

Supporting Information for:

Probing the Stereoselectivity OleD-Catalyzed Glycosylation with Cardiotonic

Steroids

Xue-Lin Zhu^{1,†}, Chao Wen^{1,†}, Qing-Mei Ye^{2,†}, Wei Xu¹, Deng-Lang Zou¹, Guang-Ping Liang¹, Fan Zhang¹,
Wan-Na Chen¹, Ren-Wang Jiang^{1*}

¹ *Guangdong Province Key Laboratory of Pharmacodynamic Constituents of TCM and New Drugs Research, College of Pharmacy, Jinan University, Guangzhou 510632, P. R. China.*

² *Department of Pharmacy, Hainan General Hospital, Haikou 570311, P. R. China.*

[†] *These authors contribute equally to this work.*

^{*} *Ren-Wang Jiang, e-mail: trwjiang@jnu.edu.cn (R.W.J.)*

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1. Determination of kinetic parameters.

Assays were performed in a final volume of 200 μ L 50 mM Tris-HCl (pH 8.0), and contained constant concentrations of OleD (ASP) (40 μ g) and UDPG (2.5 mM) while varying the concentration (0.01-1.2 mM) of **2 β** , **2 α** , **4 β** and **4 α** . Aliquots (100 μ L) were removed every 15 min, mixed with an equal volume of ice cold methanol, and centrifuged at 10,000 g for 10 min. Supernatants were analyzed by analytical reverse-phase HPLC. Conversion rate is calculated by the corresponding HPLC peak area percentage using the Agilent Chromatography Workstation Software. All experiments were performed in triplicate. Initial velocities were fitted to the Michaelis-Menten equation using Origin Pro 7.0 software.

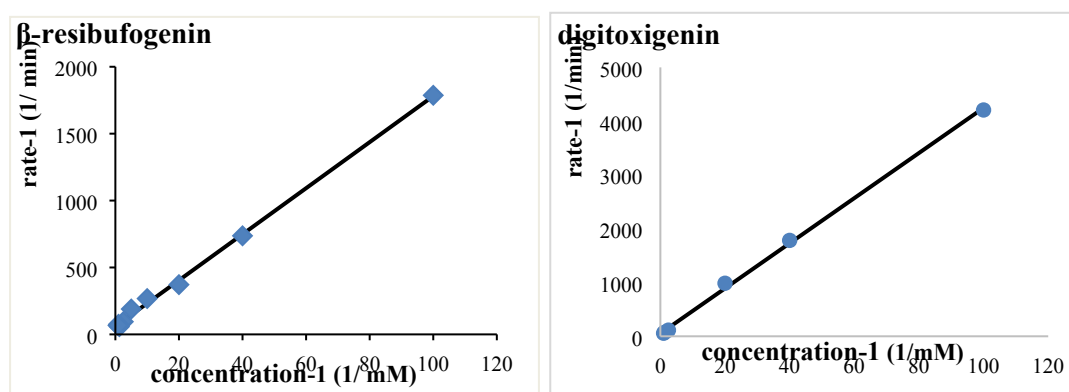


Figure S1. The Determination of K_m for the **2 β** and **4 β** .

2. Assessment of inhibition of NKA activity by CTS

NKA from pig kidney microsomal membranes was purified by differential centrifugation as reported. Stock solutions of the inhibitors, typically 10 mM, were prepared in DMSO. In brief, NKA is preincubated at 37 $^{\circ}$ C for 2 hours in the presence of 3 mM $MgCl_2$, 3 mM Na-phosphate and 40 mM Tris-HCl (pH 7.0) with increasing

concentrations of inhibitor. The residual NKA activity is subsequently determined by 40-fold dilution into a standard ATPase assay medium in triplicate. For each inhibitor of concentration the residual activity was determined in three independent experiments.

The maximal DMSO concentration of 2.5% after dilution into the NKA incubation medium was 2.5%. Control experiments showed that incubation with 2.5% DMSO for 2 hours at 37 °C leads to less than 10% inhibition of NKA activity.

3. Spectral data and characterization of **1 α** , **2 α** , **3 α** , **1 β -glu**, **2 β -glu**, **2 β -diglu**, **β -glu**, **3 β -diglu**, **4 β -glu**.

1 α : ^1H NMR (CD_3OD , 400 MHz) δ : 8.01 (1H, dd, $J = 9.7, 2.5$ Hz, H-22), 7.45 (1H, d, $J = 2.4$ Hz, H-21), 6.30 (1H, d, $J = 9.8$ Hz, H-23), 3.58 (1H, m, H-3), 2.58 (1H, m, H-17), 2.26-2.11 (2H, m), 1.92-1.62 (11H, m), 1.55-1.36 (9H, m), 1.29-1.19 (1H, m), 1.09-1.01 (2H, m), 0.95 (3H, s, H-19), 0.73 (3H, s, H-18); ^{13}C NMR (CD_3OD , 100 MHz) δ : 164.8 (C-24), 150.5 (C-21), 149.37 (C-22), 125.0 (C-20), 115.4 (C-23), 86.1 (C-14), 72.3 (C-3), 52.3 (C-17), 49.8 (C-13), 43.2 (C-9), 43.1 (C-5), 41.9 (C-12), 37.7 (C-8), 37.0 (C-4), 36.3 (C-1), 35.9 (C-10), 33.2 (C-15), 31.3 (C-2), 29.9 (C-16), 28.4 (C-6), 23.8 (C-19), 22.8 (C-7), 22.5 (C-11), 17.3 (C-18). HRESIMS m/z 386.5343 [$\text{M} + \text{H}$] $^+$ (calcd for $\text{C}_{24}\text{H}_{34}\text{O}_4$, 386.5541).

In the ^1H -NMR spectrum of **1 α** , the chemical shift of hydrogen at C-3 resonates at δ 3.65 with a multiplet pattern, which indicates that the hydroxyl group should be α -oriented because the α -oriented proton H-3, adopting the axial position, is split by two axial protons (H-2 α and H-4 α) and two equatorial proton (H-2 β and H-4 β) resulting large aa and small ae couplings. In contrast, in the ^1H -NMR spectrum of **1 β** , the

hydrogen at C-3 resonates at δ 4.13 with a broad singlet pattern.

2 α : ^1H NMR (CD_3OD , 400 MHz) δ : 7.91 (1H, dd, $J = 9.8, 2.4$ Hz, H-22), 7.47 (1H, d, $J = 2.4$ Hz, H-21), 6.28 (1H d, $J = 9.8$ Hz, H-23), 3.62 (1H, s, H-15), 3.57 (1H, m, H-3), 2.61 (1H, d, $J = 10.0$ Hz, H-10), 2.44 (1H, d, $J = 10.4$ Hz, H-16), 2.04-1.63 (9H, m), 1.63-1.28 (9H, m), 0.99 (3H, s), 0.79 (3H, s); ^{13}C NMR (CD_3OD , 100 MHz) δ : 164.5 (C-24), 151.8 (C-21), 149.6 (C-22), 124.5 (C-20), 115.3 (C-23), 75.8 (C-14), 72.2 (C-3), 61.1 (C-15), 48.6 (C-17), 46.3 (C-13), 43.1 (C-5), 41.3 (C-9), 40.1 (C-12), 36.9 (C-4), 36.1 (C-1), 36.0 (C-10), 35.2 (C-8), 33.2 (C-16), 31.3 (C-2), 27.5 (C-6), 23.8 (C-19), 22.1 (C-11), 22.0 (C-7), 17.1 (C-18). HRESIMS m/z 385.5243 [$\text{M} + \text{H}$] $^+$ (calcd for $\text{C}_{24}\text{H}_{32}\text{O}_4$, 385.5241).

In the ^1H -NMR spectrum of **2 α** , the chemical shift of hydrogen at C-3 resonates at δ 3.57 with a multiplet pattern, which indicates that the hydroxyl group should be α -oriented because the α -oriented proton H-3, adopting the axial position, is split by two axial protons (H-2 α and H-4 α) and two equatorial proton (H-2 β and H-4 β) resulting large aa and small ae couplings. In contrast, in the ^1H -NMR spectrum of **2 β** , the hydrogen at C-3 resonates at δ 4.06 with a broad singlet pattern.

