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Design, synthesis and biological evaluation of amino acids-oleanolic acid conjugates as influenza virus inhibitors

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Graphical abstract

Abstract

Viral entry inhibitors are of great importance in current efforts to develop a new generation of anti-influenza drugs. Inspired by the discovery of a series of pentacyclic triterpene derivatives as entry inhibitors targeting the HA protein of influenza virus, we designed and synthesized 32 oleanolic acid (OA) analogues in this study by

conjugating different amino acids to the 28-COOH of OA. The antiviral activity of these compounds was evaluated in vitro. Some of these compounds revealed impressive anti-influenza potencies against influenza A/WSN/33 (H1N1) virus. Among them, compound **15a** exhibited robust potency and broad antiviral spectrum with IC_{50} values at the low-micromolar level against four different influenza strains. Hemagglutination inhibition (HI) assay and docking experiment indicated that these OA analogues may act in the same way as their parent compound by interrupting the interaction between HA protein of influenza virus and the host cell sialic acid receptor via binding to HA, thus blocking viral entry.

Keywords: Influenza A virus; Entry inhibitor; Oleanolic acid; Amino acids; Hemagglutinin

1. Introduction

Influenza A virus (IAV) can cause substantial morbidity and mortality through routine seasonal spread and seasonal epidemics, and therefore it can cause a serious threat to human health.[1,2] IAV infects 3 million people leading to approximately 250,000 to 500,000 deaths every year worldwide.[3,4] Subtypes of influenza A virus are classified based on the antigenicity of two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA).[5] HA binds to sialic acid-containing receptors on target cells to initiate virus infection, whereas NA cleaves sialic acids from cellular receptors and extracellular inhibitors to facilitate progeny virus release.[6] The high mutation rate of the RNA genome of IAV promotes antigenic diversity and new subtypes, allowing the virus to avoid vaccines and become resistant to antiviral drugs.[7] Hence, the development of novel anti-influenza virus agents with high efficiency and

broad antiviral spectrum is highly importance. Recently, antiviral therapeutics are available targeting two of the three major influenza virus surface proteins: the NA and the M2 ion channel.[8-10] Unfortunately, because most of the currently circulating influenza viruses are resistant to M2 inhibitors, amantadine and rimantadine are no longer recommended as common clinical anti-influenza treatments.[11] Furthermore, mutant viruses resistant to NA inhibitors have also been detected in humans.[12,13]

IAV infection begins with the binding of HA protein to its cellular receptor, sialic acid (SA) linked glycoproteins, followed by fusion of the viral and endosomal membranes. Because it is the first step of viral infection, a favorable target for drug discovery is to inhibit IAV entry.[14,15] Thus, effective blocking of the interaction between HA and SA could lead to suppression of viral infection.[16,17]

In our previous research, we revealed that oleanolic acid (OA), a kind of pentacyclic triterpene natural product, and its analogues, show biological activity against A/WSN/33 (H1N1) virus invasion into MDCK cells.[18,19] Mechanistically, such pentacyclic triterpenes and their derivatives interrupt the interaction between viral HA protein and SA receptor by binding to HA, thus blocking the attachment of viruses to host cells.

Herein, we designed and synthesized a series of OA derivatives, in which different amino acids were coupled to the C-28 carboxyl group of OA via an amide bond. Through the introduction of different functional groups, we aimed to enhance the affinity of the compound with its target protein HA so as to increase its anti-IAV activity. All the synthesized compounds were tested, and we found that compound **15a** showed significantly higher anti-IAV activity than its precursor and the results are discussed below.

