Supplementary Materials

Liquid-phase and ultrahigh-frequency-acoustofluidics (UHFA)-based solid-phase synthesis of biotin tagged 6'/3'-sialyl-*N*-acetylglucosamine by sequential one-pot multienzyme (OPME) system

Mengge Gong[1,2], Tiechuan Li[3], Lina Wu[2], Zhenxing Zhang[4], Lishi Ren[2], Hongzhi Cao[5], Xuexin Duan[3], Meishan Pei[1,*], Yuguang Du[2,*], Jian-Jun Li[2,*]

[1] School of Chemistry and Chemical Engineering, University of Jinan,Jinan 250022, China.

[2] National Key Laboratory of Biochemical Engineering, National Engineering Research Center for Biotechnology (Beijing), Key Laboratory of Biopharmaceutical Production & Formulation Engineering, PLA, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China.

[3] State Key Laboratory of Precision Measuring Technology and Instruments, Tianjin University, Tianjin 300072, China.

[4] Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China.

[5] National Glycoengineering Research Center, State Key Laboratory of Microbial Technology, Shandong University, Qingdao 266237, China.

* Corresponding authors: Meishan Pei, Phone: +86 531 89736800, Fax: +86 531 89736800, E-mail: chm_peims@ujn.edu.cn; Yuguang Du, Phone: +86 10 8254 5070,

Fax: +86 10 8254 5039, E-mail: ygdu@ipe.ac.cn; Jian-Jun Li, Phone: +86 10 8254 5039, Fax: +86 10 8254 5039, E-mail: jjli@ipe.ac.cn.

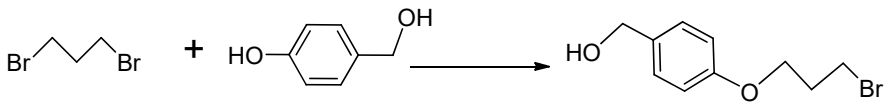
Contents

1. Synthetic procedures for biotin-tagged *N*-acetylglucosamine (Biotin-GlcNAc)
2. **Figure S1.** Purification of biotinlated *N*-acetylglucosamine (Biotin-GlcNAc) by reverse HPLC
3. **Figure S2.** HPLC analysis of purified biotinylated *N*-acetylglucosamine (Biotin-GlcNAc)
4. **Figure S3.** MS analysis of purified Biotin-GlcNAc
5. **Figure S4.** SDS PAGE analysis of purified GalE
6. **Figure S5.** SDS PAGE analysis of purified NmLgtB
7. **Figure S6.** SDS PAGE analysis of purified NmCSS
8. **Figure S7.** SDS PAGE analysis of purified Pd26ST
9. **Figure S8.** SDS PAGE analysis of purified PmST1 (M144D)
10. **Figure S9.** MS analysis of purified Compound I (C I)
11. **Figure S10.** HPLC analysis of OPME 2-catalyzed synthesis of compound II (C II)
12. **Figure S11.** MS analysis of purified Compound II (C II)
13. **Figure S12.** HPLC analysis of OPME 3-catalyzed synthesis of compound III (C III)
14. **Figure S13.** MS analysis of purified Compound III (C III)

15. **Figure S14.** MS analysis of traditional solid-phase sequential OPME synthesis of Compound I (C I)
16. **Figure S15.** MS analysis of traditional solid-phase sequential OPME synthesis of Compound II (C II)
17. **Figure S16.** ^{1}H NMR analysis of purified Compound I (C I)
18. **Figure S17.** ^{13}C NMR analysis of purified Compound I (C I)
19. **Figure S18.** ^{1}H NMR analysis of purified Compound II (C II)
20. **Figure S19.** ^{13}C NMR analysis of purified Compound II (C II)
21. **Figure S20.** ^{1}H NMR analysis of purified Compound III (C III)
22. **Figure S21.** ^{13}C NMR analysis of purified Compound III (C III)

1. Synthetic procedures for biotin-tagged *N*-acetylglucosamine (Biotin-GlcNAc) (compound 6)

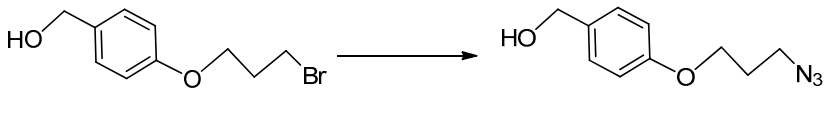
1.1 Synthesis of compound 1



1

Under the condition of nitrogen atmosphere, *p*-hydroxymethyl phenol (4.00 g, 32.22 mmol), K_2CO_3 (4.67 g, 33.82 mmol) and 1,3-dibromo propane (9.85 ml, 96.44 mmol) in the dry acetone (30 ml) were refluxed for 4.5 hr, then the reaction mixture was filtered to remove undissolved materials and the filtrate was evaporated to dryness. Compound 1 was purified by a silica gel column (1:3→1:2 ethyl acetate/petroleum ether) and was obtained as a white solid after evaporation (yield ~40%), which was taken directly to the next step.

1.2 Synthesis of compound 2

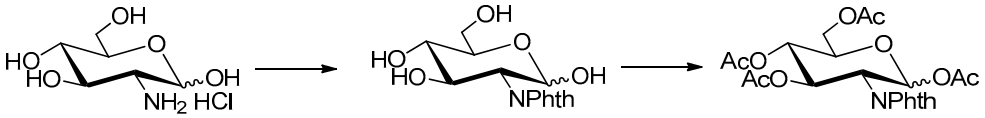


1 **2**

Compound 1 (3.00 g, 12.24 mmol) and NaN_3 (4 g, 61.2 mmol) were stirred overnight at 80°C in DMF (30 ml). Then DMF was evaporated to dryness, and the residue was redissolved in acetone and filtered, and the filtrate was evaporated to dryness. Compound 2was purified by a silica gel column (1: 5 ethyl acetate/petroleum ether), and was obtained as a colorless oily liquid (yield ~95%). ^{1}H NMR ($CDCl_3$) δ 7.30 (d, J = 8.4 Hz, 2H), 6.90 (d, J = 8.5 Hz, 2H), 4.62 (s, 2H), 4.05 (t, J = 5.9 Hz, 2H),

3.52 (t, *J* = 6.6 Hz, 2H), 2.06 (m, 2H).

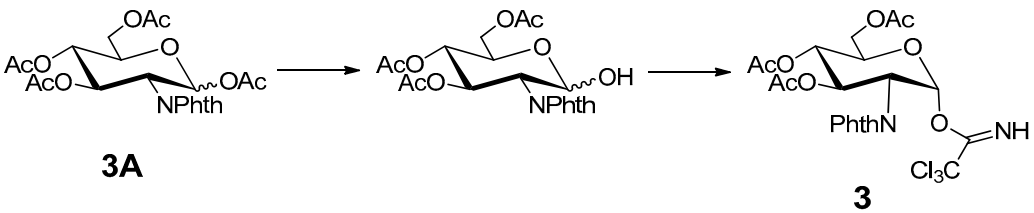
1.3 Synthesis of compound 3A



3A

Glucosamine hydrochloride (20.0 g, 92.75 mmol) and $NaHCO_3$ (15.6 g, 185.50 mmol) were dissolved in water (150 mL),and *o*-phthalic anhydride (13.8g, 92.75 mmol) was added and stirred overnight at room temperature[1]. After 3 M HCl (50 mL) was added to stop the reaction, the solvent was evaporated to dryness under reduced pressure. The obtained solid was dried under vacuum at 45°C, and redissolved in pyridine (150 mL) and cooled to 0°C. Then acetic anhydride (50 mL) was added, and the reaction was stirred at room temperature. After TLC showed that the reaction was complete (about 8 hr), the solvent was evaporated to dryness under reduced pressure. The obtained syrup was dissolved in dichloromethane (300 mL) and washed 3 times with 1M HCl, and once with saturated NaCl. After the obtained organic phase was dried over anhydrous Na_2SO_4, the solvent was evaporated to dryness under reduced pressure. The residue was purified on a silica gel column (2:1 → 1:1 ethyl acetate/ petroleum ether) to yield an α / β mixture of compound 3A (yield~92%) as a white foamy solid, and directly used for the next reaction.

