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Introducing N-glycans into natural products through a chemoenzymatic approach

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Dedicated to Professor Yongzheng Hui on the occasion of his 70th birthday

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ABSTRACT

The present study describes an efficient chemoenzymatic method for introducing a core N-glycan of glycoprotein origin into various lipophilic natural products. It was found that the *endo*-β-*N*-acetylglucosaminidase from *Arthrobactor protophormiae* (Endo-A) had broad substrate specificity and can accommodate a wide range of glucose (Glc)- or *N*-acetylglucosamine (GlcNAc)-containing natural products as acceptors for transglycosylation, when an N-glycan oxazoline was used as a donor substrate. Using lithocholic acid as a model compound, we have shown that introduction of an N-glycan could be achieved by a two-step approach: chemical glycosylation to introduce a monosaccharide (Glc or GlcNAc) as a handle, and then Endo-A catalyzed transglycosylation to accomplish the site-specific N-glycan attachment. For those natural products that already carry terminal Glc or GlcNAc residues, direct enzymatic transglycosylation using sugar oxazoline as the donor substrate was achievable to introduce an N-glycan. It was also demonstrated that simultaneous double glycosylation could be fulfilled when the natural product contains two Glc residues. This chemoenzymatic method is concise, site-specific, and highly convergent. Because N-glycans of glycoprotein origin can serve as ligands for diverse lectins and cell-surface receptors, introduction of a defined N-glycan into biologically significant natural products may bestow novel properties onto these natural products for drug discovery and development.

1. Introduction

Glycosylation constitutes an elegant strategy that nature takes to expand the structural diversity and functions of biological molecules. For example, attachment of sugars to lipophilic natural products such as steroids and flavonoids represents a common mechanism by which living organisms modulate the physicochemical properties and biological activities of these compounds.[1–4] On the other hand, it is well known that the asparagine-linked (N-linked) glycans of glycoproteins can serve as specific ligands for lectins and/or cell-surface receptors to participate in many important biological processes such as cell adhesion, host–pathogen interaction, development, and immune response.[5–9] As the sugars found in small-molecule natural products are usually different from the conserved N-glycans of glycoproteins, we reasoned that transforming the sugars in glycosylated natural products with typical N-glycan of glycoprotein origin, or introducing N-glycan into non-glycosylated secondary metabolites, would bestow novel properties onto these natural products, for example, enhanced solubility, glycan-mediated cellular targeting, altered mechanism of action, and overall improved pharmacological properties. However, pure chemical synthesis of complex sugar-containing natural products is still a difficult task, as stepwise chemical glycosylation would involve tedious protecting group manipulations and lengthy synthetic schemes. Direct glycosylation of natural products by glycosyltransferases or glycosidases provides an attractive alternative that can greatly simplify the synthetic scheme.[10–14] We have previously reported an efficient chemoenzymatic method for introducing N-glycans into GlcNAc-tagged polypeptides to make homogeneous glycopeptides and glycoproteins.[15–19] This method is based on the transglycosylation activity of *endo*-β-*N*-acetylglucosaminidases and the use of synthetic sugar oxaozlines as the activated donor substrates. Notably, the Endo-A from *Arthrobactor protophormiae*[20,21] and Endo-M from *Mucor hiemalis*[22–24] could accept a range of modified and truncated oligosaccharide oxazolines as donor substrates for transglycosylation.[15–19,25,26] In addition, by using a novel endoglycosynthase EndoM-N175A, a full-size natural N-glycan can be efficiently introduced into polypeptide to form a natural N-glycopeptide.[27] In contrast to the glycosyltransferase approach that adds monosaccharides one at a time, the unique advantage of this endoglycosidase-catalyzed transglycosylation is the single-step, site-specific attachment of a pre-assembled oligosaccharide into the acceptor in a stereospecific manner to form a natural glycosidic linkage, without the need for any protecting groups.[19] We describe in this

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paper an extension of this chemoenzymatic approach to the synthesis of novel glycosylated small-molecule natural products with defined N-glycans. We have found that Endo-A has a broad substrate specificity and is able to accommodate different types of glucose (Glc)- or *N*-acetylglucosamine (GlcNAc)-tagged natural products as acceptor substrates for transglycosylation, making it possible to introduce a defined N-glycan into natural products in a highly convergent fashion.

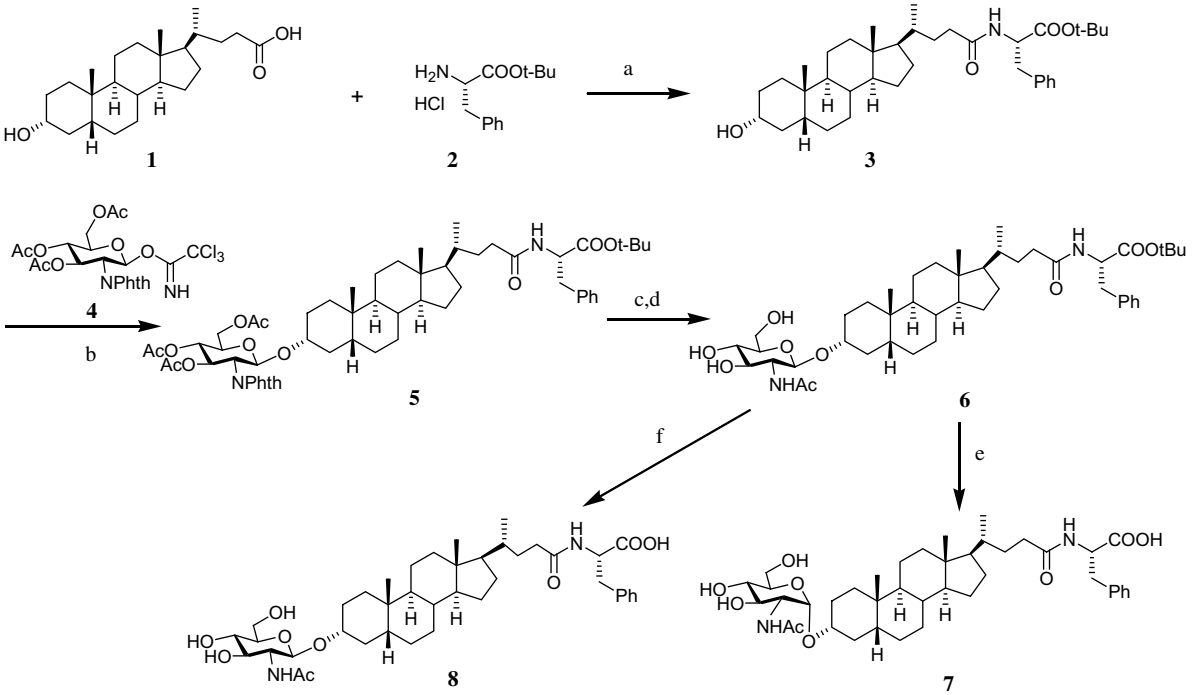
2. Results and discussion

2.1. Introduction of a monosaccharide handle into natural products

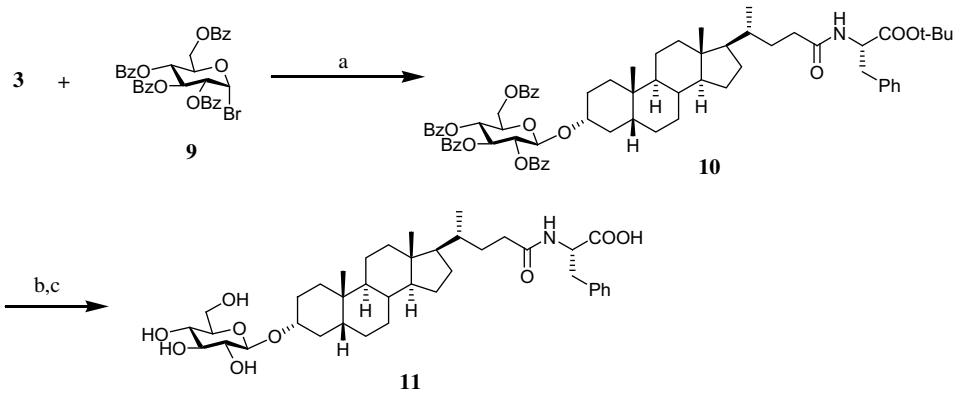
Previous studies have implicated that the endoglycosidases Endo-A and Endo-M were able to transfer an N-glycan to some monosaccharides, including Glc, GlcNAc, and their derivatives, to form a new glycosidic linkage.[28–30] An interesting application of this enzymatic transglycosylation for synthesizing novel glycosylated cyclodextrin was recently reported.[31] But enzymatic glycosylation of a broad range of natural products using this chemoenzymatic approach has not been investigated. For those natural products that do not carry sugars, monosaccharide moieties such as Glc and GlcNAc need to be introduced, which would serve as a handle for the transglycosylation to introduce a defined N-glycan. Either chemical glycosylation or enzymatic glycosylation[11,14,32–34] could be applied for introducing monosaccharide tags. Here we took lithocholic acid as a model natural product to introduce a Glc and GlcNAc tag to its 3-hydroxyl group by chemical glycosylation. Lithocholic acid is a bile acid derivative that has a free hydroxyl group at the 3-position and a carboxyl group at C-24. Its metabolic form includes amidation at the 24-carboxyl with glycine or taurine and glycosylation at 3-hydroxyl with GlcNAc, Glc or glucuronic acid.[35] To facilitate its UV detection during transformations, we replaced the glycine moiety in the native conjugate by a phenylalanine.