2. Results and Discussion

2.1. Chemistry

Amino acids are usually considered as useful modules to synthesize biologically active compounds. The introduction of such molecules with a variety of different functional groups might enhance the binding affinity of certain compounds to the target protein and thereby enhance their biological activity. Herein, we designed and synthesized a series of OA derivatives, in which different amino acids were coupled to the 28-COOH of OA via an amide bond. The general synthesis for compounds **2-11** were conducted according to the following procedures (Scheme 1). Compound **1** was obtained with over 85% yield by stirring OA with O-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU) and N,N-Diisopropylethylamine (DIEA) in THF for 4h.[20] Treatment of intermediate **1** with the corresponding amino acid methyl esters in the presence of Na_2CO_3 in DMF afforded target compounds **2-11**[21]. After that, the methyl esters of compounds **2-11** were hydrolyzed by NaOH in THF/CH_3OH (1:1), which yielded compounds **2a-11a**, respectively.

However, it is difficult to obtain compounds **12–17** by the same strategy, owing to the steric hindrance and decreased nucleophilicity. Therefore, 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide (EDC) was used as the coupling reagent to conjugate amino acid methyl esters and 28-COOH of OA. As shown in Scheme 2, compounds **12-17** were synthesized by condensation of OA 28-COOH and certain amino acid methyl esters in the presence of EDC and Triethylamine (TEA) in dry THF. Using the same strategy which yielded **2a-11a** from **2-11**, compounds **12a-17a** can be obtained in high yields. All the synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR and ESI-HRMS.

2.2. Anti-influenza virus activity of OA-amino acid conjugates

The CellTiter-Glo® screening, an assay which monitors cell viability, was utilized to evaluate the antiviral activity of the synthesized conjugates and to exclude compounds with significant toxicity toward MDCK cells[22]. As shown in Figure 1A, all the OA-amino acid conjugates exhibited increased cytotoxicity compared to OA. Interestingly, in addition to compounds **7** (the OA-serine methyl ester conjugate), **9** (the OA-histidine methyl ester conjugate), **13a** (the OA-aspartic acid conjugate) and **15a** (the OA-arginine conjugate), compounds with the carboxyl group of the amino acids protected by the methyl ester, all exhibited significant less cytotoxic than those with free carboxyl group (**2-6**, **8**, **10-17**) at 100 μM. This result indicates that the substituted groups of amino acid modules have a critical effect on the toxicity of the conjugates.

In the antiviral experiment, the activities of all the compounds against the influenza A/WSN/33 (H1N1) virus were evaluated by the CPE reduction assay, and the inhibition rate was shown in Figure 1B. Compared with DMSO which is used as a negative control, influenza A/WSN/33(H1N1) virus causes a severe CPE in MDCK-infected cells. As shown in Figure 1B, most of the compounds displayed higher inhibition rate than OA. However, when compared with Figure 1A, this observation might reflect cellular toxicity rather than potency enhancement. According to the results, compound **15a**, whose viability and inhibition rate were both over 75%, was selected for anti-H1N1 dose-response assay. We also inquired whether **15a** could exert broad antiviral spectrum, and it was evaluated against three strains of influenza A and B viruses (Table 1). Among them, one belongs to influenza A type, and the other two viruses belong to the Yamagata and Victoria lineages of the influenza B virus. All of them are oseltamivir (OSV) resistant strains.

The IC_{50} values of compound **15a**, along with the positive control (oseltamivir phosphate, OSV-p), were determined and listed in Table 1. We found compound **15a** displayed anti-influenza virus activity with IC_{50} values in the range of 6.64−61.58 μM. Furthermore, compound **15a** was effective for all tested strains including the OSV-resistant A/Texas/50/2012 strain and even the influenza B virus (BX-35 and BX-51B). Such a broad anti-influenza spectrum is significantly different from that of oseltamivir, which is much less effective against A/Texas/50/2012 (H3N2), BX-35 and BX-51B viruses (IC_{50} > 100 μM).