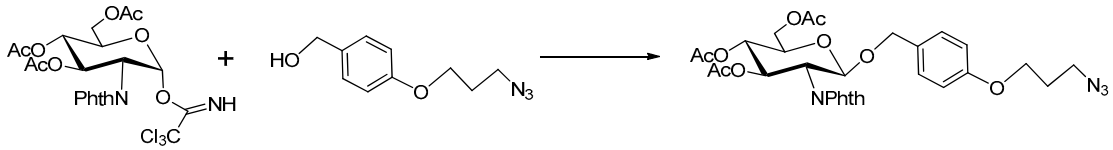
1.4 Synthesis of compound 3



3A **3**

Compound 3A (17 g, 35.6 mmol) was dissolved in 200 ml THF (tetrahydrofuran). 6.62 ml benzylamine was added under ice bath and the reaction was stirred at room temperature for 8 hr. The solvent was then evaporated to dryness, and redissolved in dichloromethane, and washed in sequence with 1M HCl, saturated $NaHCO_3$ and saturated NaCl, and dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was dissolved in 200 ml dry dichloromethane under nitrogen atmosphere. Five equivalent of trichloroacetonitrile and 0.2 equivalent of DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene) were added under condition of ice bath, and the reaction was stirred overnight at room temperature[2]. Then reaction solution was evaporated to dryness, and purification of the residue on a silica gel column (1:2 → 1:1 ethyl acetate / petroleum ether) yielded compound 3 as a pale yellow foamy solid. ^{1}H NMR (CDCl3) δ 8.66 (s, 1H), 7.84 (m, 2H), 7.73 (m, 2H), 6.62 (d, J = 8.9 Hz, 1H), 5.92 (dd, J = 9.1, 11.2 Hz, 1H), 5.28 (dd, J = 9.1, 10.3 Hz, 1H), 4.63 (dd, J = 8.95, 10.7 Hz, 1H), 4.40 (dd, J = 4.3, 12.5 Hz, 1H), 4.21 (dd, J = 2.3, 12.5 Hz, 1H), 4.08 (m, 1H), 2.13 (s, 3H), 2.04 (s, 3H), 1.90 (s, 3H).

1.5 Synthesis of compound 4

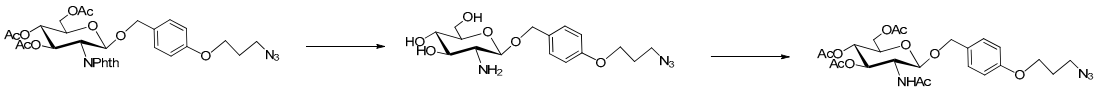


3 2 4

Compound 2 (2 g, 9.6 mmol) and trimethylsilyl triflate (TMSOTf) (110 μl, 0.6 mmol) were dissolved in 30 ml dry dichloromethane at -20°C[3]. Then compound 4 (2.2 g, 3.8 mmol) was slowly added dropwise, and the reaction was continued for 3 hr. The reaction was stopped by adding equivalent of trimethylamine, and the solvent was

evaporated. Compound 4 (2.1 g) was purified by a silica gel column (1:3 → 1:2 ethyl acetate / petroleum ether) as a white foamy solid. ^{1}H NMR ($CDCl_3$) δ 7.78 (br, 2H), 7.72 (m, 2H), 6.99 (d, *J* = 8.6 Hz, 2H), 6.58 (d, *J* = 8.5 Hz, 2H), 5.78 (dd, *J* = 9.2, 10.8 Hz, 1H), 5.35 (d, *J* = 8.5 Hz, 1H), 5.18 (dd, *J* = 9.1, 10.2 Hz, 1H), 4.77 (d, *J* = 11.9 Hz, 1H), 4.46 (d, *J* = 11.9 Hz, 1H), 4.34 (m, 2H), 4.20 (dd, *J* = 2.4, 12.2 Hz, 1H), 3.93 (m, 2H), 3.91 (m, 1H), 3.49 (t, *J* = 6.6 Hz, 2H), 2.13 (s, 3H), 2.03 (s, 3H), 2.01 (m, 2H), 1.85 (s, 3H).

1.6 Synthesis of compound 5

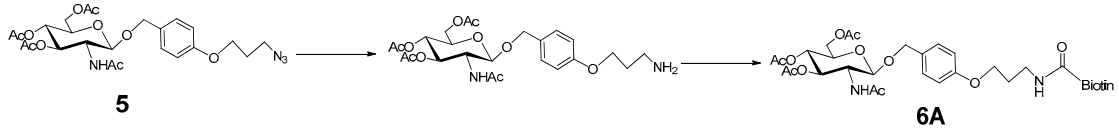


4 **5**

Compound 4 (1.6 g, 2.6 mmol) and ethylenediamine (10.3 ml, 156 mmol) were dissolved in 25 ml of ethanol and stirred overnight at 85°C. Then the solvent was evaporated to dryness, and the residue was dissolved in 50 ml of pyridine. Then acetic anhydride (5.8 ml, 61.4 mmol) was added, and the reaction was lasted overnight at room temperature. The solvent was evaporated to dryness, and the residue was redissolved in dichloromethane, and washed in sequence with 1M HCl three times and saturated NaCl once. After the obtained organic phase was dried over anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure. Purification of the residue on a silica gel column (1:1 → 2:1 ethyl acetate / petroleum ether) afforded compound 5 (1 g) as a white foam solid. ^{1}H NMR ($CDCl_3$) δ 7.22 (d, *J* = 8.5 Hz, 2H), 6.87 (d, *J* = 9.0 Hz, 2H), 5.29 (d, *J* = 9.0 Hz, 1H), 5.18 (dd, *J* = 9.5, 10.5 Hz, 1H), 5.08 (t, *J* = 9.5, 1H), 4.81 (d, *J* = 11.5 Hz, 1H), 4.59 (d, *J* = 8.5, 1H), 4.54 (d, *J* = 12.0, 1H), 4.27 (dd, *J* = 4.5, 12.5 Hz, 1H), 4.17 (dd, *J* = 2.5, 12.5 Hz, 1H), 4.05 (t, *J* = 6.0, 2H), 3.94 (m, 1H), 3.65 (m,

1H), 3.52 (t, *J* = 6.5, 2H), 2.11 (s, 3H), 2.06 (m, 2H), 2.01 (s, 6H), 1.91 (s, 3H).