3 α : ^1H NMR (CD_3OD , 400 MHz) δ : 8.04 (1H, d, $J = 9.6$ Hz, H-22), 7.39 (1H, s, H-21), 6.26 (1H, d, $J = 9.6$ Hz, H-23), 5.51 (1H, d, $J = 10.3$ Hz, H-16), 3.76 (1H, s, H-15), 3.58 (1H, m, H-3), 2.95 (1H, d, $J = 9.3$ Hz, H-17), 2.20-2.01 (1H, td, H-8), 1.81 (3H, s, COCH_3), 1.79-1.04 (15H, m), 0.99 (3H, s, H-19), 0.83 (3H, s, H-18); ^{13}C NMR (CD_3OD , 100 MHz) δ : 171.6 (COCH_3), 164.0 (C-24), 153.5 (C-22), 150.9 (C-21), 118.4 (C-20), 114.1 (C-23), 76.6 (C-16), 73.4 (C-14), 72.2 (C-3), 60.8 (C-15),

51.4 (C-17), 46.3 (C-13), 43.0 (C-5), 41.1 (C-9), 40.7 (C-12), 36.9 (C-4), 36.1 (C-1), 36.0 (C-10), 34.7 (C-8), 31.2 (C-2), 27.4 (C-6), 23.7 (C-19), 21.9 (C-7), 21.9 (C-11), 20.4 (COCH₃), 17.5 (C-18). HRESIMS m/z 443.4345 [M + H]⁺ (calcd for C₂₆H₃₄O₆, 443.5134).

In the ¹H-NMR spectrum of **3α**, the chemical shift of hydrogen at C-3 resonates at δ 3.58 with a multiplet pattern, which indicates that the hydroxyl group should be α-oriented because the α-oriented proton H-3, adopting the axial position, is split by two axial protons (H-2α and H-4α) and two equatorial protons (H-2β and H-4β) resulting large aa and small ae couplings. In contrast, in the ¹H-NMR spectrum of **3β**, the hydrogen at C-3 resonates at δ 4.07 with a broad singlet pattern.

1β-glu : ¹H NMR (CD₃OD, 400 MHz) δ: 8.03 (1H, dd, *J* = 9.7, 2.3 Hz, H-22), 7.45 (1H, d, *J* = 1.8 Hz, H-21), 6.30 (1H, d, *J* = 9.6 Hz, H-23), 4.37 (1H, d, *J* = 7.8 Hz, sugar H-1), 4.12 (1H, m, H-3), 3.88 (1H, d, *J* = 11.9, 1.8 Hz), 3.71 (1H, dd, *J* = 5.4 Hz), 3.40-3.20 (4H, m), 2.56-2.52 (1H, m, H-17), 2.26-2.11 (2H, m), 1.92-1.22 (23H, m), 1.09-1.01 (2H, m), 0.95 (3H, s, H-19), 0.73 (3H, s, H-18); ¹³C NMR (CD₃OD, 100 MHz) δ: 164.8 (C-24), 150.5 (C-21), 149.37 (C-22), 125.0 (C-20), 115.4 (C-23), 102.7 (sugar C-1), 86.1 (C-14), 78.2 (sugar C-5), 77.84 (sugar C-2), 75.6 (sugar C-4), 75.2 (sugar C-3), 71.8 (C-3), 61.7 (sugar C-6), 52.3 (C-17), 49.8 (C-13), 43.2 (C-9), 43.1 (C-5), 41.9 (C-12), 37.7 (C-8), 37.0 (C-4), 36.3 (C-1), 35.9 (C-10), 33.2 (C-15), 31.3 (C-2), 29.9 (C-16), 28.4 (C-6), 23.8 (C-19), 22.8 (C-7), 22.5 (C-11), 17.3 (C-18). HRESIMS m/z 549.3035 [M + H]⁺ (calcd for C₃₀H₄₄O₉, 549.3021).

2β-glu: ¹H NMR (CD₃OD, 400 MHz) δ: 7.91 (1H, dd, *J* = 9.8, 2.4 Hz, H-22), 7.46

(1H, d, $J = 2.4$ Hz, H-21), 6.27 (1H d, $J = 9.8$ Hz, H-23), 4.32 (1H, d, $J = 7.8$ Hz, sugar H-1), 4.08 (1H, m, H-3), 3.86 (1H, d, $J = 11.9$ Hz), 3.67 (1H, dd, $J = 11.9, 5.4$ Hz), 3.62 (1H, s, H-15), 3.39-3.17 (7H, m), 2.61 (1H, d, $J = 10.0$ Hz, H-10), 2.44 (1H, d, $J = 10.4$ Hz, H-16), 2.04-1.43 (15H, m), 1.40-1.05 (4H, m), 0.99 (3H, s), 0.79 (3H, s); ^{13}C NMR (CD_3OD , 100 MHz) δ : 164.5 (C-24), 151.8 (C-21), 149.6 (C-22), 124.5 (C-20), 115.3 (C-23), 102.7 (sugar C-1), 78.2 (sugar C-5), 77.84 (sugar C-2), 75.8 (C-14), 75.4 (sugar C-4), 75.2 (sugar C-3), 71.7 (C-3), 62.8 (sugar C-6), 61.2 (C-15), 48.6 (C-17), 46.3 (C-13), 40.7 (C-12), 40.1 (C-9), 37.4 (C-10), 36.3 (C-5), 35.0 (C-4), 33.2 (C-8), 31.1 (C-1), 30.8 (C-16), 27.5 (C-2), 27.0 (C-6), 24.1 (C-19), 22.2 (C-11), 21.7 (C-7), 17.2 (C-18). HRESIMS m/z 547.2902 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{30}\text{H}_{42}\text{O}_9$, 547.2938).

2 β -diglu: HRESIMS m/z 709.3421 $[\text{M} + \text{H}]^+$.

3 β -glu : ^1H NMR (CD_3OD , 400 MHz) δ : 7.91 (1H, dd, $J = 9.8, 2.4$ Hz, H-22), 7.47 (1H, d, $J = 2.4$ Hz, H-21), 6.28 (1H d, $J = 9.8$ Hz, H-23), 4.34 (1H, d, $J = 7.8$ Hz, sugar H-1), 4.10 (1H, m, H-3), 3.87 (1H, dd, $J = 11.8, 1.8$ Hz), 3.76 (1H, s, H-15), 3.69 (1H, dd, $J = 11.8, 5.4$ Hz), 3.58 (1H, m, H-3), 3.45-3.17 (5H, m), 2.95 (1H, d, $J = 9.3$ Hz, H-17), 2.15-2.01 (1H, td, H-8), 1.85 (3H, s, COCH_3), 1.81-1.04 (19H, m), 0.99 (3H, s, H-19), 0.83 (3H, s, H-18); ^{13}C NMR (CD_3OD , 100 MHz) δ : 171.6 (COCH_3), 164.0 (C-24), 153.5 (C-22), 150.9 (C-21), 118.4 (C-20), 114.1 (C-23), 102.7 (sugar C-1), 78.2 (sugar C-5), 77.84 (sugar C-2), 76.6 (C-16), 75.4 (sugar C-4), 75.2 (sugar C-3), 73.4 (C-14), 71.7 (C-3), 62.8 (sugar C-6), 60.8 (C-15), 51.4 (C-17), 46.3 (C-13), 40.5 (C-5), 40.7 (C-9), 37.3 (C-12), 36.3 (C-4), 36.1 (C-1), 36.0 (C-10),

31.7 (C-8), 31.2 (C-2), 27.4 (C-6), 23.7 (C-19), 21.9 (C-7), 21.9 (C-11), 20.4 (COCH₃), 17.5 (C-18). HRESIMS m/z 605.2996 [M + H]⁺ (calcd for C₃₂H₄₄O₁₁, 605.2938).

