# Chemical N-Glycosylation by Asparagine under Integrated Microfluidic/ Batch Conditions

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*Received 16 February 2009*

**Abstract:** An integrated microfluidic/batch system was applied to the chemical N-glycosylation by the asparagine amide group, a key glycosyl bond-formation reaction in the synthesis of *N*-glycopeptides. By applying the advantageous features of microfluidic conditions, that is, efficient mixing and rapid heat transfer, the GlcNTrocβAsn and the Fucα(1–6)GlcNTrocβAsn fragments were efficiently prepared.

**Key words:** N-glycosylation, asparagine, microreactor, oligosaccharide, *N*-glycopeptide

Chemical N-glycosylation by asparagine residues is a challenging topic in *N*-glycopeptides synthesis[1] due to the inherently low nucleophilicity of the amide nitrogen toward glycosylation. Kahne and co-workers[2a] initially reported N-glycosylation through amide functions by utilizing N-silylated acetamide to enhance the nucleophilicity of the amide nitrogen.[2b] Takahashi and co-workers have recently reported a more direct and efficient N-glycosylation of the glycosyl imidates via protected asparagine derivatives.[3] Their protocol utilizes *N*-phenyltrifluoroacetimidate[4] as a leaving group, TMSOTf as an activator, and nitromethane as a solvent; glucosyl, glucosaminyl, mannosyl, and galactosyl imidates have been N-glycosylated in 68–99% yields, especially by the protected monoasparagine residue, Z-Asn(OAll).

Our research on the solid-phase synthesis of *N*-glycans and *N*-glycopeptides requires a sufficient amount of the GlcNTrocβAsn and Fucα(1–6)GlcNTrocβAsn motifs.[5] Although Takahashi's protocol is very attractive, nitromethane, which is the optimum solvent for efficient N-glycosylation, is explosive, and not suitable for large-scale synthesis. Herein, we report a practical and high-yielding N-glycosylation in dichloromethane using integrated microfluidic/batch conditions. This method is successful on a few gram-scale N-glycosylation without nitromethane, and is readily applicable to *N*-glycans and/or *N*-glycopeptides synthesis.

We initially examined N-glycosylation of the suitably protected *N*-Troc glucosaminyl *N*-phenyltrifluoroacetimidate (**1a**) with Z-L-Asn(OBn) (**2**) under batch conditions according to Takahashi's procedure (Table 1). Besides nitromethane, dichloromethane, and propionitrile were investigated as more general solvents for the preparative-scale synthesis of the *N*-glycopeptides fragments. When 1.5 equivalents of glycosyl donor **1a** with respect to the asparagine derivative were treated with 0.2 equivalent of TMSOTf in dichloromethane at room temperature for 12 hours, desired *N*-glycoside **3a** was obtained in 61% yield (entry 1), which is comparable to the reported results.[3] Because TLC analysis indicated that the donor decomposed during the reaction, the donor solution was slowly added to a premixed solution of the acceptor and TMSOTf, but the reaction did not proceed (entry 2). Employing excess donor (3.0 equiv) and an activator (0.5 equiv) did not increase product formation (50%, entry 3), and the yield decreased when propionitrile was used as the solvent (27%, entry 4).

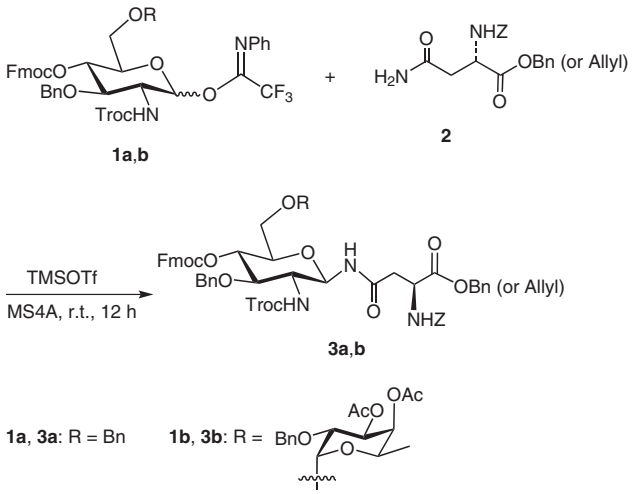
N-Glycosylation was less effective when more sterically demanding disaccharide donor, Fucα(1–6)GlcNTroc *N*-phenyltrifluoroacetimidate (**1b**), was glycosylated with asparagine amide; 35% of *N*-glycoside **3b** was produced under the optimal conditions obtained for **1a** (entry 5). Although 2.0 equivalents of acceptor **2** relative to donor **1b** resulted in an improved yield (47%, entry 6), the excess acceptor (5.0 equiv) decreased the efficiency (32%, entry 7).