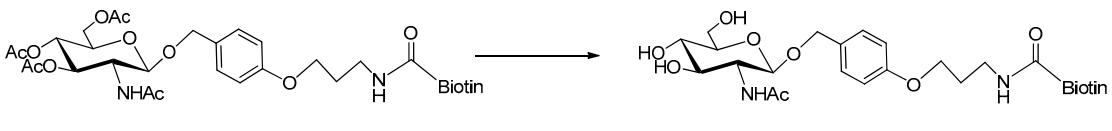
1.6 Synthesis of compound 6A



Biotin and NHS (N-Hydroxysuccinimide) were dissolved in THF (tetrahydrofuran), and then EDC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride and catalytic amount of DMAP (4- (Dimethylamino) pyridine) were added to start the reaction[4]. After the reaction was stirred overnight at room temperature, the reaction mixture was filtered and concentrated to obtain crude Biotin-NHS as a white solid, which was used directly for the next reaction.

Compound 5 (1 g, 1.86 mmol) and Ph_3P (0.73 g, 2.8 mmol) were dissolved in 50 ml THF, and 0.5 ml H_2O was added, and the reaction mixture was stirred at room temperature overnight[5]. Then Biotin-NHS (0.5 g) dissolved in 5 ml DMF was added and the reaction was lasted for 2 hr at room temperature. Then the solvent was evaporated to dryness, and purification of the residue on a silica gel column (4:1 → 3:1 dichloromethane / methanol) gave compound 6A (1.1 g) as a white solid, which was used directly for the next reaction.

1.8 Synthesis of Compound 6 (Biotin-GlcNAc)



Compound 6A was dissolved in methanol, and sodium methoxide was added to adjust pH to 9.0 ~ 10.0, and the reaction mixture was stirred at room temperature for 2

hr. After the reaction mixture was neutralize with H-type ion exchange resin, it was filtered and evaporated to dryness. Compound 6 was purified by reverse HPLC (detection wavelength: 230 nm, C_{18} column). HPLC conditions were used as follows:flow rate at 1 ml / min; A: H_2O, B: acetonitrile,B within 30 min from 5% to 50%. ^{1}HNMR ($CDCl_3$) δ 7.88 (t, *J* = 5.0 Hz, 1H), 7.22 (d, *J* = 8.6 Hz, 2H), 6.88 (d, *J* = 8.6 Hz, 2H), 6.42 (d, *J* = 32.0 Hz, 1H), 4.69 (d, *J* = 11.8 Hz, 1H), 4.43 (d, *J* = 11.85 Hz, 1H), 4.32 (t, *J* = 8.45, 1H), 4.29 (dd, *J* = 5.25, 7.5 Hz, 1H), 4.11 (m, 1H), 3.95 (t, *J* = 6.25, 2H), 3.71 (d, *J* = 11.6 Hz, 1H), 3.48 (m, 2H), 3.17 (s, 1H), 3.08 (m, 2H), 2.80 (dd, *J* = 5.1, 12.45 Hz, 1H), 2.73 (s, 2H), 2.57 (d, *J* = 13.5 Hz, 1H),2.06 (t, *J* = 7.5 Hz, 2H), 1.83 (m, 2H), 1.80 (s, 3H), 1.61 (m, 1H), 1.50 (m, 2H), 1.31 (m, 2H). MALDI-TOF-MS m/z calcd for $C_{28}H_{42}N_4O_9S$ $[M+Na]^+$ 633.72 and $[M+K]^+$ 649.72, found 632.700 and 648.680 respectively.

References

1. Iwata, R.; Doi, A.; Maeda, Y.; Wada, T. Synthesis of oligodiaminomannoses and analysis of their RNA duplex binding properties and their potential application as siRNA-based drugs. *Organic & Biomolecular Chemistry* **2015**, *13*, 9504-9515, doi:10.1039/c5ob01384d.
2. Balcerzak, A.K.; Ferreira, S.S.; Trant, J.F.; Ben, R.N. Structurally diverse disaccharide analogs of antifreeze glycoproteins and their ability to inhibit ice recrystallization. *Bioorganic & Medicinal Chemistry Letters* **2012**, *22*, 1719-1721, doi:10.1016/j.bmcl.2011.12.097.
3. Li, C.G.; Zhang, Z.X.; Duan, Q.; Li, X.B. Glycopeptide Synthesis on an Ionic Liquid Support. *Org Lett* **2014**, *16*, 3008-3011, doi:10.1021/ol501133u.
4. Canaria, C.A.; Smith, J.O.; Yu, C.J.; Fraser, S.E.; Lansford, R. New syntheses for 11-(mercaptoundecyl)triethylene glycol and mercaptododecyltriethyleneoxy biotin amide. *Tetrahedron Lett* **2005**, *46*, 4813-4816, doi:10.1016/j.tetlet.2005.04.091.
5. Pruet, J.M.; Saito, R.; Manzano, L.A.; Jasheway, K.R.; Wiget, P.A.; Kamat, I.; Anslyn, E.V.; Robertus, J.D. Optimized 5-Membered Heterocycle-Linked Pterins for the Inhibition of Ricin Toxin A. *Acs Med Chem Lett* **2012**, *3*, 588-591, doi:10.1021/ml300099t.

Figure S1. Purification of biotinlated *N*-acetylglucosamine (Biotin-GlcNAc) by reverse HPLC

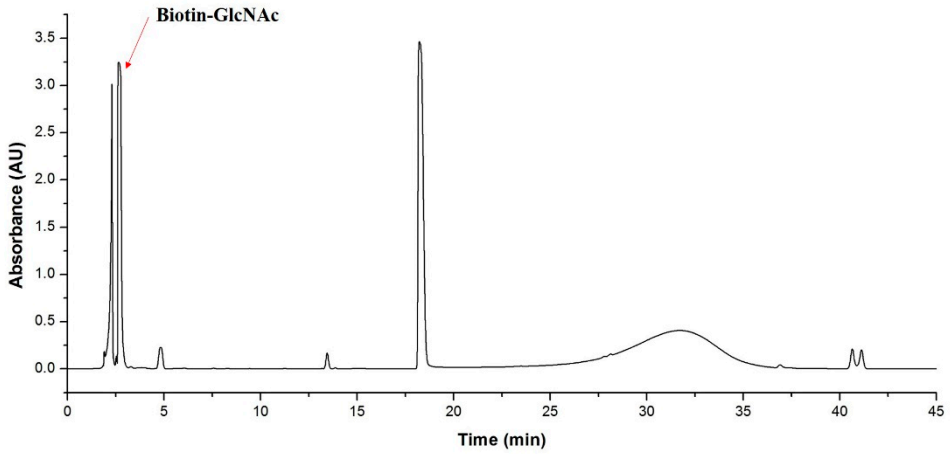


Figure S2. HPLC analysis of purified biotinylated *N*-acetylglucosamine (Biotin-GlcNAc)