3β-diglu: HRESIMS m/z 767.3479 [M + H]⁺.

4β-glu: ¹H NMR (CD₃OD, 400 MHz) δ: 5.93 (1H, s), 5.07 (1H, d, *J* = 18.4 Hz), 4.95 (1H, d, *J* = 19.8 Hz), 4.35 (1H, d, *J* = 7.8 Hz, sugar H-1), 4.11 (1H, s, H-3), 3.89 (1H, d, *J* = 2.1 Hz), 3.69 (1H, dd, *J* = 11.8, 5.4 Hz), 3.40-3.21 (5H, m), 2.95-2.79 (1H m), 2.35-2.12 (2H, m), 2.00-1.43 (19H, m), 1.40-1.22 (4H, m), 1.00 (3H, s), 0.92 (3H, s); ¹³C NMR (CD₃OD, 100 MHz) δ: 178.5 (C-23), 177.3 (C-20), 117.8 (C-22), 102.7 (sugar C-1), 86.5 (C-14), 78.2 (sugar C-5), 77.8 (sugar C-2), 75.4 (sugar C-4), 75.3 (sugar C-3), 75.2 (C-31), 71.7 (C-3), 62.8 (sugar C-6), 52.2 (C-13), 51.1 (C-17), 42.7 (C-9), 41.0 (C-8), 37.5(C-5), 36.9 (C-12), 36.3 (C-10), 33.4(C-4), 31.2 (C-15), 30.9 (C-1), 28.1 (C-2), 27.8 (C-6), 27.5 (C-16), 24.1 (C-7), 22.6 (C-7), 22.4 (C-18), 16.4 (C-19). HRESIMS m/z 537.8049 [M + H]⁺ (calcd for C₂₉H₄₄O₉, 537.8021).

4. Spectra of compounds

Figure S2. **1a** of HR-MS

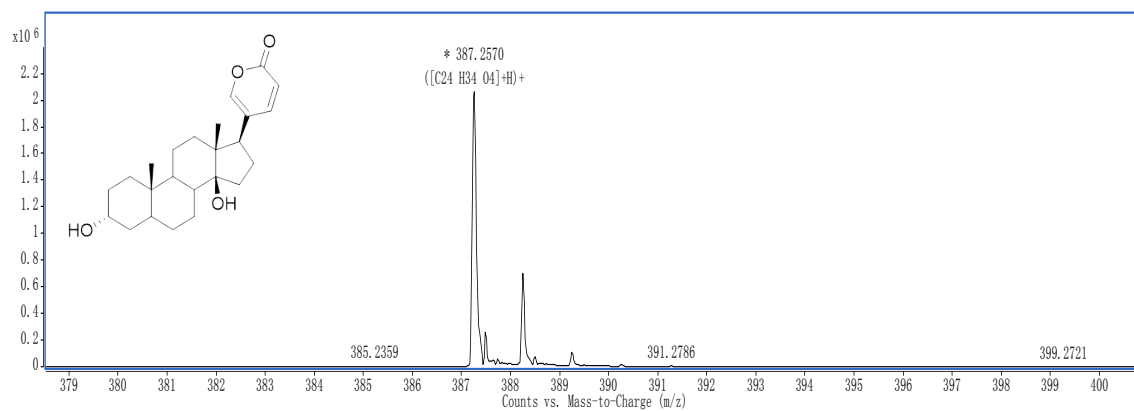


Figure S3. ¹H (CD₃OD, 400MHz)

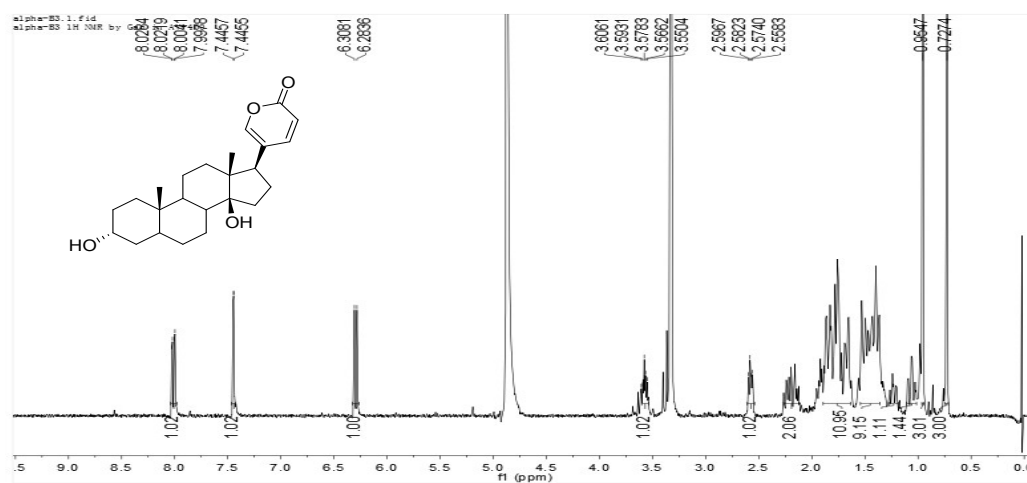


Figure S4. ¹³C (CD₃OD, 100MHz)

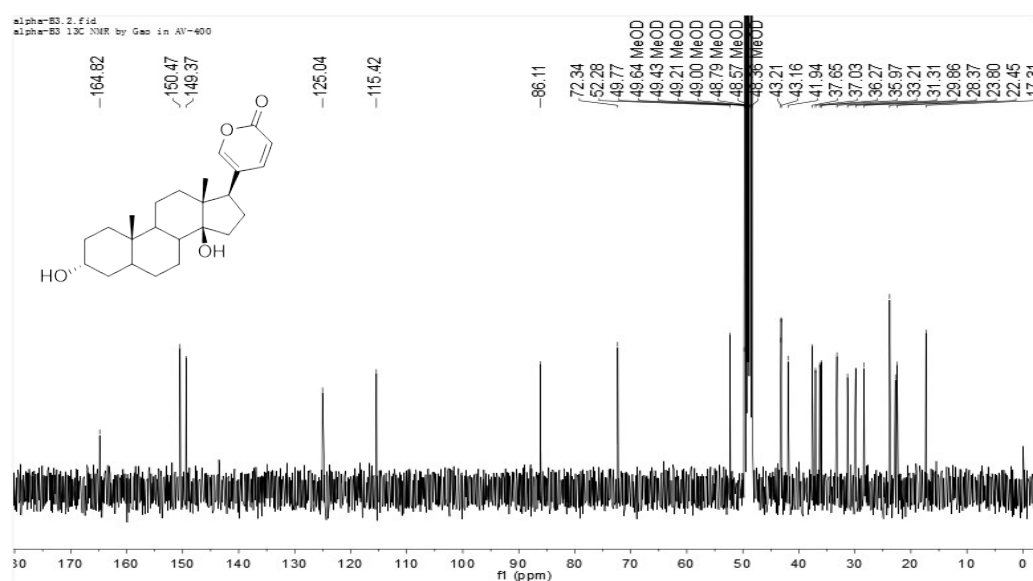


Figure S5. 2a of HR-MS

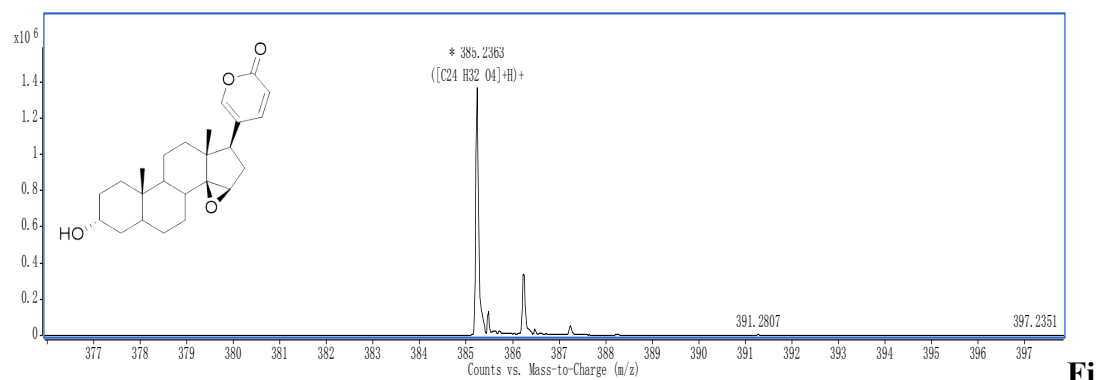


Figure S6. 1H (CD₃OD, 400MHz)

