Enzyme Catalysis

Enzymatic Synthesis of Acylphloroglucinol 3-C-Glucosides from 2-O-Glucosides using a C-Glycosyltransferase from *Mangifera indica*

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Abstract: A green and cost-effective process for the convenient synthesis of acylphloroglucinol 3-C-glucosides from 2-O-glucosides was exploited using a novel C-glycosyltransferase (MiCGTb) from *Mangifera indica*. Compared with previously characterized CGTs, MiCGTb exhibited unique de-O-glucosylation promiscuity and high regiose-lectivity toward structurally diverse 2-O-glucosides of acylphloroglucinol and achieved high yields of C-glucosides even with a catalytic amount of uridine 5'-diphosphate (UDP). These findings demonstrate for the first time the significant potential of a single-enzyme approach to the synthesis of bioactive C-glucosides from both natural and unnatural acylphloroglucinol 2-O-glucosides.

Sugar moieties are often essential for the physiological activity, specificity and pharmacological properties of many natural products.^[1] The majority of natural product glycosylations involve the O-glycosidic bond, which, however, is usually sensitive to spontaneous or enzyme-catalyzed hydrolysis in vivo.^[2] As isosteric O-glycoside mimics, C-glycosides not only maintain their efficacy and pharmacological properties but also exhibit outstanding resistance to glycosidic bond cleavage.^[3] For example, 3-C-glucoside of phloretin (1a, nothofagin) exhibits more selective and stable inhibition of human sodium-glucose co-transporter 2 (SGLT2) activities for the treatment of type 2 diabetes than 2-O-glucoside of phloretin (1, phlorizin).^[4] However, natural C-glycosides appear to be comparatively rare.^[2] Chemical C-glycosylation also remains restricted by such disadvantages as poor regio- and stereoselectivities and the protection and deprotection of functional groups.^[3b,5] Enzymatic Cglycosylation catalyzed by specific C-glycosyltransferases (CGTs; EC 2.4) can alleviate these disadvantages, making these enzymes powerful tools.^[6] In the past few years, studies on CGTs from microbes and plants have attracted increasing interest and achieved great progress in the C-glycosylation of both nat-

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Supporting information for this article can be found under http:// dx.doi.org/10.1002/chem.201600411. ural and unnatural products.^[7] However, the majority of known CGTs exhibit relatively narrow substrate selectivity and require rather expensive or rare nucleotide-activated sugar donors (NDP-sugar), limiting their availability and scope for creating structurally diverse *C*-glycosides with potent biological activities as drug leads.

Significant progress has been made recently using the reaction reversibility of O-glycosyltransferases (OGTs) in the glycodiversfication of natural and unnatural products by performing sugar and aglycon exchange reactions in vitro.^[8] Although several CGTs have been cloned and their catalytic function has been verified, strategies for C-glycodiversification through a combinatorial approach from natural and unnatural O-glycosides in vitro remain scarce. To date, only two examples have been described of synthesizing 3-C-glucoside (1 a, nothofagin) from 2-O-glucoside (1, phlorizin), using OsCGT coupled with an OGT or an engineered dual-specific O/CGT.^[9] We also recently reported a benzophenone CGT that generates C-glycosides using a simple sugar donor.^[7] However, these combinatorial approaches to the C-glycodiversification of natural and unnatural products have limited general applicability because of the specificity of the CGTs, low catalytic efficiency or the generation of byproducts. Therefore, the exploitation of a universally applicable green chemistry approach for the generation of bioactive C-glycosides from naturally abundant or easily synthesized O-glycosides is highly desired. Herein, we report for the first time an applicable green and cost-effective process for the generation of bioactive 3-C-glucosides from 2-O-glucosides through six pairs of acylphloroglucinol 2/4-O-glucosides using a novel C-glycosyltransferase, MiCGTb, from Mangifera indica. This work exposes an unexpected de-O-glucosylation promiscuity and high regioselectivity of MiCGTb toward structurally diverse 2-O-glucosides of acylphloroglucinol and highlights the first highly efficient synthesis of C-glucosides by de-O-glucosylation and C-glucosylation strategies using a single C-glycosyltransferase.