The synthesis of the GlcNAc-containing lithocholic acid derivative (**8**) is summarized in Scheme 1. Lithocholic acid (**1**) was coupled with phenylalanine *tert*-butyl ester (**2**) using DCC as the coupling reagent to give compound **3** in 95% yield. The conjugate **3** was then glycosylated with 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthlamido-β-D-glucopyranosyl trichloroimidate (**4**)[36] in the presence of $BF_3{\cdot}Et_2O$ to afford the β-glycoside **5** in 45% yield. Treatment of **5** with ethylenediamine to remove the phthaloyl group with simultaneous O-deacetylation, followed by selective N-acetylation of the resulting amino group, gave the GlcNAc-containing compound **6** in 88% yield over two steps. Initial attempts to remove the *tert*-butyl group by treatment of **6** with 20% trifluoroacetic acid (TFA) in CH_2Cl_2 at rt gave a product, compound **7**, in which the *tert*-butyl group was selectively removed. But, surprisingly, 2D NMR analysis of **7** revealed that the resulting glycoside was converted to the α-isomer (H-1 of the GlcNAc, δ 4.72 ppm, $J_{1,2}$ = 3.6 Hz). Milder conditions (5–10% TFA in CH_2Cl_2) resulted in incomplete removal of the *tert*-butyl group, but yet still led to significant formation of the α-anomer. Clearly, a TFA-promoted isomerization of the β-glycosidic bond was difficult to avoid in this case. Finally, a successful deprotection of the *tert*-butyl group was achieved by treatment of **6** with formic acid without anomeric isomerization, giving the desired β-glycoside **8** in excellent yield (H-1 of the GlcNAc in **8**, δ 4.32 ppm, $J_{1,2}$ = 8.0 Hz) (Scheme 2).

To introduce a glucose moiety into the lithocholic acid, the lithocholamide derivative **3** was glycosylated with the O-benzoylated glucopyranosyl bromide **9** in the presence of AgOTf and 2,6-di-*tert*-butylpyridine to afford compound **10**, in which the newly formed glycosidic bond was determined to be the desired β-linkage. After de-O-benzoylation, the *tert*-butyl group in **10** was successfully removed by treatment with 20% TFA in CH_2Cl_2 to give glycoconjugate **11** in 80% yield over two steps. In contrast to the GlcNAc-containing compound **6**, which resulted in the isomerization of the glycosidic linkage upon treatment with 20% TFA, the β-glucoside linkage in **10** could withstand 20% TFA treatment without isomerization. In comparison, it appears that the 2-acetamido group in **6** promotes the β- to α-glycosidic conversion under the action of TFA.



Scheme 1. Synthesis of GlcNAc-bile acid phenylalanine conjugate **7**. Reagents and conditions: (a) DCC, NHS, DIPEA, CH_2Cl_2, rt, overnight, 95%; (b) $BF_3{\cdot}Et_2O$, 4 Å MS, CH_2Cl_2, −20 °C, 2 h, 45%; (c) ethylene diamine, *n*-BuOH, 80 °C, 3 h; (d) Ac_2O, 0.1 M $NaHCO_3$, rt, 1 h, 88% for 2 steps; (e) 20% TFA, CH_2Cl_2, rt, 2 h, 70%; (f) HCO_2H, rt, 5 h, 90%.

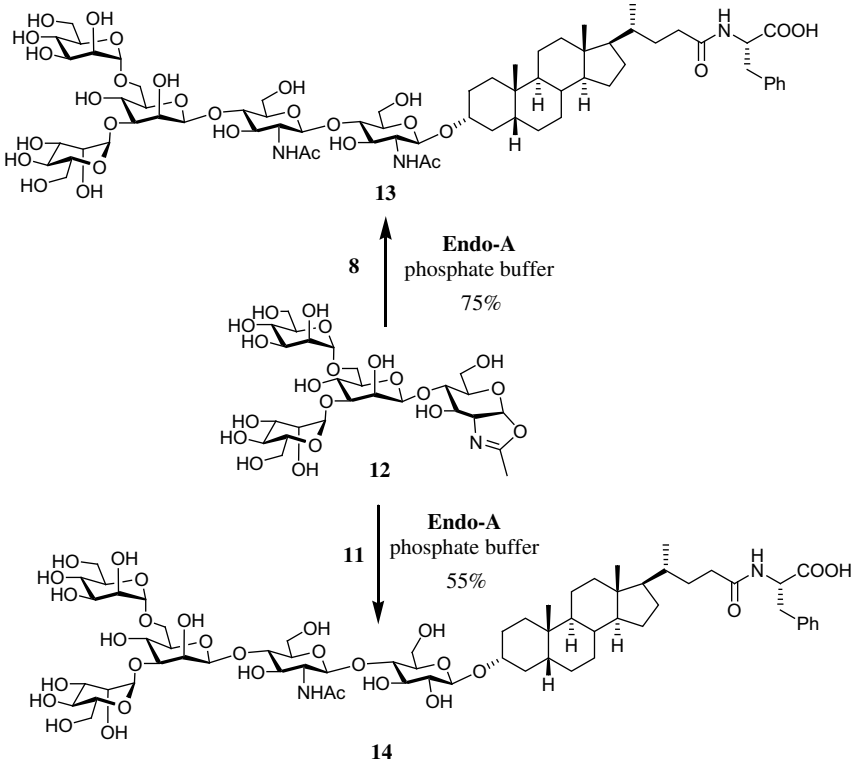


Scheme 2. Synthesis of Glc-bile acid phenylalanine conjugate **11**. Reagents and conditions: (a) 2,6-di-*tert*-butylpyridine, AgOTf, 4 Å MS, CH_2Cl_2, rt, overnight, 56%; (b) NaOMe, MeOH, rt, 1 h; (c) 20% TFA in CH_2Cl_2, rt, 3 h, 80% for 2 steps.

2.2. Chemoenzymatic transglycosylation to GlcNAc- and Glc-containing lithocholamides

Previous studies on substrate specificity of Endo-A have shown that some glucose-containing compounds such as *p*-nitrophenyl glucoside, as well as free glucose and GlcNAc, could be acceptors for Endo-A catalyzed transglycosylation.[28–30] However, it was not clear whether the Glc or GlcNAc in context of a steroid could efficiently serve as an acceptor substrate for Endo-A. To examine the feasibility of these Glc- and GlcNAc-containing compounds for enzymatic transglycosylation, we have chosen the $Man_3GlcNAc$ oxazoline (**12**) as donor substrate, as this tetrasaccharide oxazoline was shown to be an excellent donor substrate for the Endo-A catalyzed transglycosylation.[15,16] Thus, the GlcNAc-containing lithocholic acid derivative (**8**) and the sugar oxaozline **12** (donor/acceptor, 2:1) were incubated with Endo-A in a phosphate buffer (pH 7.0) at 23 °C, and the reaction was monitored by analytical RP-HPLC. It was found that compound **8** was a good substrate for Endo-A and, after 2 h under the above-mentioned conditions, the transglycosylation product **13** was obtained in 75% yield (Scheme 3).

The transglycosylation product was characterized by ESI-MS and NMR spectroscopic analysis. The observed data [m/z = 1416.70 for $[M+H]^+$] was in good agreement with the calculated molecular mass (M = 1415.68), indicating that the product is the adduct of the tetrasaccharide oxazoline and the acceptor **8**. On the other hand, ^1H NMR analysis revealed a doublet at δ 4.38 ppm with a relatively large coupling constant ($J_{1,2}$ = 8.0 Hz) assigned for the H-1 of the second GlcNAc, suggesting that the transferred glycan was attached in the desired β-glycosidic linkage. It should be noted that the signals for H-1 and H-1′ were almost overlapping at δ 4.38–4.39 ppm as doublets. The



Scheme 3. Transglycosylations on GlcNAc/Glc-bile acid phenylalanine conjugates by Endo-A.