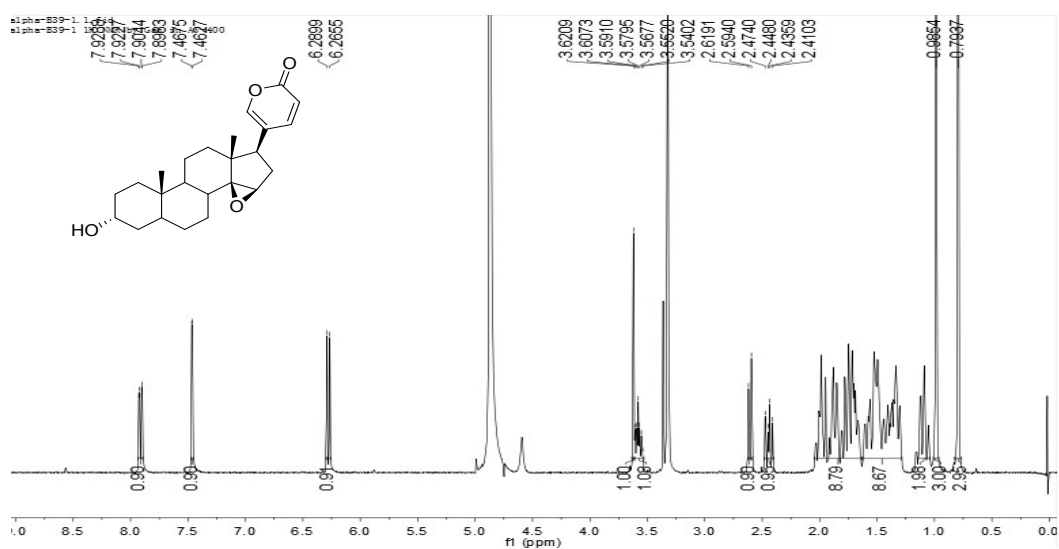


Figure S7. ^{13}C (CD₃OD, 100MHz)

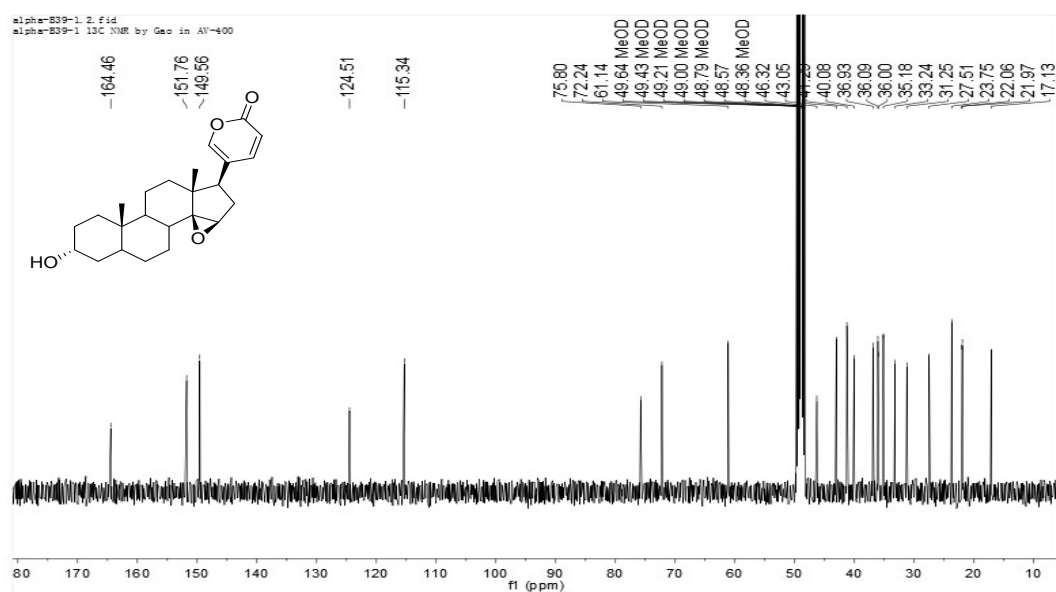
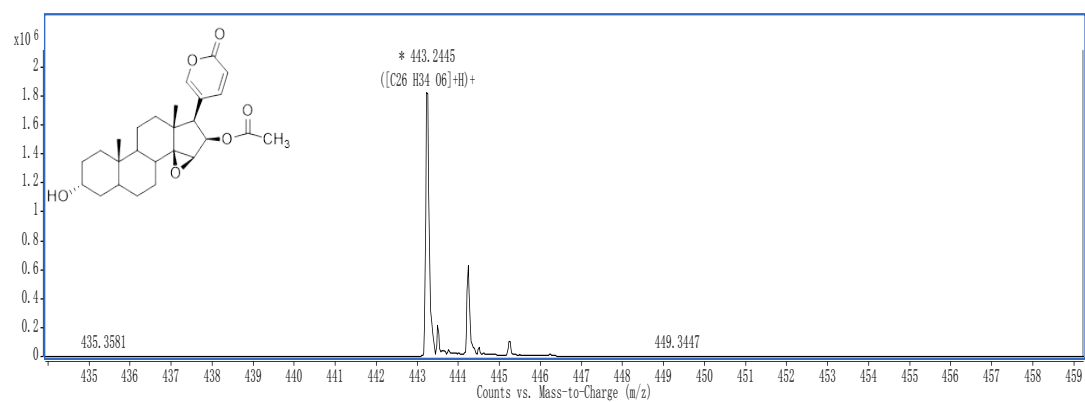


Figure S8. 3a of HR-MS



F

figure S9. ¹H (CD₃OD, 400MHz)

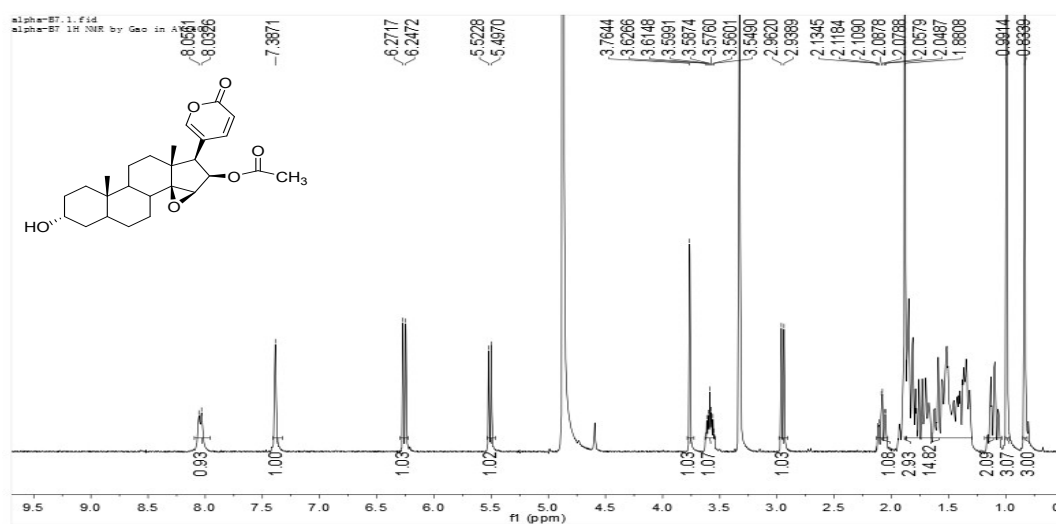


Figure S10. ¹³C (CD₃OD, 100MHz)

