time courses for 24 h were recorded and a summary of conversions after 6h reflecting the overall trend is depicted in Fig. S7. An increase of UDP concentration from 0.5 to 5 mM as well as rising phlorizin to 5 mM enhanced the formation of nothofagin (Fig. S7a). While presenting a proof of principle, a maximum yield of around 30 µM was by far too low for synthetic use. In all cases more than 90% of the starting material was still present as phlorizin or phloretin (Fig. S7b-c) and only small amounts of 4'-*O*- and 3'-*C*-glycoside (nothofagin) were found. Highest conversion to nothofagin (4.6 %) was obtained when 0.5 mM phlorizin and 5 mM UDP were applied which served as a starting point for optimizations.

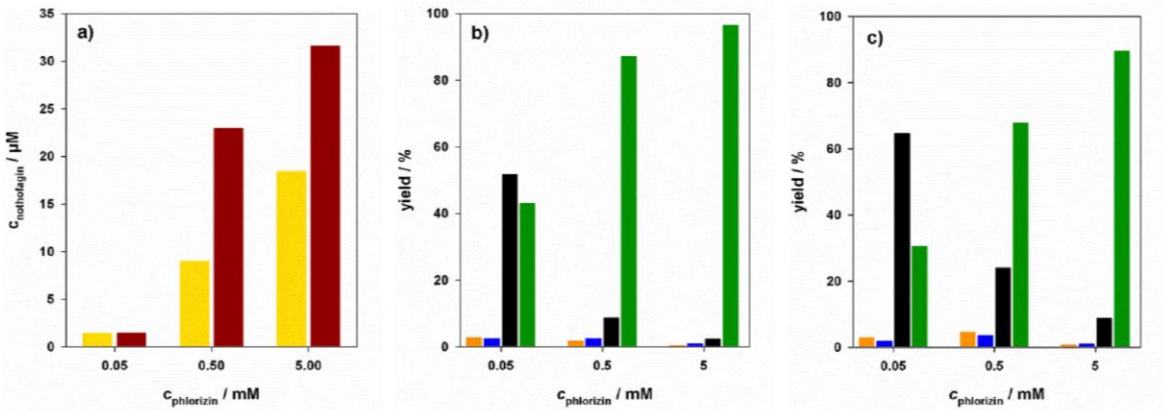


Fig. S7 Yields after 6 h of *O*- to *C*-glycoside rearrangement catalysed by *Os*CGT_I121D (1.25 mU mL^{-1}, pH 7.0) using various substrate concentrations. a) Nothofagin formed from different phlorizin concentrations using 0.5 (yellow) and 5 mM (red) UDP, respectively; Distribution of phloretin and glycosides in presence of 0.5 mM (b) and 5 mM (c) UDP: phlorizin (green), phloretin (black), nothofagin (orange), 4'-*O*-glucoside (blue).

Optimisation of the *O*- to *C*-rearrangement with wild type glycosyltransferases revealed that well balanced *O*- and *C*-glycosyltransferase activities and a suitable equilibrium constant of *O*-glycosylation are key for efficient conversions. Besides substrate concentrations, pH is the key parameter to influence the ratio of these enzymatic activities. Therefore, conversion of 0.5 mM phlorizin in presence of 5 mM UDP was evaluated at pH 6.0, 7.0 and 8.0. The effect of pH on yields after 6 h of incubation (Fig. S8) was in general comparable to results with wild type enzymes (Fig. 2a). At low pH formation of the aglycon was favoured but *C*-glycosylation was impaired. Best yield of *C*-glycoside as well as a favourable ratio of 3'-*C*- to 4'-*O*-glycoside were obtained at pH 7.0.

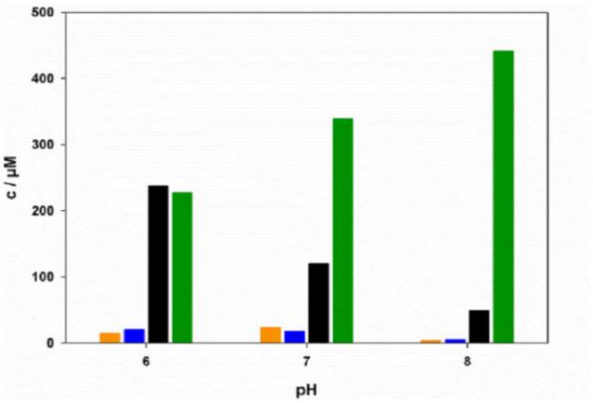


Fig. S8 Distribution of phloretin (glycosides) after 6 h of rearrangement by *Os*CGT_I121D (1.25 mU mL^{-1}) at various pH (50 mM BisTris, 0.5 mM phlorizin, 5 mM UDP): phlorizin (green), phloretin (black), nothofagin (orange), 4'-*O*-glucoside (blue).

Since accumulation of the aglycon phloretin was omnipresent we hypothesized that availability of UDP-glucose could limit *C*-glycosylation. Therefore addition of UDP-glucose (0, 0.5, 1 and 5 mM) was tested. Addition of UDP-glucose should boost final *C*-glycosylation but disfavour initial phloretin production. To analyse potential improvement on *C*-glycoside formation, we focussed on the final yields after 43 h (Fig. S9) rather than on effects on the initial rate. The expected negative effect of UDP-glucose on deglycosylation of phlorizin was detrimental using 5 mM UDP-glucose. At low concentrations (0.5, 1 mM), however, the positive effect of UDP-glucose on *C*-glycosylation prevailed. Although the highest nothofagin yield was obtained in presence of 0.5 mM UDP-glucose we decided to continue working with 1 mM UDP-glucose. We anticipated it to be beneficial for maximizing nothofagin formation since phloretin concentrations (~70 µM) did not seem to be limiting. By doubling the amount of enzyme and increasing the reaction time to 70 h it was finally possible to obtain 70% yield (0.35 mM) in *O*- to *C*-glycoside rearrangement catalysed by *Os*CGT_I121D.

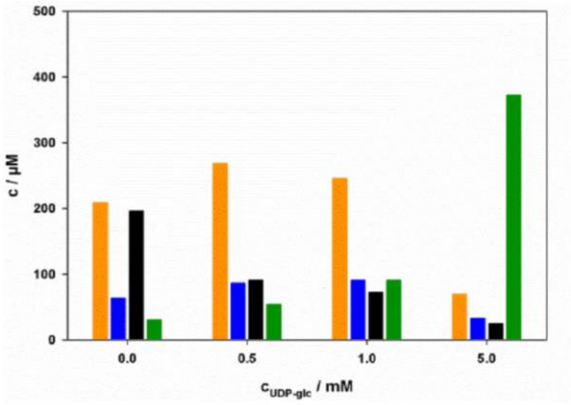


Fig. S9 Distribution of phloretin (glycosides) after 43 h of rearrangement by *Os*CGT_I121D (1.25 mU mL^{-1}) in presence of different UDP-glucose concentrations (0.5 mM phlorizin, 5 mM UDP): phlorizin (green), phloretin (black), nothofagin (orange), 4'-*O*-glucoside (blue).

3. References

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4. Appendix

4.1 Maps of expression vectors

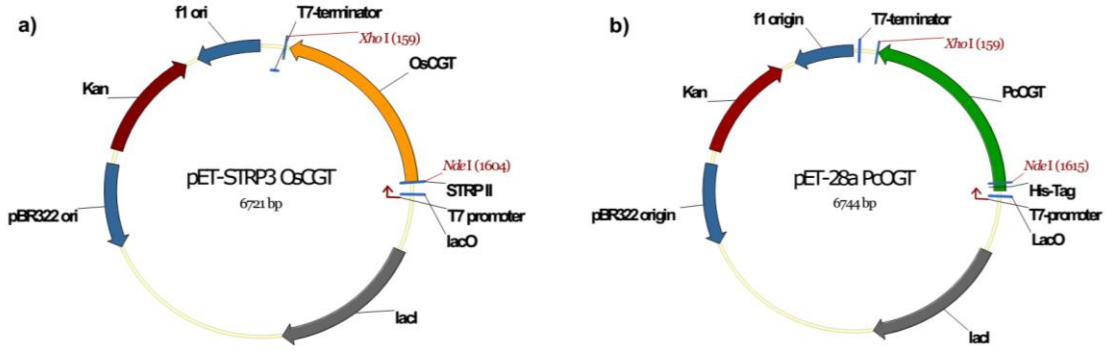

Fig. S10 Map of vectors for *Os*CGT (pET-STRP3) (a) and *Pc*OGT (pET-28a) (b) expressions. Genes are inserted into *Nde*I and *Xho*I sites for expression with N-terminally fused *Strep*-tag II and His-tag, respectively; f1 ori: f1 phage origin of replication, Kan: kanamycin resistance gene, pBR322 ori: pBR322 origin of replication, lacI: lactose repressor gene, lacO: lactose operator, STRP II: *Strep*-tag II, OsCGT: *Os*CGT gene (GenBank: FM179712), PcOGT: *Pc*OGT gene (GenBank: FJ854496)

4.2 DNA sequence of *Os*CGT in pET-Strep3 expression vector

Bold:	confirmed by sequencing
CAPITAL LETTERS:	Expressed open reading frame (*Os*CGT + *Strep*-tag II)
Grey:	*Strep*-tag II

>pET-STRP3_*Os*CGT

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4.3 DNA sequence of *Pc*OGT

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Towards the synthesis of glycosylated dihydrochalcone natural products using glycosyltransferase-catalysed cascade reactions

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Towards the synthesis of glycosylated dihydrochalcone natural products using glycosyltransferase-catalysed cascade reactions†

Alexander Gutmann,[a] Linda Bungaruang,[a] Hansjoerg Weber,[b] Mario Leypold,[b] Rolf Breinbauer[b] and Bernd Nidetzky*[a,c]

Regioselective *O*-β-D-glucosylation of flavonoid core structures is used in plants to create diverse natural products. Their prospective application as functional food and pharmaceutical ingredients makes flavonoid glucosides interesting targets for chemical synthesis, but selective instalment of a glucosyl group requires elaborate synthetic procedures. We report glycosyltransferase-catalysed cascade reactions for single-step highly efficient *O*-β-D-glucosylation of two major dihydrochalcones (phloretin, davidigenin) and demonstrate their use for the preparation of phlorizin (phloretin 2′-*O*-β-D-glucoside) and two first-time synthesised natural products, davidioside and confusoside, obtained through selective 2′- and 4′-*O*-β-D-glucosylation of the dihydroxyphenyl moiety in davidigenin, respectively. Parallel biocatalytic cascades were established by coupling uridine 5′-diphosphate (UDP)-glucose dependent synthetic glucosylations catalysed by herein identified dedicated *O*-glycosyltransferases (OGTs) to UDP dependent conversion of sucrose by sucrose synthase (SuSy; from soybean). The SuSy reaction served not only to regenerate the UDP-glucose donor substrate for OGT (up to 9 times), but also to overcome thermodynamic restrictions on dihydrochalcone β-D-glucoside formation (up to 20% conversion and yield enhancement). Using conditions optimised for overall coupled enzyme activity, target 2′-*O*- or 4′-*O*-β-D-glucoside was obtained in ≥88% yield from reactions consisting of 5 mM dihydrochalcone acceptor, 100 mM sucrose, and 0.5 mM UDP. Davidioside and confusoside were isolated and their proposed chemical structures confirmed by NMR. OGT-SuSy cascade transformations present a green chemistry approach for efficient glucosylation in natural products synthesis.

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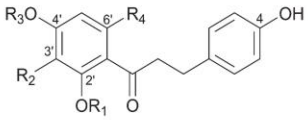
Introduction

Flavonoids are a large and structurally diverse group of natural polyphenols.[1,2] They are widely distributed in edible plants and therefore constitute an important part of the human diet.[3,4] Some flavonoids are strong antioxidants and thus serve as powerful inhibitors of lipid peroxidation.[5,6] Dietary intake of flavonoids has been correlated with reduced risk of chronic diseases, coronary heart disease in particular.[7] Dihydrochalcones present a major sub-class of flavonoids and are characterized structurally by two phenolic rings connected through a flexible open-chain three-carbon linker (Table 1).[8] Many dihydrochalcones, such as the common phloretin (**1**), which is abundantly present in the leaves and peel of apples,[9,10] exhibit a wide spectrum of interesting and pharmacologically relevant bioactivities.[11] Next to a general antioxidative property,[12] **1** shows antithrombotic[13] and hepatoprotective properties,[14]

Table 1 Major dihydrochalcones and their glucosides



Dihydrochalcone	R_1	R_2	R_3	R_4
Phloretin (**1**)	H	H	H	OH
Davidigenin (**2**)	H	H	H	H
Phlorizin (**3**)	Glucose	H	H	OH
Trilobatin (**4**)	H	H	Glucose	OH
Davidioside (**5**)	Glucose	H	H	H
Confusoside (**6**)	H	H	Glucose	H
Nothofagin (**7**)	H	Glucose	H	OH

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[c]*Austrian Centre of Industrial Biotechnology, Petersgasse 14, 8010 Graz, Austria*
†Electronic supplementary information (ESI) available: Protein gel; HPLC traces: distinction of **2** glucosides, purified **5** and **6**; *Pc*OGT-*Gm*SuSy conversions at various pH; NMR data. See DOI: 10.1039/C4GC00960F

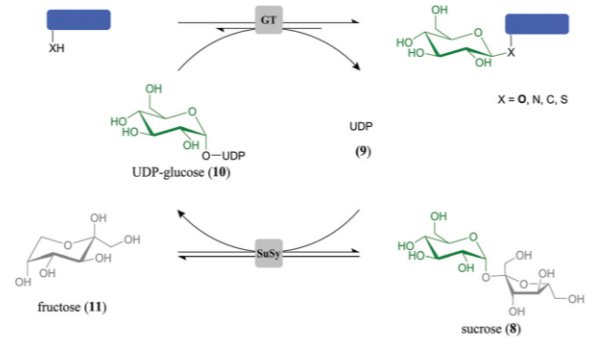
potential suppression of metabolic carcinogen activation[15] and effects intracellular drug accumulation.[16] Davidigenin (**2**), the 4′-deoxy analogue of **1**, is broadly distributed among different plant families.[17] As an antispasmodic compound present in or derived from traditional medicines it is applied to the treatment of intestinal disorders and asthma.[17] **2** also exhibits weak antibacterial activity[18] and shows antidiabetic activity.[19]

Glycosylation is an important biological mechanism for the structural and functional diversification of natural flavonoids.[20,21] Generally, the attachment of sugar residue(s) to a flavonoid core increases compound bioavailability due to strong water solubility enhancement.[22,23] Furthermore, physiological and pharmacological properties are often beneficially altered as consequence of glycosylation.[24,25] Taste properties (*e.g.* sweetness, bitterness) and colour can also be modified by glycosylation.[26,27] Interestingly, compared to other flavonoid sub-classes that show broadly diverging and often complex glycosylation patterns, glycosylated dihydrochalcones occur primarily as mono-β-D-glucopyranosides.[8] Sugar attachment to dihydrochalcones often results from a phenolic *O*-glycosidic bond, although aromatic C-glucosylation is also found in certain compounds.[8,28] Phloretin is naturally present mainly as the 2′-*O*-β-D-glucoside, commonly referred to as phlorizin (**3**).[29] Due to inhibition of cellular glucose transport **3** was considered for type II diabetes treatment.[29] The corresponding 4′-*O*-β-D-glucoside, which is called trilobatin (**4**), is less common.[30] Davidioside (**5**) is the 2′-*O*-β-D-glucoside of **2**, while the 4′-*O*-β-D-glucoside is called confusoside (**6**). Compounds **5** and **6** were previously isolated from plant extracts and characterized structurally.[31,32] However, except for a low yielding total synthesis of **6**, preparation of glucosides of **2** was not reported.[33]

Due to their various prospective applications as fine chemicals, pharmaceutical ingredients and food additives, dihydrochalcone glucosides **3–6** are interesting targets for large-scale preparation.[29,34,35] However, their isolation from natural material is complicated by occurrence in complex multi-component mixtures and at relatively low abundance.[30–32,36] Selective glucosylation of **1** and **2** would provide convenient access to **3–6** through bottom-up synthesis. Process chemistry for this purpose is not well developed, though, and elaborate procedures involving a substantial amount of protecting group chemistry are required for precise instalment of the glucosyl group.[37] Alternative biotransformations using cultured plant cells were successfully applied for direct glucosylation of a variety of phenolic compounds including several flavonoids.[38,39] However, presumably due to a mixture of native plant glucosyltransferases, control of regioselectivity was difficult and typically mixtures of various glucosides were obtained. Isolated enzymes in contrast have earned green credentials for being able to cope with issues of reactivity and selectivity in glycosylation reactions.[40,41] Glycosyltransferases (GTs) are widely applied for glycosylation of flavonoids, whereas use of glycoside hydrolases, glycosynthases and transglycosidases is so far limited by a narrow substrate range and typically low yields.[26,41,42] GTs utilise an activated donor substrate, typically a nucleoside diphosphate (NDP)-sugar, for transfer of a glycosyl residue onto certain position(s) of the acceptor molecule.[43] The impressive flexibility displayed by several promiscuous GTs allowed glycosylation of a wide range of acceptors, but distinction between different phenolic hydroxyls in flavonoid acceptors still presents a notable challenge.[44–47]

Practical enzyme catalysts for selective glucosylation of **1** or **2** are to be established. Up to now only one GT from apple (*Malus* × *domestica*) and its ortholog from pear (*Pyrus communis*) (99% sequence identity) were reported to selectively produce analytical amounts of phlorizin (**3**) by glucosylation of **1**.[48,49] Other recently reported glucosylations of **1** by plant GTs yielded mixtures of monoglucosides.[49,50] An even more promiscuous bacterial GT glucosylated **1** at 2′-, 4′- and 4-OH, producing a complex mixture of glucosidic compounds including two di- and a triglucoside.[51] Therefore, this emphasises that selectivity-based GT enzyme selection is of key importance for successful development of a biocatalytic synthesis process. Furthermore, dedicated reaction engineering is necessary to solve the problem of cost-effective supply of the NDP-sugar substrate.[52]

Herein we present identification of GT biocatalysts for selective transformation of **1** into **3**, and of **2** into **5** or **6**. A parallel GT cascade reaction (Scheme 1) was established to enable dihydrochalcone glucosylation from sucrose (**8**) as a highly expedient and inexpensive glucosyl donor substrate. The overall bi-enzymatic transformations were carried out in the presence of catalytic amounts of uridine 5′-diphosphate (UDP) (**9**) to generate and constantly recycle *in situ* the UDP-glucose (**10**) utilised for glucoside synthesis. Applying reaction conditions optimised for enzyme activity and designed to overcome thermodynamic limitations, target *O*-β-D-glucoside was obtained as single transfer product in ≥88% yield and could be isolated readily. We propose Scheme 1 to be a generally applicable green chemistry approach for efficient glucosylation in natural products synthesis.



Scheme 1 GT-catalysed cascade reaction for synthesis of natural product glucosides. Coupling of a "synthetic" GT with SuSy in one pot enables cost-effective *in situ* formation of glucosyl donor substrate **10** from **8** and catalytic amounts of **9**. End-product inhibition by **9** is obviated.

Results and discussion

Identification of GT enzymes catalysing regioselective glucosylation of phloretin (1) and davidigenin (2)

A GT from pear (*Pyrus communis*; *Pc*OGT) was recently reported to be highly selective for transferring the glucosyl moiety of UDP-glucose (**10**) to the 2′-OH of **1**, producing **3**.[49,53] However, evidences were only from thin layer chromatography[49] or from initial-rate experiments carried out at low conversion (≤25%) of both donor and acceptor substrate.[53] Furthermore, the concentrations of **1** used (300 and 100 μM, respectively) were too low to be of synthetic relevance. A number of studies show that GT regioselectivity may change substantially depending on degree of conversion or on initial substrate concentration.[46,53] Therefore, this necessitated rigorous evaluation of *Pc*OGT as catalyst for synthesis of **3** at elevated substrate concentration.

Using recombinant *Pc*OGT (0.1 U mL^{-1}) purified from an *Escherichia coli* overexpression culture (ESI,† Fig. S1), transformation of **10** and **1** (5 mM each, pH 6.5) into phloretin glucoside(s) was examined in dependence of reaction progress until attainment of apparent equilibrium (~70% conversion of **1**). Product analysis was done using a reversed-phase HPLC method in which the monoglucosidic regio-isomers **3** and **4** were baseline separated (Fig. 1a). Only **3** and no **4** was detected at all times (Fig. 1b). Moreover, close balance between **1** consumed and **3** formed was consistent with enzymatic reaction exclusively at the acceptor's 2′-OH.