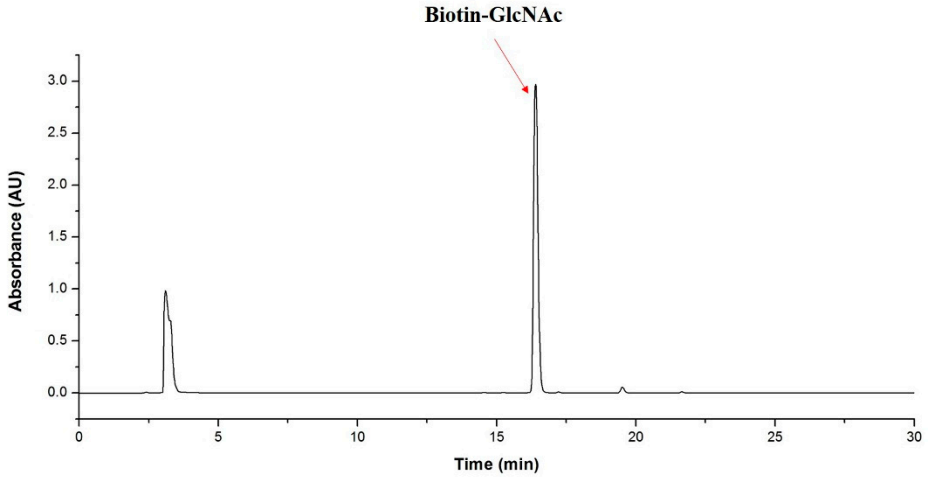


Figure S3. MS analysis of purified Biotin-GlcNAc

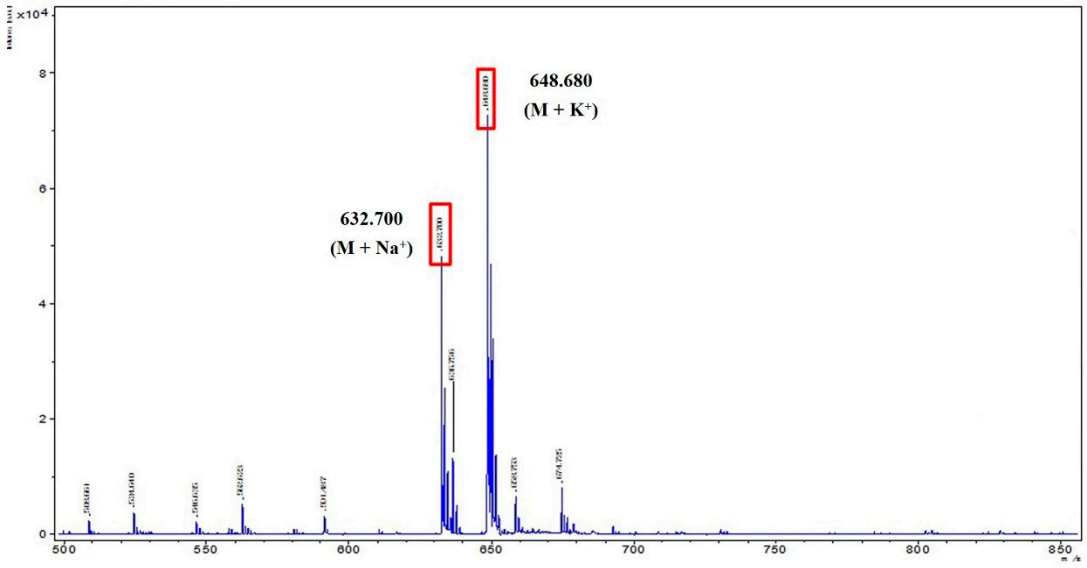


Figure S4. SDS PAGE analysis of purified GalE. Lane 1, Protein molecular weight marker; Lane 2, flow through when loaded; Lane 3, cell debris; Lane 4, eluents of buffer A (50 mM Tris/HCl, pH7.9, + 0.5 M NaCl); Lane 5,6 eluents of of buffer A + 20 mM imidazole; Lanes 7-9, eluents of buffer A + 200 mM imidazole.

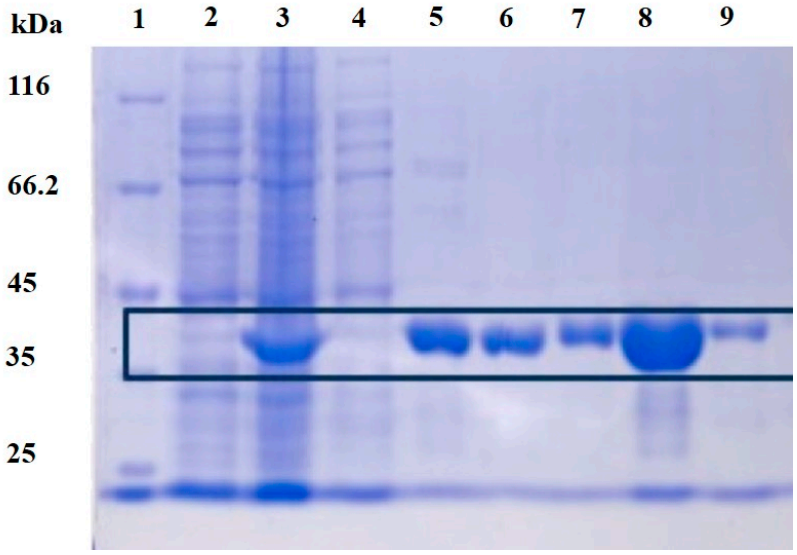


Figure S5. SDS PAGE analysis of purified NmLgtB.Lane 1, Protein molecular weight marker; Lane 2,3, eluents of buffer A (50 mM Tris/HCl, pH7.9, + 0.5 M NaCl) + 50 mM imidazole; Lane 4-8, eluents of buffer A + 160 mM imidazole; Lane 9-17, eluents of buffer A + 200 mM imidazole; Lane 18, eluents of buffer A; Lane 19, cell debris; Lane 20, flow through when loaded.

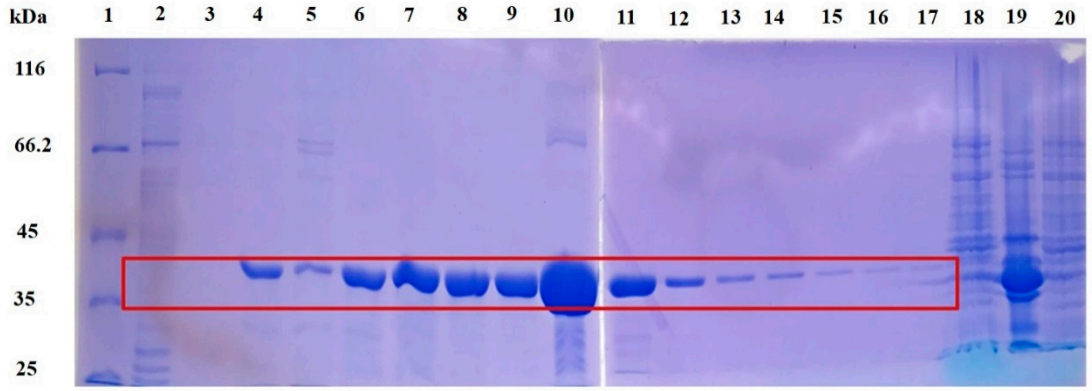


Figure S6. SDS PAGE analysis of purified NmCSS. Lane 1, Protein molecular weight marker; Lane 2, crude protein; Lane 3, flow through when loaded; Lane 4, eluents of buffer A (50 mM Tris/HCl, pH7.9, + 0.5 M NaCl); Lane 5 eluents of of buffer A + 20 mM imidazole; Lane 6, eluents of buffer A + 60 mM imidazole; Lanes 7,8, eluents of buffer A + 160 mM imidazole.