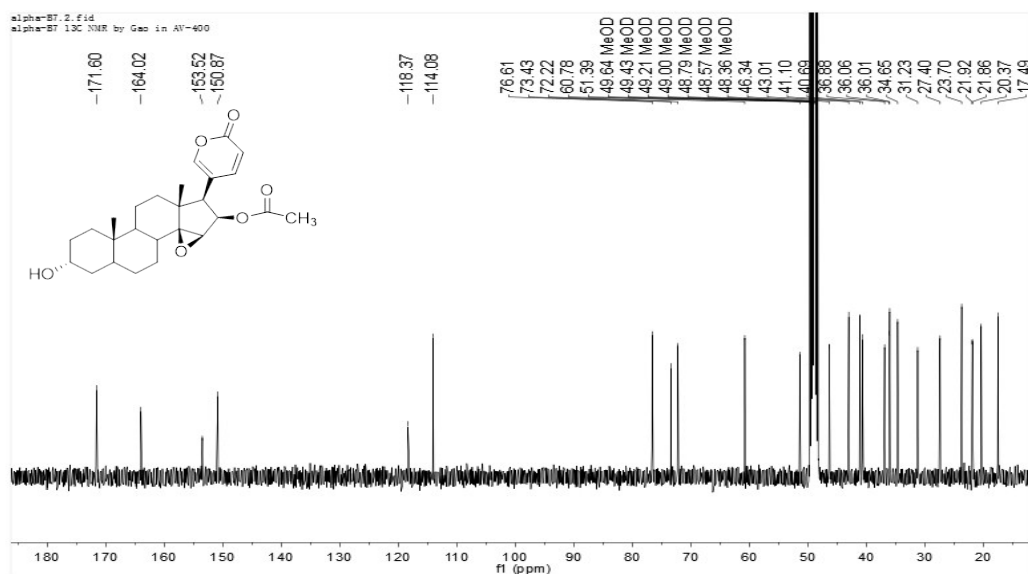


Figure S11. 1β-glu of HR-MS

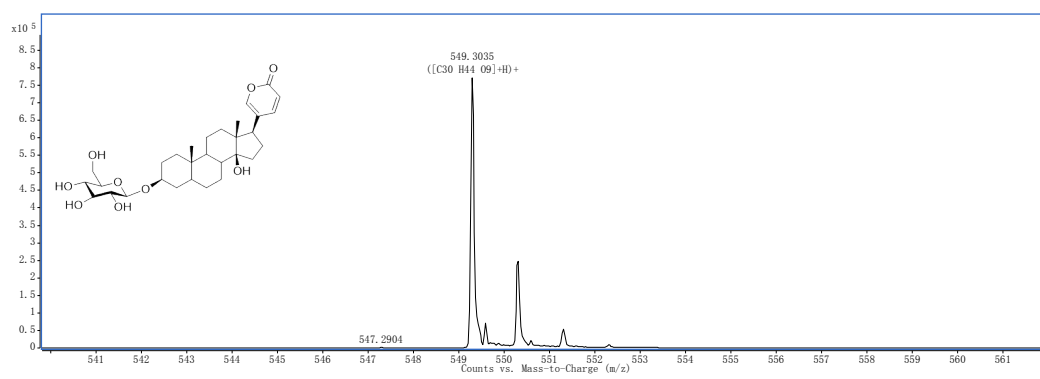


Figure S12. ¹H (CD₃OD, 400MHz)

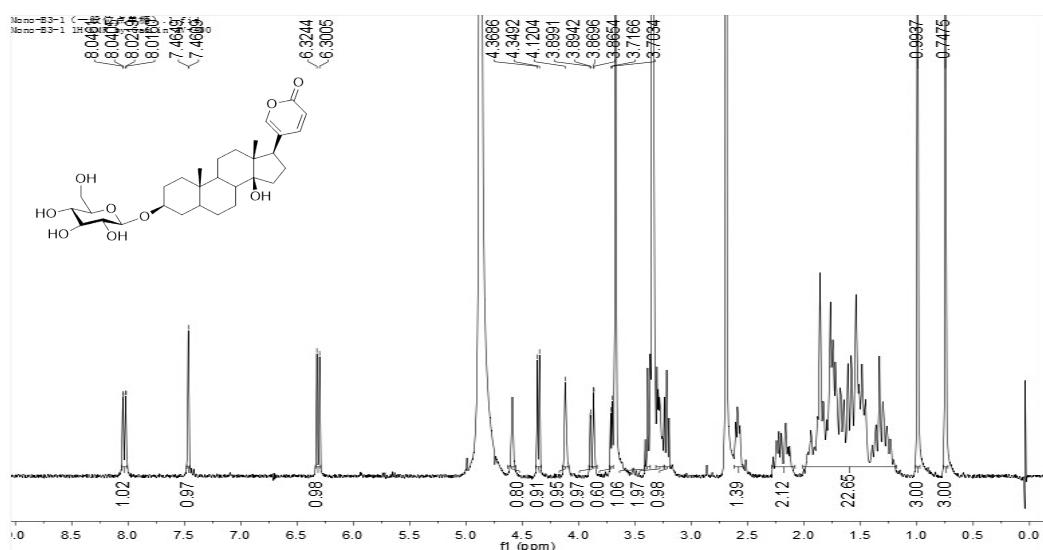


Figure S13. ¹³C (CD₃OD, 100MHz)

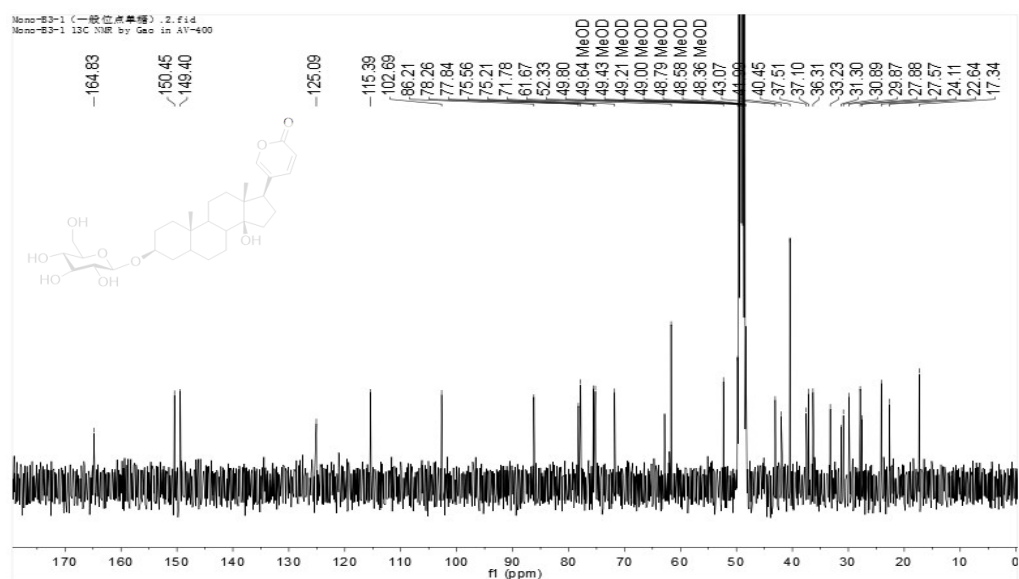


Figure S14. HSQC (CD₃OD, 400MHz)