*Pc*OGT was then examined for glucosylation of **2** from **10** (each 5.0 mM, pH 7.5). UV absorbance traces from HPLC analysis of samples taken at different times revealed gradual consumption of **2** with concomitant appearance of a major and a minor signal eluting at positions consistent with glucosidic products (Fig. 1c). According to HPLC peak areas around 80% of **2** were converted to the major glycoside and less than 2% of the by-product were formed when equilibrium was obtained. NMR data recorded from the reaction mixture gave preliminary evidence that the 2′-OH of **2** had been glycosylated, thereby indicating that regioselectivity was maintained upon exchange of acceptor substrate. Specific activities of *Pc*OGT related to glucosyl acceptor substrate were determined from the time courses of consumption of **1** and **2**, and **1** (1.76 U mg^{-1} protein) was an about 500-fold better substrate than **2**.

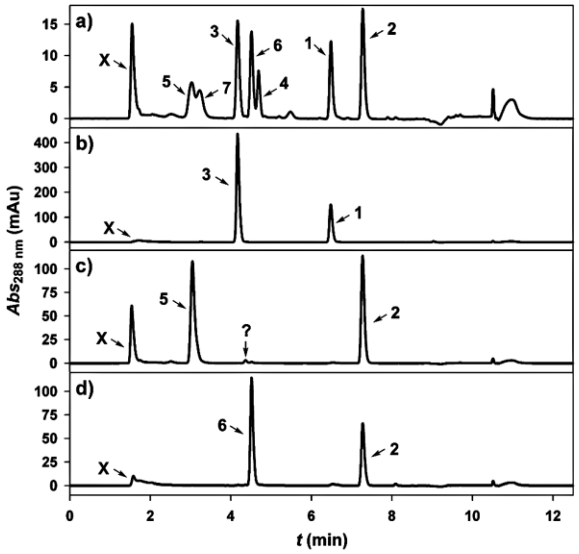


Fig. 1 Reversed-phase C-18 HPLC-analysis of dihydrochalcone (glucosides) (X = unbound compounds including **9** and **10**). (a) Separation of glucosides of **1** (**3**, **4**, **7**) and **2** (**5**, **6**) is achieved. (b) Conversion of **1** by *Pc*OGT. (c) Conversion of **2** by *Pc*OGT (? = unknown by-product). (d) Conversion of **2** by *Os*CGT I121D.

In search of a differently regioselective GT catalysing glucosyl transfer to the 4′-OH of **1** and **2**, we tested a recently described variant of a *C*-glycosyltransferase from rice (*Oryza sativa*; *Os*CGT) that had its Ile^{121} replaced by Asp.[53] Highly purified mutant enzyme was used in all experiments (ESI,† Fig. S1). Wild-type *Os*CGT was shown to catalyse glucosyl transfer from **10** to the aromatic 3′-*C* of **1**, thus producing the *C*-β-D-glucoside nothofagin (**7**).[53,54] The particular residue substitution in *Os*CGT causes change in reaction selectivity such that O-glucosylations of the 2′-OH and 4′-OH occur next to the native C-glucosylation of **1**.[53] Using **1** at different concentrations between 2.5 μM and 2.5 mM, it was found that the enzymatic conversion resulted invariably in a mixture of glucosidic products that contained **3** or **7** as the main constituent whereas **4** was present only as a by-product (≤15% of total). It was not possible to enhance the relative abundance of **4** by running the reaction at different pH in the range 6.2–10.5. Ability of the *Os*CGT variant to synthesise substantial amounts of **4** is nevertheless remarkable, and the question of whether isolation of **4** from the enzymatically prepared mixture of phloretin glucosides would still be practical was left for consideration in the future. Notably, though, the *Os*CGT variant was complementarily regioselective to *Pc*OGT in the glucosylation of **2**, as shown in Fig. 1d. The main *Pc*OGT glucosylation product, likely **5**, was completely absent from the reaction sample obtained with the mutated *Os*CGT. However, a single new product peak appeared. Its area increased proportionally to the decreasing peak area of the **2**. Interestingly, the retention time was very similar to that of the trace product formed from **2** by *Pc*OGT, however HPLC analysis of a mixture of both conversions clearly reveals them to be distinct (ESI,† Fig. S2). Based on preliminary NMR data of the reaction mixture it was assumed, and will be confirmed later, that **6** was formed in the reaction of the GT mutant. Specific activity of the *Os*CGT variant for glucosylation of **2** was determined as 22 ± 3 mU mg^{-1}.

In summary, therefore, GT catalysts for selective glucosylation of **1** and **2** have been identified, and they were used further for synthesis of **3**, **5** and **6**.

Thermodynamic analysis of glucosylation of phloretin (1)

Glucosylation of **1** or **2** from **10** is an equilibrium-controlled process.[55] Thermodynamic restrictions on the synthesis of dihydrochalcone β-D-glucosides therefore required clarification.

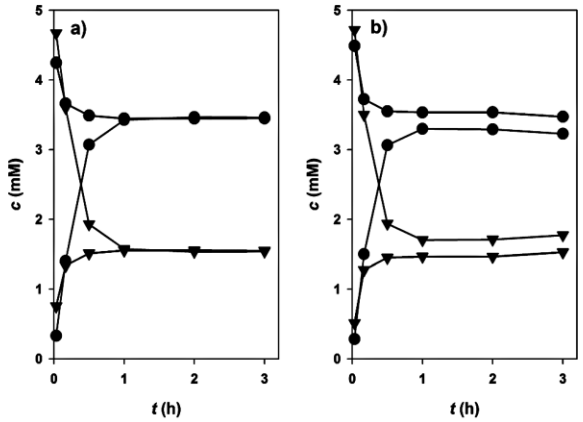


Fig. 2 Phlorizin (**3**) synthesis (grey, 5 mM **1** and **10**) and degradation (black, 5 mM **3** and **9**) by *Pc*OGT (pH 6.5) level out at the same equilibrium concentrations. (a) **1** (triangles), **3** (circles); (b) **10** (triangles), **9** (circles).

Fig. 2 displays results of time-course analyses for synthesis and degradation of **3**, catalysed by *Pc*OGT at pH 6.5 and 30 °C. All compounds present in the reaction were quantified (**1**, **3**, **9**, **10**). Close balance for the proposed overall conversion, **1** + **10** ↔ **3** + **9**, was obtained at each time and in each direction of reaction. Therefore, this indicated absence of enzyme-catalysed or spontaneous side reactions, such as hydrolysis of **10** for example. Reactions run in forward and reverse direction levelled out at exactly the same end concentrations of **1** and **3** within limits of experimental error (Fig. 2a), clearly suggesting that the true thermodynamic equilibrium had been attained. It was affirmed that addition of fresh enzyme to a reaction mixture at (apparent) equilibrium in Fig. 2 did, as expected, not induce further concentration changes. An equilibrium constant (K_{eq}) of 4.6 (±0.7) was calculated from the data. Under the conditions used, therefore, only about 70% of substrate **1** can be converted to product **3**. Development of a glucosyl transfer cascade (Scheme 1) that involved *in situ* generation of the UDP-sugar substrate **10** from sucrose (**8**) and UDP (**9**) was highly useful to overcome these thermodynamic restrictions.

Glucosyl transfer cascade for efficiency-enhanced glucosylation of phloretin (1)

The general principle of the glucosyl transfer cascade is shown in Scheme 1. Two parallel glucosyl transfer reactions are connected in a one-pot biotransformation *via* their common **10**/**9** substrate/product pair. Conversion of **8** and **9** is catalysed by sucrose synthase (SuSy) and yields **10** and D-fructose (**11**). The SuSy reaction, which represents the invariant part of the proposed cascade, is flexibly coupled to different GT-catalysed glucosylations of target acceptors under utilisation of **10** as the donor substrate. Benefit of performing synthetic glucosylations through SuSy-GT cascades as compared to single GT reactions is manifested in significant improvement of key parameters of overall transformation efficiency. First of all, cost-effective supply of **10** is accomplished using **8** as a highly expedient and inexpensive donor substrate. Only catalytic amounts of **9** as compared to stoichiometric amounts of **10** are required in the process. By using **8** in suitable excess over the acceptor substrate, thermodynamic restrictions on acceptor glucosylation are overcome effectively (see later). Equilibrium of the SuSy reaction ($K_{eq} \geq 0.5$) favours formation of **10** in a wide pH range (pH ≤ 7.5).[52] Finally, problems of pronounced end-product inhibition by **9**, which have severely restricted direct synthetic use of several flavonoid glycosyltransferases in the past, are brought under control.[56,57] Continuous removal of **9** due to formation of **10** decreases the steady-state concentration of **9** in the reaction to a value even smaller than the one established from the low (catalytic) amount that was initially added.

To optimise conditions for synthesis of **3**, the coupled reaction of SuSy and *Pc*OGT was studied at different pH values in the range 5.7–8.5. Recombinant SuSy from soybean (*Glycine max*; *Gm*SuSy) purified from an *E. coli* expression culture was used (ESI,† Fig. S1). The applied concentrations of **1**, **8** and **9** were 5.0 mM, 100 mM and 0.5 mM, respectively. Despite addition of 20% DMSO as co-solvent, poor aqueous solubility of **1** restricted use of concentrations higher than about 5 mM. The time course of **3**-formation was measured at each pH (ESI,† Fig. S3). The data was used for calculation of initial production rates of **3** (r_3), final yields at reaction equilibrium (24 h) and space-time yields (STY) for a target product concentration of 4.0 mM (Fig. 3). The optimum pH range for high r_3 was between pH 6.2 and 7.0 (Fig. 3a). Except for pH 5.7 and 8.5 where r_3 was small and reaction equilibrium was therefore not attained within the 24 h timespan of the experiment, the final concentrations of **3** were around 4.4 mM, equivalent to a yield of ~90% based on conversion of **1** (Fig. 3b). With increasing pH a marginal improvement of equilibrium concentrations of **3** from 4.36 (pH 6.2) to 4.46 (pH 7.8) was observed. However, STY for formation of 4 mM **3** (Fig. 3a) confirmed that pH dependency of initial rates is predominantly determining efficiency of conversions. A clear optimum of STY was found

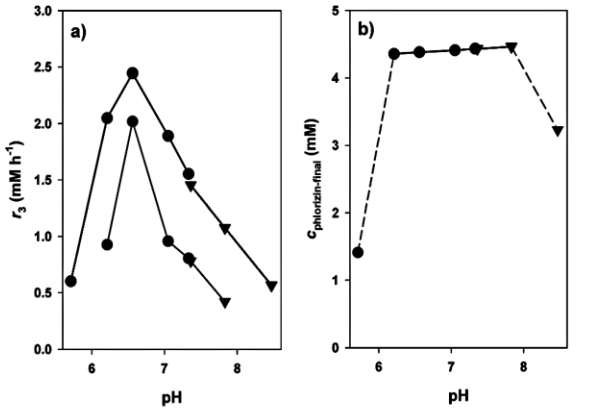


Fig. 3 Influence of pH on efficiency of *Pc*OGT-*Gm*SuSy cascade conversions using BisTris (circles) and TAPS (triangles) buffers (5 mM **1**, 0.5 mM **9**, 100 mM **8**). (a) Initial rate of formation of **3** (black), STY for formation of 4 mM **3** (grey). (b) Final equilibrium concentrations of **3** after 24 h (grey: equilibrium was not reached).

around pH 6.5 with more than 50% loss at pH 6.2 and 7.1, respectively. Overall, compared to synthesis of **3** directly from **1** and **10** (each 5 mM), introduction of the SuSy-GT cascade represented a substantial yield enhancement from ~70 to ~90%. The effect of "thermodynamic push" from **8** on glucoside product formation is noted. It is explained from the net reaction of the glucosyl transfer cascade, which is sucrose (**8**) + phloretin (**1**) ↔ phlorizin (**3**) + D-fructose (**11**).

To further examine the role of **8** in driving the glucosylation of **1**, the cascade reaction was performed at pH 6.5 under conditions where both **8** (100 mM) and **10** (5 mM) were present as donor substrates for glucosylation of **1** (5 mM). Fig. 4 shows the full time course of the conversion along with the corresponding time courses from the coupled reaction without supplementation of **10** (but 0.5 mM **9**) and the direct glucosylation in the absence of *Gm*SuSy. Final conversions, initial rates and space time yields are summarised in Table 2. Product formation in the early reaction phase of cascade reactions benefited somewhat (~25% enhancement of r_3) from the presence of external **10**, probably because the *in situ* produced steady-state concentration of **10** was not sufficient for *Pc*OGT to become fully saturated with glucosyl donor substrate. Use of 5 mM **10** instead of 0.5 mM **9** in SuSy-GT cascade reactions furthermore caused moderate gain in final conversion (~3%) and a more significant improvement of STY for production of 4.0 mM of **3** by about 35%. However, in our opinion a roughly 30% reduced reaction time to obtain a very similar product concentration is not compensating additional costs of replacing UDP with 10-times higher concentrations of the more expensive **10**. Both SuSy-*Pc*OGT cascade reactions clearly outperformed direct glycosylation of **1** by *Pc*OGT. By addition of *Gm*SuSy the final concentration of **3** was increased from 3.61 mM to 4.44 (0.5 mM **9**) and 4.60 mM (5 mM **10**), respectively.

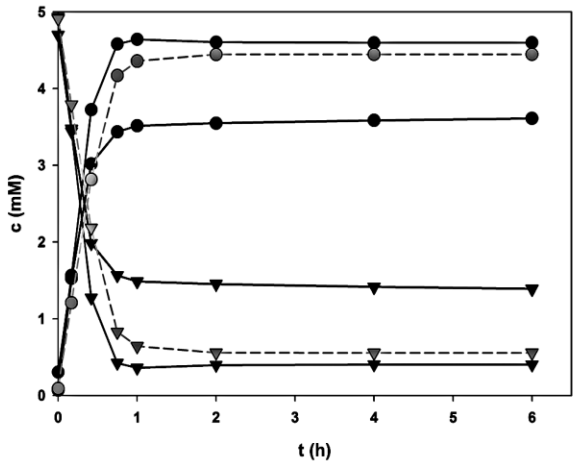


Fig. 4 *Pc*OGT catalysed formation of **3** (circles) from 5 mM **1** (triangles) at pH 6.5: black: without *Gm*SuSy, 5 mM **10**; dark grey: *Gm*SuSy, 5 mM **10**; light grey (dashed): *Gm*SuSy, 0.5 mM **9**.

Table 2 Parameters of direct/GT-cascade catalysed synthesis of **3**, **5** and **6**[a]

		3[b]	**5**[c]	**6**[d]
GT 5 mM **10**	Conversion[e] (%)	72.2	79.6	88.3
	$r_{glucoside}$[f] (mM h^{-1})	6.9	1.3	1.9
	STY[g] (mM h^{-1})	nd	nd	1.2
GT-*Gm*SuSy 5 mM **10**	Conversion[e] (%)	91.9	91.4	95.3
	$r_{glucoside}$[f] (mM h^{-1})	8.2	1.9	2.2
	STY[g] (mM h^{-1})	7.6	0.8	1.4
GT-*Gm*SuSy 0.5 mM **9**	Conversion[e] (%)	88.9	88.0	91.7
	$r_{glucoside}$[f] (mM h^{-1})	6.5	1.7	1.3
	STY[g] (mM h^{-1})	5.6	0.7	1.0

[a] Data extracted from Fig. 4 and 5. [b] 0.1 U mL^{-1} *Pc*OGT (0.1 U mL^{-1} *Gm*SuSy), pH 6.5, 5 mM **1**. [c] 0.1 U mL^{-1} *Pc*OGT, (0.1 U mL^{-1} *Gm*SuSy), pH 7.5, 5 mM **2**. [d] 0.04 U mL^{-1} *Os*CGT I121D (0.04 U mL^{-1} *Gm*SuSy), pH 7.5, 5 mM **2**. [e] At equilibrium after 6 (**3**), 24 (**5**) or 20 (**6**) h of conversion. [f] Rate of glucoside formation during initial 25 (**3**) or 60 min (**5**, **6**). [g] STY for formation of 4 mM glucoside (nd: less than 4 mM product).

Synthesis of davidoside (5) and confusoside (6) *via* glucosyl transfer cascade reaction

In order to compensate in some degree the 200-fold decrease in specific activity of *Pc*OGT caused by change of acceptor substrate from **1** to **2**, the pH of the cascade reaction was raised to 7.5 where *Pc*OGT is optimally active and the specific **2** glucosylation rate was therefore enhanced around 2.5-fold as compared to pH 6.5. The Ile^{121} → Asp variant of *Os*CGT is also best active at a pH of around 7.5.[53] Loss in specific activity of *Gm*SuSy caused by the rise in pH was negligible in comparison (~35%). The optimum pH of conversion of **8** by *Gm*SuSy was recently determined to be about 6.0.[52]

Relevant time courses for glucosylation of **2** by *Pc*OGT and *Os*CGT variant are shown in Fig. 5 and summarised in Table 2. The substrate concentrations used were the same as before in the conversions of **1**. The concentration of **2** (5.0 mM) was set according to aqueous solubility in the water-DMSO solvent used. Coupled enzyme reactions carried out in the presence and absence of added **10** are compared to the single enzyme reaction. Confusoside (**6**) was always obtained as single glucosyl transfer product in conversions with *Os*CGT I121D (Fig. 5b). However, in *Pc*OGT catalysed conversions besides davidioside (**5**) a second compound, distinct from **6**, accumulated during the first 4 h and diminished again upon prolonged incubation (Fig. 5a). Very low concentrations (~0.08 mM, <2% of total products) prevented structural analysis of the by-product by NMR. However, most likely the by-product is the third potential mono *O*-glucoside of **2**, resulting from glucosylation at the 4-OH. Its decline in favour of formation of **5** at longer incubation times is explained by reversibility of the glycosylations.[55] This causes accumulation of the thermodynamically favoured product (here **5**) at equilibrium conditions. Final mixture of the conversion of **2** contained the main product **5** in about 50-fold excess over the by-product, clearly demonstrating that highly regiospecific glucosylation of **2** can be achieved by *Pc*OGT. Comparison of the different reaction conditions used revealed that the initial conversion of **2** proceeded slightly faster and the overall conversion was ~3.5%

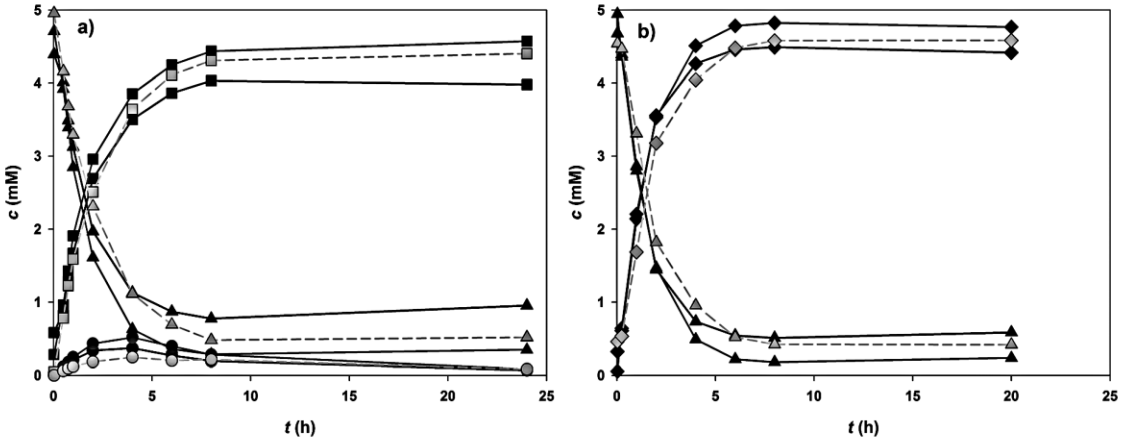


Fig. 5 Glucosylation of 5 mM **2** (triangles) at pH 7.5: black: without *Gm*SuSy, 5 mM **10**; dark grey: *Gm*SuSy, 5 mM **10**; light grey (dashed): *Gm*SuSy, 0.5 mM **9**; (a) *Pc*OGT forms **5** (squares) and an unknown compound (circles). (b) *Os*CGT I121D only produces **6** (diamonds).

higher in GT-*Gm*SuSy cascade reactions that contained external **10**. Benefit of coupling the synthetic glucosylation to conversion of **8** was again manifested primarily in the final product concentration, which was enhanced by 12% (**5**) and 7% (**6**), respectively in the SuSy-GT cascade reaction as compared to the single GT reference reaction. Product yields in coupled enzyme conversions of **2** were 88% or higher. The STYs of glucosylations of **2** were 5.5 (*Os*CGT variant) and 8 times (*Pc*OGT) lower than those for glucosylation of **1**.

Product isolation and NMR spectroscopic characterization

The two products synthesised by glucosylation of 5 mM **2** were isolated from 5 mL reaction mixtures using preparative reversed phase HPLC. **5** and **6** were obtained as white powder after freeze-drying and recovered in ≥80% yield. Identities of **5** and **6** were unambiguously assigned from results of a detailed NMR spectroscopic characterization that involved besides ^{1}H and ^{13}C NMR, HMQC (only **5**), COSY and HMBC. Results are summarized in Table S1 and Scheme S1 (ESI†). Although separation was achieved, concentrations of the by-product of **2** glucosylation by *Pc*OGT were too low for NMR analysis. Davidioside (**5**) and confusoside (**6**) were recovered from glucosylation of **2** by *Pc*OGT and *Os*CGT variant as single compounds at purities >98% based on HPLC peak areas (ESI,† Fig. S4).