β-(1→4)-glycosidic linkage type of the newly formed glycosidic bond was confirmed by the apparent NOE correlation between H-1′ and H-4 by the NOESY analysis of compound **13** (Fig. 1).

Interestingly, it was found that the α-linked GlcNAc derivative (**7**) was also an acceptor substrate of Endo-A. Thus, incubation of **7** with oxazoline **12** and Endo-A gave the corresponding transglycosylation product in 48% yield [ESI-MS: calculated molecular mass, $M = 1415.68$; found, 1416.87 $[M+H]^+$]. These experiments suggest that the enzyme has a flexibility to accommodate both the α- and β-linked GlcNAc moiety for transglycosylation. Similarly, incubation of the Glc-containing compound **11** and oxazoline **12** (donor/acceptor, 2:1) with Endo-A in a phosphate buffer (pH 7.0) for 2 h gave the transglycosylation product **14** in 55% yield (Scheme 3). Again, the β-(1→4)-glycosidic linkage for the newly formed glycosidic bond in compound **14** was confirmed by the ^{1}H NMR and NOESY analysis (Fig. 2). A strong NOE between the H-1′ and H-4 protons clearly suggest that the GlcNAc and the Glc moieties were linked through the expected β-(1→4)-glycosidic bond. Comparison of the enzymatic transglycosylation yields with compounds **7** and **11** indicates that the GlcNAc derivative was a relatively better acceptor substrate for Endo-A than the Glc-derivative. These experiments substantiated a broad substrate specificity of Endo-A, implicating its feasibility for glycosylating a wide range of sugar-containing natural products.

2.3. Chemoenzymatic transglycosylation of other natural products containing a Glc moiety

Encouraged by the positive results from the enzymatic transglycosylation with the synthetic GlcNAc- and Glc-containing steroids,

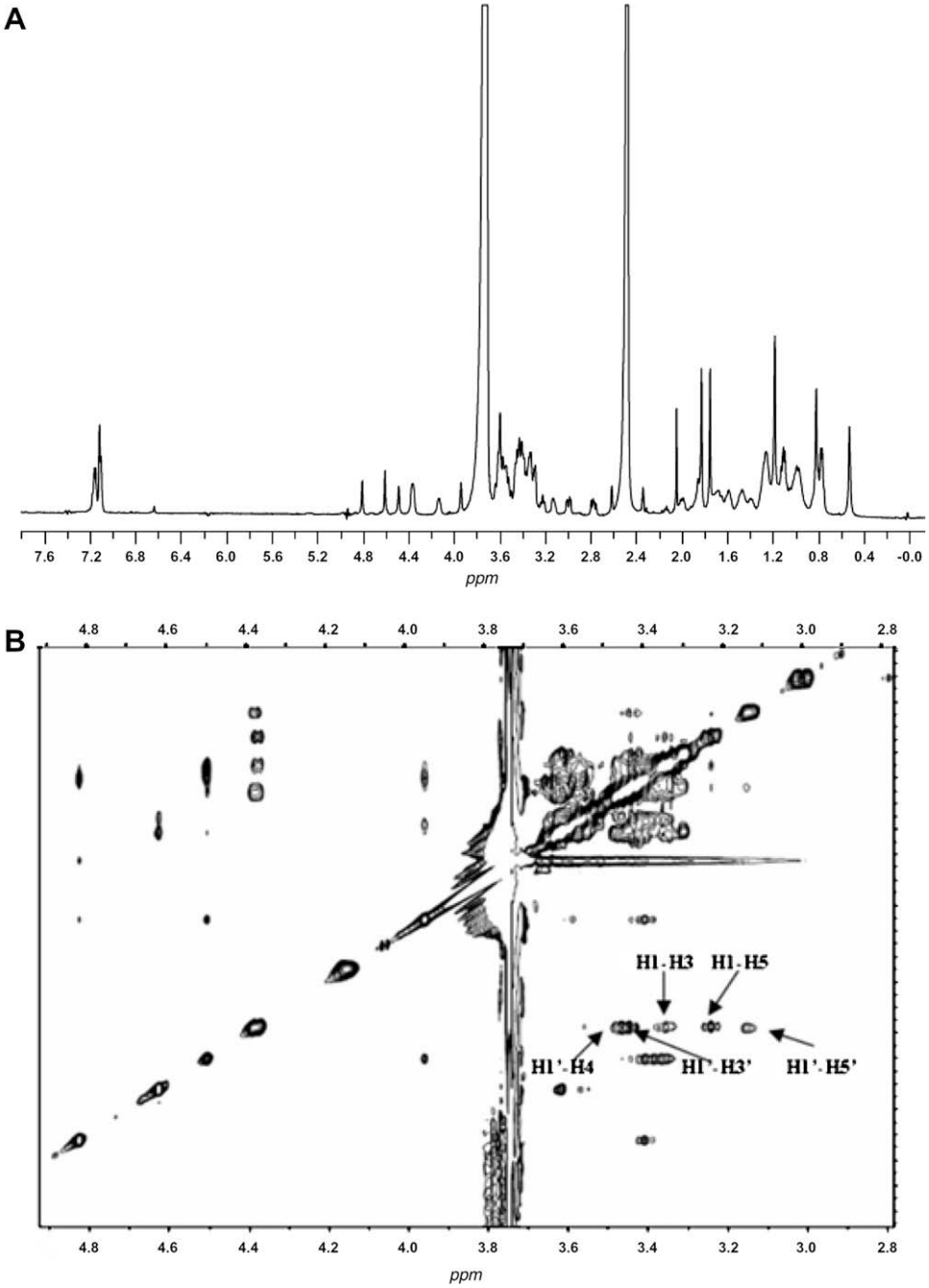


Figure 1. ^{1}H NMR (A) and NOESY (B) of the transglycosylation product **13**.