In our ongoing attempt to identify novel CGTs with specific catalytic properties from *M. indica* leaves, as described in our previous work,^[7] a *MiCGT* homologue gene with 90% identity (Figure S1 in the Supporting Information), named *MiCGTb* (GenBank: KT989668), was successfully cloned and heterologously expressed in *E. coli* (Figure S2 in the Supporting Information). Similarly to MiCGT, MiCGTb also exhibited catalytic promiscuity and regio- and stereospecificity toward numerous natural and unnatural acceptors with uridine 5'-diphosphate (UDP)-glucose (Table S2 in the Supporting Information). Moreover, MiCGTb likewise generates only *C*-glucosides with a 2,4,6-

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tri-hydroxylacetophenone-type structure. Therefore, MiCGTb can also be validated as a benzophenone CGT due to its catalytic characteristics of C-glycosylation.^[7]

Unexpectedly, the C-glucosylated product turned out to be 3-C-glucoside nothofagin (1a), when 2-O-glucoside phlorizin (1) and UDP-glucose were incubated with MiCGTb at pH 6.6. The same result was also obtained when MiCGTb and 1 were incubated with UDP (Figure 1). However, this conversion of 1 into 1a was not observed with only MiCGT (Figure S3 in the Supporting Information) or OsCGT.^[9c] These unique results inspired our interest in how this enzymatic conversion of 2-Ointo 3-C-glucoside occurred: through chemical Fries-type O/Cglucosidic bond rearrangement or an aglycone intermediate followed by a Friedel-Crafts-like reaction.^[6c, 9c, 10] To verify these predictions, a mixture of 1, UDP and MiCGTb was incubated at 30 °C from 10 to 60 min, and the time course of the reaction was monitored by HPLC, which clearly indicated the formation of aglycone phloretin (1b, Figure 1B). In the control experiments, no reaction was detected at all in the absence of either MiCGTb or UDP. In light of these results, the conversion is unambiguously considered to proceed via the intermediary release of 1b and the formation of UDP-glucose, followed by regio- and stereoselective C-glucosylation. However, given that the O-glucosylation activity toward 1b with UDP-glucose by MiCGTb was not detected (Figure S4 in the Supporting Information), the above reaction seems implausible based on the



Figure 1. Transformation of phlorizin (1) to nothofagin (1 a) using a single *C*-glycosyltransferase MiCGTb. A) The reaction catalyzed by recombinant MiCGTb. B) HPLC chromatograms of 1 and the enzyme products 1 a and 1 b. All reactions were performed at pH 6.6 and 30 °C. The HPLC conditions are provided in Table S1 in the Supporting Information.

established protocols of OGTs' reversible reaction.^[8b,11] This transformation reveals that MiCGTb exhibits an unexpected de-*O*-glucosylation capability toward **1** in the presence of UDP except for its *C*-glucosylation activity, in which UDP is the "trigger" in the de-*O*-glucosylation process (thus catalyzing the release of **1b** and formation of UDP-glucose).^[8] Although the mechanism of aryl-*C*-glycosylation remains unclear, these results lend strong support to the *C*-glycosylation through a direct Friedel–Crafts-like reaction.^[2a, 3b, 6c, 10]