Conclusions

Glucosyltransferases catalysing highly regiospecific β-D-glucosyltransfer from UDP-glucose (**10**) to the 2′-OH of the dihydrochalcones **1** and **2**, and to the 4′-OH of **2** were identified. So far no glycosyltransferase for selective synthesis of trilobatin (**4**) by glucosylation of the 4′-OH of **1** was found, but it presumably exists in *Malus trilobata* which only forms **4** but not **3**.[30] Maximum glucoside product concentrations were mainly restricted by aqueous solubility of the comparably hydrophobic dihydrochalcone acceptors. Optimised use of co-solvent,[58] running the reaction in an aqueous-organic two-phase system[59] and feeding the acceptor substrate in accordance to reaction progress[52] are all viable strategies to enhance the effective acceptor concentration in the reaction. However, detailed reaction engineering for targeted "de-bottlenecking" of an enzymatic production was beyond the scope of this study. Alternative isolation of single flavonoids from complex mixtures of phenolic compounds in plant materials involves time consuming multi-step procedures and large quantities of organic solvents. With the notable exception of highly abundant **3**, no yields above 5 mg g^{-1} dry weight were reported for extraction of **4**–**6** from plant leaves.[30–32,36] Due to simplification of work-up typical yields of the herein described GT-SuSy cascade reactions of around 2 g L^{-1} (~4.5 mM) seem sufficient to compete with extraction from natural sources. Furthermore, natural availability is no longer a bottleneck on supply of aglycons **1** and **2** because they are readily obtained by high yielding single step Friedel–Crafts acylation.[60] A two-enzyme one-pot glucosyl transfer cascade for convenient synthesis of the corresponding dihydrochalcone β-D-glucosides (**3**, **5**, **6**) was developed. Coupling of the synthetic enzymatic glucosyl transfer to conversion of **8** and **9** catalysed by SuSy presents a generally applicable strategy to provide UDP-glucose (**10**) donor substrate in a cost-effective manner and to cope with issues of unfavourable thermodynamics and UDP (**9**) product inhibition. It can be applied as a universal approach for green enzymatic synthesis of flavonoid glucosides.

Experimental

Materials

Unless otherwise indicated, all chemicals were from Sigma-Aldrich (Vienna, Austria) in the highest purity available. Phloretin (98%) was from AK Scientific (Union City, CA, US) and phlorizin dihydrate (≥98%), from Carl Roth (Karlsruhe, Germany). *Strep*-Tactin® Sepharose® and desthiobiotin were

from IBA (Goettingen, Germany). BCA Protein Assay Kit was from Thermo Scientific (Waltham, MA, US).

Synthesis of davidigenin (2)

Synthesis of **2** was done in a single step from 1,3-benzenediol (resorcinol) and 3-(4-hydroxyphenyl)propanoic acid (phloretic acid) using an adopted Friedel–Crafts acylation method.[60] Phloretic acid (6 mmol, 990 mg) and resorcinol (6 mmol, 661 mg) were stirred in 4 mL BF_3Et_2O (32 mmol) at 90 °C for 120 min under argon. Complete conversion of resorcinol was verified by TLC. The mixture was poured into 200 mL 10% aqueous NaOAc and stirred for 2 h at room temperature. The solution was 3 times extracted with 200 mL EtOAc. Combined EtOAc was washed with 40 mL water and 40 mL brine before drying over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was chromatographed over silica gel column using cyclohexane–EtOAc mixtures. **2** (61% isolated yield) was confirmed by ^{1}H- and ^{13}C-NMR spectroscopy (Bruker AVANCE III 300 spectrometer).

Enzyme production

Escherichia coli BL21-Gold (DE3) expression strains for *Pc*OGT (UGT88F2; GenBank: FJ854496),[53] *Os*CGT (GenBank: FM179712) Ile^{121} to Asp mutant[53] and *Gm*SuSy (GenBank: AF030231)[52] were described elsewhere in detail. Enzyme expression in lysogeny broth (LB)-medium as N-terminal *Strep*-tag II fusion proteins and enzyme purification by affinity chromatography on *Strep*-Tactin® Sepharose® columns was also reported elsewhere.[52] Expected molecular mass and purity of enzymes were affirmed by SDS polyacrylamide gel electrophoresis (PAGE). Aliquots of final preparations were stored at −70 °C and thawed only once prior to their use.

Activity assays

Sucrose cleavage by *Gm*SuSy was measured spectrophotometrically using a discontinuous two-step enzymatic assay. Oxidation of **10**, formed from **8** (100 mM) and **9** (0.5 mM), to UDP-α-D-glucuronic acid is coupled to reduction of two NAD^+ molecules to NADH by human UDP-α-D-glucose 6-dehydrogenase (hUGDH) as described elsewhere in detail.[52] In short, reactions were started by *Gm*SuSy addition and samples of 150 μL were stopped by heating (95 °C, 5 min). Precipitated protein was removed by centrifugation (13 200 rpm, 20 min) and 100 μL of the supernatant were mixed with 400 μL of measuring solution (2.5 mM NAD^+, 0.05% Triton™ X-100 in 100 mM HEPES, pH 8.0) in a Half Micro Cuvette. Absorbance at 340 nm was measured before and after incubation with 1.5 mU of hUGDH and concentration of **10** was calculated from the increase of absorbance (ε_{NADH} = 6220 M^{-1} cm^{-1}). Typically 4 distinct measurements were used to calculate linear initial rates. One unit of *Gm*SuSy was defined as the amount of enzyme producing 1 μmol **10** (2 μmol NADH) per minute under following conditions: 30 °C, pH of cascade reactions (6.5 or 7.5) 0.5 mM **9**, 100 mM **8** in 50 mM BisTris, 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% DMSO.

An reversed phase HPLC-based assay for quantification of dihydrochalcones (**1**, **2**) and their glycosides (**3**–**6**) was used to determine activities of *Pc*OGT and the *Os*CGT variant.[53] In short, conversions of 5 mM dihydrochalcone with 0.5 mM **10** were started by GT addition and conducted under agitation (400 rpm) and temperature control (30 °C) using a thermomixer. Typically 4 aliquots of 100 μL were taken within 60 min to determine linear initial rates. Reactions were stopped by mixing with 100 μL acetonitrile. Precipitated protein was removed by centrifugation (13 200 rpm, 20 min) before applying 5–10 μL of supernatant to HPLC analysis. An Agilent 1200 HPLC equipped with a Chromolith® Performance RP-18e column (100 × 4.6 mm) was used at 35 °C, and UV-detection at 288 nm was applied. A water (A) to acetonitrile (B) gradient (0.1% trifluoroacetic acid each) was applied for elution: 20–47.5% B (7.5 min, 1 mL min^{-1}), 47.5–100% B (0.05 min, 1 mL min^{-1}), 100% B (1.45 min, 1.5 mL min^{-1}), 100–20% B (0.05 min, 1.5 mL min^{-1}), 20% B (2.45 min, 1.5 mL min^{-1}). One unit of GT activity was defined as the amount of enzyme glucosylating 1 μmol **1** per min under following conditions: 30 °C, 5 mM **1**, 0.5 mM **10** in 50 mM BisTris, pH 6.5 (*Pc*OGT) or 7.5 (*Os*CGT I121D) containing 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% DMSO.

Equilibrium constant (K_{eq}) of glucosylation of 1 by *Pc*OGT

K_{eq} of *Pc*OGT was determined by running glucosylation of **1** in forward (5 mM **1**, 5 mM **10**) and reverse direction (5 mM **3**, 5 mM **9**) until no further conversion was observed. Reactions were run at 30 °C and started by addition of 100 mU mL^{-1} *Pc*OGT (50 mM BisTris, pH 6.5, 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% DMSO). Concentrations of **1** and **3** were determined by the HPLC based GT activity assay.

The same samples (10 μL) were applied for quantification of **9** and **10** using an anion exchange HPLC protocol. An Agilent 1200 HPLC system was used for separation on an Agilent ZORBAX SAX (4.6 × 250 mm) column at 30 °C. **9** and **10** were monitored by UV detection at 254 nm. Using 20 and 500 mM potassium phosphate buffer (pH 6.8) as solvent A and B, respectively following gradient was applied at a constant flow rate of 1.5 mL min^{-1}: 0–100% B (7 min), 100% B (2 min), 100–0% B (0.05 min), 0% B (3.95 min).

Formation of 3, 5 and 6 by direct GT and GT-*Gm*SuSy cascade reactions

5 mM **1** and **2** were converted in 50 mM BisTris at pH 6.5 and 7.5, respectively. All reactions contained 13 mM $MgCl_2$, 50 mM KCl, 0.13% BSA and 20% DMSO. As glucose source conversions without *Gm*SuSy contained 5 mM **10** while for those with *Gm*SuSy either 5 mM **10** and 100 mM **8** or 0.5 mM **9** and 100 mM **8** were used. Conversions were started by addition of 100 mU mL^{-1} *Pc*OGT (and 100 mU mL^{-1} *Gm*SuSy) or 40 mU mL^{-1} *Os*CGT I121D (and 40 mU mL^{-1} *Gm*SuSy). Samples were incubated at 30 °C and the GT HPLC protocol was applied for sampling after distinct incubation times and quantification of **1**, **2**, **3**, **5** and **6**.

Coupled glucosylation of 5 mM **1** by *Pc*OGT and *Gm*SuSy (100 mU mL^{-1} each) in presence of 0.5 mM **9** and 100 mM **8** was studied at various pH as described for standard conditions. Buffers were prepared in steps of 0.5 pH units (BisTris pH 5.5–7.5, TAPS pH 7.5–8.5) and added to a final concentration of 50 mM. Actual pH in conversions was calculated as the average of measurements in beginning and at the end of conversions.

Isolation and identification of 5 and 6

5 and **6** from glucosylation of **2** by *Pc*OGT and *Os*CGT I121D mutant were purified by preparative reversed phase C-18 HPLC on an Agilent 1200 system equipped with a SphereClone 5 μm ODS(2) (250 × 10.0 mm) column. Water was used as solvent A and acetonitrile as solvent B (0.1% formic acid each). Separation of **5** and **6** from other compounds was achieved by step gradients from 10% to 100% B at room temperature. **5** eluted at 25% and **6** at 35% B. After removing acetonitrile under reduced pressure water was removed by freeze drying. Purity and identity were confirmed by HPLC and NMR. ^{1}H-NMR, ^{13}C-NMR, COSY, HMBC and HMQC (**5** only) were recorded on a Varian Unity Inova 500 MHz spectrometer.

Acknowledgements

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Electronic Supplementary Material (ESI) for Green Chemistry.

Electronic Supplementary Information

Towards the synthesis of glycosylated dihydrochalcone natural products using glycosyltransferase-catalysed cascade reactions

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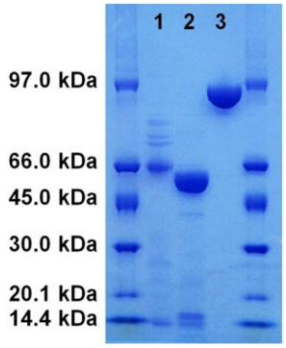


Fig. S1 SDS-PAGE of enzymes from *E. coli* overexpression cultures purified by *Strep*-tag affinity chromatography; lane 1: *Pc*OGT (55.4 kDa), lane 2: *Os*CGT I121D (51.3 kDa), lane 3: *Gm*SuSy (94.1 kDa)

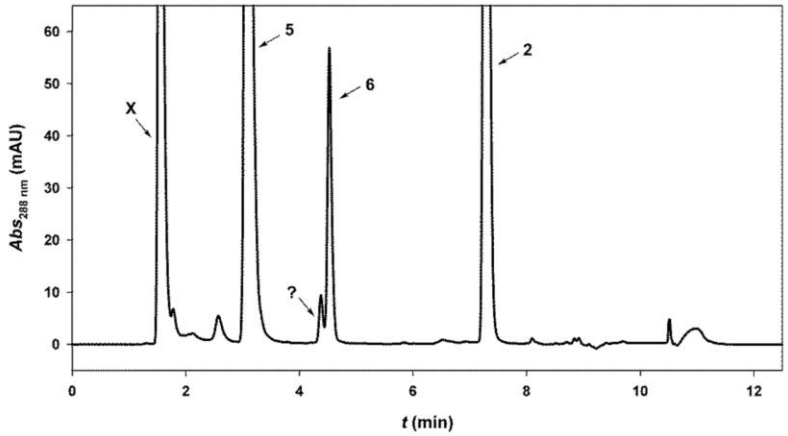


Fig. S2 Reversed-phase C-18 HPLC-analysis of a mixture of glucosylations of **2** by *Pc*OGT and *Os*CGT I121D clearly shows that the minor product of the *Pc*OGT reaction (**?**) is distinct from confusoside (**6**), formed by the *Os*CGT variant.

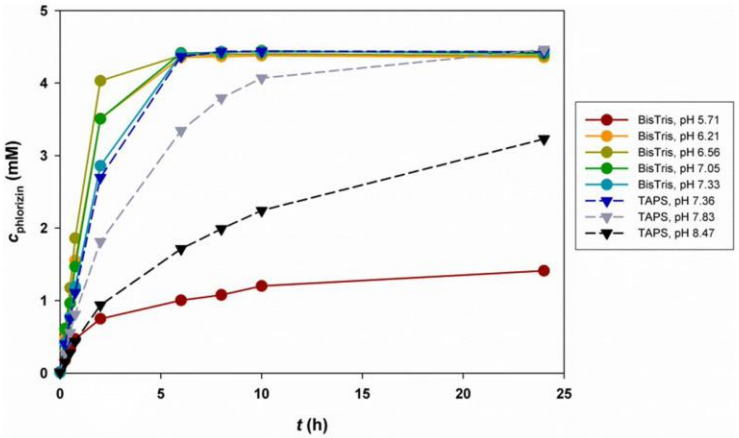


Fig. S3 Time courses of **3** formation through glucosylation of 5 mM **1** by coupled *Pc*OGT-*Gm*SuSy reaction (0.5 mM **9**, 100 mM **8**) using BisTris and TAPS reaction buffers at various pH.

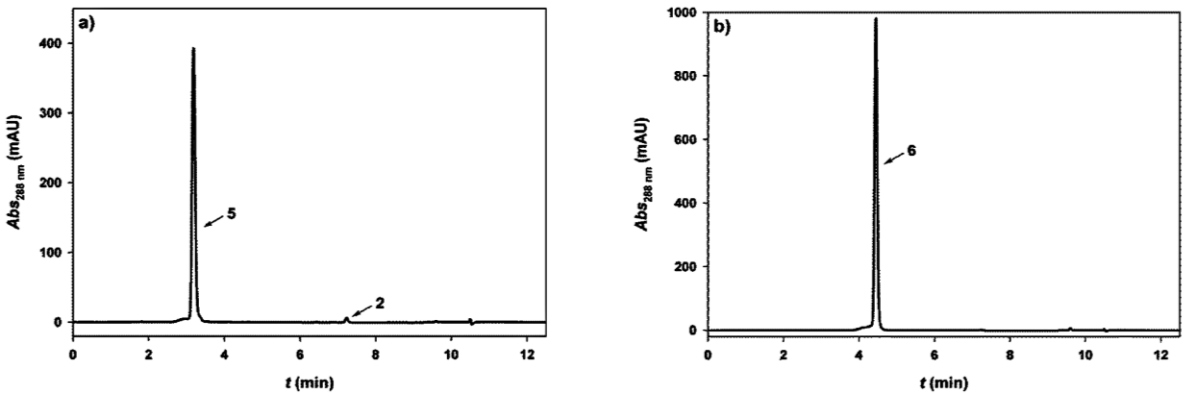
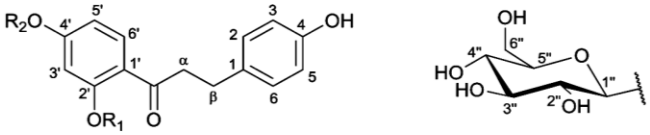


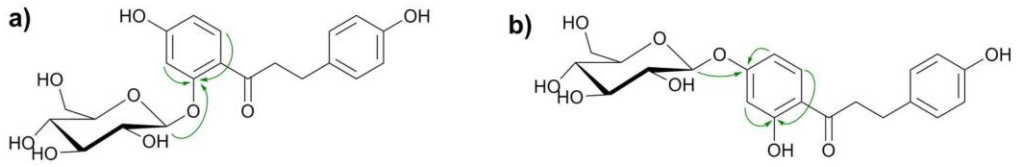
Fig. S4 Reversed-phase C-18 HPLC-analysis of (a) davidioside (**5**) and (b) confusoside (**6**) after purification by preparative HPLC confirms them to be of high purity (>98% based on HPLC peak area).

Table S1 ^{1}H and ^{13}C-NMR spectral data of davidigenin (**2**), davidioside (**5**) and confusoside (**6**)



nr	davidigenin (2) (R_1, R_2 = H)[a]		davidioside (5) (R_1 = glucose, R_2 = H)[b]		confusoside (6) (R_1 = H, R_2 = glucose)[b]	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	133.0		133.8		133.4	
2 / 6	129.2	7.07 (2H, d, *J* = 8.5 Hz)	130.3	7.03 (2H, d, *J* = 8.4 Hz)	130.4	7.05 (2H, d, *J* = 8.4 Hz)
3 / 5	115.0	6.68 (2H, d, *J* = 8.5 Hz)	116.1	6.67 (2H, d, *J* = 8.2 Hz)	116.2	6.69 (2H, d, *J* = 8.4 Hz)
4	155.5	9.17 (1H, s)	156.4		156.7	
C=O	203.9		202.9		206.2	
α	39.4	3.22 (2H, t, *J* = 7.6 Hz)	46.2	~3.3[c]	41.2	3.23 (2H, t, *J* = 7.3 Hz)
β	29.1	2.83 (2H, t, *J* = 7.4 Hz)	31.0	2.86 (2H, t, *J* = 7.5 Hz)	30.9	2.92 (2H, t, *J* = 7.3 Hz)
1'	112.5		121.9		116.0	
2'	164.7	10.62 (1H, s)	160.3		165.8	
3'	102.4	6.26 (1H, d, *J* = 2.2 Hz)	103.9	6.70 (1H, d, *J* = 2.0 Hz)	105.1	6.58 (1H, d, *J* = 2.2 Hz)
4'	164.3	12.65 (1H, s)	164.4		165.1	
5'	108.1	6.37 (1H, dd, *J* = 8.74, 2.3 Hz)	110.8	6.50 (1H, dd, *J* = 8.5, 1.9 Hz)	109.4	6.62 (1H, dd, *J* = 8.9, 2.3 Hz)
6'	131.0	7.81 (1H, d, *J* = 8.8 Hz)	133.2	7.58 (1H, d, *J* = 8.6 Hz)	133.1	7.81 (1H, d, *J* = 8.9 Hz)
1"			102.6	4.99 (1H, d, *J* = 7.1 Hz)	101.3	5.00 (1H, d, *J* = 7.2 Hz)
2"			74.9		74.7	
3"			78.3	3.33-3.48-(4H, unresolved)	77.9	3.45-3.50 (3H, unresolved)
4"			71.2		71.2	
5"			78.4		78.3	3.41 (1H, m)
6"			62.6	3.91 (1H, dd, *J* = 12.4, 2.0 Hz) 3.72 (1H, dd, *J* = 12.1, 5.7 Hz)	62.4	3.89 (1H, dd, *J* = 12.1, 2.1 Hz) 3.70 (1H, dd, *J* = 12.3, 5.5 Hz)

[a] ^{1}H: 300.36 MHz, ^{13}C: 75.53 MHz; (DMSO-d_6, δ in ppm)
[b] ^{1}H: 499.89 MHz, ^{13}C: 125.70 MHz; (CD_3OD, δ in ppm)
[c] overlap with MeOH signal



Scheme S1 Key HMBC couplings to identify (a) davidioside (**5**) and (b) confusoside (**6**), respectively