2.3. Hemagglutination Inhibition Assay

As we know, the transmembrane viral envelope protein HA plays an important role at the early stage of influenza virus infection. Therefore, hemagglutination inhibition (HAI) assay was applied to investigate whether compound **15a** targets HA protein. In this assay, 2-fold serial dilutions of compound **15a** were made from 100 μM to 1.56 μM. In parallel, inhibition of hemagglutination by anti-HA antibody was used as the positive control. As shown in Figure 2, **15a** displayed a similar capability as anti-HA antibody in effectively inhibiting influenza virus-induced aggregation of chicken erythrocytes. This result suggests that **15a** and anti-HA antibody may have the same target, HA, and thus blocks the interactions between viruses and target cells.

2.4. Docking calculation

Considering that HA may be a potential target for OA derivatives, we investigated the binding pocket and binding free energy of compound **15a** and HA through blind docking calculations (Figure 3). Compound **15a**

was docked into the influenza HA protein, which was obtained from the RCSB Protein Data Bank, using AutoDock 4.2 program. The docked conformations of HA-**15a** were determined based on the minimum free energy analyses. The computer-aided docking data suggests that compound **15a** exhibits a similar binding mode as compound **Y3**, by occupying the binding pocket for sialic acid receptor with an estimated binding energy of -9.57 kcal/mol and an inhibition constant (Ki) of 24.11 nM. This means compound **15a** can bind tightly to HA protein. The docking result indicated that the 3-OH of the OA moiety formed two hydrogen bonds with S193 residues within the HA1 conserved domain associated with sialic acid binding. For the arginine moiety, the carboxyl and the sub-amino groups formed three hydrogen bonds with residue A137 and Q226, which are also critical residues involved in sialic acid binding.

3. Conclusion

In summary, to find compounds with higher anti-IAV activity and broader antiviral spectrum, we have designed and synthesized 32 OA analogues by conjugating different amino acids to the 28-COOH of OA. Compound **15a** exhibited better inhibitory potency against influenza A/WSN/33 (H1N1) virus, with an IC_{50} value of 6.64 μM. Moreover, it also showed a broad anti-influenza spectrum, with IC_{50} values of 14.05 μM, 61.58 μM and 45.8 μM against A/Texas/50/2012, BX-35 and BX-51B, better than that of OSV-p (>100 μM, >100 μM and >100 μM, respectively). Hemagglutination inhibition assay indicated that these compounds may target the HA protein of influenza virus, and docking studies suggested that these compounds may occupy HA's conserved binding pocket for sialic acid receptor, disrupting the interaction between HA and the SA receptor and thus blocking the attachment of viruses to

host cells.

4. Experimental protocols

4.1.Chemistry

General Procedure A for the Synthesis of OA O-methylation amino acids conjugates (Compounds **2-11**)

To Compound **1** (1 equiv.) and Na_2CO_3 (1.5 equiv.) stirring in 8 mL DMF was added O-methylation amino acid (1.5 equiv.). The mixture was stirred at room temperature for 20 min. After completion (TLC), the solvent was removed under reduced pressure. The mixture was resolved in AcOEt and washed with water and brine twice. The organic layer was dried over Na_2SO_4, then filtered and concentrated. The crude residue was purified by column chromatography.

General Procedure B for the Synthesis of OA O-methylation amino acids conjugates (Compounds **12-17**)

OA (1 equiv.) was added to a stirred solution of O-methylation amino acid (1.5 equiv.) and TEA (1.5 equiv.) in THF. After 10 min, EDC (1.5 equiv.) was added to the stirred solution, and the mixture was then stirred at room temperature overnight. The mixture was resolved in AcOEt and washed with water and brine twice. The organic layer was dried over Na_2SO_4, then filtered and concentrated. The crude residue was purified by column chromatography.

General Procedure C for the Synthesis of OA amino acids conjugates (Compounds **2a-17a**)

To the O-methylationamino acids conjugates stirring in THF/CH_3OH (1:1) was added sodium hydroxide (cat.). The mixture was stirred at rt. After completion (TLC), the reaction mixture was neutralized with HCl (1 M). Water was added, and the resulting suspension was filtered.