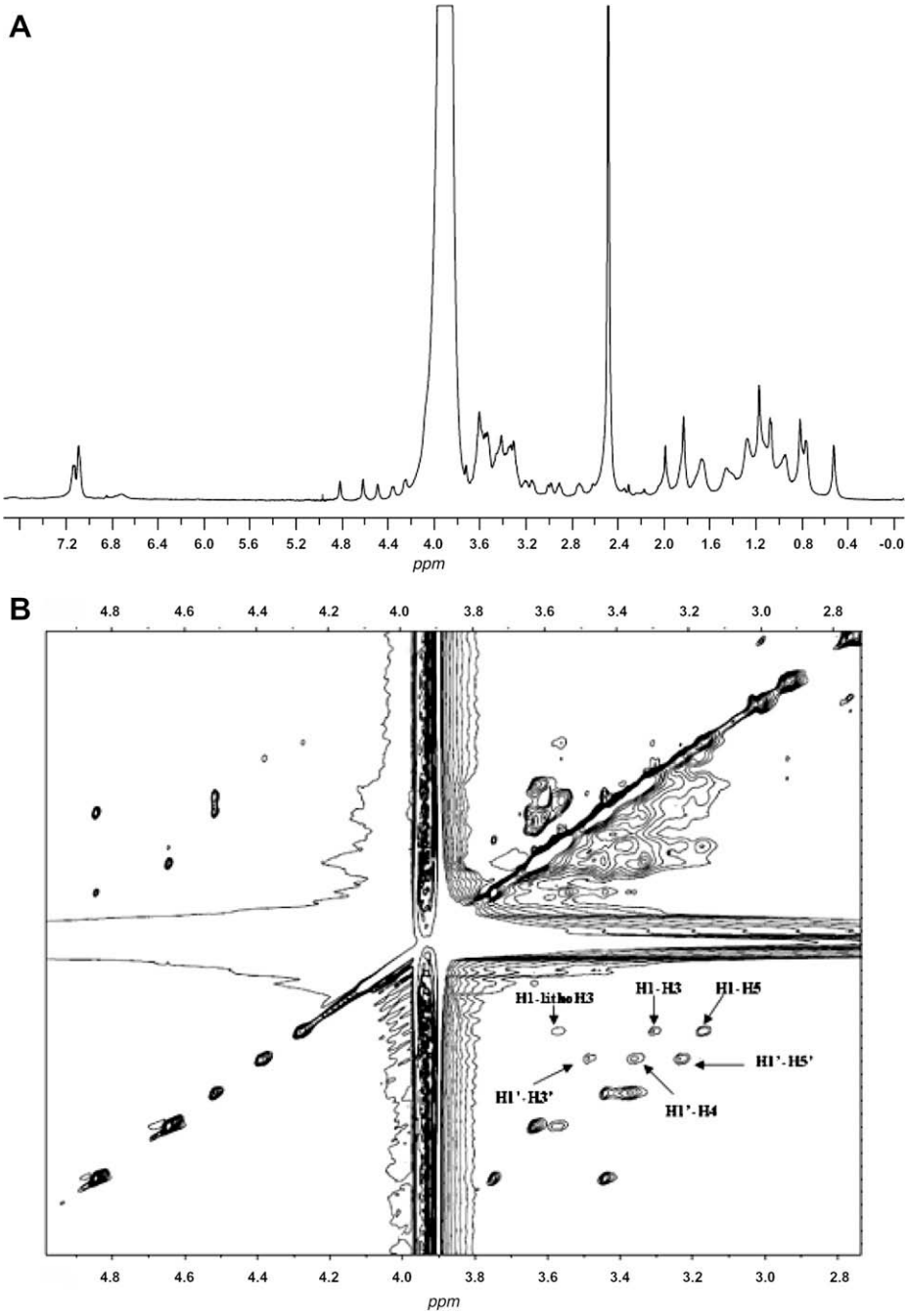
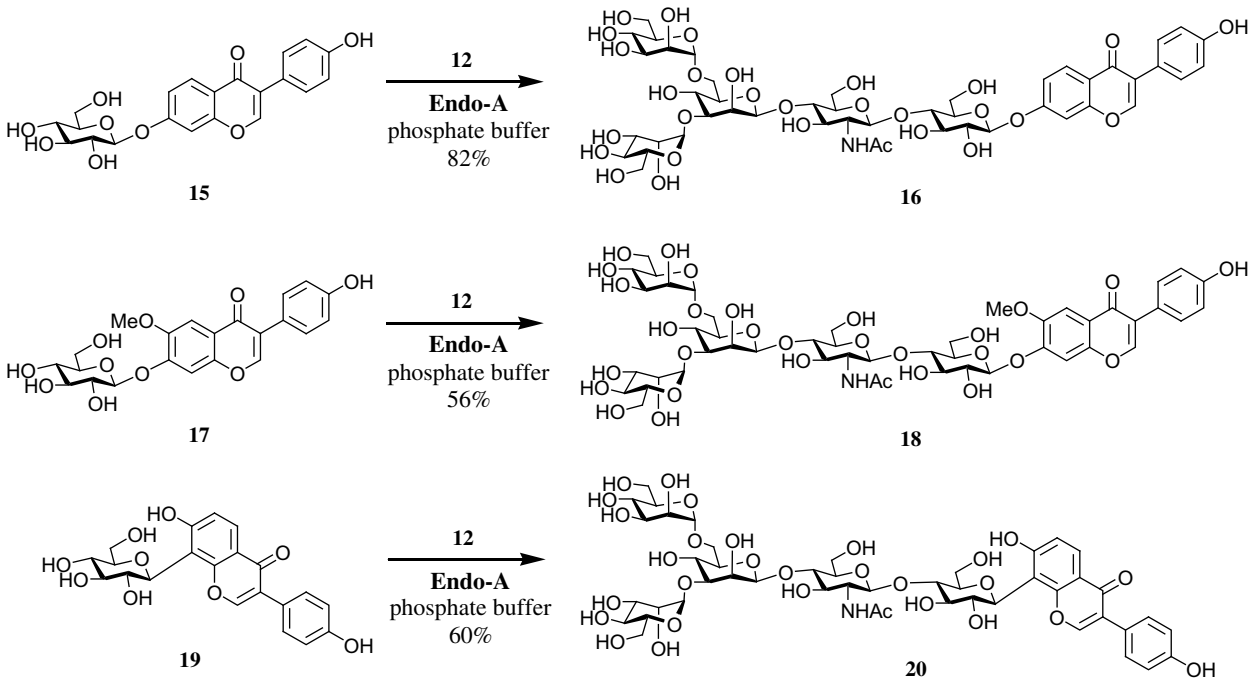


Figure 2. ^{1}H NMR (A) and NOESY (B) of the transglycosylation product **14**.

we next examined the enzymatic reactions with an array of commercially available natural products, which already contain a sugar moiety in the structure. First, we tested a few flavonoid glycosides (Scheme 4). Flavonoids occur widely in plants and have been implicated as potential therapeutics.[37–40] Daidzin (**15**),[41] glycitin (**17**),[42] and puerarin (**19**)[43] are three flavonoid glucosides extracted from *Pueraria*, which exhibit the activities on reduction of bone loss and lipid metabolism abnormality.[44] All the enzymatic reactions were performed in a phosphate buffer at 23 °C on an analytical scale using the tetrasaccharide oxazoline **12** as the donor substrate, and a fixed molar ratio (2:1) of donor/acceptor was applied. The reaction was monitored by reverse-phase HPLC for 2 h and the transglycosylation product was isolated and was subjected to ESI-MS analysis.

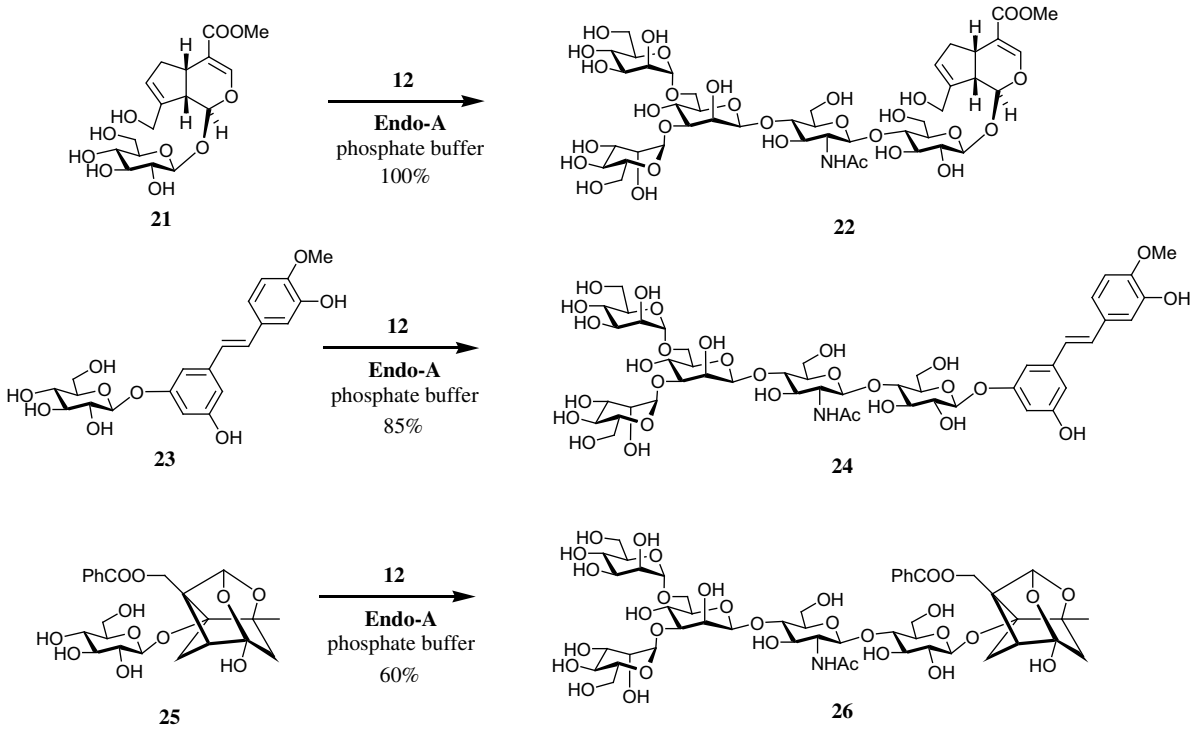
It was found that all the three flavonoid glucosides (**15**, **17**, and **19**) were substrates for the Endo-A-catalyzed transglycosylation, giving the corresponding transglycosylation products **16**, **18**, and **20**, respectively (Scheme 4). In comparison, daidzin **15** achieved a higher yield of transglycosylation (82%) than glycitin (**17**) (56%) and puerarin (**19**) (60%) under the same reaction conditions. Interestingly, the only difference between **15** and **17** is the presence of an extra methoxyl group at the 6-position of the benzene ring in glycitin (**17**). A plausible explanation is that the 6-methoxy group proximal to the 7-glucoside may exert partially steric hindrance for enzyme recognition, thus making glycitin (**17**) a less active substrate for transglycosylation. Puerarin (**19**) is actually a C-linked glucoside. Yet it could also serve as a substrate for the Endo-A catalyzed transglycosylation. The relatively low transglycosylation



Scheme 4. Transglycosylations on flavonoids natural products with a terminal glucose moiety.

yield of **19** in comparison with that of **15** might be again attributed to the presence of a proximal hydroxyl group at the 7-position, but not necessarily due to the nature of C-linked glycosidic bond. C-linked GlcNAc and Glc-derivatives as acceptors for endoglycosidase-catalyzed transglycosylation were previously reported.[21,30,45] These results further demonstrate the wide substrate tolerance of Endo-A.