Time-course TLC analyses of entry 6 provided information about the intermediates during the course of the reaction (Figure 1). Namely, the addition of TMSOTf into a mixture of the donor and acceptor at room temperature (5 min TLC) provided several intermediates, which presumably consisted of the silylated acceptor, *O*-glycoside, *N*-glycoside, hydrolyzed donor, etc. However, the mixture of these products ultimately converged into desired *N*-glycoside **3b**, decomposed donor, and unconsumed acceptor **2** (12 h TLC). Although the initial intermediates detected on the 5 min TLC were not examined in detail, we hypothesized that the heat generated during the initial mixing might yield a mixture of the intermediates, resulting in the decomposition of the donor and eventually the decreased yield of *N*-glycoside **3b**. Surprisingly, more of the acceptor was recovered upon slowly adding a Lewis acid at 0 °C than the reaction performed at room temperature in entry 6 (less than 10% of **3b**).

Based on the observations in Table 1, which suggest that efficient mixing and heat transfer may be responsible for N-glycosylation, we decided to examine the microfluidic conditions. A continuous flow microreactor, an innovative technology, has been used to realize efficient mixing

*SYNLETT* 2009, No. 10, pp 1571–1574
Advanced online publication: 02.06.2009
DOI: 10.1055/s-0029-1217343; Art ID: U01809ST

**Table 1** N-Glycosylation with Asparagine Amide[a] under Batch Conditions




| Entry | Donor (equiv) | Acceptor **2** (equiv) | TMSOTf (equiv) | Solvent | Yield (%)[b] |
|---|---|---|---|---|---|
| 1 | **1a** (1.5) | 1.0 | 0.2 | $CH_2Cl_2$ | 61 |
| 2[c] | **1a** (1.5) | 1.0 | 0.2 | $CH_2Cl_2$ | 0 |
| 3 | **1a** (3.0) | 1.0 | 0.5 | $CH_2Cl_2$ | 50 |
| 4 | **1a** (1.5) | 1.0 | 0.2 | EtCN | 27 |
| 5 | **1b** (1.5) | 1.0 | 0.2 | $CH_2Cl_2$ | 35 |
| 6 | **1b** (1.0) | 2.0 | 0.2 | $CH_2Cl_2$ | 47 |
| 7 | **1b** (1.0) | 5.0 | 0.2 | $CH_2Cl_2$ | 32 |

[a] Benzyl ester was used for entries 1–4, and allyl ester was used for entries 5–7.
[b] Isolated yields.
[c] Donor solution was added slowly to a premixed solution of the acceptor and TMSOTf.

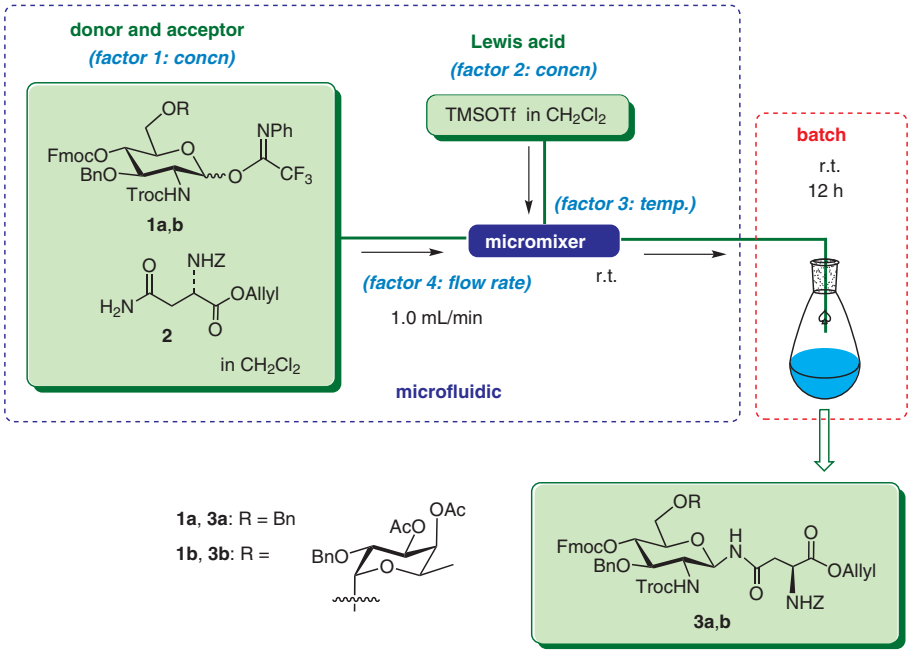
and rapid heat transfer in organic syntheses.[6,7] Once the reaction conditions are optimized for a small-scale operation, the same conditions are directly applicable to large-scale synthesis under the flow process. We have recently applied a microfluidic system to cation-mediated reactions[8] to realize an improved α-sialylation,[8b] dehydration,[8c] and reductive opening of the benzylidene acetal groups in sugar.[8d] Moreover, we have developed an integrated microfluidic/batch system to manage reactions requiring a long reaction time after micromixing. This protocol has been applied to practical β-mannosylation.[8e] Because the inefficiency of all these cation-mediated reactions under the batch process are due to inefficient mixing with the acid reagents, we envisioned that microfluidic conditions might also lead N-glycosylation to take place efficiently even in dichloromethane. Hence, this protocol was used in the preparative synthesis of *N*-glycosides **3a**,**b**.