Compound 2 Prepared from O-methylation glycine (133.5 mg, 1.5 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **2** as a white solid (418.9 mg, 78%). m.p.: 133.3–135.1 °C; ^{1}H NMR (600 MHz, $(CD_3)_2SO$) δ: 0.64-1.08 (m, 7×CH_3), 1.08-2.00 (m, other aliphatic ring protons) 2.76 (d, J=9.6 Hz, 1H) 2.97-3.00 (m, 1H) 3.59 (s, 3H) 3.64 (dd, J=5.52, 17.04 Hz, 1H) 3.80 (dd, J=6, 16.98 Hz, 1H), 4.29 (d, J=5.16 Hz, 1H) 5.17 (t, J=3.36 Hz, 1H), 7.76 (t, J=5.7 Hz, 1H). ^{13}C NMR (150 MHz, $(CD_3)_2SO$) δ: 15.1, 16.1, 16.6, 18.0, 22.4, 22.9, 23.5, 25.6, 26.8, 27.0, 28.2, 30.4, 32.4, 32.5, 32.9, 33.6, 36.6, 38.1, 38.4, 38.9, 40.4, 40.9, 41.2, 45.2, 46.1, 47.1, 51.5, 54.8, 76.8, 121.4, 144.0, 170.5, 176.9. ESI-HRMS (m/z): $[M]^+$ calcd for $C_{33}H_{53}NO_4$, 527.3975; found, 527.3965.

Compound 2a Prepared from **2** (200 mg, 0.37 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **2a** as a white solid (181 mg, 93%). m.p.: 173.2–175.2°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.64-1.08 (m, 7×CH_3), 1.08-2.00 (m, other aliphatic ring protons) 2.76 (dd, J=3.84, 13.38 Hz, 1H) 2.97-3.00 (m, 1H) 3.56 (dd, J=3.54, 17.28 Hz, 1H) 3.73 (dd, J=6, 17.28 Hz, 1H) 4.28 (d, J=4.44 Hz, 1H) 5.19 (t, J=3.36 Hz, 1H) 7.57 (t, J=5.58 Hz, 1H) 12.37 (s, 1H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.1, 16.1, 16.6, 18.0, 22.5, 23.0, 23.5, 25.7, 26.9, 27.0, 28.2, 30.4, 32.4, 32.5, 32.9, 33.6, 36.6, 38.1, 38.4, 38.9, 40.5, 40.9, 41.2, 45.1, 46.1, 47.2, 54.8, 76.8, 121.5, 144.0, 171.5, 176.7. ESI-HRMS (m/z): $[M+H]^+$ calcd for $C_{32}H_{52}NO_4$, 514.3896; found, 514.3897.

Compound 3 Prepared from O-methylation alanine (154.5 mg, 1.5 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **3** as a white solid (443.6 mg, 82%). m.p.: 191.4–192.1°C; ^{1}H NMR (400 MHz,

$CDCl_3$) δ: 0.69-1.15 (m, 7×CH_3), 1.15–2.00 (m, other aliphatic ring protons) 1.37 (d, J = 7.0 Hz 3H), 2.63 (d, J = 10.08 Hz 1H) 3.18-3.22 (m, 1H), 3.72 (s, 3H), 4.41-4.48 (m, 1H), 5.42 (brs, 1H), 6.57 (d, J =5.56 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.5, 15.7, 16.7, 18.2, 18.8, 23.7, 23.8, 23.8, 25.9, 27.3, 27.5, 28.2, 30.9, 32.7, 33.0, 33.4, 34.3, 37.1, 38.7, 38.9, 39.6, 42.1, 42.2, 46.4, 46.6, 47.7, 48.5, 52.5, 55.3, 79.1, 123.4, 144.0, 173.8, 177.6. ESI-HRMS (m/z): $[M]^+$ calcd for $C_{34}H_{55}NO_4$, 541.4131; found, 541.4136.