In addition to the flavonoid glucosides, we also tested three other plant extracts: Geniposide (**21**)[46] was extracted from *Gardenia jaminoides*, a Chinese herb; Rhaponticin (**23**)[47] was extracted from rhubarb; and Paeoniflorin (**25**)[48] was extracted from *Paeonia lactiflora*. All the three natural products have shown diverse biological activities. It was found that these three natural product glucosides were substrates for Endo-A, and yet a different reactivity



Scheme 5. Transglycosylations on more natural products with glucose.

for these structurally distinct compounds were observed (Scheme 5). Interestingly, while the geniposide (**21**) could quickly achieve a quantitative conversion to the transglycosylation product **22**, rhaponticin **23** and paeoniflorin **25** gave a 85% and 60% yield of the transglycosylation product **24** and **26**, respectively, within 2 h incubation with Endo-A. The relatively low transglycosylation yield for **25** might be attributed to the steric hindrance of the acceptor, whereas the reason for the unusual high reactivity of **21** is not clear. Probably the proximal primary hydroxyl group in the aglycon portion may assist enzyme recognition and/or facilitate the substrate–enzyme binding. Further testing of an expanded library of compounds with diverse structures may provide an answer to this interesting question.

2.4. Double transglycosylation to luteolin 3′,7-diglucoside

Luteolin 3′,7-diglucoside (**27**) is a flavonoid extracted from *Reseda luteola* that shows antioxidant activity.[49,50] Compound **27** contains two β-glucoside moieties. Therefore, it would be interesting to test its behavior in the Endo-A catalyzed transglycosylation. The Endo-A catalyzed reaction between **27** and oxazoline **12** was performed under the same conditions as described above for the other natural product glycosides (Scheme 6). The enzymatic reaction was monitored by reverse-phase HPLC, and the new products were isolated for ESI-MS analysis. It was observed that when compound **27** was incubated with 2 mol equiv of the oxazoline **12** in the presence of Endo-A for 2 h, the mono-transglycosylated product (Fig. 3, peak 2) was formed as the major product (about 60%) as validated by ESI-MS (data not shown), together with the doubly transglycosylated product (Fig. 2, peak 3) (about 25%). However, when an excess of donor substrate (**12**) (4 mol equiv in total) was supplemented to the reaction and the mixture was incubated for 4 h, further conversion could be achieved and the doubly transglycosylated product **28** was isolated in 70% yield (Fig. 3, peak 3). The identity of the transglycosylation product **28** was confirmed by its HR-MS: calculated, $[M+H]^+ = 1989.6385$; found, 1989.6369 $[M+H]^+$. These results suggest that the enzymatic transglycosylation may be equally efficient for simultaneous transfer of multiple N-glycans to a natural product when more than one monosaccharide tags, for example, terminal glucose moieties, are present in the structure. We assume that the transferred glycans were attached to the glucose moieties in the expected β-(1→4)-glycosidic linkages, as clearly demonstrated for the glycosylated lithocholic acid derivatives **13** and **14**. This assumption was also based on previous studies, indicating that when a Glc or GlcNAc moiety was used as an acceptor, the Endo-A demonstrated a strict stereospecificity to form a new β-(1→4)-glycosidic linkage, which was determined by detailed NMR analysis and enzymatic transformations of the transglycosylation products.[15,17,18,51]

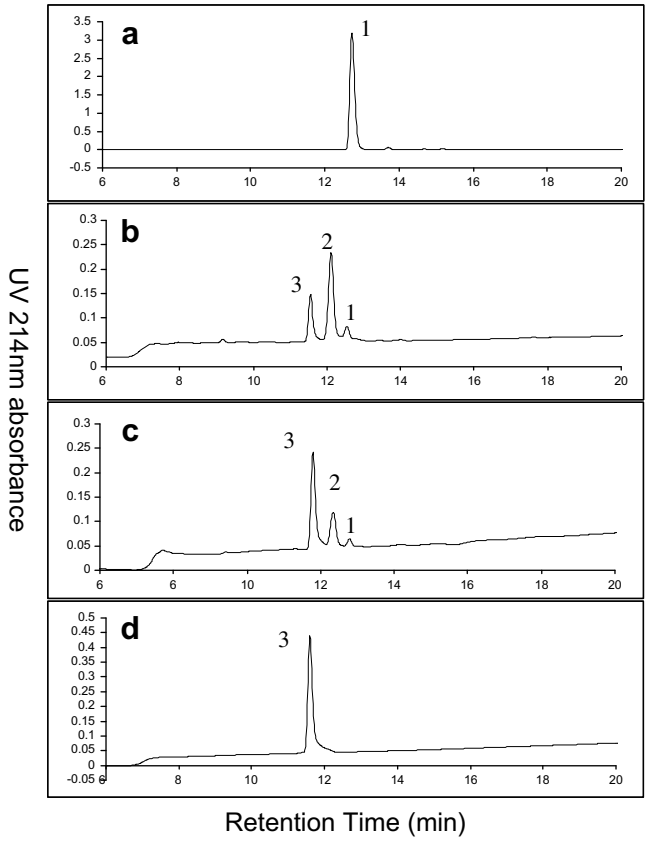
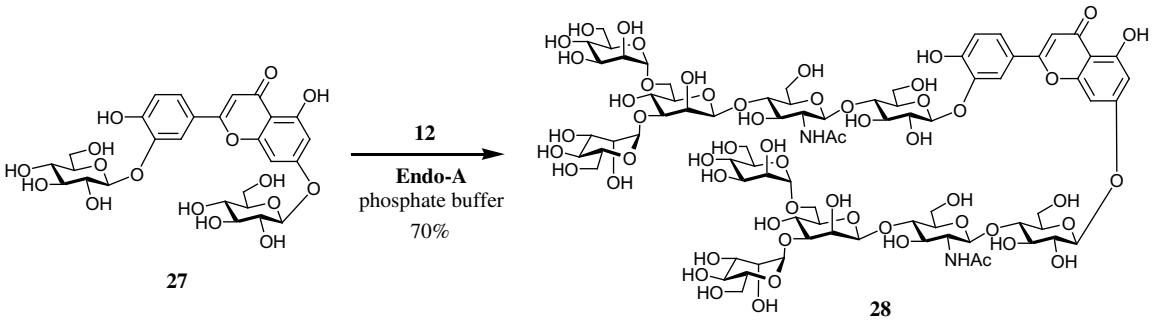


Figure 3. HPLC monitoring of Endo-A catalyzed transglycosylation with luteolin 3′,7-di-O-glucoside (**27**). A solution of oxazoline **12** and luteolin glucoside **27** (donor/acceptor 2:1) in a phosphate buffer (50 mM, pH 7.0) was incubated with Endo-A. The reaction progress was monitored by analytical HPLC: (a) 0 min; (b) 2 h incubation; (c) another 2 h incubation with an addition of 2 mol equiv of donor substrate; and (d) purified glycosylated product **28**. Peak 1, compound **27**; peak 2, mono-transglycosylated product; peak 3, doubly transglycosylated product **28**.

3. Conclusion

An efficient enzymatic transglycosylation for introducing a core N-glycan into various lipophilic natural products was described. The present study reveals a broad acceptor substrate specificity of the endoglycosidase Endo-A, making it possible to attach an N-glycan to a range of natural products in a site-specific and highly convergent fashion. N-Glycans of glycoprotein origin can serve as ligands for diverse lectins and cell-surface receptors. Thus, introduction of a defined N-glycan into biologically significant natural



Scheme 6. Transglycosylations on doubly substituted natural product **27**.

products may bestow novel properties onto these natural products, for example, leading to enhanced solubility, specific cellular targeting, altered mechanism of action, and overall improvement of pharmacological properties. Together with our previous results that Endo-A could accommodate various selectively modified sugar oxazolines as donor substrates, the broad acceptor substrate specificity revealed by the present study should facilitate a quick generation of an expanded glyco-randomized natural product library valuable for drug discovery and development.