Because the current N-glycosylation requires a long reaction time (Table 1), a microfluidic apparatus was constructed based on our previous experiences with microfluidic β-mannosylation.[8e] Namely, the reaction solution prepared in the micromixing system was subsequently inserted into a batch system, and conventionally stirred in a flask for hours to complete the reaction (Table 2). Small quantities of materials were used to determine the optimal conditions, that is, substrate concentrations, mixing speed, temperature, and flow rate (optimization factors 1–4, in Table 2). Thus, a dichloromethane solution of glucosaminyl donors **1a**,**b** and asparagine acceptor **2** with various concentrations (optimization factor 1) were mixed with a TMSOTf solution in dichloromethane to determine the optimal concentration (factor 2) at the appropriate temperature (factor 3) using an IMM micromixer[9] at various flow rates (factor 4). Unlike the β-mannosylation case[8e] where micromixing occurs at a very low temperature (–90 °C) using a Comet X-01 micromixer[10] with a channel width of ca. 500 μm, we took advantage of the more delicate microstructure of IMM (40 μm) and assumed that solution blockage might not be a severe problem due to the relatively good solubility of both the donor and acceptor in dichloromethane above room temperature. For the rapid optimization of the microfluidic conditions, the product yields were initially estimated by the TLC stain contrasts using *ImageJ 1.40*.

Table 2 shows representative data obtained by the microfluidic N-glycosylation of **1a** and **1b** with asparagine derivative **2**, when micromixing was performed at room temperature, and the flow rate and the concentration of TMSOTf were fixed at 1.0 mL/min and 43 mM, respectively. All the entries in Table 2 using batch stirring at room temperature required 12 hours to complete the N-glycosylation. When the concentrations of monosaccharide imidate **1a** and acceptor **2** were adjusted to 86 mM (1.0 equiv) and 172 mM (2.0 equiv), respectively, GlcN-TrocβAsn fragment **3a** was obtained in 60% (entry 1). A slight excess of donor **1a** relative to **2**, that is, 130 mM of **1a** (1.5 equiv) and 86 mM (1.0 equiv) of **2**, gave similar results (55%, entry 2). However, applying more of donor **1a** (259 mM, 3.0 equiv) completely consumed the asparagine acceptor, and desired *N*-glycoside **3a** was produced in 81% yield (entry 3).

An efficient microfluidic N-glycosylation was also realized using disaccharide imidate **1b** as a donor; but the micromixing between 22 mM (1.0 equiv) of **1b** and 43 mM (2.0 equiv) of **2** provided *N*-glycoside **3b** only in 27% yield (entry 4). However, doubling the concentrations for both the reactants dramatically enhanced the yield (84%, entry 5). Further trials with higher concentrations of the reactants, that is, 86 mM of the donor and 172 mM of the acceptor, caused the microchannel to become blocked due to the limited solubility of donor **1b**. The batch and microfluidic reactions displayed vastly different TLC behaviors (Figure 1). The batch reaction provided several intermediates on the 5 min TLC, but gradually produced *N*-glycoside **3b** as well as decomposed materials. On the other hand, the 5 min TLC under the microfluidic conditions detected only a few spots with obvious structures, including the starting donor, the acceptor, and *N*-glycoside;