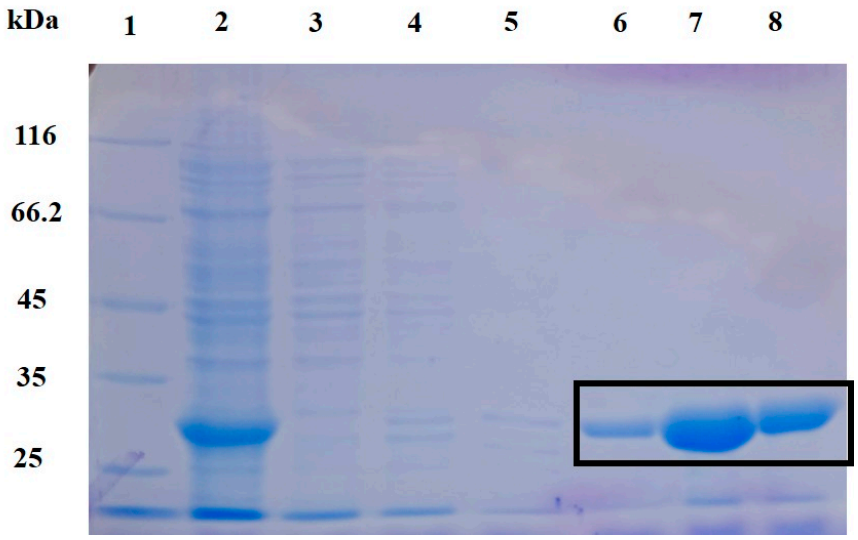


Figure S7. SDS PAGE analysis of purified Pd26ST. Lane 1, Protein molecular weight marker; Lane 2, flow through when loaded; Lane 3, crude protein; Lane 4, eluents of buffer A (50 mM Tris/HCl, pH7.9, + 0.5 M NaCl); Lane 5 eluents of of buffer A + 20 mM imidazole; Lane 6,7, eluents of buffer A + 60 mM imidazole; Lanes 8,9, eluents of buffer A + 160 mM imidazole.

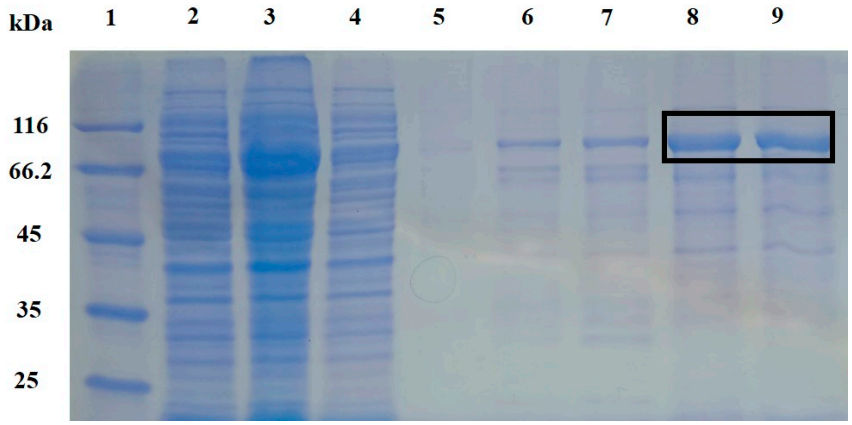


Figure S8. SDS PAGE analysis of purified PmST1 (M144D). Lane 1, Protein molecular weight marker; Lane 2, flow through when loaded; Lane 3, crude protein; Lane 4, eluents of buffer A (50 mM Tris/HCl, pH7.9, + 0.5 M NaCl); Lane 5 eluents of buffer A + 20 mM imidazole; Lane 6,7, eluents of buffer A + 60 mM imidazole; Lanes 8,9, eluents of buffer A + 160 mM imidazole.

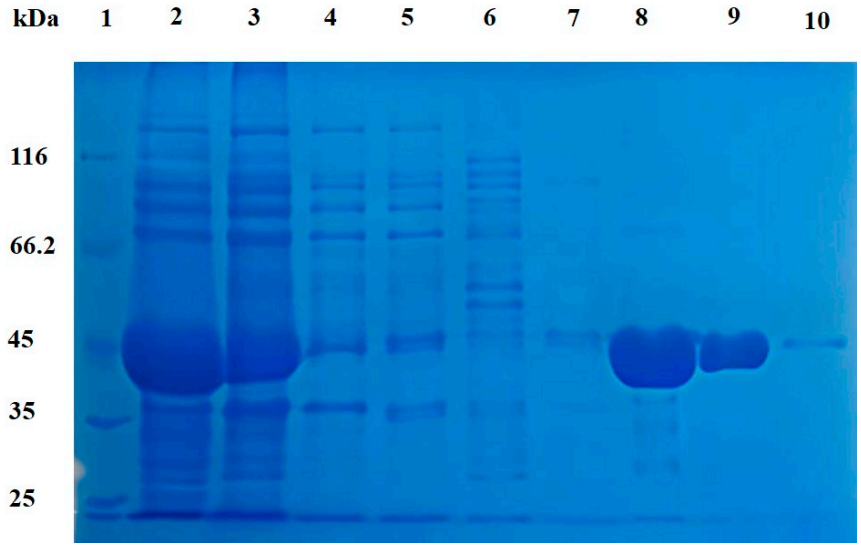


Figure S9. MS analysis of purified Compound I (C I)

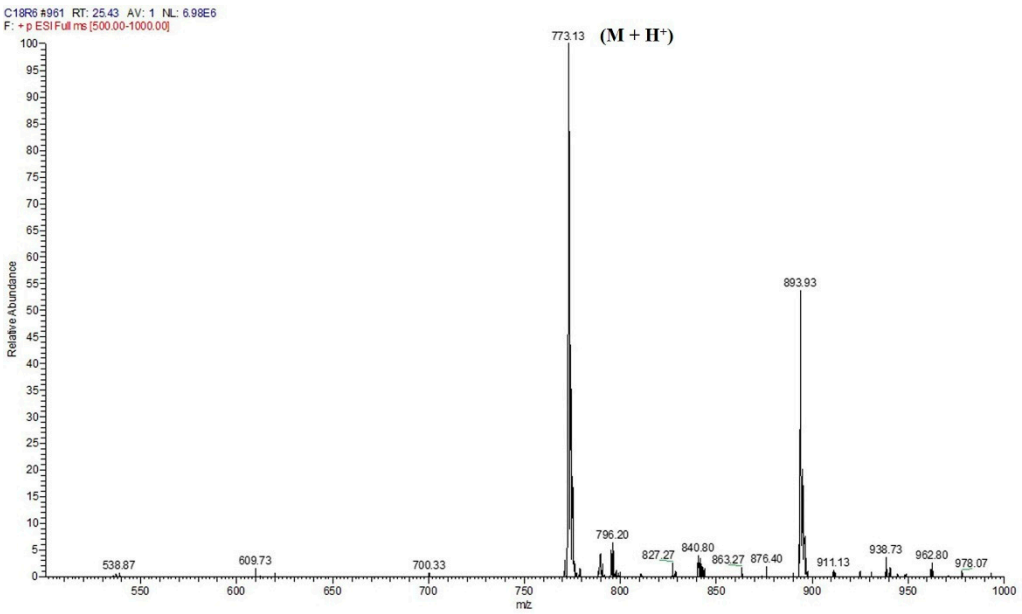


Figure 10. HPLC analysis of OPME 2-catalyzed synthesis of compound II (C II)