4. Experimental

4.1. Materials

Lithocholic acid (**1**), 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide (**9**), daidzin (**15**), glycitin (**17**), peurarin (**19**), and rhaponticin (**23**) were purchased from Sigma–Aldrich and used as received. Geniposide (**21**) and paeoniflorin (**25**) were purchased from AXXORA Life Sciences Inc. Luteolin-3′,7-diglucoside (**27**) was purchased from ChromaDex Corporate. The recombinant wild type Endo-A was overproduced in *Escherichia coli* and purified by affinity chromatography according to the literature.[52] All other reagents were purchased from Sigma–Aldrich and used as received.

4.2. Analytical methods

Analytical RP-HPLC was performed on a Waters 626 HPLC instrument with a Symmetry300™ C18 column (5.0 μm, 4.6 × 250 mm) at 40 °C. The column was eluted with a linear gradient 0–60% MeCN containing 0.1% trifluoroacetic acid (TFA) for 20 min at a flow rate of 1 mL/min (*Method A*) or a linear gradient 0–90% MeCN containing 0.1% trifluoroacetic acid (TFA) for 10 min at a flow rate of 1 mL/min and then isocratic 90% MeCN containing 0.1% TFA for 20 min (*Method B*). Preparative HPLC was performed on a Waters 600 HPLC instrument with a preparative C18 column (Symmetry300, 19 × 300 mm). The column was eluted with a suitable gradient of water–acetonitrile containing 0.1% TFA. NMR spectra were measured with JEOL ECX 400 MHz. The chemical shifts were assigned in ppm. ESI-MS Spectra were measured on a micromass ZQ-4000 single quadruple mass spectrometer. High-resolution mass spectra (HR-MS) were measured on a QSTAR/Pulsar (Applied Biosystems/MDS Sciex) ESI mass spectrometer.

4.3. *N*-(L-Phenylalanine-*tert*-butyl ester)-lithocholamide (3)

To a solution of lithocholic acid (**1**) (376 mg, 1.0 mmol) and phenylalanine *tert*-butyl ester hydrochloride (**2**) (300 mg, 1.2 mmol) in CH_2Cl_2 (10 mL) were added *N,N′*-dicyclohexylcarbodiimide (DCC) (340 mg, 1.65 mmol), *N*-hydroxysuccimide (NHS) (160 mg, 1.39 mmol), and *N,N*-diisopropylethylamine (DIPEA) (0.5 mL, 2.87 mmol). The mixture was stirred at rt overnight. The precipitate was filtered out and the filtrate was concentrated under vacuum. The residue was subject to column chromatography on silica gel using EtOAc–hexanes (1:2, v/v) as eluent to give the coupling product **3** (550 mg, 95%) as a white powder. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.30–7.12 (m, 5H, Ph-ArH), 6.00 (d, 1H, J = 8.0 Hz, NH), 4.74 (dd, 1H, J = 6.4 Hz, 13.6 Hz, Phe-αH), 3.62 (m, 1 H, 3-H of lithocholamide), 3.06 (d, 2H, J = 6.8 Hz, Phe-βH), 1.39 (s, 9H, *t*-Bu), 0.89 (s, 3H, 19-CH_3), 0.87 (d, 3H, J = 6.8 Hz, 21-CH_3), 0.61 (s, 3H, 18-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.4, 170.9, 136.2, 129.6, 128.4, 127.0, 82.4, 71.9, 56.5, 56.0, 53.4, 42.8, 42.1, 40.5, 40.2, 38.1, 36.4, 35.9, 35.5, 35.4, 34.6, 33.5, 31.7, 30.5, 28.2, 28.0, 27.2, 26.5, 24.2, 23.4, 20.9, 18.4, 12.1; ESI-MS: calculated for $C_{37}H_{57}NO_4$, M = 579.43, found, 580.56 $[M+H]^+$.

4.4. 3-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-*N*-(L-phenylalanine-*tert*-butyl ester)-lithocholamide (5)

A solution of compound **3** (100 mg, 0.172 mmol) and 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl trichloroacetimidate **4** (200 mg, 0.345 mmol) in CH_2Cl_2 (5 ml) containing activated 4 Å molecular sieves (300 mg) was stirred under an atmosphere of argon at room temperature for 0.5 h. After cooling to −20 °C, $BF_3{\cdot}Et_2O$ (10 μL, 80 μmol) was added and the resulting mixture was stirred at −20 °C for 2 h. The mixture was filtered through a Celite pad and the filtrate was poured into saturated $NaHCO_3$. Then the mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated and the residue was subject to column chromatography on silica gel using EtOAc–hexanes (1:2, v/v) as eluent to provide **5** (77 mg, 45%) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.86 (m, 2H, Phthaloyl-ArH), 7.75 (m, 2H, Phthaloyl-ArH), 7.28 (m, 3H, Ph-ArH), 7.14 (m, 2H, Ph-ArH), 5.85 (d, 1H, J = 7.6 Hz, NH), 5.76 (t, 1H, J = 9.6 Hz, H-3 of Glc), 5.46 (d, 1H, J = 9.6 Hz, H-1 of Glc), 5.14 (t, 1H, J = 9.6 Hz, H-4 of Glc), 4.75 (dd, 1H, J = 6.0 Hz, 14.0 Hz, Phe-αH), 4.30 (m, 2H, H-2, H-6 of Glc), 4.15 (dd, 1H, J = 2.8 Hz, 11.6 Hz, H-6 of Glc), 3.84 (m, 1H, H-5 of Glc), 3.53 (m, 1H, 3-H of lithocholamide), 3.07 (d, 2H, J = 6.0 Hz, Phe-βH), 2.09 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.85 (s, 3H, Ac), 1.39 (s, 9H, *t*-Bu), 0.85 (d, 3H, J = 6.8 Hz, 21-CH_3), 0.83 (s, 3H, 19-CH_3), 0.56 (s, 3H, 18-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.0, 170.9, 170.8, 170.3, 169.6, 136.3, 134.3, 131.5, 129.6, 128.4, 127.0, 123.6, 97.1, 82.4, 80.5, 71.7, 71.0, 69.2, 62.4, 56.3, 56.0, 55.0, 53.3, 42.7, 42.2, 40.2, 40.0, 38.1, 35.8, 35.4, 35.1, 34.6, 34.2, 33.5, 31.7, 29.7, 28.2, 28.0, 27.2, 27.0, 26.5, 24.2, 23.3, 20.8, 20.7, 20.5, 18.4, 12.0; ESI-MS: calculated for $C_{57}H_{76}N_2O_{13}$, M = 996.53, found, 997.86 $[M+H]^+$.

4.5. 3-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-*N*-(L-phenylalanine-*tert*-butyl ester)-lithocholamide (6)

A solution of compound **5** (50 mg, 50 μmol) in *n*-BuOH (2 mL) containing ethylenediamine (2 mL) was heated at 80 °C with stirring for 3 h. The solution was concentrated and the residue was subject to preparative HPLC to afford the product with free amino and hydroxyl groups. The obtained intermediate was dissolved in a mixed solution of MeOH (2 mL) and 0.1 M $NaHCO_3$ (5 mL), to which Ac_2O (50 μL) was added. The mixture was stirred at rt for 1 h. The solution was concentrated and the residue was subject to preparative HPLC to give the *N*-acetyl derivative **6** (34.5 mg, 88% for 2 steps) as a white solid. ^{1}H NMR (DMSO-d_6 + 5% D_2O, 400 MHz): δ 7.22–7.12 (m, 5H, Ph-ArH), 4.32 (d, 1H, J = 8.8 Hz, H-1 of GlcNAc), 4.25 (m, 1H, Phe-αH), 3.62 (m, 1H, H-6, of GlcNAc), 3.38 (m, 2H, 3-H of lithocholamide, H-6 of GlcNAc), 3.25 (m, 2H, H-3, H-4 of GlcNAc), 2.99 (m, 2H, H-5 of GlcNAc, Phe-βH), 2.87 (m, 1H, H-2 of GlcNAc), 2.78 (m, 1H, Phe-βH), 1.75 (s, 3H, Ac), 1.24 (s, 9H, *t*-Bu), 0.80 (s, 3H, 19-CH_3), 0.78 (d, 3H, J = 8.0 Hz, 21-CH_3), 0.51 (s, 3H, 18-CH_3); ^{13}C NMR (DMSO-d_6 + 5% D_2O, 100 MHz) δ 173.5, 173.3, 170.4, 138.0, 129.6, 128.4, 126.8, 95.7, 77.6, 73.0, 71.0, 70.9, 61.2, 56.6, 56.2, 56.0, 54.2, 53.7, 42.8, 41.6, 37.1, 35.8, 35.5, 35.3, 34.8, 32.6, 32.5, 32.4, 32.0, 28.5, 28.2, 28.0, 27.2, 26.6, 24.3, 23.6, 23.0, 20.9, 18.7, 18.5, 12.3; ESI-MS: calculated for $C_{45}H_{70}N_2O_9$, M = 782.51, found, 783.78 $[M+H]^+$.