The biochemical characteristics of this transformation catalyzed by MiCGTb were then further investigated. The pH dependence of 1b C-glucosylation showed a broad optimum over pH 6.0-11.0, whereas the optimum conversion of 1 into 1 a was in a pH range of 4.0-6.0 (Figure 2A), which might be more suitable for the de-O-glucosylation of 1. MiCGTb displayed its maximum activity at 40 °C (Figure 2B) and was divalent cation independent (Figure 2C). Moreover, high yields of 1 a (>90%) were achieved even with a catalytic amount of UDP (Figure 2D), which was more efficient than the reported results.^[9] Kinetic analysis revealed that MiCGTb exhibited k_{cat} values of 47.3 and 0.7 min⁻¹ for 1 b and 1, respectively, with the corresponding $k_{cat}/K_{\rm M}$ values of 2.8×10^5 and $6.4 \times$ $10^3 \,\mathrm{m^{-1}\,min^{-1}}$ (Figure S5 in the Supporting Information). These results clearly demonstrate that the catalytic efficiency of directly C-glucosylation 1b appeared to be at least 40-fold higher than that of the reaction from 1 to 1a (de-O-glucosylation 1 first, and then C-glucosylation 1b), and de-O-glucosylation was thus a rate-limiting step in the conversion of 2-Ointo 3-C-glucoside. In contrast to these results, C-glucosylation was rate-limiting for this transformation when performed by the dual-specific O/CGT.^[9c] The higher yields of C-glucoside in this process most likely resulted from the higher catalytic efficiency of the C-glucosylation step, which was a large driving force promoting such conversion.



Figure 2. Yields of nothofagin (**1 a**) catalyzed by recombinant MiCGTb with different: A) pH (phlorizin (**1**): gray columns; phloretin (**1 b**): blue columns), B) temperature, and C) divalent cation after 2 h, and D) reaction times with different molar ratios of phlorizin/UDP. The pH profile of **1 b** *C*-glucosylation was tested after incubation for 10 min.

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Figure 3. Exploring the generality and regiospecificity of MiCGTb toward structurally diverse glucosides of acylphloroglucinol. A) Percent yields of 3-C-glucosylated products from six pairs of acylphloroglucinol 2/4-O-glucosides (1–12) listed in descending order of yield. B) Structures of the corresponding 2/4-O-glucosides in part A and 3-C-glucosylated products. All reactions were performed at pH 6.0 and 40 °C. The HPLC conditions are provided in Table S1.

For further exploitation of the generality and regiospecificity of the transformation of 2-O- to 3-C-glucoside, five other pairs of 2/4-O-glucosides of acylphloroglucinol were assayed with MiCGTb. In the presence of UDP, MiCGTb could efficiently catalyze the de-O-glucosylation and 3-C-glucosylation of the 2-Oglucosides (3, 5 and 7; >80% yields by HPLC) that appeared to be at least as structurally flexible as 1, while the conversion rates of 9 and 11 were drastically lower, below 35% (Figure 3). In contrast, the de-glucosylation activity of MiCGTb on the corresponding 4-O-glucosides is very low, even barely detectable in the cases of 10 and 12. Since the C-glucosylated products (1a, 3a, 7a, 9a, and 11a) were prepared and structurally characterized in our previous work,^[7] they were used as authentic compounds for the identification of the C-glucosides by HPLC (Figures S4, S6-S10 in the Supporting Information) in this study. Scaling up the reaction of 5 with the recombinant MiCGTb provided sufficient 5a for structure elucidation by MS, ¹H and ¹³C NMR analysis. The appearance of parent ion peak at m/z 371 $[M-H]^-$, which was 162 amu more than that of 5, and the characteristic fragment ions of C-glucoside, $[M-H-90]^{-1}$ and $[M-H-120]^-$, suggested that **5** a was a mono-C-glucosylated product (Figure S7 in the Supporting Information). The disappearance of H-3 signal in the ¹H NMR spectrum and the chemical shift of C-3 at $\delta = 103.6$ ppm and the anomeric carbon at $\delta = 73.6$ ppm (C-1") in ¹³C NMR spectrum further

suggested that **5a** was the 3-*C*-glucosylated product. The large coupling constant (J=9.9 Hz) of the anomeric proton (H-1") supported the formation of the β -anomer (Figures S11 and S12 in the Supporting Information). Thus, the structure of **5a** was identified as 3-*C*- β -*D*-glucoside. Hence, MiCGTb displayed catalytic promiscuity and high regioselectivity toward more linear acylphloroglucinol 2-*O*-glucosides. It is important to note that most of these *C*-glucosylated products exhibited potential and selective human SGLT2 inhibitory activities for the treatment of type 2 diabetes in vitro.^[7]