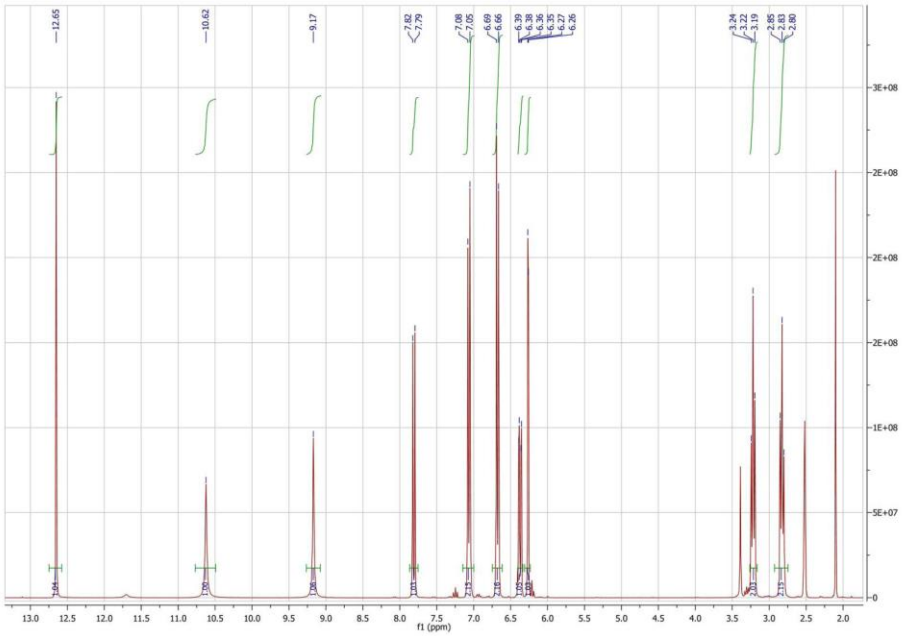


Fig. S5 ^{1}H-NMR of davidigenin (**2**)

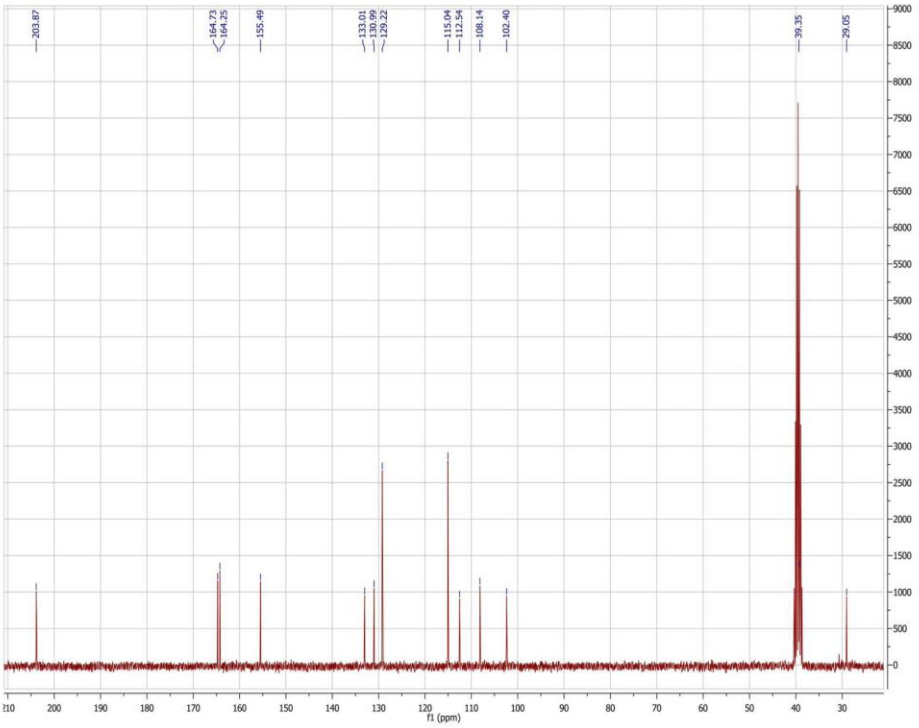


Fig. S6 ^{13}C-NMR of davidigenin (**2**)

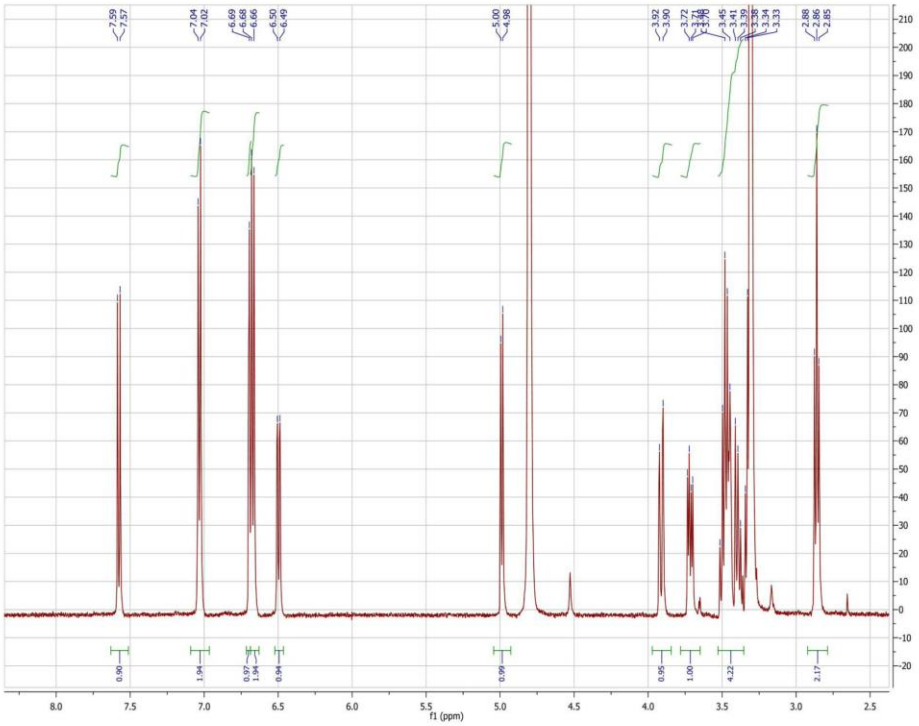


Fig. S7 ^{1}H-NMR of HPLC purified davidioside (**5**)

5

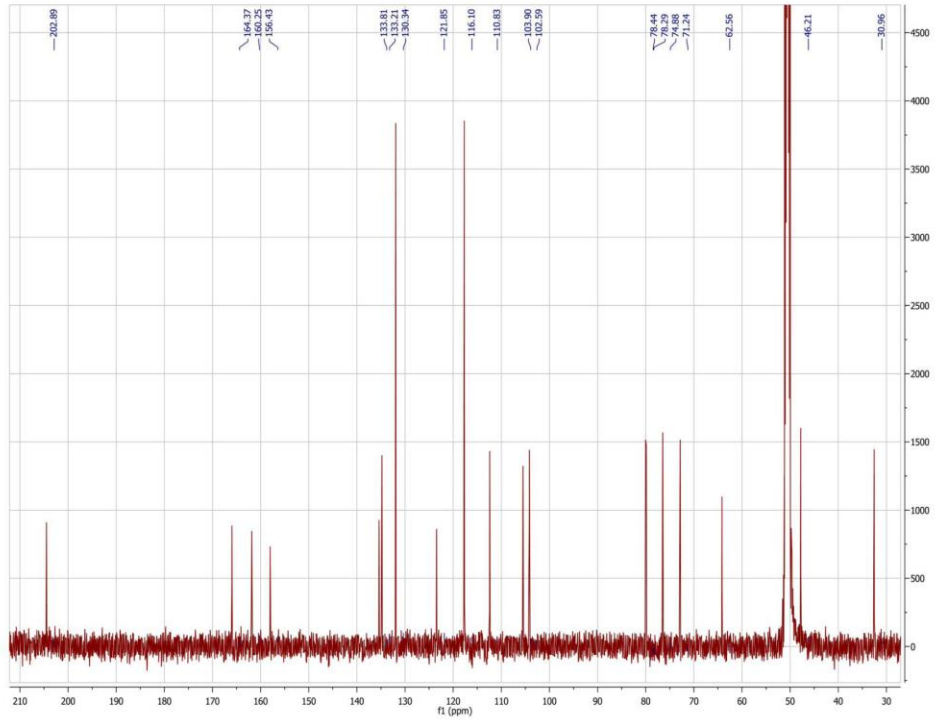


Fig. S8 ^{13}C-NMR of HPLC purified davidioside (**5**)

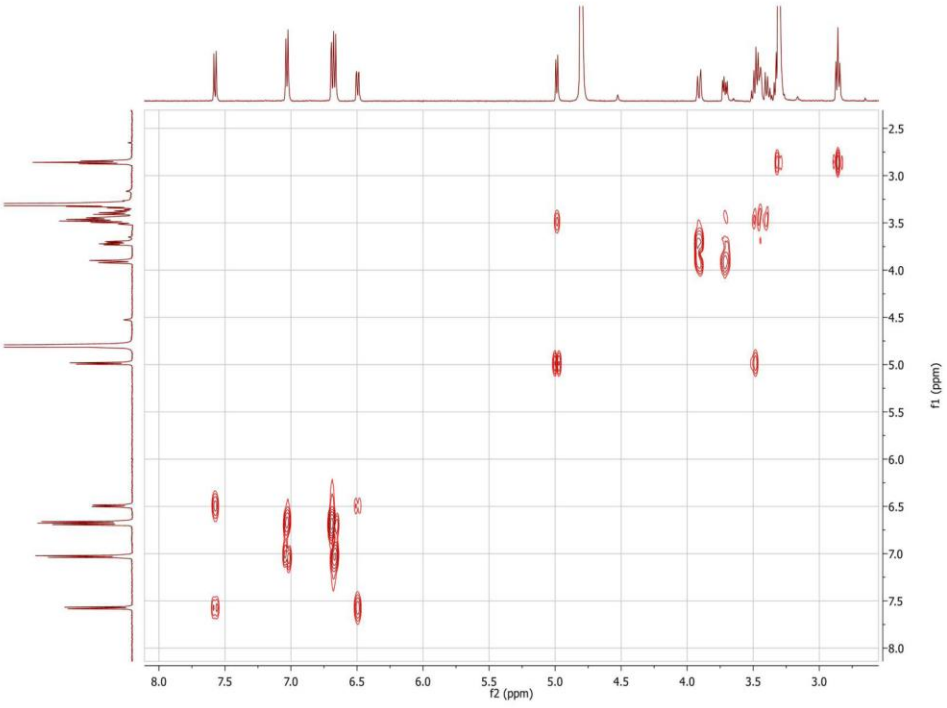


Fig. S9 2D COSY-NMR of HPLC purified davidioside (**5**)

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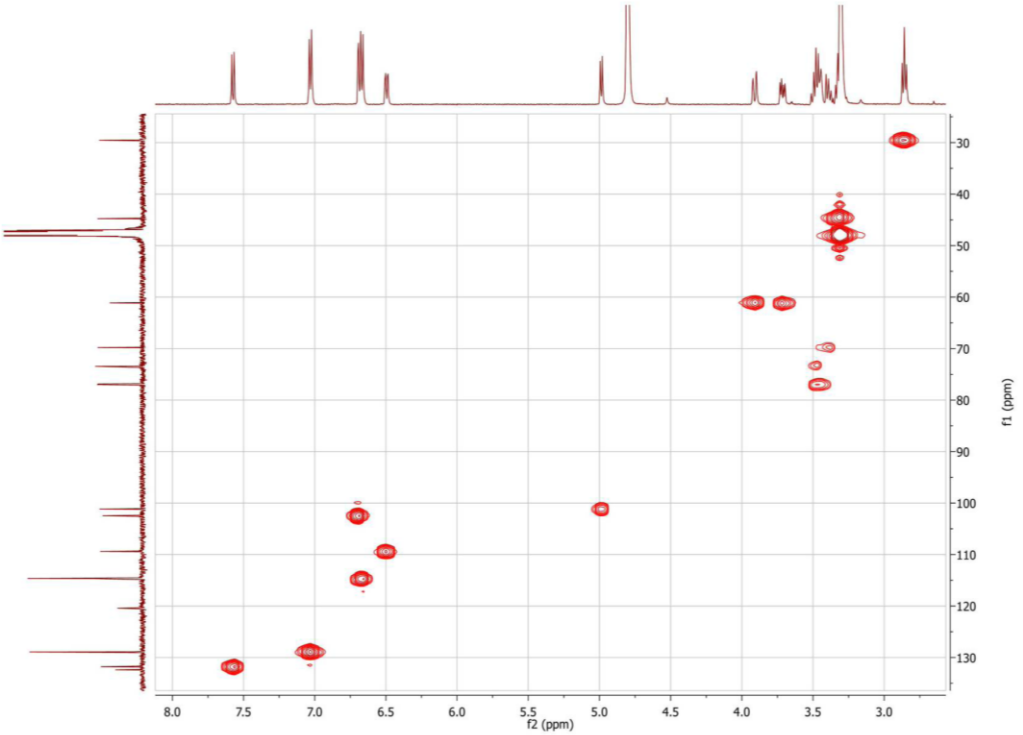

Fig. S10 2D HMQC-NMR of HPLC purified davidioside (**5**)

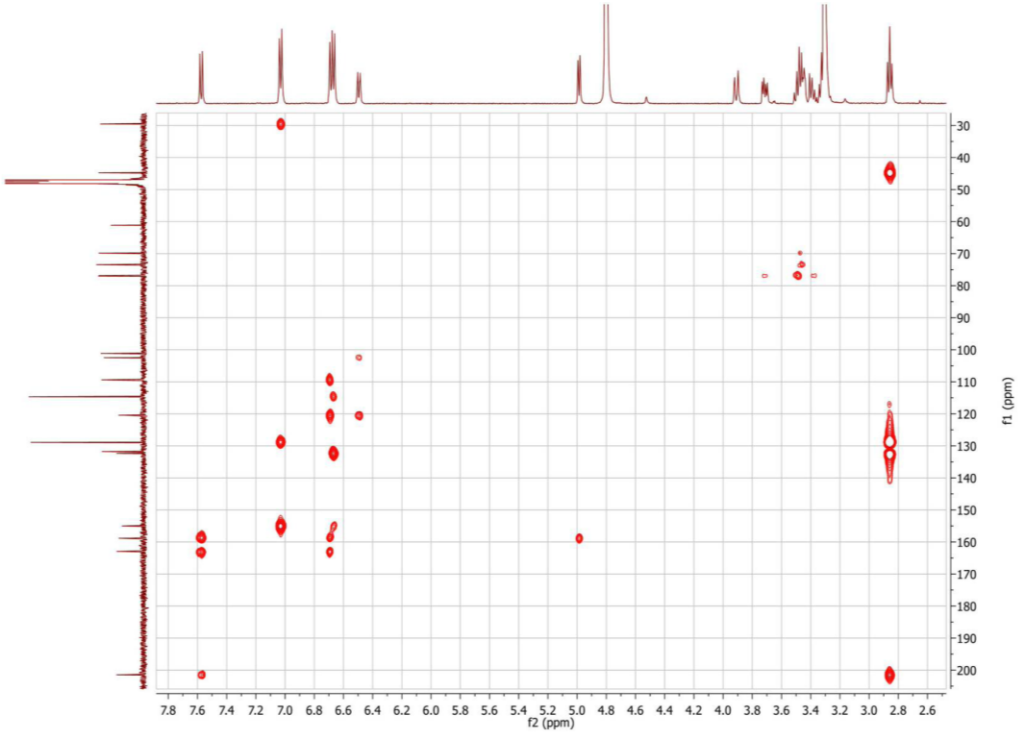

Fig. S11 2D HMBC-NMR of HPLC purified davidioside (**5**)

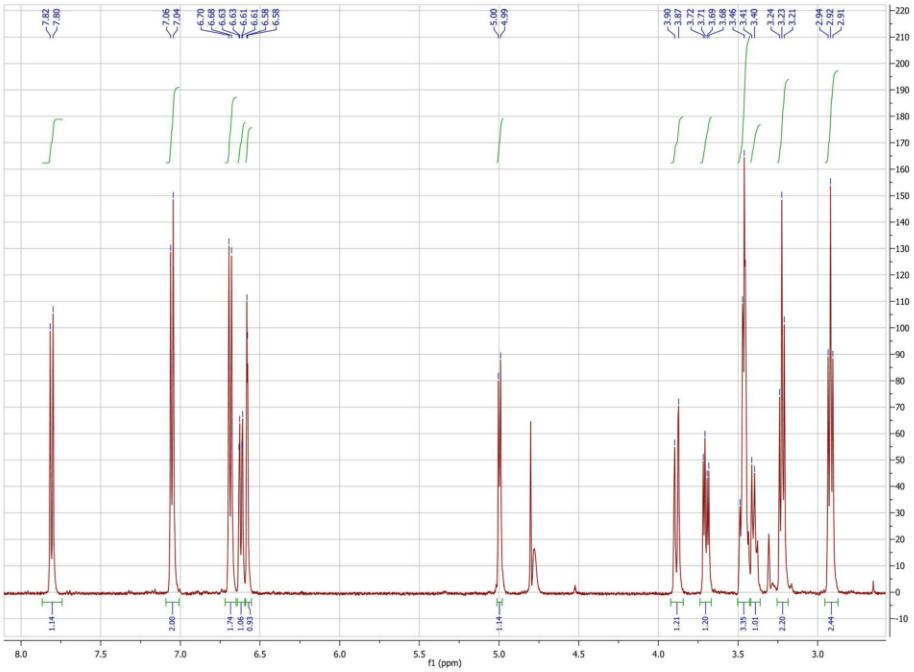

Fig. S12 ^{1}H-NMR of HPLC purified confusoside (**6**)

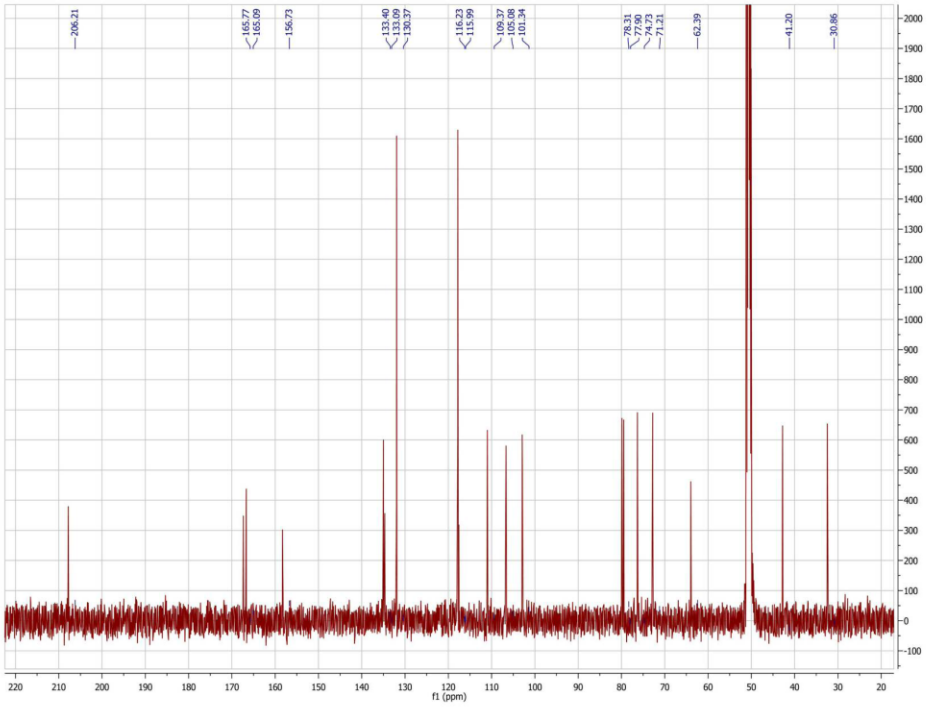

Fig. S13 ^{13}C-NMR of HPLC purified confusoside (**6**)

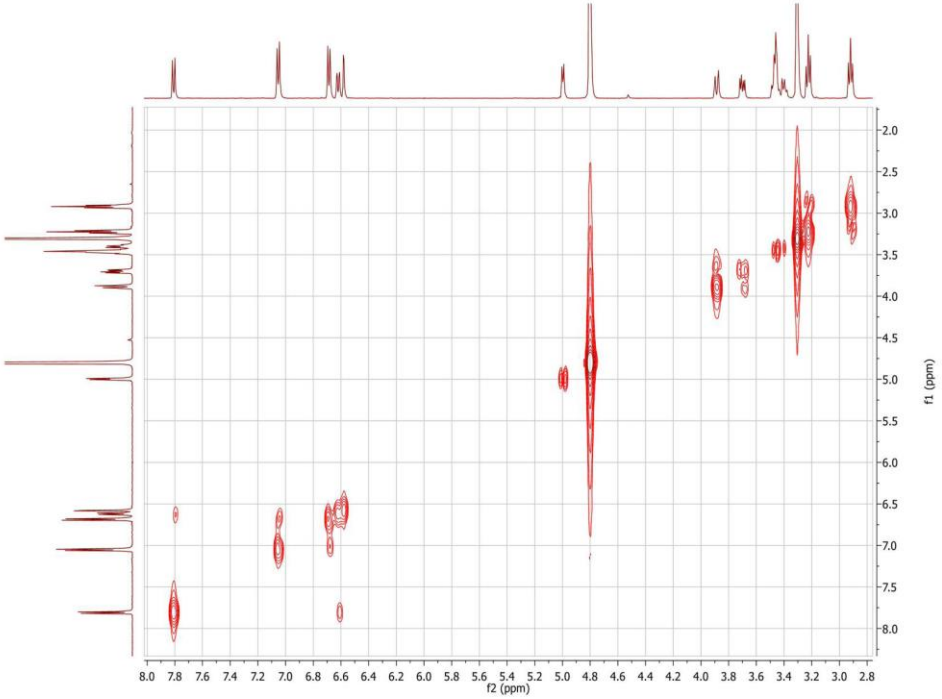

Fig. S14 2D COSY-NMR of HPLC purified confusoside (**6**)