4.6. 3-(2-Acetamido-2-deoxy–D-glucopyranosyl)-*N*-(L-phenylalanine)-lithocholamide (7)

A solution of compound **6** (20 mg, 25 μmol) in 20% TFA in CH_2Cl_2 (3 mL) was stirred at rt for 2 h. The solution was concen-

trated and the residue was subject to preparative HPLC to afford **7** (13 mg, 70%) as a white solid. ^{1}H NMR (DMSO-d_6 + 5% D_2O, 400 MHz): δ 7.23–7.12 (m, 5H, Ph-ArH), 4.72 (d, 1H, J = 3.6 Hz, H-1 of α-GlcNAc), 4.33 (m, 1H, Phe-αH), 3.55 (m, 2H, H-2, H-6 of GlcNAc), 3.40 (m, 4H, 3-H of lithocholamide, H-3, H-5, H-6 of GlcNAc), 3.08 (t, 1H, J = 8.0 Hz, H-4 of GlcNAc), 3.00 (dd, 1H, J = 2.8 Hz, 13.6 Hz, Phe-βH), 2.78 (dd, 1H, J = 8.8 Hz, 13.6 Hz, Phe-βH), 1.80 (s, 3H, Ac), 0.83 (s, 3H, 19-CH_3), 0.78 (d, 3H, J = 7.2 Hz, 21-CH_3), 0.58 (s, 3H, 18-CH_3); ^{13}C NMR (DMSO-d_6 + 5% D_2O, 100 MHz) δ 173.7, 173.5, 170.4, 138.1, 129.6, 128.6, 126.8, 96.3, 77.6, 73.1, 71.2, 70.9, 61.2, 56.6, 56.2, 56.0, 54.2, 53.7, 42.8, 41.8, 37.1, 35.8, 35.5, 35.3, 34.8, 32.6, 32.5, 32.3, 32.0, 28.5, 28.1, 27.2, 26.6, 24.3, 23.6, 23.2, 20.9, 18.7, 18.4, 12.3; ESI-MS: calculated for $C_{41}H_{62}N_2O_9$, M = 726.45, found, 727.78 $[M+H]^+$.

4.7. 3-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-*N*-(L-phenylalanine)-lithocholamide (8)

A solution of compound **6** (34.5 mg, 44 μmol) in formic acid (5 mL) was stirred at rt for 5 h. The solution was then added to a cold $NaHCO_3$ (1 M) solution and the solution was stirred at rt for 3 h. The mixture was concentrated and the residue was subject to preparative HPLC to afford **8** (12 mg, 90% based on a small recovery of starting material. ^{1}H NMR (DMSO-d_6 + 5% D_2O, 400 MHz): δ 7.22–7.10 (m, 5H, Ph-ArH), 4.32 (d, 1H, J = 8.0 Hz, H-1 of GlcNAc), 4.23 (m, 1H, Phe-αH), 3.63 (m, 1H, H-6 of GlcNAc), 3.40 (m, 2H, 3-H of lithocholamide, H-6 of GlcNAc), 3.25 (m, 2H, H-3, H-4 of GlcNAc), 3.00 (m, 3H, H-5, H-2 of GlcNAc, Phe-βH), 2.78 (m, 1H, Phe-βH), 1.75 (s, 3H, Ac), 0.81 (s, 3H, 19-CH_3), 0.74 (d, 3H, J = 6.8 Hz, 21-CH_3), 0.52 (s, 3H, 18-CH_3); ^{13}C NMR (DMSO-d_6 + 5% D_2O, 100 MHz) δ 173.7, 173.4, 170.5, 138.1, 129.6, 128.6, 126.8, 95.9, 77.6, 73.1, 71.1, 70.9, 61.2, 56.6, 56.2, 56.0, 54.2, 53.7, 42.8, 41.7, 37.1, 35.8, 35.5, 35.3, 34.8, 32.6, 32.5, 32.4, 32.0, 28.5, 28.1, 27.2, 26.6, 24.3, 23.6, 23.0, 20.9, 18.7, 18.5, 12.3; ESI-MS: calculated for $C_{41}H_{62}N_2O_9$, M = 726.45, found, 727.70 $[M+H]^+$.

4.8. 3-(2,3,4,6-Tetra-*O*-benzoyl-β-D-glucopyranosyl)-*N*-(L-phenylalanine-*tert*-butyl ester)-lithocholamide (10)

A solution of compound **3** (100 mg, 0.172 mmol), 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide **9** (235 mg, 0.346 mmol), and 2,6-di-*t*-butylpyridine (120 μL, 0.544 mmol) in CH_2Cl_2 (5 mL) containing activated 4 Å molecular sieves (300 mg) was stirred under an atmosphere of argon at rt for 0.5 h. Then, AgOTf (80 mg, 0.311 mmol) was added and the resulting mixture was stirred at rt overnight. The mixture was filtered through a Celite pad and the filtrate was poured into a saturated $NaHCO_3$ solution. The solution was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated and the residue was subject to column chromatography on silica gel using EtOAc–hexanes (1:2, v/v) as eluent to provide **10** (112 mg, 56%) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.01–7.82 (m, 8H, Bz-ArH), 7.56–7.22 (m, 15H, Bz-ArH, Ph-ArH), 7.14 (m, 2H, Ph-ArH), 5.89 (t, 1H, J = 9.6 Hz), 5.60 (t, 1H, J = 9.6 Hz), 5.48 (t, 1H, J = 9.6 Hz), 4.92 (d, 1H, J = 8.0 Hz, H-1 of Glc), 4.75 (dd, 1H, J = 6.0 Hz, 14.0 Hz, Phe-αH), 4.59 (dd, 1 H, J = 3.2 Hz, 12.4 Hz, H-6 of Glc), 4.51 (dd, 1H, J = 6.4 Hz, 12.4 Hz, H-6 of Glc), 4.15 (m, 1H, H-5 of Glc), 3.61 (m, 1H, 3-H of lithocholamide), 3.08 (d, 2 H, J = 6.8 Hz, Phe-βH), 1.40 (s, 9H, *t*-Bu), 0.85 (d, 3H, J = 6.8 Hz, 21-CH_3), 0.83 (s, 3H, 19-CH_3), 0.57 (s, 3H, 18-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.1, 170.9, 166.2, 165.9, 165.4, 165.2, 136.3, 133.5–133.1 (m), 130.2–129.6 (m), 129.0, 128.9, 128.5–128.3 (m), 127.0, 100.4, 82.4, 81.3, 73.1, 72.3, 72.2, 70.2, 63.5, 56.2, 55.9, 55.0, 53.3, 42.7, 42.3, 40.6, 40.2, 38.1, 35.8, 35.4, 35.2, 34.6, 34.3, 33.5, 31.7, 29.7, 28.2, 28.0, 27.2, 27.1, 26.4, 26.3, 24.2, 23.3, 20.9, 18.4, 12.0; ESI-MS: calculated for $C_{71}H_{83}NO_{13}$, M = 1157.59, found, 1158.81 $[M+H]^+$.