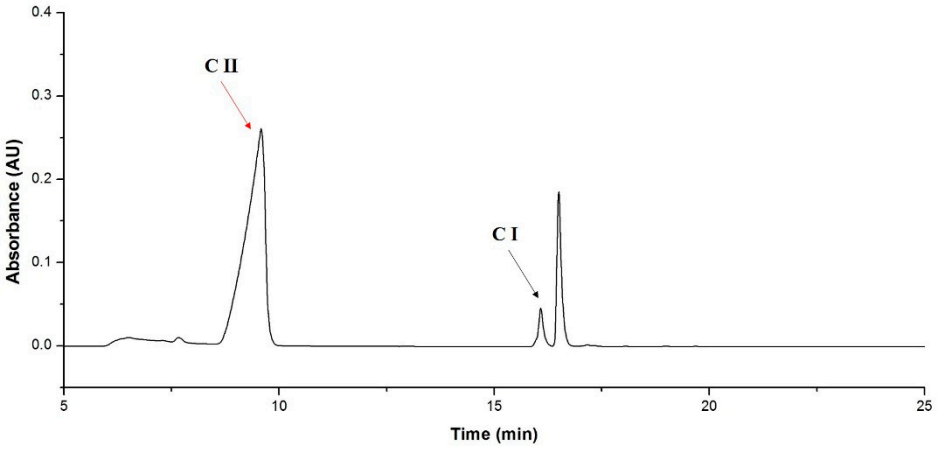


Figure S11. MS analysis of purified Compound II (C II)

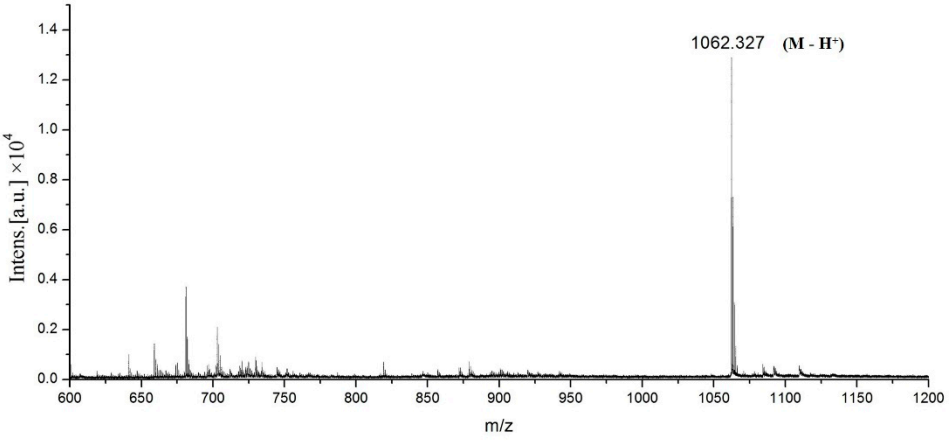


Figure S12. HPLC analysis of OPME 3-catalyzed synthesis of compound III (C III)

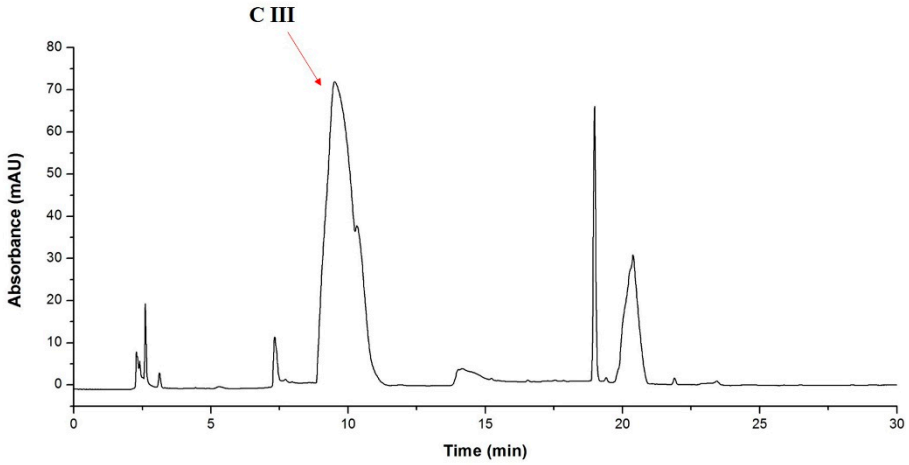


Figure S13. MS analysis of purified Compound III (C III)

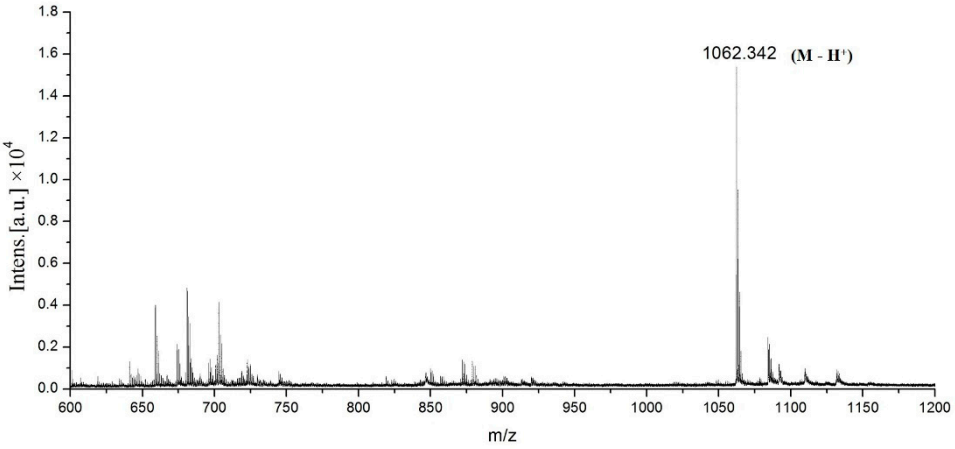


Figure S14. MS analysis of traditional solid-phase sequential OPME synthesis of Compound I (C I)

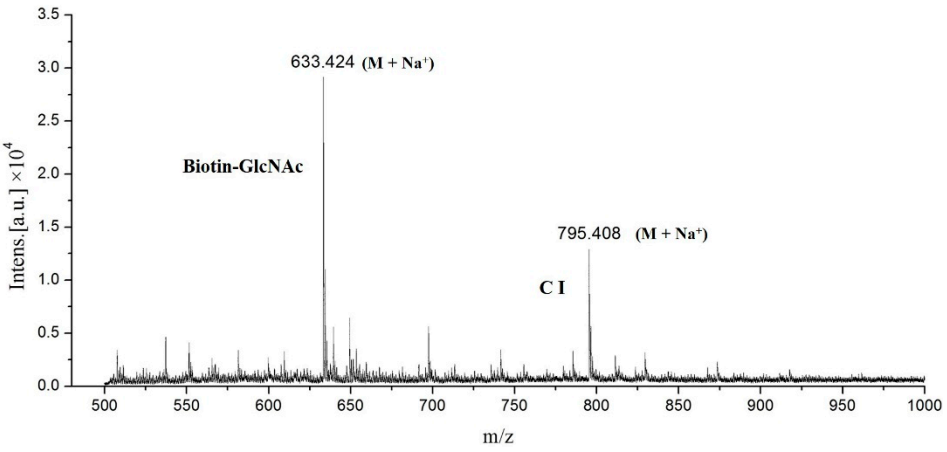


Figure S15. MS analysis of traditional solid-phase sequential OPME synthesis of Compound II (C II)