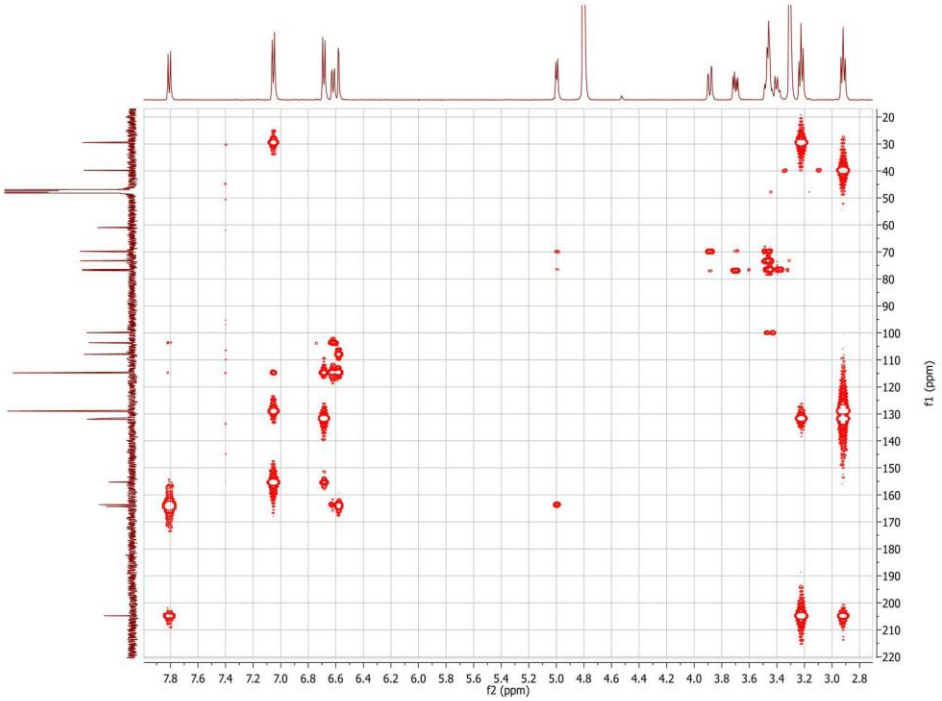

Fig. S15 2D HMBC-NMR of HPLC purified confusoside (**6**)

β-Cyclodextrin improves solubility and enzymatic *C*-glucosylation of the flavonoid phloretin

UPDATE

β-Cyclodextrin Improves Solubility and Enzymatic *C*-Glucosylation of the Flavonoid Phloretin

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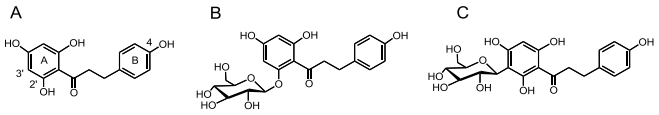
Abstract: Biocatalytic method for synthesis of the *C*-glucoside nothofagin was established by using β-cyclodextrin (β-CD), which enhanced the aqueous solubility of substrate phloretin. The phloretin/β-CD inclusion complex (1:1) was prepared by a freeze-drying method. The solubility of phloretin/β-CD complex at 30 °C was 41-fold greater than that of free phloretin. Using β-CD instead of organic cosolvent (DMSO), overall catalytic efficiency by single enzymes and the coupling enzyme cascade reactions for transformation of phloretin was increased. Conversion of phloretin/β-CD complex (50 mM phloretin) was performed without further feeding of phloretin and enzymes. High nothofagin concentrations (44 mM) and very good yield (≥ 98%) were obtained in aqueous solutions. Additionally**,** only 0.5 mM UDP were applied and UDP-glucose was therefore recycled 88 times (RC_{max} = 88). Furthermore, 66 mM nothofagin were obtained and RC_{max} was up to 132 when 100 mM phloretin were applied. Nothofagin was obtained from preparative HPLC purification with very high purity (≥ 99%) and good yield (78%). This simplified approach provides an environmentally clean process for biocatalytic synthesis of glycosylated natural products.

Keywords: carbohydrates; β-cyclodextrin; phloretin; *C*-glucoside; *C*-glycosyltransferase; sucrose synthase; UDP-glucose recycling

Flavonoids are a large group of plant derived natural products possessing antioxidative,[1] anticancer,[2] antitumor and antiviral properties.[3] These bioactivities aroused interest for application of flavonoids as ingredients in functional food and pharmaceuticals. However, their bioavailability is often limited by very poor water solubility. A commonly used strategy to circumvent solubility imposed restrictions is to decrease flavonoid hydrophobicity by glycosylation.[4]

Phloretin (Scheme 1A) is a prominent dihydrochalcone flavonoid in apple trees with potential applications in food and pharmaceutical products.[5] Phloretin is mainly found as its 2′-*O*-β-D-glucoside phlorizin (Scheme 1B) which has an improved water solubility. Furthermore the 3′-*C*-β-D-glucoside nothofagin (Scheme 1C) is known as a prominent antioxidant in rooibos tea. *C*-glucosides are generally very attractive pharmaceutical targets because they are resistant to acidic and enzymatic hydrolysis.[6] However *C*-glycosides are relatively rare in nature[7] and their chemical synthesis is challenging. Therefore, the development of biocatalytic *C*-glycoside synthesis is highly desired.

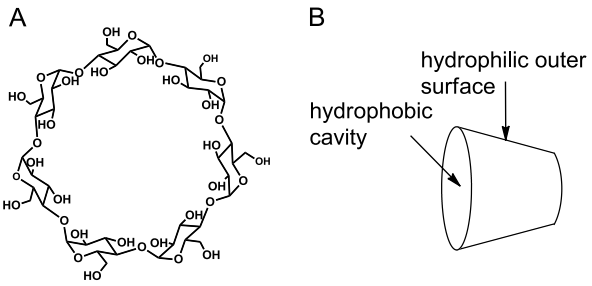


Scheme 1. Chemical structures of A) phloretin, B) phlorizin and C) nothofagin.

We have previously developed a method for selective biocatalytic synthesis of nothofagin by a *C*-glycosyltransferase from rice (*Oryza sativa*; *Os*CGT). Phloretin glycosylation was coupled in one-pot to recycling of the sugar donor uridine 5'-diphosphate (UDP)-glucose from sucrose using sucrose synthase from soybean (*Glycine max*; *Gm*SuSy).[8] Unfortunately the water solubility of phloretin is very low (< 0.5 mM) and an organic cosolvent (DMSO, 20% v/v) had to be added to achieve quantitative conversion of 5 mM phloretin. Further increase of phloretin concentrations in batch-conversions was again obviated by limited substrate solubility. Only by using 8 rounds of stepwise phloretin and enzyme feed we were able to boost nothofagin formation (44 mM). However, this fed-batch approach was inconvenient and time consuming (135 h). Since solubility improvements represent a main challenge for process intensification in natural product glycosylation reactions, the aim of the present study was to increase the phloretin solubility. For consequently improved enzymatic conversion conditions leading to reduced enzyme activity and stability had to be avoided.

Enzymatic conversions[9] and glycosylations[10] of poor water soluble compounds using cyclodextrins (CDs) as solubilizing agent have been reported. The ability of CDs in enzymatic oxidation of polynuclear aromatic hydrocarbons (PAHs) by lignin peroxidase was reported.[11] CDs are cyclic oligosaccharides, consisting of a number of α-1,4-linked D-glucopyranose units. The most common CDs are α-CD, β-CD (Scheme 2A) and γ-CD containing 6, 7 and 8 glucose units, respectively. CDs are shaped like a truncated cone (Scheme 2B) as a consequence of the chair conformation of glucopyranose units. It contains a hydrophobic cavity that entraps a hydrophobic molecule to form a host-guest complex. Due to hydroxy groups orientated on the outer surface of CDs, the formed complex becomes soluble in water.[12] The binding of a guest molecule within the host cyclodextrin is not permanent but rather dynamic. The complex formation, often a 1:1 interaction, is usually described by equation 1 (*Kc* = stability constant or binding constant), although higher order complexes are often seen.

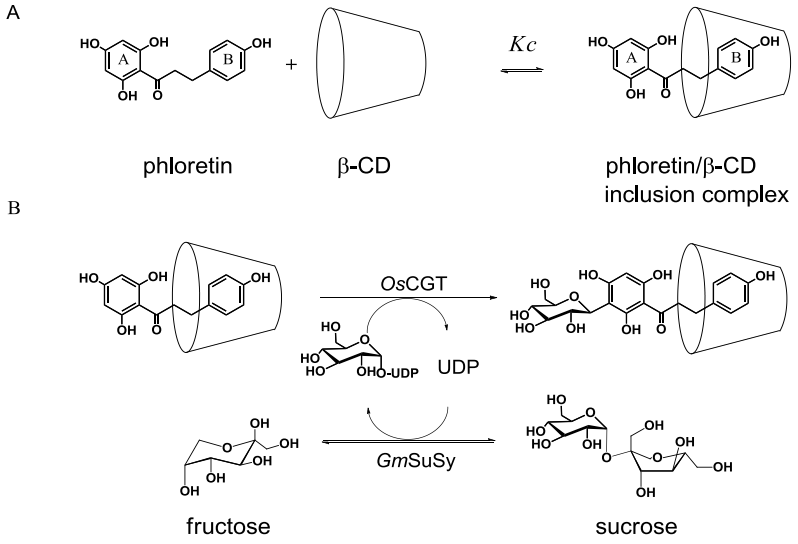
$$\text{guest}_{\text{free}} + \text{CD}_{\text{free}} \overset{Kc}{\rightleftharpoons} \text{guest/CD}_{\text{complex}} \quad (1)$$



Scheme 2. A) Chemical structure of β-CD. B) Schematic drawing of CD truncated cone shape.

Inclusion in CD is a convenient method to solve the solubility related limitations of poorly water soluble flavonoids. β-CD is most widely used due to its low cost, excellent complexing abilities, specific properties and higher productive rate.[13] Complex formation studies of dihydrochalcones, and dihydrochalcone glucosides with β-CD have been reported.[14] Characterization of the complexes revealed that the terminal phenol ring (aromatic ring B) of the dihydrochalcone was inserted into the hydrophobic cavity of β-CD. The interaction between β-CD and phlorizin was investigated in a detailed NMR study.[15] The complex was hypothesized having the phenol ring and propanol group included in the β-CD from its entrance leaving the sugar unit outside the β-CD cavity. Two inclusion possibilities were suggested using either the large or the small rim as an entrance of the inclusion. Using phlorizin as a model structure, schematic drawing of inclusion complex of phloretin/β-CD and nothofagin/β-CD are shown in Scheme 3A. Herein, we present a system consisting of one-pot conversion of a phloretin inclusion complex with β-CD for synthesis of nothofagin. We coupled *Os*CGT with *Gm*SuSy in the presence of catalytic amounts of UDP (Scheme 3B).

To find optimal conversion conditions we first determined phloretin solubility at various conditions then examined their influence on enzyme activity and stability. We used β-CD for phloretin solubility enhancement. However, β-CD shows low water solubility (18.5 g L^{-1} at 25 °C).[16] This is limiting the investigation of forming inclusion complex. It has been reported that the use



Scheme 3. A) Complex formation of phloretin with β-CD (1:1). B) Biocatalytic synthesis of nothofagin with UDP-glucose regeneration process catalyzed by *Os*CGT and *Gm*SuSy in the presence of catalytic amounts of UDP.

of cosolvents in the formation of CD inclusion complexes can improve the solubility of CD.[16b] Solubility measurements of β-CD were done in DMSO (0-50%) at 30 °C (Supporting Information, Methods). The results show that β-CD solubility increased at elevated DMSO concentration (Figure S1). While β-CD solubility was only slowly increasing from 0 % to 40% DMSO (25-44 mM) it almost doubled when DMSO was increased from 40% to 50%. Furthermore, solubility measurements were performed using various concentrations of DMSO (0-100%) in absence and presence of β-CD to identify maximum phloretin concentrations applicable in batch conversions (Supporting Information, Methods). Phloretin solubility increased with increasing DMSO content up to ~1.5 M at 80% DMSO (Figure S2; triangle). In presence of excess amounts of β-CD the phloretin solubility was largely increased at conditions of rather low phloretin solubility (≤ 40% DMSO). At increased DMSO concentrations the already high concentration of dissolved phloretin could not be further boosted (Figure S2; circle).

After determining phloretin solubility we examined enzymatic activities under these conditions. Assuming that high concentration of DMSO effect enzyme activities, the influence of DMSO was tested in conversions of 5 mM phloretin (Supporting Information, Methods). Considering that previously reported conversion experiments were performed in 20% DMSO, we used it as a control condition for comparison of reactions at 30-90% DMSO. Besides single enzyme conversions using *Os*CGT also coupled *Os*CGT-SuSy cascade reactions were tested (Supporting Information, Methods). The results (Figure 1A) indicated that DMSO up to 50% caused only minor effects on *Os*CGT activity while 60% DMSO reduced the nothofagin production around 46%. The *Os*CGT activity was completely lost at 70% DMSO. *Gm*SuSy displayed a lower cosolvent tolerance and activity in the coupling reaction was completely lost in the presence of 50% DMSO (Figure 1B). Furthermore, the initial rate in 30% DMSO was lower than in 20% DMSO. We concluded that synthesis of nothofagin by a coupled *Os*CGT-*Gm*SuSy reaction was limited to DMSO concentrations of 30% or lower which corresponds to a maximum phloretin concentration of 25 mM (see Figure S2, triangle). We considered using β-CD-DMSO mixtures for phloretin solubility enhancement and enzymatic conversion of phloretin. Due to the results of solubility measurements and enzymatic activity assay, we thus fixed the maximum concentration of DMSO to 20% for further experiments.

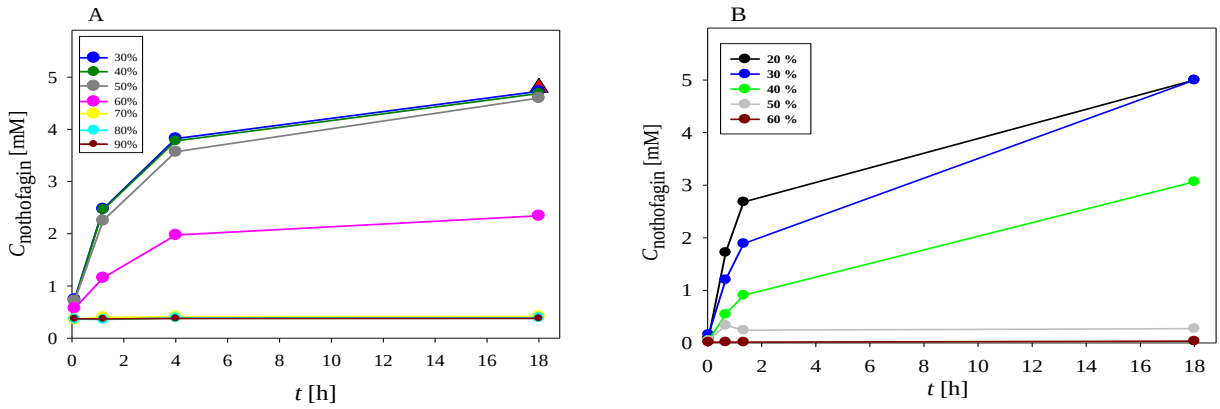


Figure 1. Time course analyses of nothofagin production at 30 °C at various DMSO concentrations catalyzed by; A) 200 mU mL^{-1} *Os*CGT using 5 mM phloretin, 7.5 mM UDP-glucose and standard reaction mixture at pH 8.5 (reactions were performed in 20% DMSO for 2 min, aliquoted to 30- 90% DMSO and incubated for 18 h. A control sample in 20% DMSO was measured at 18 h (red triangle); B) 200 mU mL $^{-1}$ *Os*CGT coupling with 200 mU mL^{-1} *Gm*SuSy using 5 mM phloretin, 0.5 mM UDP and 300 mM sucrose in standard reaction mixtures at pH 7.5.

Complexation of flavonoid with β-CD has been reported. Quercetin forms a 1:1 molar ratio inclusion complex with β-CD.[17] Hesperetin, hesperidin, naringenin and naringin also form 1:1 complexes.[18] So far there are no reports about solubility studies of phloretin with CDs. A phase solubility study according to Higuchi and Connors[19] was made to analyze the phloretin/β-CD complex formation (Supporting Information, Methods). The stability constant (K_c) from phase solubility studies is a useful index to estimate the binding strength of a guest-host complex and changes in the physicochemical properties of a guest in a CD-complex.[19] A phase solubility diagram of phloretin in aqueous β-CD solution in HEPES buffer pH 7.5 at 30 °C is shown in Figure S3 (circle). It was obtained by plotting the changes in phloretin solubility as a function of β-CD concentration. The solubility of phloretin increased linearly, a feature of the A_L-type complex,[19] Assuming the phloretin/β-CD complex is of 1:1 binding mode (see Scheme 3A). The apparent K_c was calculated from the initial linear increase of the solubility curves using following equation: K_c = slope/ $S_0 \times$ (1 - slope), where S_0 is the intrinsic solubility of phloretin in HEPES buffer pH 7.5 without β-CD and slope means the corresponding slope of the phase solubility diagram. The value of K_c was found to be 1534 M^{-1} (Table S1). The K_c value for quercetin was 402 M^{-1}.[17c] Therefore, the complexation ability of phloretin is greater than quercetin. The solubility of phloretin in HEPES buffer pH 7.5 at 30°C in the presence of 20 mM β-CD was 8.5 mM and 21 times greater than that of free phloretin (Table 1). We also performed a phase solubility study in aqueous β-CD solution in the presence of 20% DMSO (Figure S3, triangle). DMSO increases aqueous solubility of phloretin (Table 1). It can therefore enhance the apparent S_0 (Table S1). The phloretin solubility in 20% DMSO was around 2-fold greater than without DMSO.

Table 1. Phloretin solubility.[a]

Phloretin type	Solubility [mM]	Relative solubility
Free phloretin	0.4	1
Phloretin/β-CD		
β-CD[b]	8.5	21
β-CD and DMSO[c]	17.8	45
inclusion complex[d]	16.4	41
physical mixture[d]	12.3	31

[a] HEPES buffer pH 7.5 at 30 °C
[b] 20 mM β-CD
[c] 20 mM β-CD and 20% DMSO
[d] phloretin/β-CD solid powder (1:1 molar ratio)

The reaction of *Os*CGT was monitored with an HPLC assay (Supporting Information, Methods). To determine the effect of β-CD on HPLC measurements we prepared mixtures of phloretin (0.2 mM) with β-CD (0-10 mM). We tested that applied β-CD concentrations did not affect peak area and retention time of phloretin in HPLC measurements (Table S2). The effect of β-CD on enzymatic synthesis of nothofagin was investigated. We observed that *Os*CGT activity was increased (18 %) when increasing β-CD concentration from 0 mM to 10 mM (Figure S4).

Considering that solubility of phloretin with 20 mM β-CD in the presence of 20 % DMSO was enhanced around 2-fold (see Table 1), we tested effect of DMSO and β-CD on the individual enzymatic activity and stability. Table 2 presents a summary of conditions and results. Using β-CD the *Gm*SuSy activity was 4-fold higher than with DMSO. *Gm*SuSy was inhibited by DMSO even when β-CD was mixed with DMSO. The remaining activity after 4 h of *Os*CGT and *Gm*SuSy in the β-CD system was significantly higher than with DMSO system.

Activities of *Os*CGT with and without coupling to *Gm*SuSy were studied in 20 mM β-CD or 20 % DMSO. First, activities of *Os*CGT were measured at different phloretin concentrations in standard conditions at pH 8.5 (the pH optimum of *Os*CGT). With β-CD specific *Os*CGT activities were higher than with DMSO (Figure S5). Furthermore, substrate inhibition by phloretin was significantly decreased in presence of β-CD. This is tentatively explained by a reduced concentration of free phloretin due to formation of phloretin/β-CD inclusion complex. Moreover, *Os*CGT activities at elevated temperature using β-CD were 3-fold higher than in presence of DMSO (Figure S6). We then performed phloretin conversions with *Os*CGT coupled to *Gm*SuSy. 5 mM phloretin (Figure 2A) were used for comparison of conversions in presence of β-CD and DMSO. The initial rate of nothofagin production with β-CD was higher than that in presence of DMSO and the conversion was completed after 6 h. When applied DMSO the conversion did not reach equilibrium at 10 h. In Figure 2B, various concentrations of phloretin from 0.1 to 50.0 mM were applied and final nothofagin conversions were analyzed. They strongly decreased when DMSO was applied. Particularly at high phloretin concentrations synthesis of nothofagin was far more efficient in presence of β-CD.