Compound 3a Prepared from **3** (200 mg, 0.36 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **3a** as a white solid (161.8 mg, 83%). m.p.: 170.4–171.8°C; ^{1}H NMR (400 MHz, CD_3OD) δ: 0.75-1.18 (m, 7×CH_3), 1.18-2.15 (m, other aliphatic ring protons), 2.76 (d, J=10.48 Hz, 1H), 3.15 (dd, J=5.6, 12 Hz, 1H), 4.31 (q, J=7.08 Hz, 1H), 5.38 (s, 1H), 7.38 (d, J=6.12 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ: 15.9, 16.3, 17.5, 18.2, 19.5, 24.0, 24.0, 24.6, 26.4, 27.9, 28.5, 28.7, 31.6, 33.5, 33.9, 35.1, 38.1, 39.8, 39.9, 40.7, 43.0, 47.4, 47.5, 48.9, 49.3, 49.5, 56.7, 79.7, 124.6, 144.7, 175.9, 179.8. ESI-HRMS (m/z): $[M+H]^+$ calcd for $C_{33}H_{54}NO_4$, 528.4053; found, 528.4096.

Compound 4 Prepared from O-methylation valine (196.5 mg, 1.5 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **4** as a white solid (516.1 mg, 93%). m.p.: 203.8–205°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.61-1.08 (m, 7×CH_3), 1.08-2.00 (m, other aliphatic ring protons), 2.02-2.09 (m, 1H) , 2.77 (d, J=13.64 Hz, 1H) 2.96-3.01 (m, 1H), 3.59 (s, 3H), 4.02 (t, J=7.88 Hz, 1H), 4.27 (d, J=4.36 Hz, 1H), 5.20 (s, 1H), 7.25 (d, J=7.88 Hz, 1H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.6, 16.5, 17.2, 18.5, 19.5, 19.6, 22.6, 23.4, 24.0, 26.0, 27.4, 27.4, 28.7, 30.1, 30.8, 32.6, 33.0, 33.4, 34.0, 37.0, 38.6, 38.8, 39.3, 41.0, 41.7, 45.9, 46.3,

47.6, 51.9, 55.3, 58.6, 77.3, 122.0, 144.4, 172.7, 177.1. ESI-HRMS (m/z): $[M]^+$ calcd for $C_{36}H_{59}NO_4$, 569.4444; found, 569.4452.

Compound 4a Prepared from **4** (200 mg, 0.34 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **4a** as a white solid (167.9 mg, 86%). m.p.: 211.4–214.2°C; ^{1}H NMR (400 MHz, CD_3OD) δ: 0.75-1.18 (m, 7×CH_3), 1.18-2.11 (m, other aliphatic ring protons), 2.74 (d, J=11.08 Hz, 1H), 3.14 (dd, J=4.6, 11.76 Hz, 1H), 3.71 (s, 1H), 4.18 (d, J=4.84 Hz, 1H), 5.42 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ: 14.5, 14.9, 16.3, 17.6, 18.0, 18.1, 22.5, 22.8, 23.2, 24.9, 26.5, 27.2, 27.3, 30.2, 31.2, 32.1, 32.6, 33.0, 33.7, 36.7, 38.4, 38.5, 39.3, 41.6, 41.8, 46.3, 46.5, 47.7, 55.3, 58.4, 78.3, 123.6, 142.9, 178.4. ESI-HRMS (m/z): $[M+H]^+$ calcd for $C_{35}H_{58}NO_4$, 556.4366; found, 556.4344.