4.9. 3-β-D-Glucopyranosyl-*N*-(L-phenylalanine)-lithocholamide (11)

To a solution of compound **10** (100 mg, 86 μmol) in MeOH (5 mL) was added a solution of NaOMe in MeOH (0.5 M, 0.1 mL, 50 μmol). The solution was stirred at rt for 1 h. The base was neutralized by Dowex 50 W-X8 (H^+ form), and the solution was filtered and the filtrate was concentrated. The residue was purified by preparative HPLC to give *N*-(L-phenylalanine *t*-butyl ester)-lithocholamide-3-β-D-glucoside. The obtained intermediate was treated with 20% TFA in CH_2Cl_2 at rt for 3 h. The solution was then concentrated and the residue was subject to preparative HPLC to provide **11** (47 mg, 80% for 2 steps) as a white solid. ^{1}H NMR (DMSO-d_6 + 5% D_2O, 400 MHz): δ 7.16 (m, 5H, Ph-ArH), 4.32 (q, 1H, J = 4.8 Hz, Phe-αH), 4.18 (d, 1H, J = 7.6 Hz, H-1 of Glc), 3.57 (m, 2H, H-6 of Glc, 3-H of lithocholamide), 3.38 (dd, 1H, J = 5.6 Hz, 12.0 Hz, H-6 of Glc), 3.10 (t, 1H, J = 8.4 Hz, H-3 of Glc), 2.99 (m, 3H, H-5, H-4 of Glc, Phe-βH), 2.87 (t, 1H, J = 8.4 Hz, H-2 of Glc), 2.77 (dd, 1H, J = 9.6 Hz, 13.6 Hz, Phe-βH), 0.80 (s, 3H, 19-CH_3), 0.76 (d, 3H, J = 6.4 Hz, 21-CH_3), 0.50 (s, 3H, 18-CH_3); ^{13}C NMR (DMSO-d_6 + 5% D_2O, 100 MHz) δ 173.9, 173.6, 138.0, 129.5, 128.7, 127.0, 100.7, 77.5, 76.7, 73.7, 70.3, 61.4, 56.5, 55.9, 55.8, 53.8, 42.7, 42.3, 41.8, 40.4, 37.0, 35.7, 35.1, 34.6, 34.2, 32.5, 31.9, 28.1, 27.1, 26.7, 26.5, 24.2, 23.5, 20.8, 18.7, 12.2; ESI-MS: calculated for $C_{39}H_{59}NO_9$, M = 685.42, found, 686.74 $[M+H]^+$.

4.10. Transglycosylation with GlcNAc-containing lithocholic acid derivative (8). Preparation of glycosylated lithocholic acid derivative (13) carrying a core N-pentasaccharide

A solution of compound **8** (1 mg, 1.37 μmol) and oxazoline **12** (2 mg, 2.9 μmol) in a phosphate buffer (50 mM, pH 7.0, 150 μL) containing 20% DMSO was incubated with Endo-A (60 mU) at 23 °C. The reaction was monitored by analytic HPLC (method B). After 2 h, the reaction solution was directly subject to preparative HPLC to afford **13** (1.46 mg, 75%). ^{1}H NMR (DMSO-d_6 + 5% D_2O, 400 MHz): δ 7.20–7.11 (m, 5H, Ph-ArH), 4.81 (s, 1H, H-1 of Man^4), 4.61 (s, 1H, H-1 of $Man^{4'}$), 4.47 (m, 1H, H-1 of Man^3), 4.39 (d, 1H, J = 8.0 Hz, H-1 of $GlcNAc^1$), 4.38 (d, 1 H, J = 8.0 Hz, H-1 of $GlcNAc^2$), 4.27 (m, 1H, Phe-αH), 2.98 (dd, 1H, J = 4.8 Hz, 13.6 Hz, Phe-βH), 2.76 (dd, 1H, J = 7.2 Hz, 13.6 Hz, Phe-βH), 1.81 (s, 3H, Ac), 1.74 (s, 3H, Ac), 0.79 (s, 3H, 19-CH_3), 0.75 (d, 3H, J = 8.8 Hz, 21-CH_3), 0.50 (s, 3H, 18-CH_3); HR-MS (ESI): calculated for $C_{67}H_{106}N_3O_{29}$, $[M+H]^+$ = 1416.6912; found, 1416.7057; Analytical RP-HPLC (method B), t_R = 11.86 min.

4.11. Transglycosylation with the Glc-containing lithocholic acid derivative (11). Preparation of glycosylated lithocholic acid derivative (14) carrying a novel $Man_3GlcNAcGlc$ glycan

A solution of compound **11** (1 mg, 1.46 μmol) and oxazoline **12** (2 mg, 2.9 μmol) in a phosphate buffer (50 mM, pH 7.0, 150 μL) containing 20% DMSO was incubated with Endo-A (60 mU) at 23 °C. After 2 h, the reaction mixture was subject directly to preparative HPLC to afford **14** (1.1 mg, 55%). ^{1}H NMR (DMSO-d_6 + 5% D_2O, 400 MHz): δ 7.18–7.11 (m, 5H, Ph-ArH), 4.80 (s, 1H, H-1 of Man^4), 4.60 (s, 1H, H-1 of $Man^{4'}$), 4.47 (m, 1H, H-1 of Man^3), 4.35 (d, 1H, J = 8.4 Hz, H-1 of $GlcNAc^1$), 4.24 (d, 1H, J = 8.8 Hz, H-1 of Glc^2), 4.22 (m, 1H, Phe-αH), 2.99 (dd, 1H, J = 4.8 Hz, 13.6 Hz, Phe -βH), 2.76 (dd, 1H, J = 8.8 Hz, 13.6 Hz, Phe-βH), 1.81 (s, 3H, Ac), 0.80 (s, 3H, 19-CH_3), 0.75 (d, 3H, J = 7.2 Hz, 21-CH_3), 0.50 (s, 3H,

18-CH_3); HR-MS (ESI): calculated for $C_{65}H_{102}N_2O_{29}Na$, $[M+Na]^+$ = 1397.6466; found, 1397.6548; Analytical RP-HPLC: method B, t_R = 11.87 min.

4.12. Transglycosylation with natural products 15, 17, 19, 21, 23, and 25. A general procedure

A solution of the respective natural product (0.1 μmol) and the $Man_3GlcNAc$ oxazoline donor (**12**) (0.2 μmol) in a phosphate buffer (50 mM, pH 7.0, 10 μL) containing 20% DMSO (to improve the solubility of the lipophilic natural product) was incubated with Endo-A (10 mU) at 23 °C. After incubation for 2 h, the reaction was analyzed by analytic HPLC and the transglycosylation product was isolated for HR-MS analysis.

4.12.1. Daidzin-GlcNAcMan3 (16)

HR-MS (ESI): calculated for $C_{47}H_{63}NO_{29}Na$, [M+Na] = 1128.3383; found, 1128.3405 $[M+Na]^+$; analytical RP-HPLC: method A, t_R = 11.48 min.

4.12.2. Glycitin-GlcNAcMan3 (18)

HR-MS (ESI), calculated for $C_{48}H_{64}NO_{30}Na$, [M+Na] = 1157.3410; found, 1157.3388 $[M+Na]^+$; analytical RP-HPLC: method A, t_R = 11.63 min.

4.12.3. Puerarin-GlcNAcMan3 (20)

HR-MS (ESI), calculated for $C_{47}H_{64}NO_{29}$, [M+H] = 1106.3564; found, 1106.3494 $[M+H]^+$; analytical RP-HPLC: method A, t_R = 10.91 min.

4.12.4. Geniposide-GlcNAcMan3 (22)

HR-MS (ESI), calculated for $C_{43}H_{68}NO_{30}$, [M+H] = 1178.3826; found, 1178.3825 $[M+H]^+$; analytical RP-HPLC: method A, t_R = 11.33 min.

4.12.5. Rhaponticin-GlcNAcMan3 (24)

HR-MS (ESI), calculated for $C_{47}H_{68}NO_{29}$, [M+H] = 1110.3877; found, 1110.3803 $[M+H]^+$; analytical RP-HPLC: method A, t_R = 13.62 min.

4.12.6. Paeoniflorin-GlcNAcMan3 (26)

HR-MS (ESI), calculated for $C_{49}H_{72}NO_{31}$, [M+H] = 1170.4088; found, 1170.3985 $[M+H]^+$; analytical RP-HPLC: method A, t_R = 11.78 min.

4.13. Double transglycosylation with the luteolin 3′,7-di-O-glucoside (27)

A solution of oxazoline (**12**) (0.2 μmol) and luteolin 3′,7-di-O-glucoside (**27**) (0.1 μmol) in a phosphate buffer (50 mM, pH 7.0, 10 μL) containing 20% DMSO was incubated with Endo-A (10 mU) at 23 °C for 2 h. Then an excess of donor substrate **12** (0.2 μmol) was added and the reaction mixture was incubated for another 2 h. The reaction was analyzed by RP-HPLC and the transglycosylation products were collected and characterized by HR-MS analysis. HR-MS (ESI) of **28**: calculated for $C_{79}H_{117}N_2O_{56}$, [M+H] = 1989.6369; found, 1989.6385 $[M+H]^+$; analytical RP-HPLC: method A, t_R = 11.84 min.

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