Table 2. Enzyme activity and stability.[a]

Solvent	Specific activity[Umg^{-1}]		Remaining [%][b]	
	*Gm*SuSy[c]	*Os*CGT[d]	*Gm*SuSy	*Os*CGT
Buffer	17.0 ± 0.6	-[e]	88	-[e]
β-CD	16.0 ± 0.7	3.8 ± 0.1	98	83
DMSO	4.2 ± 0.8	2.7 ± 0.1	27	57
β-CD/DMSO	4.5 ± 0.9	3.0 ± 0.3	31	60

[a] Reaction conditions: 30 °C, 50 mM HEPES buffer pH 7.5, 50 mM KCl, 13 mM $MgCl_2$, 0.13% BSA and 20 mM CD or 20% DMSO.
[b] Activity remaining after 4 h.
[c] Conversions of 300 mM sucrose and 2 mM UDP.
[d] Conversions of 5 mM phloretin and 2 mM UDP-glucose.
[e] Solubility limited.

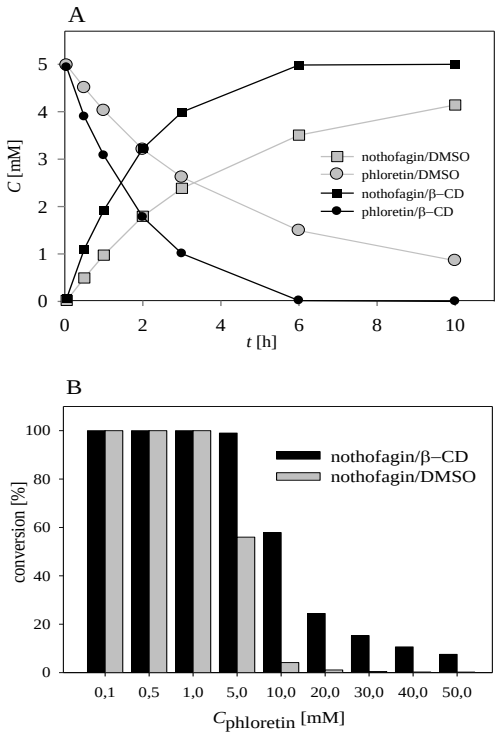


Figure 2. The beneficial effect of β-CD on enzymatic conversions of phloretin catalyzed by *Os*CGT coupled with *Gm*SuSy in a standard reaction mixture pH 7.5 (0.5 mM UDP, 300 mM sucrose) at 30 °C using 20 mM β-CD or 20% DMSO; A) time course analysis of the conversions of 5 mM phloretin (100 mU mL^{-1} *Os*CGT, 100 mU mL^{-1} *Gm*SuSy); B) final conversions at different phloretin concentrations after 24 h (50 mU mL^{-1} *Os*CGT, 50 mU mL^{-1} *Gm*SuSy), showing reactions in DMSO are more limited than β-CD at high phloretin concentrations (≥ 5 mM) by substrate inhibition and solubility.

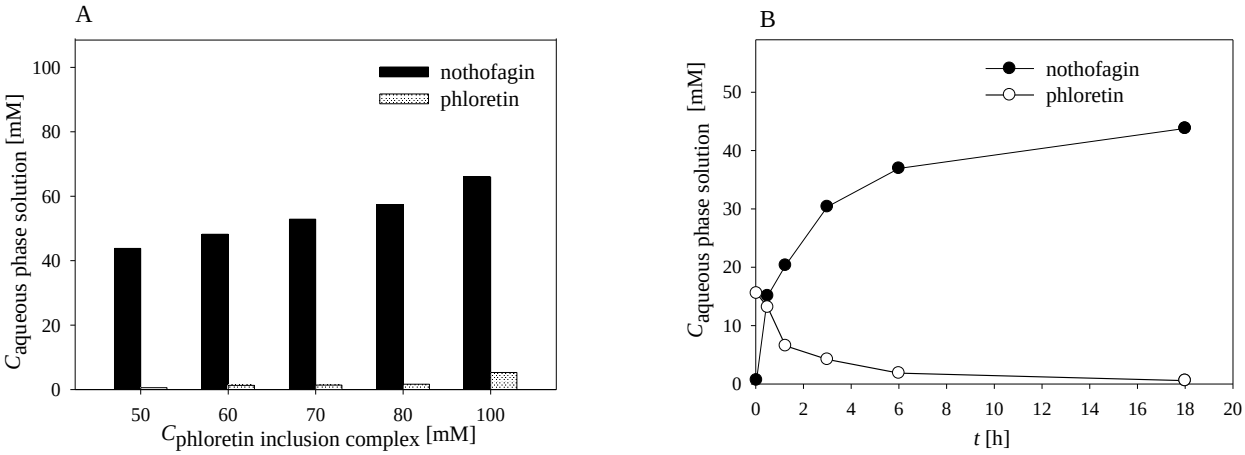


Figure 3. Batch conversions of phloretin/β-CD (lyophilized solid powder) to nothofagin aqueous solutions catalyzed by *Os*CGT coupling with *Gm*SuSy using 0.5 mM UDP and 300 mM sucrose in standard conditions; A) final conversions of 50 to 100 mM phloretin after 18 h (10 U mL^{-1} *Os*CGT and 2 U mL^{-1} *Gm*SuSy); B) time course analysis for conversion of 50 mM phloretin (5 U mL^{-1} *Os*CGT and 1 U mL^{-1} *Gm*SuSy.

Besides enzyme inactivation in presence of DMSO the reduced substrate inhibition in presence of β-CD is responsible for this effect. We thus performed nothofagin synthesis using phloretin/β-CD inclusion complex without adding DMSO. However, the solubility of phloretin in the presence of 20 mM β-CD was 8.5 mM. The idea was to apply a solid powder of inclusion complex of phloretin with β-CD (> 10 mM phloretin) to the conversion reactions. We therefore prepared solid inclusion complex of phloretin with β-CD at a 1:1 molar ratio using a freeze-drying method. The phloretin concentration of the lyophilized solid powder was measured spectrophotometrically. The powder contained 27 mg of phloretin per 100 mg of complex. We also prepared solid powder of physical mixtures of phloretin and β-CD. The solubility measurements of solid powder of lyophilized phloretin/β-CD inclusion complex, physical mixtures and free phloretin were performed in water and HEPES buffer pH 7.5. Phloretin solubility in water was lower than in HEPES buffer pH 7.5 (Figure S7). Phloretin/β-CD inclusion complex exhibited higher solubility than the corresponding physical mixtures and free phloretin (see Table 1).

Lyophilized solid inclusion complexes were used in batch conversions for nothofagin synthesis (Supporting Information, method). Optimization of the phloretin/β-CD concentration (5 to 200 mM phloretin) revealed that 50 mM phloretin were most promising for efficient high yielding batch conversions (Figure 3A). 44 mM nothofagin were obtained in aqueous solution (98.7 % nothofagin and only 1.3% phloretin; see Table S3). Most of the remaining unconverted phloretin was found as precipitate. Using 100 mM phloretin/β-CD enzymes had to be fed to finally obtain 66 mM nothofagin (Figure S8). The higher amount of nothofagin in solution was accompanied with an increased loss of phloretin in the insoluble fraction and reduced enzymatic efficiency. When 200 mM of the phloretin complex with β-CD were applied the conversion was slow and only around 15 mM nothofagin were obtained (Figure S9). We noticed that the aqueous volume was limited when a lot of solid powder was applied.

We concluded that optimal condition for conversion of phloretin forming complex with β-CD at 1:1 molar ratio was 50 mM phloretin. Batch conversions using 50 mM phloretin for synthesis of nothofagin by *Os*CGT coupling with *Gm*SuSy were performed. The time course of the reaction is depicted in Figure 3B. After biocatalytic synthesis, nothofagin was purified by HPLC (Supporting Information, Methods and Figure S10-S12). Nothofagin with high purity (≥ 99%) and good yield (78%) was obtained. High concentrations (44 mM or 19.2 g L^{-1}) of nothofagin were obtained from the conversion of 50 mM/β-CD inclusion complex (Table 3). Space-time yield (STY) was 5.5-fold higher than in fed-batch conversion. The RC_{max} was also increased up to 88. Moreover, we reached the highest RC_{max} number (132) when

Table 3. Nothofagin synthesis using β-CD in comparison to DMSO.

Reaction condition	β-CD[a]		DMSO[b]	
Phloretin [mM]	50	100	5	45
Phloretin feed [time]	0	0	0	8
Enzyme feed [time]	0	1	0	8
$C_{nothofagin}$[c] [mM]	44	66	5	44
Nothofagin[d] [%]	99	92	99	99
t [h]	18	36	6	135
Space-time yield[e] [g L^{-1} h^{-1}]	1.1	0.8	0.4	0.2
RC_{max}[f]	88	132	5	44

[a] present work: lyophilized solid powder of phloretin/β-CD inclusion complex, 300 mM sucrose, 0.5 mM UDP, 50 mM HEPES pH 7.5, 50 mM KCl, 13 mM $MgCl_2$, 1.3% BSA.

[b] previous work: phloretin aqueus solution in 20% DMSO, 300 mM sucrose, 1 mM UDP, 50 mM HEPES pH 7.5, 50 mM KCl, 13 mM $MgCl_2$, 0.13% (w/v) BSA.

[c] Final nothofagin concentration in aqueous solution.

[d] Based on final nothofagin and phloretin concentrations in aqueous solutions.

[e] Total amount of nothofagin in aqueous solution (g L^{-1}) per total reaction time.

[f] Maximum number of UDP-glucose regeneration cycles (mM nothofagin produced per mM UDP added).

enzymes were fed in the conversion reaction of 100 mM phloretin/β-CD complex. The number of UDP-glucose regeneration cycles was 3-fold higher than a previous report.

The results indicate that β-CD not only solubilizes the substrate but also enhances biocatalyst efficiency.[20] There are reports of the use of CDs for bioconversions of poor water soluble compounds. Complexation with β-CD substantially enhanced the conversion lipophilic steroid drug in an aqueous fermentation system.[21] β-CD was used to enhance the solubility of flavonoid substrates. Enzymatic hydrolysis of icariin and naringin (~17 mM) by snailase has been reported.[22] The higher bioconversion efficiency of substrate/β-CD complex was obtained when compared with bioconversion of free substrate. We described enzymatic process of glucosylation of phloretin/β-CD inclusion complex for the production of nothofagin. This is the first synthetic use of a *C*-glucosyltransferse for the conversion of flavonoid-like phloretin complex.

In conclusion, we present a procedure using β-CD for solubility enhancement of the poorly water soluble substrate phloretin for enzymatic synthesis of nothofagin. Conversion of phloretin/β-CD inclusion complex was achieved by coupling two enzymes in a one-pot cascade reaction. High nothofagin concentrations were obtained in batch conversions. Using phloretin/β-CD for nothofagin synthesis allows faster and more efficient conversions than by using phloretin in DMSO solution. The methodology describes a highly efficient and very convenient synthesis of flavonoid natural products.

Experimental Section

Conversions of phloretin/β-CD inclusion complex

The inclusion complex of phloretin with β-CD was prepared at a molar ratio of 1:1 utilizing freeze-drying. Phloretin dissolved in ethanol was dispersed into β-CD aqueous solution. The suspension was stirred continuously at room temperature for 24 h and it was cooled to 4 °C. The obtained suspension was filtered through a 0.22 µm membrane filter and the filtrate was lyophilized. Conversions of phloretin/β-CD inclusion complex by *Os*CGT coupling with *Gm*SuSy were performed at 30°C in 2 mL tubes using a rotator wheel at 20 rpm. Reaction mixtures, unless otherwise indicated, contained 50 mM phloretin, 0.5 mM UDP, 300 mM sucrose, 50 mM HEPES buffer pH 7.5, 13 mM $MgCl_2$, 50 mM KCl and 1.3% BSA. Samples were aliquoted and solid particles were removed by centrifugation. The aqueous solutions were mixed with acetonitrile/DMSO (1:3) to stop the reactions. Precipitation proteins were removed by centrifugation. Phloretin and nothofagin in the supernatant were analysed using a reversed phase C18 HPLC-assay. Nothofagin was isolated and the purity was confirmed by HPLC.

A detailed description of phloretin solubility study, preparation of the phloretin/β-CD inclusion complex, enzyme assays, effects of β-CD and DMSO on enzymatic synthesis of nothofagin, analytical methods and product isolation can be found in Supporting Information.

Acknowledgements

Financial support from the Austrian Science Fund (FWF) and the EU FP7 project "SuSy" (Sucrose Synthase as Effective Mediator of Glycosylation) is gratefully acknowledged. L.B. is supported from a scholarship of Ramkhamhaeng University (Bangkok Thailand).

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β-Cyclodextrin Improves Solubility and Enzymatic *C*-Glucosylation of the Flavonoid Phloretin

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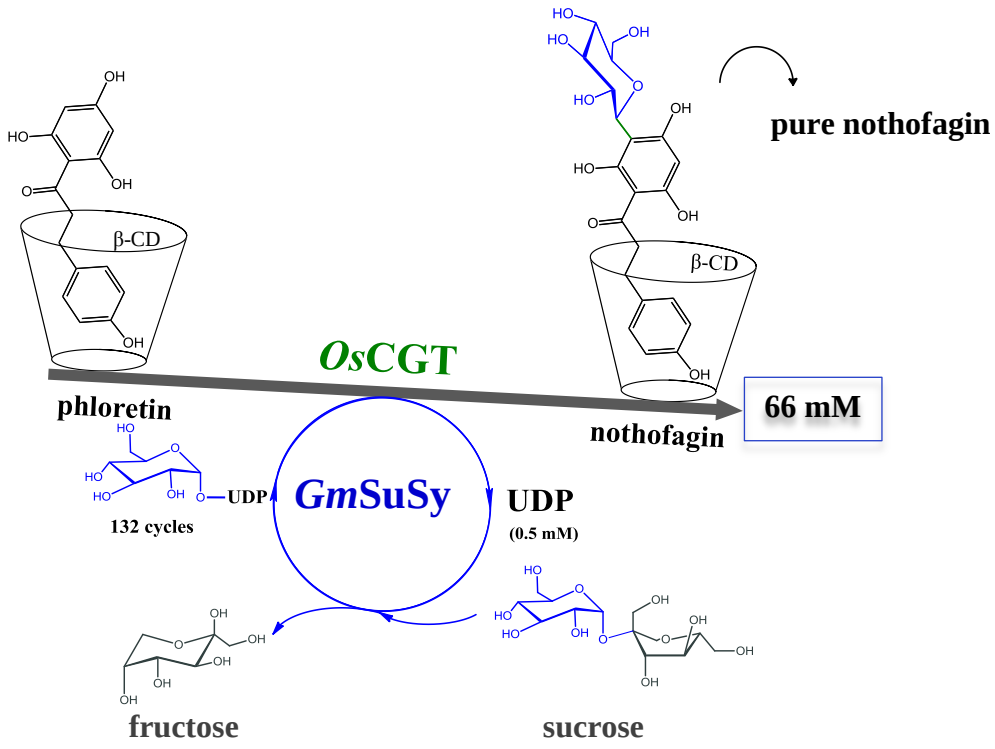

Supporting Information

β-Cyclodextrin Improves Solubility and Enzymatic *C*-Glucosylation of the Flavonoid Phloretin

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Methods

1. Materials

Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich (Vienna, Austria) in the highest purity available and were used without further purification. Phloretin (98%) was purchased from Carbosynth (Berkshire, UK). *Strep*-Tactin® Sepharose® and desthiobiotin were from IBA (Goettingen, Germany). BCA protein assay kit was from Thermo Scientific (Waltham, MA, US). Minisart®-RC4 0.45 µm syringe membrane filter and cellulose acetate 0.22 µm membrane filter were from Sartorius Stedim Biotech GmbH (Goettingen, Germany).

2. β-CD solubility in DMSO water mixtures

β-CD solubility was tested in various DMSO concentrations from 0 to 50%. Excess amounts of β-CD were transferred to 1.5 mL centrifuge tubes containing 1 mL double distilled water and shaken at 30 °C and 1000 rpm in a thermomixer (Eppendorf, Hamburg, Germany) for 4 h. The concentration of β-CD was measured by discoloration of phenolphthalein at 550 nm due to complexation with β-CD as described elsewhere.[1]

3. Phloretin solubility in aqueous solutions containing β-CD and DMSO

Phase solubility study of phloretin with β-CD was performed according to the method of Higuchi and Connors.[2] Excess amounts of phloretin were added to 1.5 mL centrifuge tubes containing 1 mL of β-CD aqueous solutions (from 0 to 20 mM) in 50 mM HEPES buffer pH 7.5. The suspensions were shaken at 30 °C and 1000 rpm in a thermomixer for 24 h. After reaching equilibrium, samples were centrifuged at 30 °C and 15000 rpm for 20 min. Supernatant was filtered through a Minisart®-RC4 0.45 µm syringe membrane filter. All procedures were conducted at 30 °C to avoid precipitation of phloretin. Suitable dilutions were spectrophotometrically analyzed at 288 nm using a micro-volume spectrophotometer (DeNovix DS-11, Wilmington, USA). The concentration of phloretin was quantified by interpolation of the corresponding standard curve. A phase solubility study was also conducted using β-CD as described in the presence of 20% DMSO. All experiments were carried out in triplicates. Phloretin solubility was also measured in various DMSO concentrations from 0 to 100% in the absence and presence of excess amounts of β-CD. Samples were diluted using 80% DMSO. Phloretin concentrations were analyzed by HPLC (Agilent 1200, Santa Clara, CA, USA).

4. Preparation of phloretin/β-CD solid powder

The inclusion complex of phloretin with β-CD was prepared using a freeze drying method.[3] Phloretin and β-CD with 1:1 molar ratio were accurately weighed separately. Phloretin (0.2057 g) was dissolved in 2 mL ethanol and was added drop wise to the solution of β-CD (0.8513 g) in 50 mL water. The suspension was stirred continuously at room temperature for 24 h and it was cooled to 4 °C for 12 h. The precipitates were then filtered through a 0.22 µm cellulose acetate membrane filter and dried in freeze dryer at -55 °C (Chaist Alpha1-4/Loc-1m, B. Braun Biotech International). The powder obtained was diluted appropriately in 80% DMSO and the phloretin concnetration was determined by HPLC. The lyophilized solid powder of phloretin/β-CD inclusion complex was stored at -20 °C. Physical mixtures of phloretin and β-CD were prepared by mixing phloretin and β-CD at a molar ratio of 1:1. The mixtures were blended in a mortar for 30 min. To determine phloretin/β-CD solubility, excess amounts of solid powder of inclusion complex, physical mixtures and free phloretin were added to water or HEPES buffer pH 7.5. Phloretin concentrations were measured as described above.

5. Enzyme production

Escherichia coli BL21-Gold (DE3) expression strains for *Os*CGT (GenBank: FM179712)[4,5] and *Gm*SuSy (GenBank: AF030231)[6] were described elsewhere in detail. Enzyme expression as N-terminal *Strep*-tag II fusion protein and enzyme purification by *Strep*-Tactin® Sepharose® affinity chromatography were also reported elsewhere in detail.[6] Protein concentrations and purities were determined using the BCA method and SDS-PAGE, respectively. Aliquots of enzyme preparations were stored at -70 °C and thawed only once prior to their use.

6. Enzymatic assays

*Os*CGT activity

*Os*CGT activity assay was performed in 1.5 mL tubes by using aqueous solutions of 5 mM phloretin with 20 mM β-CD, 2 mM UDP-glucose, 50 mM HEPES buffer pH 7.5, 50 mM KCl, 13 mM $MgCl_2$ and 0.13 % BSA. All experiments were carried out in triplicates. Reactions were started with the addition of *Os*CGT and incubated in a thermomixer at 30 °C and 300 rpm. Samples were stopped by mixing an aliquot of 50 µL with 50 µL acetonitrile. At least four samples were taken during the linear initial rate to determine activities. Precipitated proteins were removed by centrifugation at 15000 rpm for 20 min. All quantifications of phloretin and nothofagin were carried out by HPLC. One unit of *Os*CGT was defined as the amount of enzyme producing 1 µmol nothofagin per min at 30 °C under above conditions. The specific activity of *Os*CGT was expressed as unit per milligram of purified protein.

*Gm*SuSy activity

*Gm*SuSy activity was measured in 1.5 mL reaction tubes by using standard condition; 300 mM sucrose, 2 mM UDP, 20 mM β-CD, 50 mM HEPES buffer pH 7.5, 50 mM KCl, 13 mM $MgCl_2$ and 0.13 % BSA. All experiments were carried out in triplicates. Reactions were started with the addition of *Gm*SuSy. Samples were incubated and further processed as described for the *Os*CGT activity assay. UDP and UDP-glucose were determined based on HPLC-assay. One unit of *Gm*SuSy was defined as the amount of enzyme producing 1 µmol UDP-glucose per min at 30 °C under above conditions. The *Gm*SuSy specific activity was expressed as unit per milligram of purified protein.