**Table 2** N-Glycosylation with Asparagine Amide under Microfluidic Conditions[a]




| Entry | Donor (mM) | Acceptor **2** (mM) | TMSOTf (mM) | Yield (%)[b] |
|---|---|---|---|---|
| 1 | **1a** (86) | 172 | 43 | 60 |
| 2 | **1a** (130) | 86 | 43 | 55 |
| 3 | **1a** (259) | 86 | 43 | 81 |
| 4 | **1b** (22) | 43 | 43 | 27 |
| 5 | **1b** (43) | 86 | 43 | 84 |

[a] Solutions flowed through a Teflon tube (ϕ = 1.0 mm) at a rate of 1.0 mL/min, and micromixing was performed at r.t. The Lewis acid concentration was fixed at 43 mM.
[b] Yields were estimated based on TLC stain contrast detected by *ImageJ 1.40* (see Figure 1).

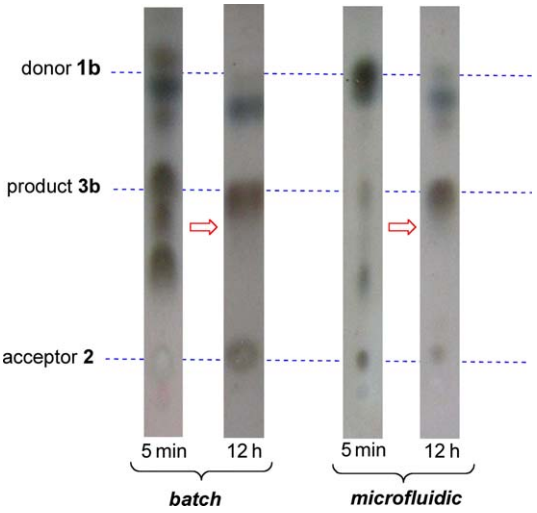



**Figure 1** TLC analysis of batch and microfluidic N-glycosylation of disaccharide **1b** with asparagine **2** (eluent, hexane–EtOAc = 1:1)

*N*-glycoside was then gradually and cleanly produced over 12 hours. Thus, efficient microfluidic mixing as well as rapid heat transfer might be achieved for the current N-glycosylation with the asparagine amide.

Preliminary optimized conditions in Table 2 were then applied to the preparative scale synthesis of *N*-glycosides **3a** and **3b**; continuously pumping the stock solutions of the substrates and the Lewis acid activator into the integrated microfluidic/batch system gave isolated yields of 85% for **3a** and 84% for **3b**.[11]

In summary, we established an efficient N-glycosylation of glycosyl *N*-phenyltrifluoroacetimidates with asparagine amide under integrated microfluidic/batch conditions. The optimal conditions were rapidly determined using a fluidic system and simple TLC monitoring. The subsequent preparative-flow reaction led to the reproducible synthesis of GlcNTrocβAsn and the Fucα(1–6)GlcNTrocβAsn derivative in high yields, and is applicable to our *N*-glycopeptides synthesis. The success of the protocol must be due to efficient mixing and rapid heat transfer temperature control, which inhibit decomposition of the reactants and/or the formation of complicated glycosyl intermediates during the batch mixing between the donor, acceptor, and activator.

## Acknowledgment

This work was partly supported by Grants-in-Aids for Scientific Research No. 19681024 and 19651095 from the Japan Society for the Promotion of Science, Collaborative Development of Innovative Seeds from Japan Science and Technology Agency (JST), New Energy and Industrial Technology Development Organization (NEDO, project ID: 07A01014a), Research Grants from Yamada Science Foundation as well as the Molecular Imaging Research Program, and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

## References and Notes

(1) (a) *Introduction to Glycoscience Synthesis of Carbohydrates, In Comprehensive Glycoscience, from Chemistry to Systems Biology*, Vol 1; Kamerling, J. P.; Boons, G.-J.; Lee, Y. C.; Suzuki, A.; Taniguchi, N.; Voragen, A. G. J., Eds.; Elsevier: Oxford, **2007**. (b) Tanaka, K.; Fukase, K. *Polymer-Supported and Tag-Assisted Methods in Oligosaccharide Synthesis in Glycoscience: Chemistry and Chemical Biology*, 2nd ed., Vol. I-III; Fraser-Reid, B. O.; Tatsuta, K.; Thiem, J., Eds.; Springer: New York, **2009**.