To gain additional information on the substrate specificity toward the nucleoside 5'-diphosphate (NDP) moiety, the nucleotide specificity of MiCGTb was subsequently probed with 1 and four other commercially available NDPs under the optimized conditions. To our delight, MiCGTb displayed broad substrate specificity in recognizing deoxythymidine 5'-diphosphate (dTDP), adenosine 5'-diphosphate (ADP), cytidine 5'-diphosphate (CDP) or guanosine 5'-diphosphate (GDP) and their corresponding activated glucose, albeit with a much lower conversion rate. As shown in the representative time course of the synthesis of 1a from 1 (Figure S13 in the Supporting Information), the substrate specificity of MiCGTb to five different types of NDP was as follows: 95% yields were observed with UDP in 1 h or dTDP in 6 h, respectively, but less than 20% yields were observed with ADP, CDP, or GDP after 18 h incubation, indicat-



ing that UDP is a more suitable substrate for the de-O-glucosylation and C-glucosylation of MiCGTb. As expected, **1b** was likewise observed when **1** was incubated with dTDP, ADP, CDP, or GDP, which further supported the hypothesis of the transformation of 2-O- to 3-C-glucoside through the intermediate **1b**. Collectively, these results offer vast combinatorial potential for in vivo C-glycodiversification from natural and unnatural Oglucosides with different NDPs of the host strains.^[12]

In summary, an applicable green and cost-effective process for the convenient synthesis of acylphloroglucinol 3-C-glucosides from 2-O-glucosides using a novel C-glycosyltransferase, MiCGTb, from M. indica was presented. Compared with previously characterized CGTs, MiCGTb exhibited unique de-O-glucosylation promiscuity and high regioselectivity toward structurally different acylphloroglucinol 2-O-glucosides, with high tolerance toward five different NDP moieties, rendering it a powerful biocatalyst for the cost-effective enzymatic synthesis of bioactive C-glucosides without adding activated sugars. The studies described herein not only indicate the significant potential of a single-enzyme approach to the synthesis of bioactive C-glucosides from both natural and unnatural acylphloroglucinol 2-O-glucosides with catalytic amounts of NDP but also offer strong support for C-glycosylation through a direct Friedel-Crafts-like reaction. Given the versatility of MiCGTb for de-O-glucosylation and C-glucosylation, further systematic structural investigation together with site-directed mutation are imperative for elucidating the catalytic mechanism and substrate promiscuity and for addressing how the enzyme governs such conversion. The unique de-O-glucosylation of MiCGTb reported here hints more exciting and novel CGTs from plants as alternative tools for the C-glycosylation of bioactive natural and unnatural products and would also facilitate further enzyme engineering for the exploitation of promiscuous biocatalysts in combinatorial biosynthesis and/or metabolic engineering.

Experimental Section

Chemical reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), J&K Scientific Ltd. (Beijing, China), and InnoChem Science & Technology Co., Ltd. (Beijing, China). MiCGTb was amplified by PCR, and the gene fragment was then inserted into plasmid pET-28a. The resultant plasmid was then transformed into E. coli (TransGen Biotech, China) for heterologous expression. Enzymatic products were detected on an Agilent 1200 series HPLC system (Agilent Technologies, Germany) coupled with a LCQ Fleet ion trap mass spectrometer (Thermo Electron Corp., USA) equipped with an electrospray ionization (ESI) source. Compounds were characterized by ¹H NMR spectroscopy at 400 MHz on Mercury-400 spectrometer and ¹³C NMR spectroscopy at 150 MHz on Bruker AVIIIHD spectrometer, and HRESIMS on an Agilent Technologies 6520 Accurate Mass Q-TOF LC/MS Spectrometer. A detailed description of the experimental procedures as well as the analytical protocols can be found in the Supporting Information.

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