7. HPLC-based assays

Determination of phloretin and nothofagin

A HPLC-assay was used for determination of phloretin and nothofagin concentrations and it was also used for measurement of nothofagin production rates in *Os*CGT conversions as described elsewhere in detail.[6] The measurement procedure was developed and performed on an Agilent 1200 HPLC equipped with a Chromolith® Performance RP-18e column (100 x 4.6 mm). 5 µl of a suitable dilution of sample were applied on the column and it was thermostatically controlled at 35 °C. The separation was monitored by UV detection at 288 nm. Water and acetonitrile (each containing 0.1% formic acid) were used as solvent A and B, respectively. A 10 min long linear gradient from 10 to 40% B (1 mL min^{-1}) was used for produce separation. It was followed by 0.05 min of a linear gradient from 40 to 100 % B (1.5 mL min^{-1}) and 1.45 min of isocratic flow at 100% B (1.5 mL min^{-1}) to wash off hydrophobic compounds. A 0.05 min linear gradient from 100 to 10 % B (1.5 mL min^{-1}) was applied to equilibrate the column.

Determination of UDP and UDP-glucose

Determination of UDP and UDP-glucose concentrations was done by HPLC. 5 µl of sample were applied on a Shimadzu HPLC, RID-10A (Columbi, Maryland, U.S.A) equipped with a Kinetex™ C18 column (5 µm, 100 Å, 50×4.6 mm) which was thermostatically controlled at 35 °C. Using 20 mM potassium phosphate buffer pH 5.9 with 40 mM tetrabutylammonium bromide as solvent A and acetonitrile as solvent B an isocratic flow (12.5% B, 2 mL min^{-1}) was applied. Separation was monitored by UV detection at 262 nm.

8. Effect of β-CD on HPLC measurement

All samples from enzymatic assays and conversion reactions were diluted before *HPLC* analysis and the concentrations of β-CD were less than 10 mM. The effect of β-CD on HPLC measurement (peak area and retention time) was determined. Phloretin complex with β-CD was obtained using 0.2 mM phloretin with 0 to 10 mM β-CD. The samples were measured on an Agilent 1200 HPLC as described and double distilled water was injected two times before application of a new sample to wash the column.

9. Effect of β-CD and DMSO on enzymatic synthesis of nothofagin

*Os*CGT activity at different temperature

Optimum temperature of nothofagin synthesis by *Os*CGT was determined at optimum pH (pH 8.5)[6] in 20% DMSO by measurement of linear initial rates at 10 °C to 70 °C. Reactions contained 1 mM phloretin, 2 mM UDP-glucose, 50 mM Tris-HCl buffer pH 8.5, 50 mM KCl, 13 mM $MgCl_2$, 0.13 % BSA and 20% DMSO. *Os*CGT (20 mU mL^{-1}) was added for starting the reactions. Samples were aliquoted and measured by HPLC. To test the effect of β-CD and DMSO on *Os*CGT activity at high temperature, *Os*CGT assays were performed as described at 40, 45, 50 and 60°C by using 20 % DMSO or 10 mM β-CD.

*Os*CGT activity at different β-CD and DMSO concentrations

To test the effect of β-CD on *Os*CGT activity, reaction mixtures of 1 mM phloretin, 2 mM UDP-glucose, 50 mM KCl, 13 mM $MgCl_2$, 0.13 % BSA and 50 mM Tris-HCl pH 8.5 were prepared with different β-CD concentrations from 0 to 10 mM. *Os*CGT (100 mU mL^{-1}) was added for starting the reactions. Samples were incubated at 30°C and 300 rpm. The initial rate measurement was performed as described. The effect of DMSO was also tested with different DMSO concentrations from 30 to 80% by using 5 mM phloretin and 5 mM UDP-glucose. Reactions were prepared in 20% DMSO. *Os*CGT (200 mU mL^{-1}) was added to start the reaction. After 2 min samples were aliquoted to new tubes and conversion were monitored at final DMSO concentrations ranging from 30 to 80%.

*Os*CGT activity at different phloretin concentrations

The *Os*CGT inhibition by phloretin was determined using linear initial rate measurement at 30°C in standard conditions containing 20 mM β-CD or 20 % DMSO. Phloretin stock solutions were prepared in ethanol (1, 10 and 100 mM) and aliquoted for preparation of final concentrations from 0.01 mM to 7.5 mM in 20 mM β-CD and 0.01 mM to 10 mM in 20 % DMSO. Ethanol was evaporated at 37 °C. The standard reaction mixtures were added and the reactions were started by adding *Os*CGT.

Comparison of activity and stability

To test the effect of β-CD and DMSO on individual enzyme activity and stability, initial rate measurements were performed. 500 µL standard enzyme assay conditions were prepared at pH 7.5 in the presence of 20 mM β-CD or 20 % DMSO or the mixtures of β-CD and DMSO (without UDP-glucose for *Os*CGT or without UDP for *Gm*SuSy) using 10 U mL^{-1} of enzyme. Samples were aliquoted 225 µl for testing enzyme activity and 225 µl for testing enzyme stability. Aliquoted samples were incubated at 30 °C for 15 min and 4 h for determination of enzyme activity and stability, respectively. The reactions were started by adding 25 µl UDP-glucose (2 mM) or UDP (2 mM). *Os*CGT and *Gm*SuSy activity assays were performed using described methods. All experiments were carried out in triplicates.

Determination of *Os*CGT coupled with *Gm*SuSy

Unless otherwise mentioned, *Os*CGT coupled with *Gm*SuSy for conversions of phloretin were performed using 20 mM β-CD or 20 % DMSO in the reaction mixtures of 0.5 mM UDP, 300 mM sucrose, 50 mM HEPES buffer pH 7.5, 50 mM KCl, 13 mM $MgCl_2$, 0.13 % BSA. Enzymes were added to start (100 mU mL^{-1} *Os*CGT and 100 mU mL^{-1} *Gm*SuSy) measuring time courses of conversions of 5 mM phloretin. 50 mU mL^{-1} *Os*CGT and 50 mU mL^{-1} *Gm*SuSy were used for conversions of 0.1 to 50 mM phloretin. The various phloretin concentrations were prepared from phloretin solution in ethanol which was removed in a thermomixer at 37°C. Phloretin was redissolved in aqueous solution of β-CD or DMSO at 30°C and 1000 rpm.

10. Conversion optimization of phloretin/β-CD inclusion complex

In order to identify the optimal condition for batch conversions of phloretin/β-CD, various concentrations of phloretin (5 to 200 mM) forming inclusion complex with β-CD were tested. The conversion reactions were performed in standard reaction mixtures (0.5 mM UDP, 300 mM sucrose, 50 mM HEPES buffer pH 7.5, 13 mM $MgCl_2$, 50 mM KCl with 1.3% BSA) and reactions were started by adding *Os*CGT and *Gm*SuSy. The samples of 5 to 25 mM phloretin/β-CD were incubated in a thermomixer at 30 °C and 300 rpm while the samples of 50 to 200 mM phloretin/β-CD were incubated on a rotator wheel at 30 °C and 20 rpm. Reactions were aliquoted and solid compounds were removed by centrifugation at 30 °C and 15000 rpm for 20 min. The aqueous solutions were mixed with acetonitrile/DMSO (1:3) to stop the reaction. Precipitated proteins were removed by centrifugation (15000 rpm, 20 min). Phloretin and nothofagin concentrations were measured in suitable dilutions of the supernatant as described. Enzymes were fed and samples were measured until the reactions reached the equilibrium.

11. Batch conversions of phloretin/βCD inclusion complex

Batch conversions of phloretin/β-CD inclusion complex by *Os*CGT (5 U mL^{-1}) coupled with *Gm*SuSy (1 U mL^{-1}) were performed at 30°C for 18 h using a rotator wheel (20 rpm). Reaction tubes (2 mL) contained 1.5 mL standard reaction mixtures; 50 mM phloretin, 0.5 mM UDP, 300 mM sucrose, 50 mM HEPES buffer pH 7.5, 13 mM $MgCl_2$, 50 mM KCl and 1.3% BSA. Samples of 5 µl were centrifuged. 1 µl of aqueous solutions was mixed with 39 µl of acetonitrile/DMSO (1:3). Samples were prepared and measured using HPLC-assay as described.

12. Product isolation and identification

Nothofagin was generated from the inclusion complex using batch conversions as described. The produced nothofagin was purified by preparative reversed phase C18 HPLC on an Agilent 1200 system equipped with a SphereClone 5 µm ODS (2) (250 x 10.0 mm) column. After conversions of phloretin/βCD inclusion complex, precipitated compounds were removed by centrifuge at 15000 rpm for 30 min. Proteins were removed by centrifugal concentrators with a Molecular Weight Cut off of 10 kDa. 250 µl of 44 mM nothofagin were applied on the column. Water and acetonitrile (0.1% formic acid each) were used as solvent A and solvent B, respectively. Separation of nothofagin was achieved by step gradients from 10% to 100% B and nothofagin was eluted at 20% B. Purity was confirmed by Agilent 1200 HPLC equipped with a Chromolith® Performance RP-18e column as described. Fractions containing pure nothofagin were combined. Nothofagin was finally isolated from aqueous solution by removal of acetonitrile and water under reduced pressure. After freeze drying, nothofagin was obtained as white powder. Purified product was previously identified as nothofagin by ^{1}H and ^{13}C NMR as described elsewhere in detail.[7]

Results

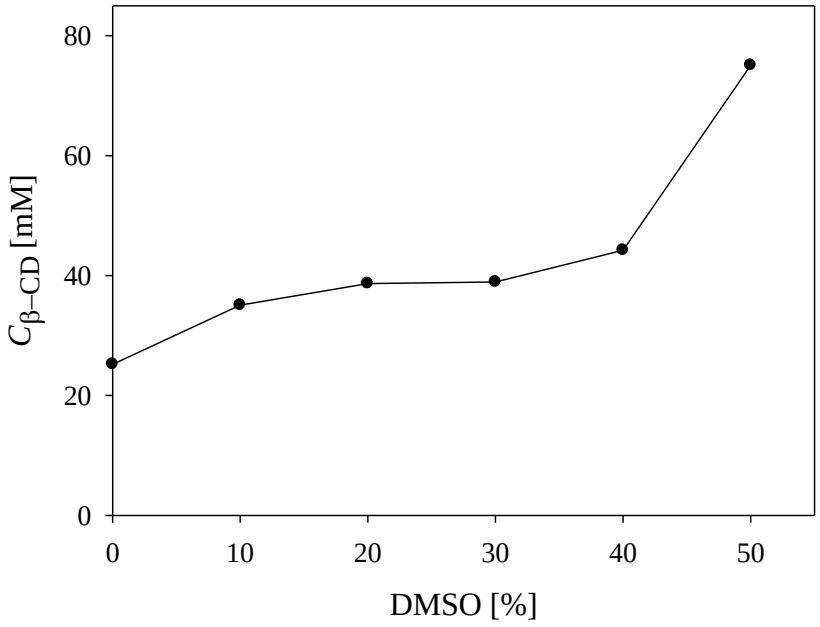


Figure S1. Effect of DMSO on β-CD solubility at 30 °C in water. The β-CD solubility increased at elevated DMSO concentration. It was slowly increasing from 0% to 40% DMSO (25 - 44 mM β-CD). β-CD solubility in 50% DMSO was ~1.7-fold higher than 40% DMSO.

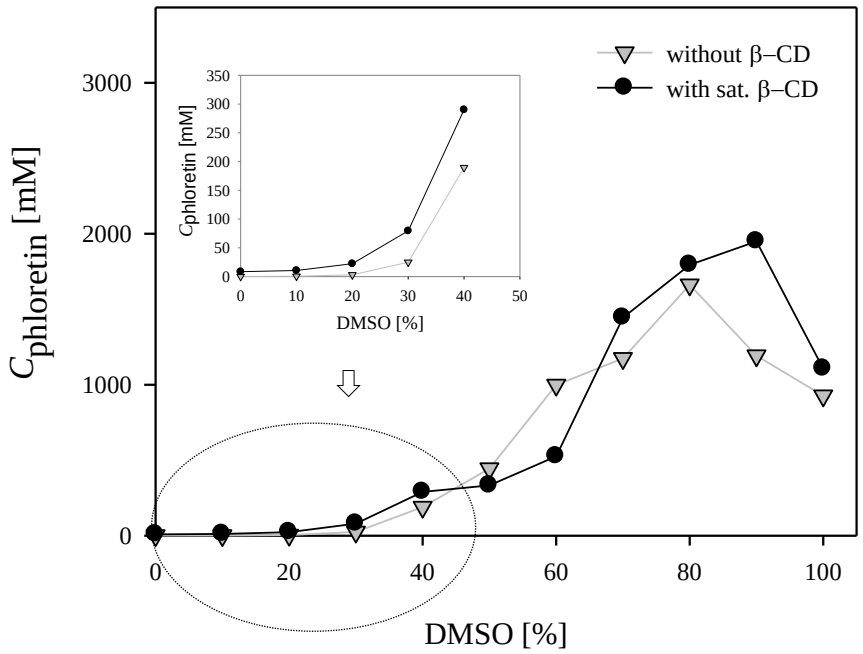


Figure S2. Solubility of phloretin at 30 °C using various DMSO concentrations in water in the absence and presence of excess amounts of β-CD.

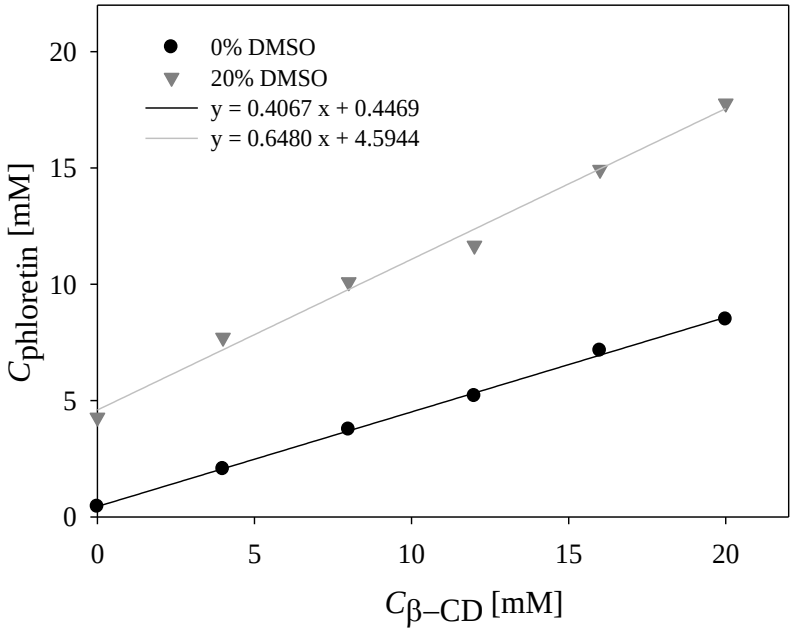


Figure S3. Phase solubility diagram of phloretin with increasing concentration of β-CD in HEPES buffer pH 7.5 at 30 °C in the absence and presence of 20% DMSO.

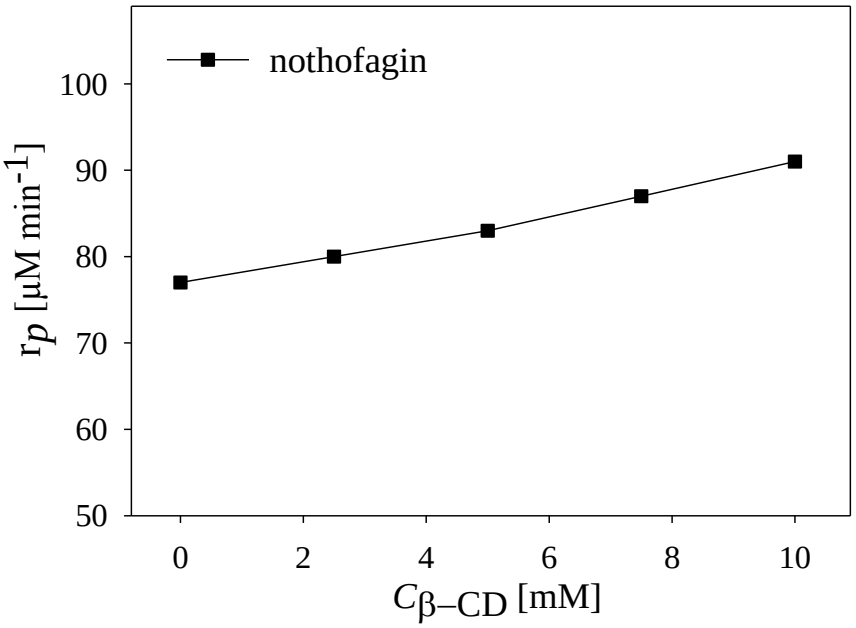


Figure S4. Effect of β-CD on *Os*CGT activity showing the nothofagin production rate increase with increasing β-CD concentrations.

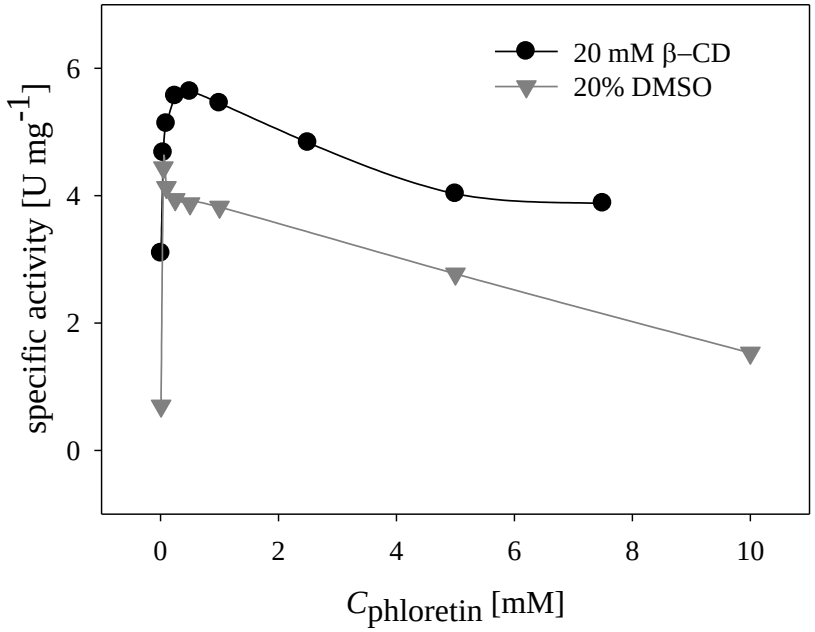


Figure S5. Effect of phloretin concentrations on *Os*CGT activity in 20 mM β-CD and 20% DMSO. Note: No specific activity data point at 10 mM phloretin-20 mM β-CD because of solubility limit.

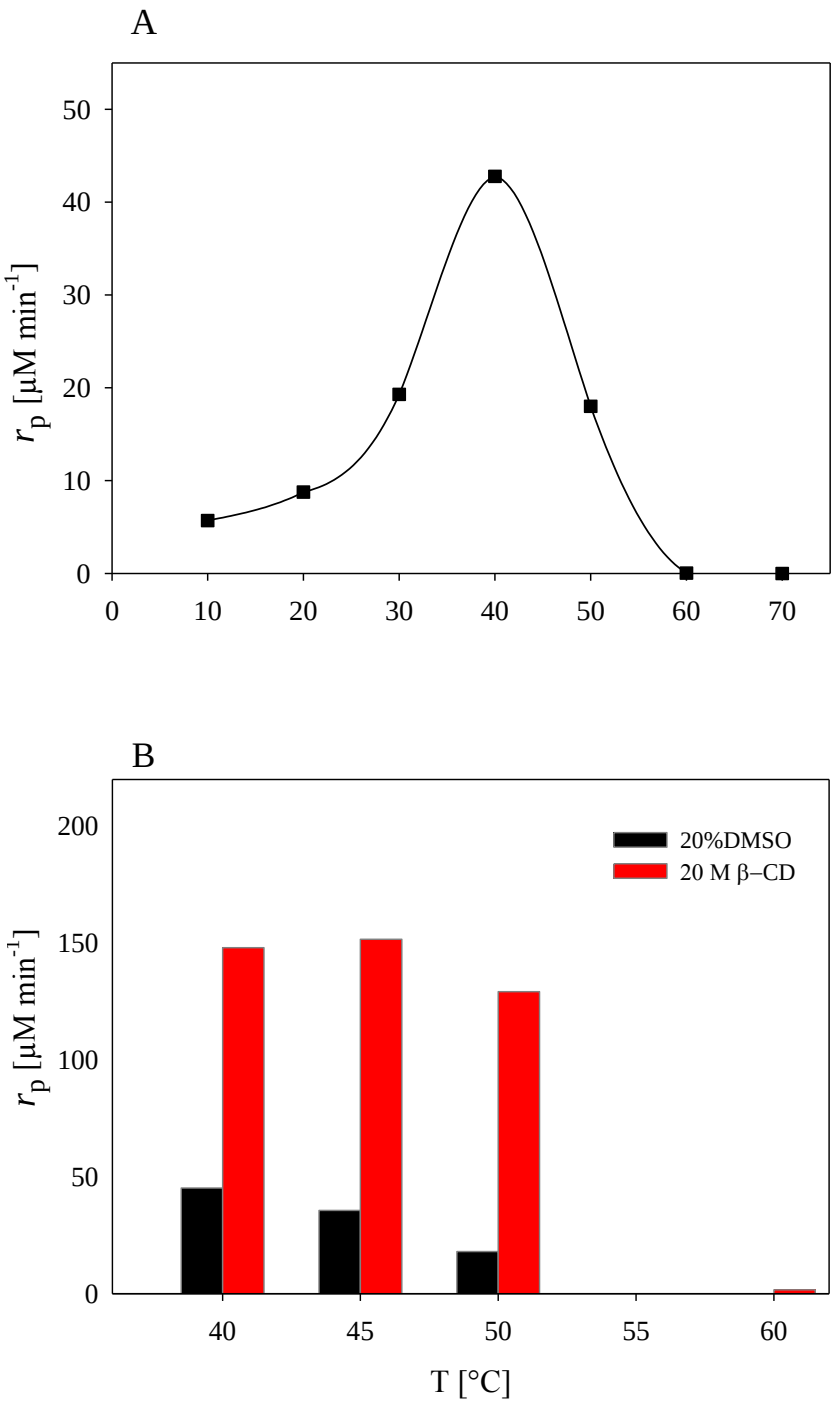


Figure S6. The nothofagin production rate (r_p) in *Os*CGT standard reaction using A) 20% DMSO at different temperature showing the optimum temperature at 40 °C and B) the comparison of 20 mM β-CD with 20% DMSO at high temperature.