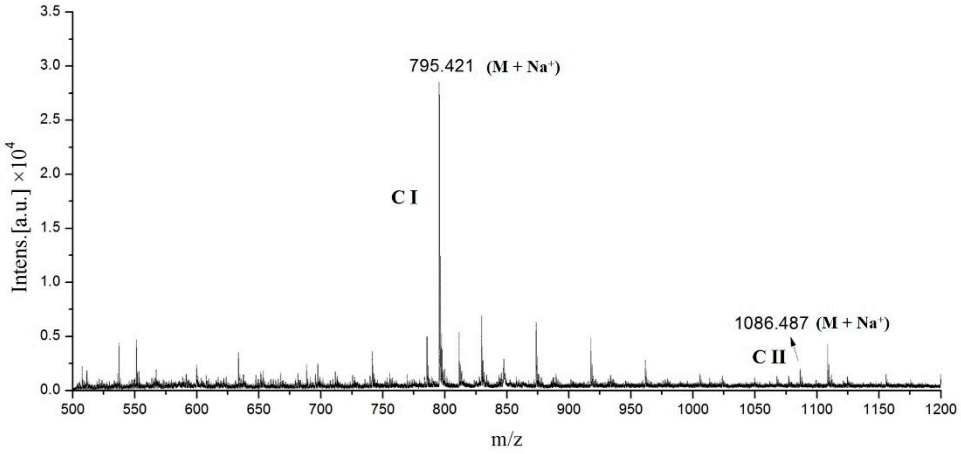


Figure S16. ^{1}H NMR analysis of purified Compound I (C I)

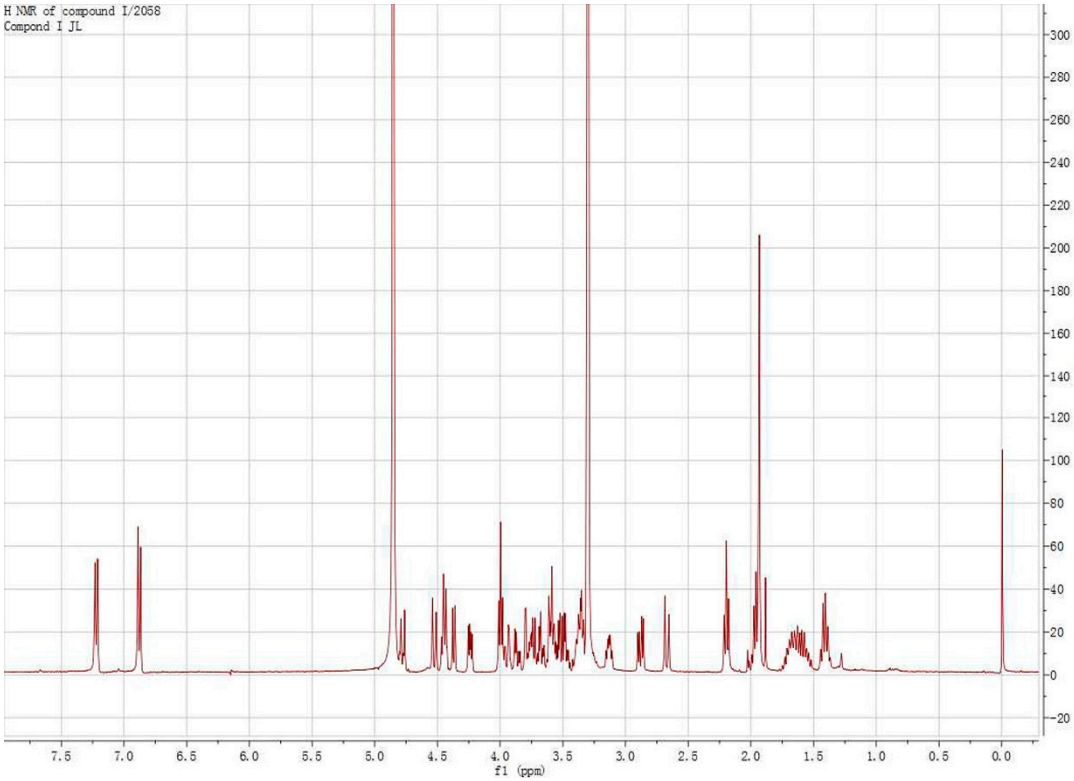


Figure S17. ^{13}C NMR analysis of purified Compound I (C I)

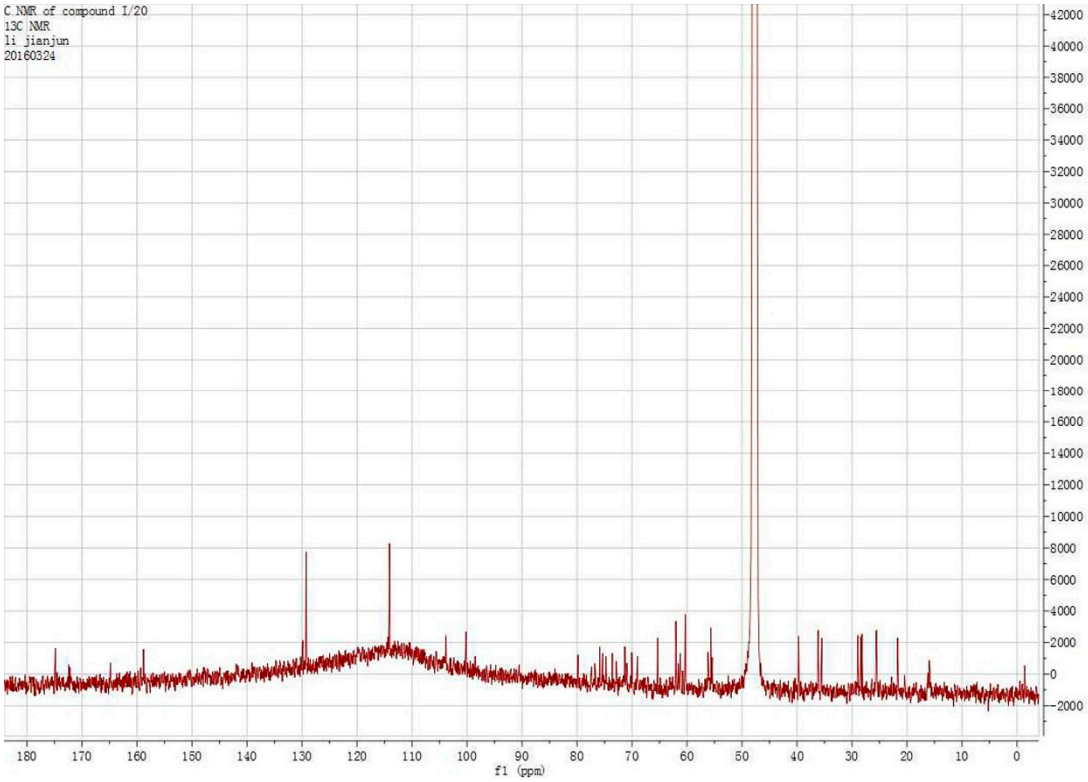


Figure S18. ^{1}H NMR analysis of purified Compound II (C II)