(2) (a) Kahne, D.; Walker, S.; Cheng, Y.; Engen, D. V. *J. Am. Chem. Soc.* **1989**, *111*, 6881. (b) Garcia, B. A.; Gin, D. Y. *J. Am. Chem. Soc.* **2000**, *122*, 4269.

(3) Tanaka, H.; Iwata, Y.; Takahashi, D.; Adachi, M.; Takahashi, T. *J. Am. Chem. Soc.* **2005**, *127*, 1630.

(4) (a) Yu, B.; Tao, H. *Tetrahedron Lett.* **2001**, *42*, 2405. (b) Adinolfi, M.; Barone, G.; Iadonisi, A.; Schiattarella, M. *Synlett* **2002**, 269. (c) Cai, S.; Yu, B. *Org. Lett.* **2003**, *5*, 3827.

(5) Tanaka, K.; Fujii, Y.; Tokimoto, H.; Mori, Y.; Tanaka, S.; Bao, G.-m.; Siwu, E. R. O.; Nakayabu, A.; Fukase, K. *Chem. Asian J.* **2009**, *4*, 574; based on the established solid-supported protocol, N-glycosylation strategy with the optimal protection groups on **1a**,**b** and **2** will be applied to the future glycopeptide synthesis.

(6) For representative reviews, see: (a) *Microreaction Technology*; Ehrfeld, W., Ed.; Springer: Berlin, **1998**. (b) *Microsystem Technology in Chemistry and Life Sciences*; Manz, A.; Becker, H., Eds.; Springer: Berlin, **1998**. (c) Ehrfeld, W.; Hessel, V.; Lowe, H. *Microreactors*; Wiley-VCH: Weinheim, **2000**. (d) Hessel, V.; Hardt, S.; Lowe, H. *Chemical Micro Process Engineering*; Wiley-VCH: Weinheim, **2004**. (e) Yoshida, J.-I.; Suga, S.; Nagaki, A. *J. Synth. Org. Chem. Jpn.* **2005**, *63*, 511.

(7) For recent applications, especially to oligosaccharides synthesis, see: (a) Pennemann, H.; Hessel, V.; Loewe, H. *Chem. Eng. Sci.* **2004**, *59*, 4789. (b) Jahnisch, K.; Hessel, V.; Loewe, H.; Baerns, M. *Angew. Chem. Int. Ed.* **2004**, *43*, 406. (c) Nagaki, A.; Togai, M.; Suga, S.; Aoki, N.; Mae, K.; Yoshida, J.-I. *J. Am. Chem. Soc.* **2005**, *127*, 11666. (d) Ratner, D. M.; Murphy, E. R.; Jhunjhunwala, M.; Snyder, D. A.; Jensen, K. F.; Seeberger, P. H. *Chem. Commun.* **2005**, 578. (e) Flogel, O.; Codee, J. D. C.; Seebach, D.; Seeberger, P. H. *Angew. Chem. Int. Ed.* **2006**, *45*, 7000. (f) Geyer, K.; Seeberger, P. H. *Helv. Chim. Acta* **2007**, *90*, 395. (g) Carrel, F. R.; Geyer, K.; Jeroen, D. C.; Codee, J. D. C.; Seeberger, P. H. *Org. Lett.* **2007**, *9*, 2285. (h) Usutani, H.; Tomida, Y.; Nagaki, A.; Okamoto, H.; Nokami, T.; Yoshida, J. *J. Am. Chem. Soc.* **2007**, *129*, 3046. (i) Tanaka, K.; Motomatsu, S.; Koyama, K.; Fukase, K. *Tetrahedron Lett.* **2008**, *49*, 2010.

(8) (a) Fukase, K.; Takashina, M.; Hori, Y.; Tanaka, D.; Tanaka, K.; Kusumoto, S. *Synlett* **2005**, 2342. (b) Tanaka, S.; Goi, T.; Tanaka, K.; Fukase, K. *J. Carbohydr. Chem.* **2007**, *26*, 369. (c) Tanaka, K.; Motomatsu, S.; Koyama, K.; Tanaka, S.; Fukase, K. *Org. Lett.* **2007**, *9*, 299. (d) Tanaka, K.; Fukase, K. *Synlett* **2007**, 164. (e) Tanaka, K.; Mori, Y.; Fukase, K. *J. Carbohydr. Chem.* **2009**, *28*, 1.

(9) IMM micromixer: http://www.imm-main2.de/

(10) Comet X-01 micromixer: http://homepage3.nifty.com/techno-applications/ or e-mail: yukio-matsubara@nifty.com.