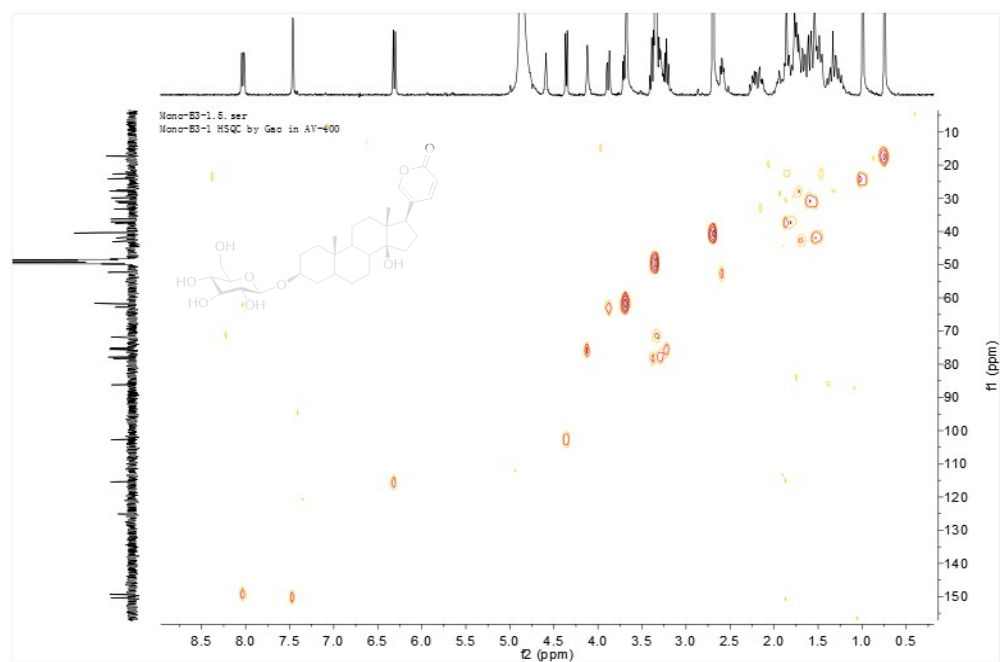


Figure S15. 2 β -glu of HR-MS

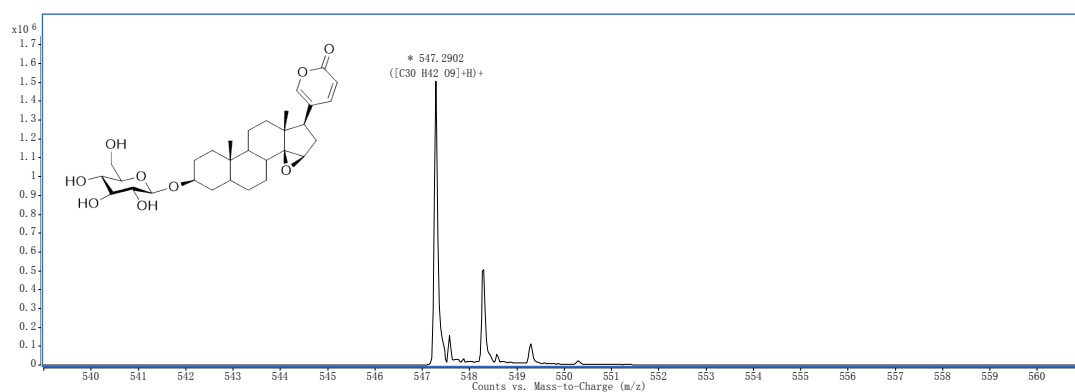


Figure S16. ^1H (CD_3OD , 400MHz)

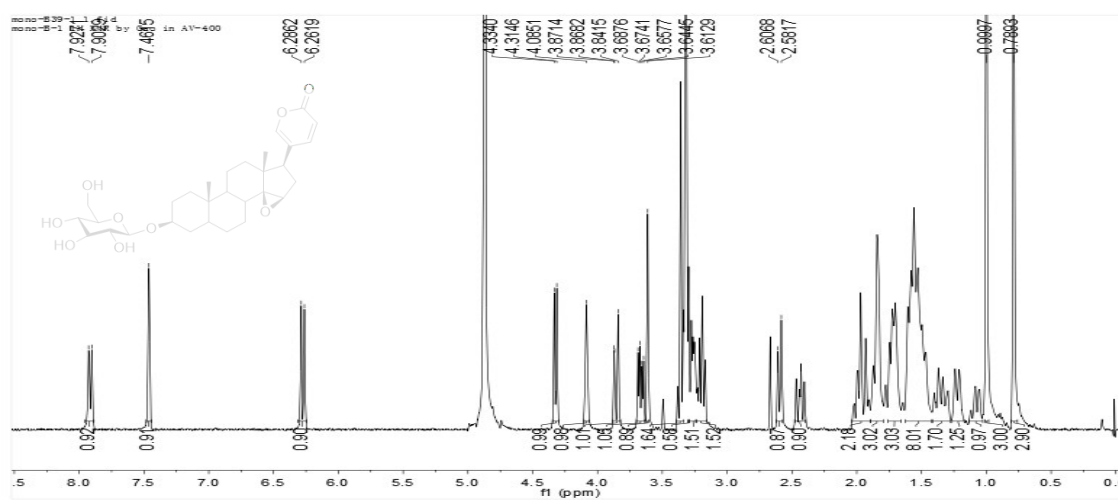


Figure S17. ^{13}C (CD_3OD , 100MHz)

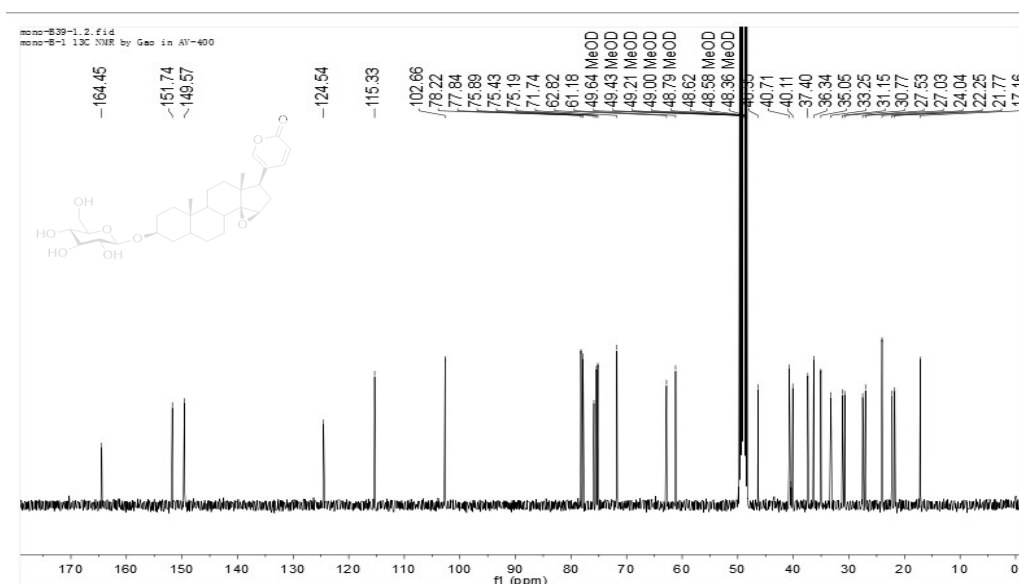


Figure S18. HSQC (CD_3OD , 400MHz)