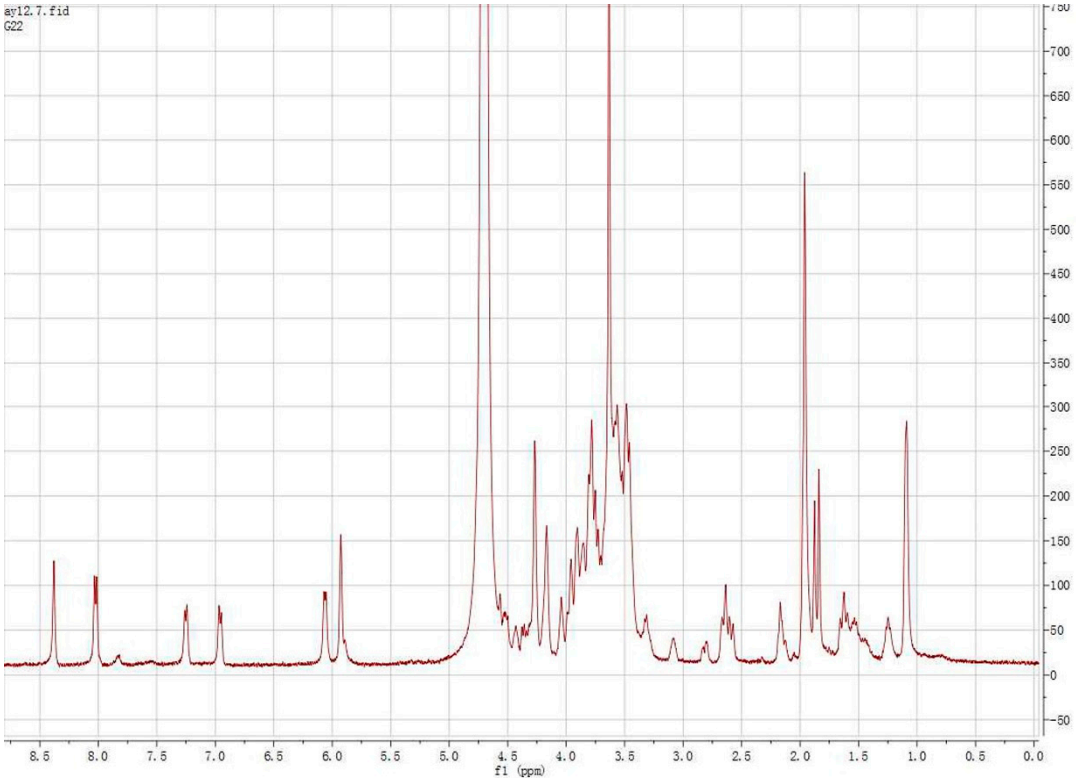


Figure S19. ^{13}C NMR analysis of purified Compound II (C II)

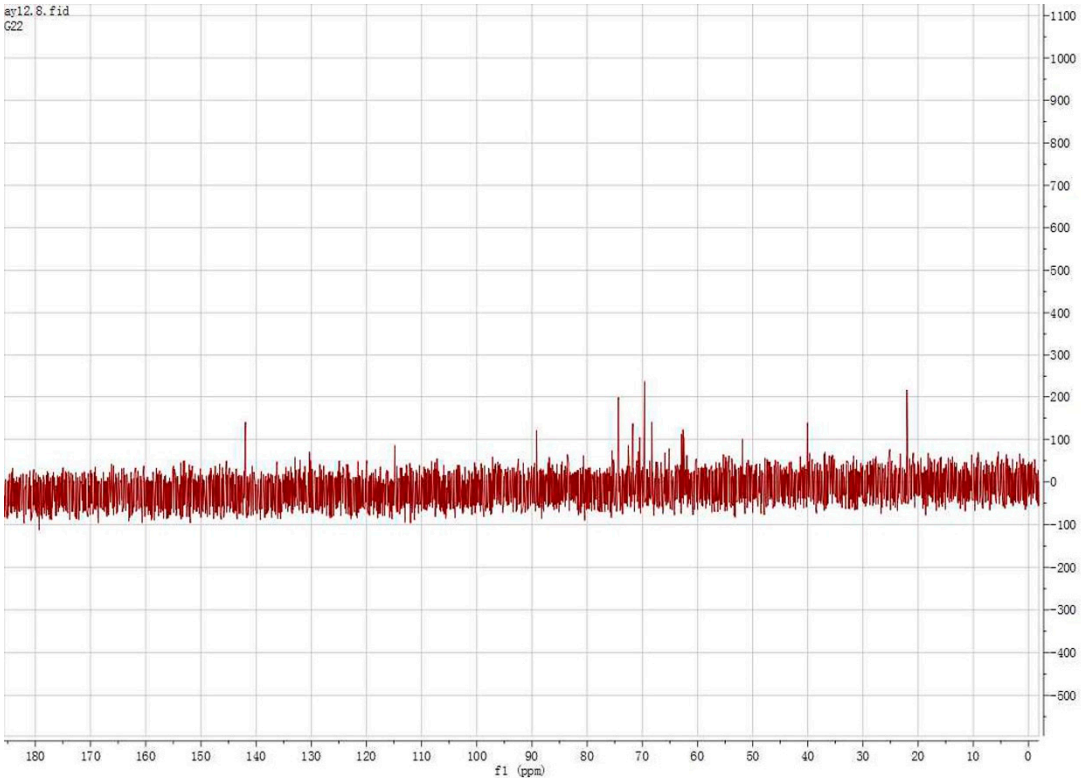


Figure S20. ^{1}H NMR analysis of purified Compound III (C III)

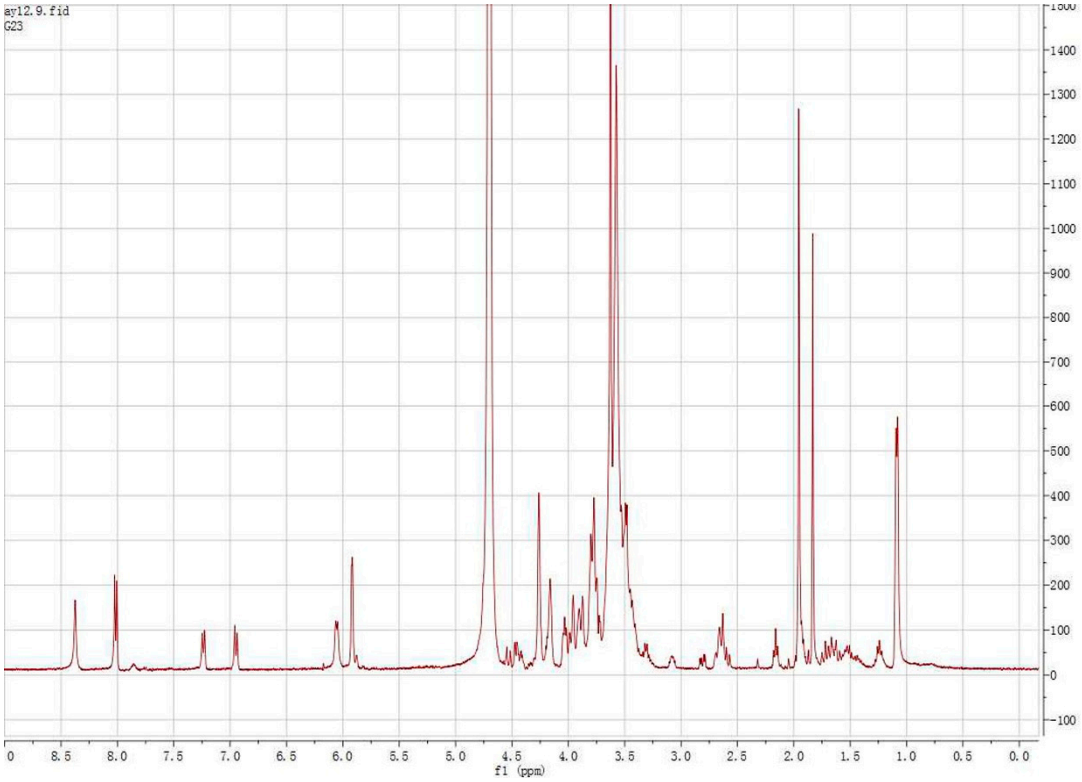


Figure S21. ^{13}C NMR analysis of purified Compound III (C III)