(11) **Procedure of N-Glycosylation Using an Integrated Microfluidic/Batch System**

A solution of TMSOTf (33 μL, 180 μmol, 43 mM) in $CH_2Cl_2$ (4.2 mL) was injected, in advance, into the micromixer by a syringe pump at a flow rate of 1.0 mL/min. Then a solution of donor **1a** (1.0 g, 1.1 mmol, 260 mM) and acceptor **2** (110 mg, 360 μmol, 86 mM) dissolved in $CH_2Cl_2$ (4.2 mL) was injected into the IMM micromixer by another syringe pump at a flow rate of 1.0 mL/min. The reaction was mixed at r.t. After the reaction mixture was allowed to flow at r.t. for an additional 94 s through a Teflon tube reactor (Φ = 1.0 mm, l = 1.0 m), the mixture was introduced into a flask, and stirred for 12 h at this temperature. Then the mixture was quenched by an aq $NaHCO_3$ solution. The resulting mixture was extracted with EtOAc, washed with brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo to give the crude product. The residue was purified by column chromatography on silica gel (from 25–33% EtOAc in hexane) to give *N*-glycoside **3a** as a white solid (376 mg, 85%). ESI-MS: *m/z* calcd for $C_{53}H_{53}Cl_3N_3O_{13}$ $[M + H]^+$: 1044.3; found: 1044.2. $^1H$ NMR (500 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.6 Hz, 1 H), 7.73 (d, *J* = 7.5 Hz, 1 H), 7.59 (d, *J* = 7.6 Hz, 1 H), 7.55 (d, *J* = 7.4 Hz, 1 H), 7.41–7.17 (m, 19 H), 6.80 (d, *J* = 8.5 Hz, 1 H), 5.91 (d, *J* = 9.2 Hz, 1 H), 5.88–5.80 (m, 1 H), 5.27 (dd, *J* = 17.2, 1.3 Hz, 1 H), 5.19 (dd, *J* = 10.5, 1.3 Hz, 1 H), 5.14–5.04 (m, 4 H), 4.89 (dd, *J* = 9.2, 9.2 Hz, 1 H), 4.75 (d, *J* = 12.1 Hz, 1 H), 4.69 (d, *J* = 12.0 Hz, 1 H), 4.68–4.58 (m, 3 H), 4.52–4.35 (m, 6 H), 4.15 (dd, *J* = 6.9, 6.9 Hz, 1 H), 3.65–3.61 (m, 3 H), 3.56–3.49 (m, 2 H), 2.86 (dd, *J* = 16.7, 3.8 Hz, 1 H), 2,68 (dd, *J* = 16.4, 4.2 Hz, 1 H).

**Data for 3b**

ESI-MS: *m/z* calcd for $C_{63}H_{67}Cl_3N_3O_{19}$ $[M + H]^+$: 1274.3; found: 1274.2. $^1H$ NMR (500 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.6 Hz, 1 H), 7.73 (d, *J* = 7.5 Hz, 1 H), 7.61 (d, *J* = 7.6 Hz, 1 H), 7.60 (d, *J* = 7.3 Hz, 1 H), 7.41–7.19 (m, 19 H), 6.89 (d, *J* = 8.2 Hz, 1 H), 5.94 (d, *J* = 8.6 Hz, 1 H), 5.86–5.79 (m, 1 H), 5.31–5.25 (m, 3 H), 5.18 (d, *J* = 11.6 Hz, 1 H), 5.13 (d, *J* = 12.2 Hz, 1 H), 5.06 (d, *J* = 12.4 Hz, 1 H), 4.99 (d, *J* = 3.5 Hz, 1 H), 4.90–4.87 (m, 2 H), 4.76 (d, *J* = 12.2 Hz, 1 H), 4.68 (dd, *J* = 12.1, 4.0 Hz, 2 H), 4.62–4.52 (m, 10 H), 4.37 (d, *J* = 11.8 Hz, 1 H), 4.23 (dd, *J* = 6.7, 6.7 Hz, 1 H), 3.80 (dd, *J* = 10.2, 3.5 Hz,1 H), 3.71 (dd, *J* = 11.8, 2.2 Hz, 1 H), 3.67–3.52 (m, 3 H), 2.85 (dd, *J* = 16.5, 3.6 Hz, 1 H), 2.69 (dd, *J* = 16.5, 3.9 Hz, 1 H), 2.09 (s, 3 H), 1.96 (s, 3 H), 1.06 (d, *J* = 6.5 Hz, 3 H).