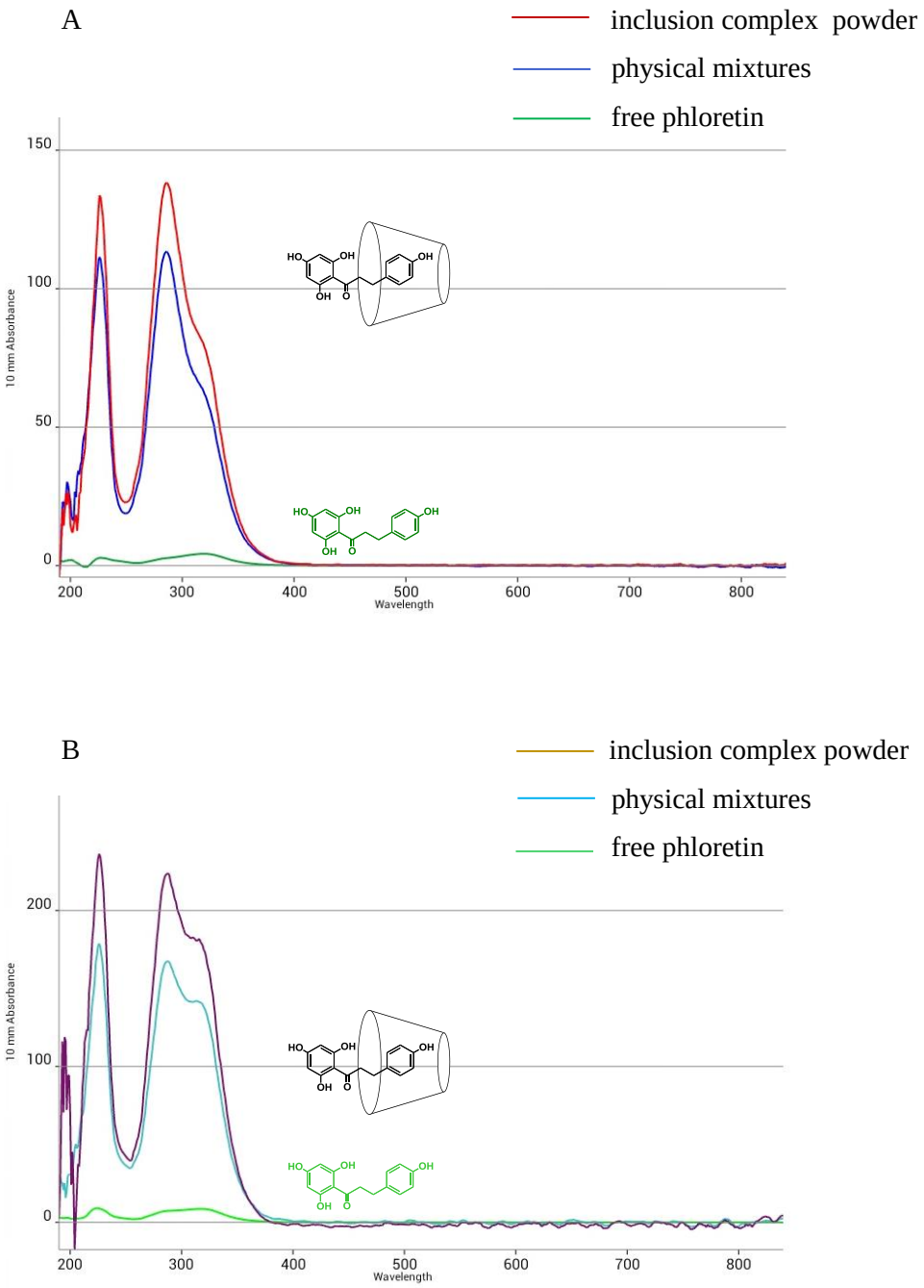


Figure S7. UV-VIS absorption spectra of free phloretin, phloretin/β-CD physical mixtures and phloretin/β-CD inclusion complex in A) water and B) HEPES buffer pH 7.5 showing an efficient of β-CD on phloretin solubility (inclusion complex > physical mixtures > free phloretin).

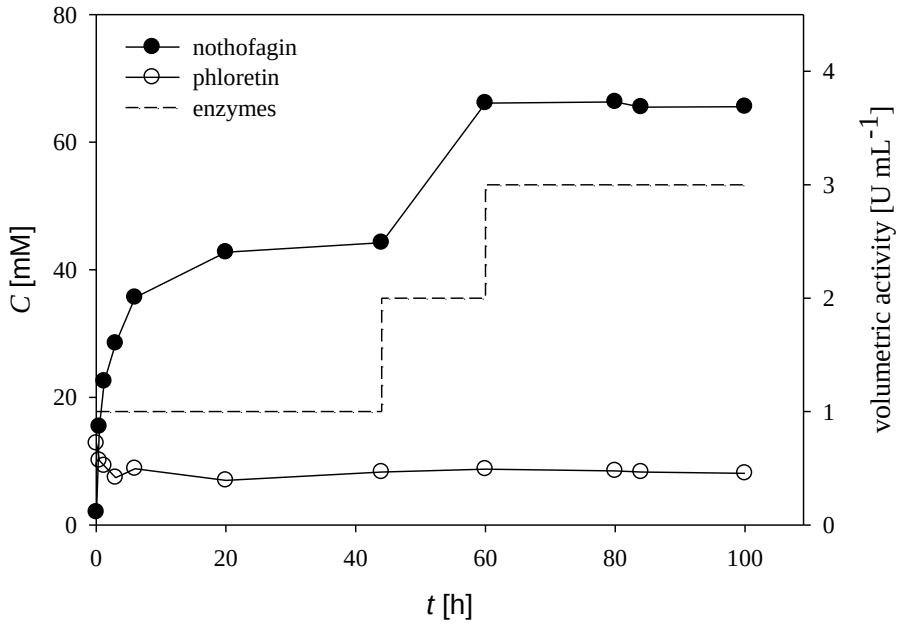


Figure S8. Time course analysis of conversion of 100 mM phloretin inclusion complex with β-CD, 0.5 mM UDP, 300 mM sucrose, 50 mM HEPES buffer pH 7.5, 13 mM $MgCl_2$, 50 mM KCl and 1.3% BSA in the coupling reaction of *Os*CGT (5 U mL^{-1}) and *Gm*SuSy (1 U mL^{-1}). Enzymes were fed (5 U mL^{-1} *Os*CGT/1 U mL^{-1} *Gm*SuSy) at 44 h and 60 h and UDP (1 mM) and sucrose (100 mM) were fed after 80 h. Nothofagin concentration in system was stable at 66 mM.

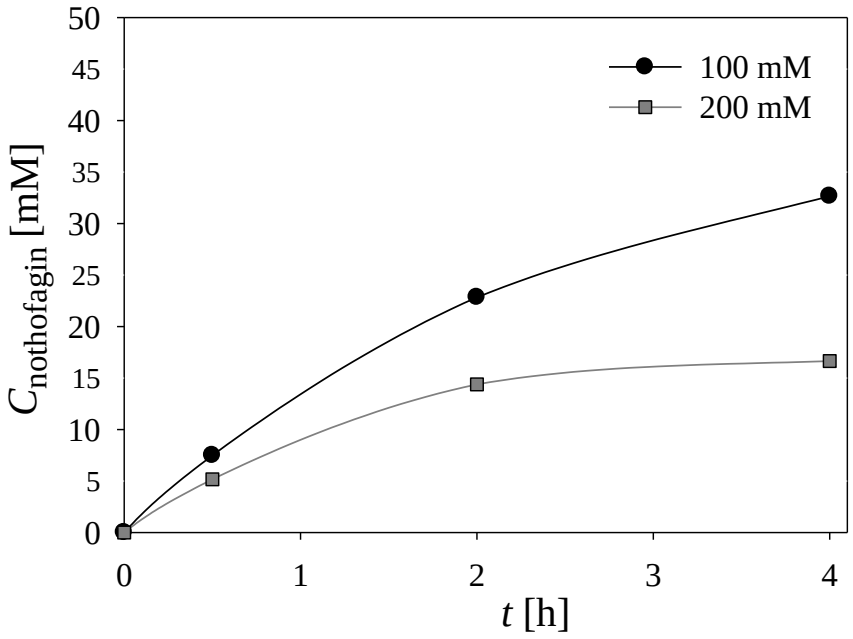


Figure S9. Time course analysis of the conversions of 100 mM and 200 mM of the phloretin inclusion complex with β-CD (0.5 mM UDP, 300 mM sucrose, 50 mM HEPES buffer pH 7.5, 13 mM $MgCl_2$, 50 mM KCl, 1.3% BSA, 5 U mL^{-1} *Os*CGT and 1 mU mL^{-1} *Gm*SuSy). The conversion of 200 mM phloretin was slower than 100 mM and the reaction reached equilibrium within 4 h. Less than 15 mM of nothofagin were formed.

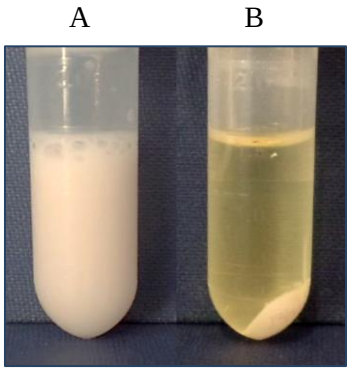
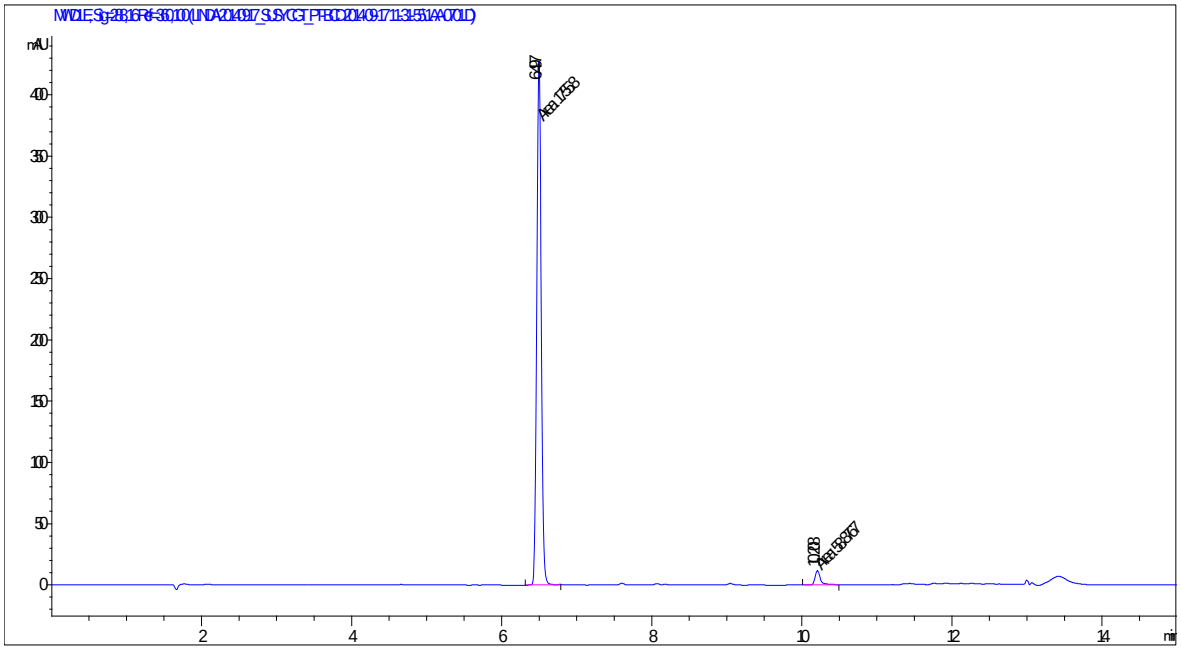


Figure S10. Conversion reaction of phloretin/β-CD inclusion complex (50 mM phloretin) (A) before adding 5 U mL^{-1} *Os*CGT and 1 U mL^{-1} *Gm*SuSy and (B) after adding enzymes and incubating at 30 °C and 20 rpm for 18 h.

A B

Figure S11. A) Aqueous solution containing 44 mM nothofagin for application on reversed phase C18 column and B) nothofagin powder as obtained after purification and lyophilization.

A



B

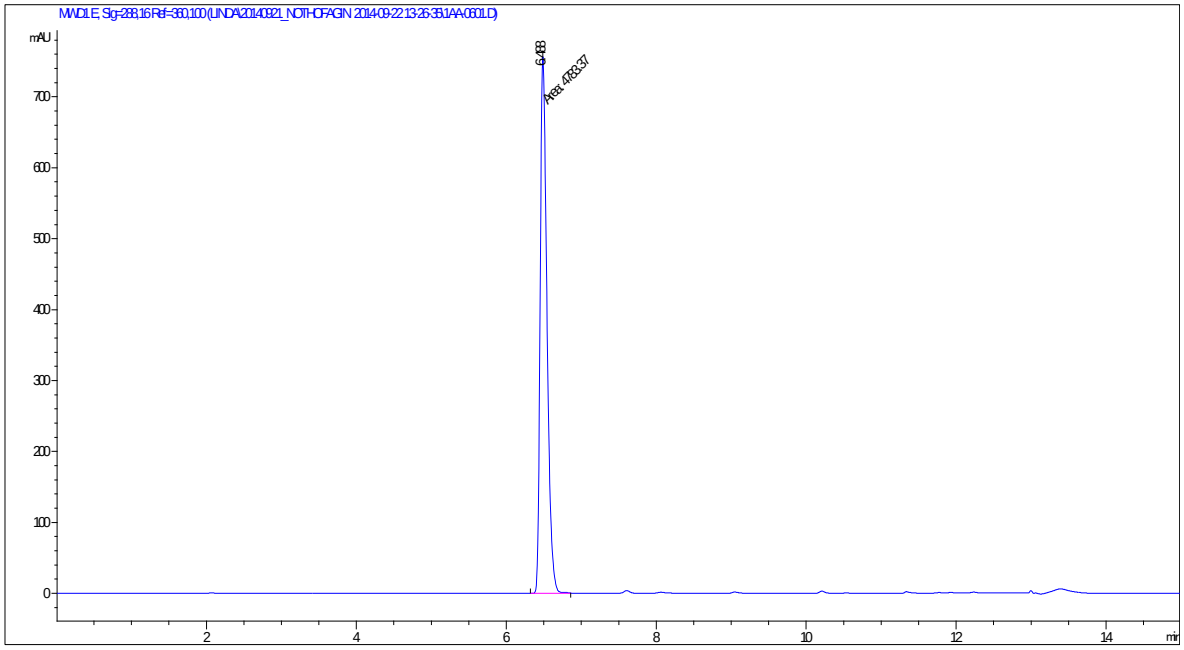


Figure S12. HPLC chromatograms of A) aqueous solution of phloretin/β-CD inclusion complex (50 mM phloretin) conversion in a coupled reaction of *Os*CGT and *Gm*SuSy (18 h) showing a large peak of nothofagin (6.5 min) and a small peak of phloretin (10.2 min). B) Aqueous solution after purification of nothofagin by reversed phase C18 HPLC.

Table S1. Stability constants of the phloretin complex with β-CD aqueous solution pH 7.5 at 30 °C in the absence and presence of 20% DMSO determined by phase solubility technique.

DMSO (%)	S_0 [mM]	Slope	R^2	K_c [a] [M^{-1}]
0	0.45	0.40±0.03	0.99	1534
20	4.59	0.65±0.03	0.99	401

[a]Solubility constants K_c were calculated from the phase solubility graph (Figure 2) using the following equation:

$$K_c = \frac{\text{slope}}{S_0\,(1-\text{slope})}$$

where S_0 is the intrinsic solubility of phloretin in HEPES buffer pH 7.5 without β-CD and slope means the corresponding slope of the phase solubility diagrams.

Table S2. HPLC peak areas and retention times of phloretin (0.2 mM) in presence of different β-CD concentrations. β-CD did not interfere with the determination of phloretin concentration.

β-CD [mM]	Retention time [min]	Peak area [mAU]	Phloretin[a] [mM]
0.00	6.4760	353.4	0.1857
2.00	6.4790	357.2	0.1877
4.00	6.4800	365.9	0.1922
6.00	6.4830	358.3	0.1882
8.00	6.4810	359.5	0.1889
10.00	6.4830	372.2	0.1955

[a] It was calculated from peak area and standard phloretin.

Table S3. Nothofagin synthesis using various phloretin concentrations (phloretin/β-CD inclusion complex) in the coupling reactions of *Os*CGT and *Gm*SuSy. Samples were performed at 30 °C using thermomixer (5 to 25 mM phloretin) and rotator wheel (50 to 100 mM).

Mixer	Phloretin [mM]	Nothofagin [mM]	Conversion [%][a]	Nothofagin in aq. Sol.[%][b]
thermomixer	5	4.6	91.0	98.6
	10	8.7	86.9	99.7
	25	20.2	80.7	99.2
rotator wheel	50	43.8	87.6	98.7
	60	48.2	80.3	97.4
	70	52.9	75.6	97.4
	80	57.4	71.8	97.2
	100	66.0	66.0	92.6

[a] nothofagin in aqueous solution compared to phloretin starting concentration

[b] nothofagin in aqueous solution compared to total amount of nothofagin plus phloretin

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Scientific record

Scientific publications

Leloir glycosyltransferases and natural product glycosylation: biocatalytic synthesis of the *C*-glucoside nothofagin, a major antioxidant of redbush herbal tea

Linda Bungaruang, Alexander Gutmann, and Bernd Nidetzky, *Adv. Synth. Catal.* **2013**, 355, 2757-2763.

A two-step *O*-to *C*-glycosidic bond rearrangement using complementary glycosyltransferase activities

Alexander Gutmann, Corinna Krump, Linda Bungaruang and Bernd Nidetzky, *Chem. Commun.* **2014**, 50, 5465-5468.

Towards the synthesis of glycosylated dihydrochalcone natural products using glycosyltransferase-catalysed cascade reactions

Alexander Gutmann, Linda Bungaruang, Hansjoerg Weber, Mario Leypold, Rolf Breinbauer and Bernd Nidetzky, *Green Chem*. **2014**, 16, 4417-4425.

β-Cyclodextrin Improves Solubility and Enzymatic *C*-Glucosylation of the Flavonoid phloretin

Linda Bungaruang, Alexander Gutmann, and Bernd Nidetzky, in preparation.

Oral presentations

Glycosyltransferase-catalyzed synthesis of a *C*-glycoside natural product

Linda Bungaruang, Alexander Gutmann and Bernd Nidetzky

17th Austrian Carbohydrate Symposium, 2013, Graz, Austria

Biocatalytic synthesis of glucoside natural products

Linda Bungaruang, Alexander Gutmann and Bernd Nidetzky

13th Bratislava Symposium on Saccharides, 2014, Bratislava, Slovakia

Poster presentations

Biocatalytic synthesis of *C*- and *O*-glycosidic natural products

Linda Bungaruang , Alexander Gutmann and Bernd Nidetzky

11th Molecular Biosciences and Biotechnology, 2014, Graz, Austria