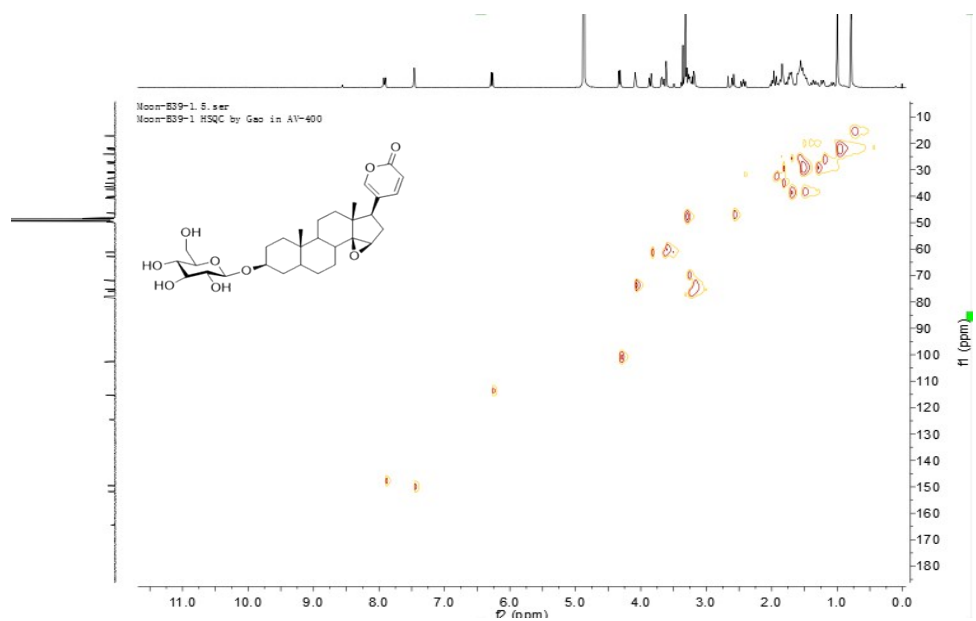


Figure S19. 2 β -diglu of HR-MS

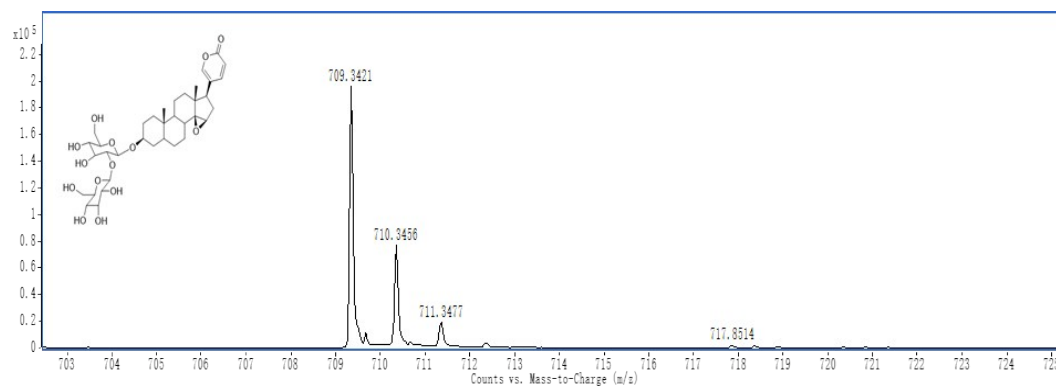


Figure S20. 3 β -glu of HR-MS

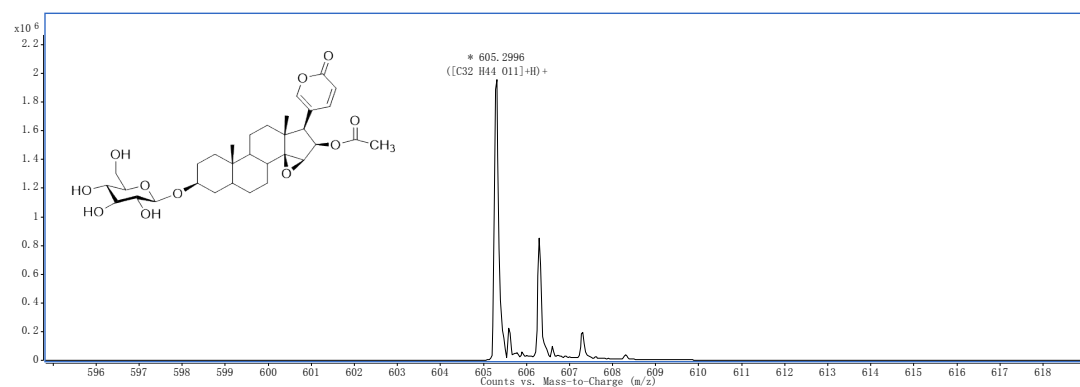


Figure S21. ^1H (CD_3OD , 400MHz)

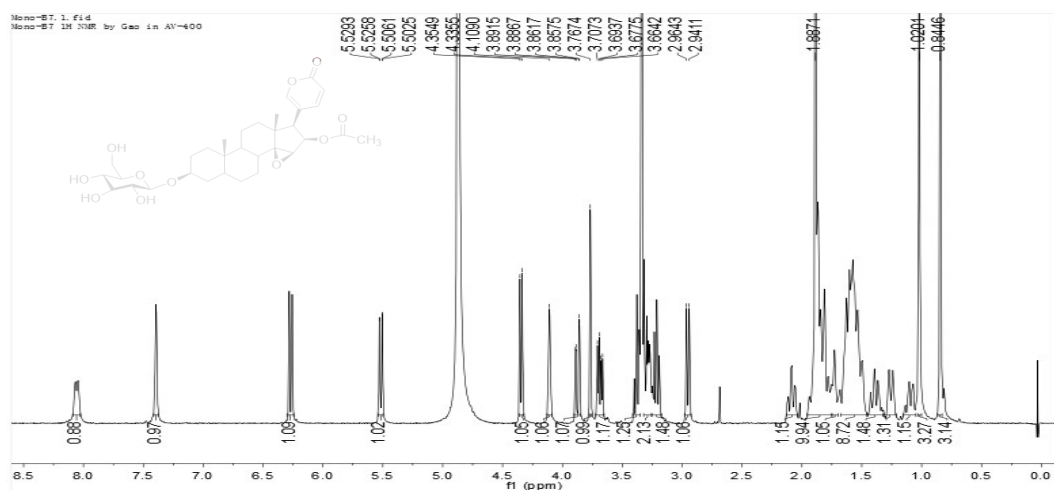


Figure S22. ^{13}C (CD_3OD , 100MHz)

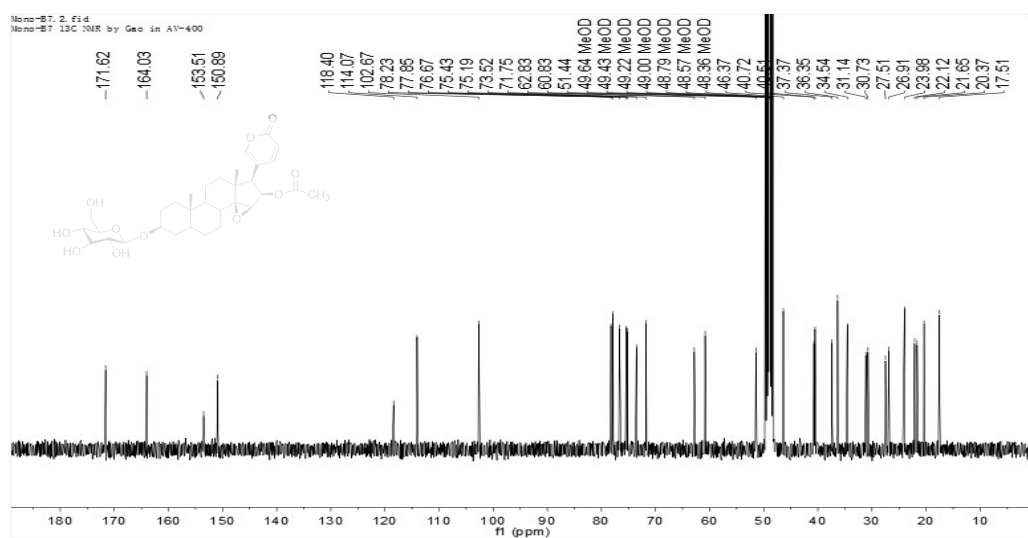


Figure S23. HSQC (CD_3OD , 400MHz)

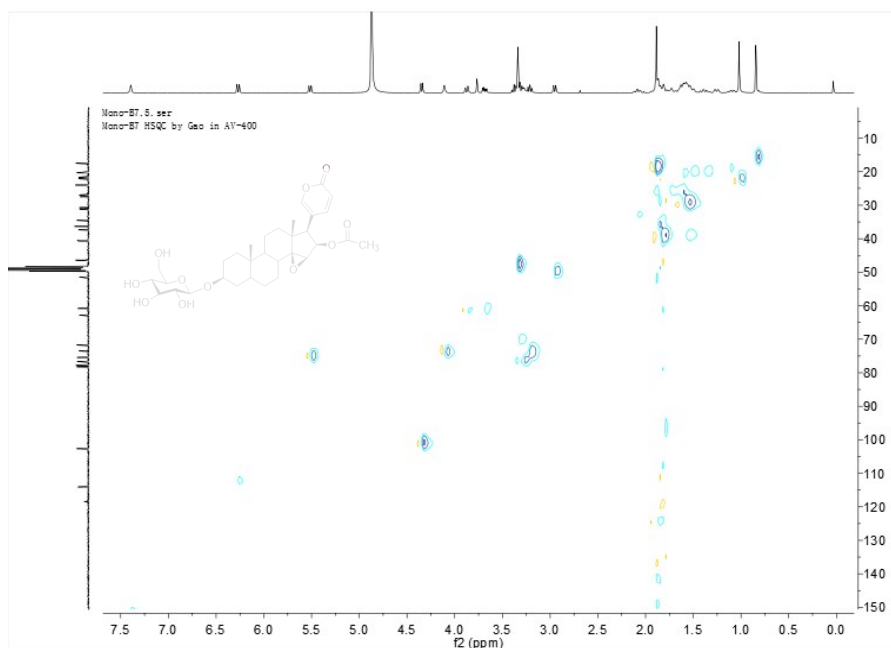


Figure S24. 3β-diglu of HR-MS

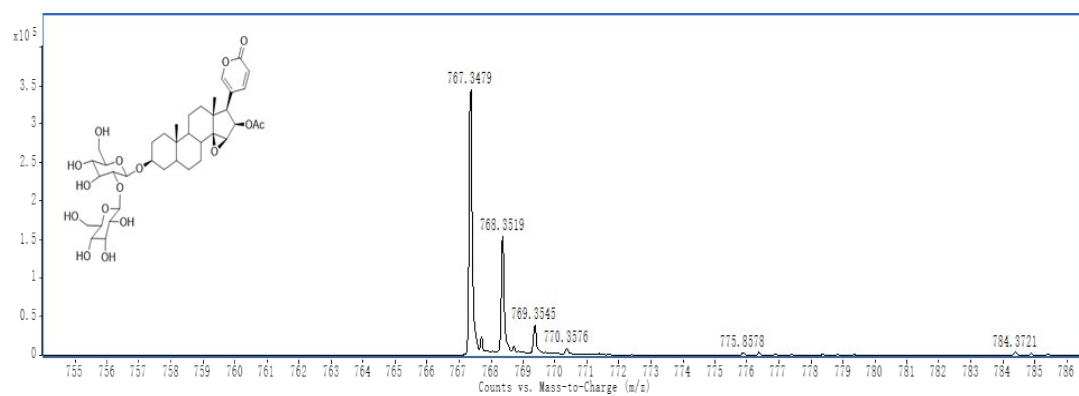


Figure S25. 4β-glu of HR-MS

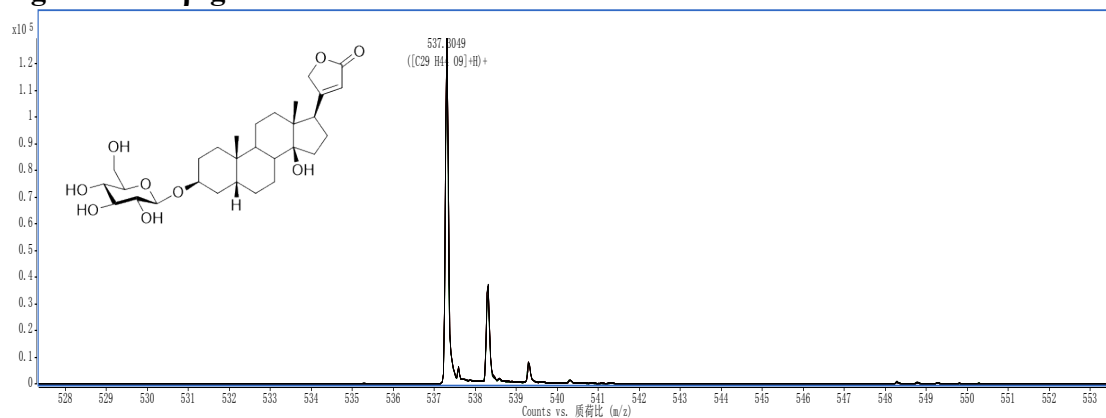


Figure S26. ^1H (CD_3OD , 400MHz)

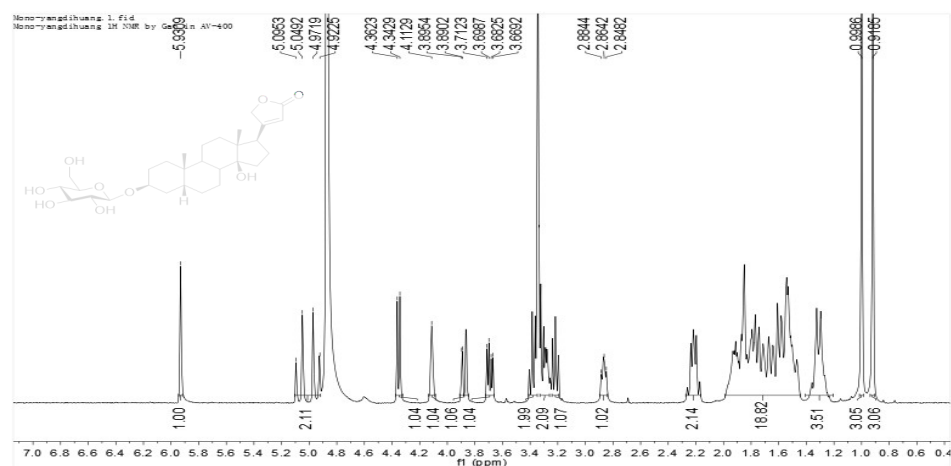


Figure S27. ^{13}C (CD_3OD , 100MHz)

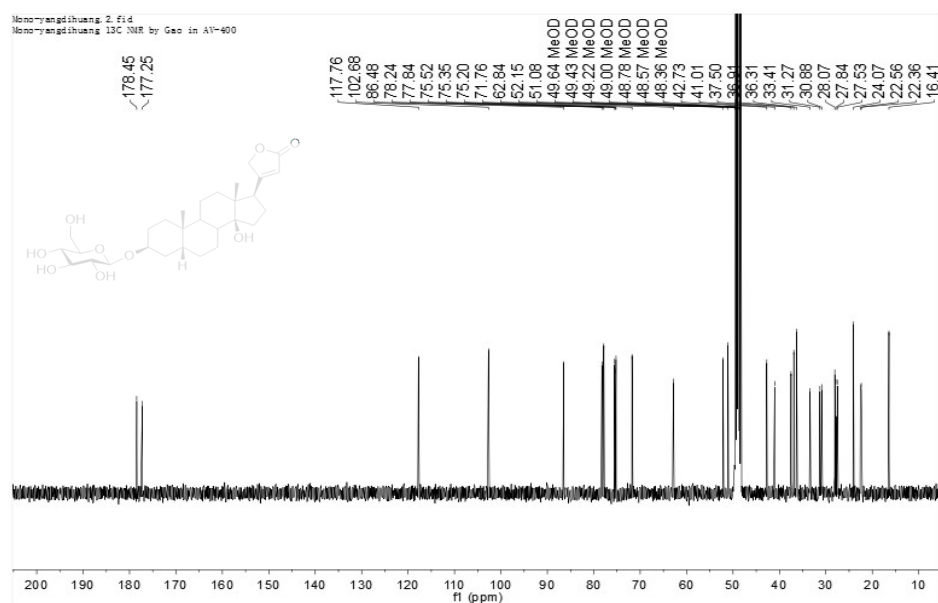


Figure S28. HSQC (CD_3OD , 400MHz)