Compound 5 Prepared from O-methylation leucine (218 mg, 1.5 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **5** as a white solid (455.2 mg, 80%). m.p.: 215.5–216.6°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.71-1.15 (m, 7×CH_3), 1.15-2.00 (m, other aliphatic ring protons), 2.61 (d, J=10.04 Hz, 1H), 3.18-3.22 (m, 1H), 3.70 (s, 3H), 4.52 (q, J=6.88 Hz, 1H), 5.41 (brs, 1H) 6.34 (d, J=6.84 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.5, 15.7, 16.8, 18.4, 22.6, 22.9, 23.7, 23.7, 24.0, 25.1, 25.8, 27.3, 27.5, 28.2, 30.8, 32.8, 33.0, 33.1, 34.3, 37.1, 38.7, 38.9, 39.5, 42.1, 42.2, 42.2, 46.5, 46.7, 47.7, 51.2, 52.2, 55.3, 79.1, 123.4, 144.0, 173.7, 177.8. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{37}H_{61}NO_4Na$, 606.4498; found, 606.4497.

Compound 5a Prepared from **5** (200 mg, 0.33 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **5a** as a white solid (181.6 mg, 93%). m.p.: >250°C; ^{1}H NMR (400 MHz, CD_3OD) δ: 0.76-1.17 (m,

7×CH_3), 1.17-2.20 (m, other aliphatic ring protons), 2.79 (d, *J*=10.04 Hz, 1H), 3.14 (dd, *J*=4.4, 10.88 Hz, 1H), 3.49-3.65 (m, 1H), 4.38 (q, *J*=3.36 Hz, 1H), 5.35 (s, 1H) 5.50 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD) *δ*: 15.9, 16.3, 17.9, 19.5, 19.6, 22.4, 23.5, 24.0, 24.01, 24.6, 26.1, 26.3, 27.9, 28.5, 28.7, 30.3, 31.6, 33.5, 34.0, 34.1, 35.2, 38.1, 39.8, 40.6, 41.9, 42.8, 43.0, 47.6, 47.6, 49.1, 52.5, 56.7, 79.7, 124.4, 144.8, 180.1. ESI-HRMS (m/z): $[M+H]^+$ calcd for $C_{36}H_{60}NO_4$, 570.4522; found, 570.4541.

Compound 6 Prepared from O-methylation isoleucine (218 mg, 1.5 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **6** as a white solid (438.1 mg, 77%). m.p.: 192.5–195.1°C; ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 0.68 (s, 1H) 0.77 (s, 1H), 0.86-1.15 (m, 7×CH_3), 1.15-2.00 (m, other aliphatic ring protons) 2.64 (d, *J*=11.06 Hz, 1H) 3.19-3.23 (m, 1H) 4.50 (dd, *J*=1.68, 6.76 Hz, 1H) 5.43 (t, *J*=3.52 Hz, 1H) 6.46 (d, *J*=7.68 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 15.2, 15.4, 15.6, 16.7, 18.3, 23.5, 23.6, 23.7, 25.6, 25.7, 27.2, 27.4, 28.1, 30.7, 32.7, 33.0, 33.1, 34.1, 37.0, 38.2, 38.5, 38.8, 39.4, 42.0, 42.2, 46.5, 46.6, 47.6, 51.9, 55.2, 56.3, 77.2, 79.0, 123.3, 143.7, 172.3, 177.5. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{37}H_{61}NO_4Na$, 606.4498; found, 606.4494.

Compound 6a Prepared from **6** (200 mg, 0.33 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **6a** as a white solid (189.4 mg, 97%). m.p.: 199.3–200.9°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) *δ*: 0.64-1.08 (m, 7×CH_3), 1.08-2.00 (m, other aliphatic ring protons), 2.71 (d, *J*=11.04 Hz, 1H), 2.96-3.00 (m, 1H), 3.94 (t, *J*=5.52 Hz, 1H), 5.23 (s, 1H), 7.17 (d, *J*=6.64H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) *δ*: 15.3, 15.5, 16.1, 16.7, 18.1, 22.8, 23.1, 23.4, 25.4, 25.6, 27.0 (2C), 28.3, 30.5, 32.7, 32.7, 32.9, 33.8 (2C), 36.6 (2C), 38.2, 38.5, 41.0, 41.5, 45.5, 46.2, 47.3, 54.9, 57.2, 76.9,

122.1, 143.4, 173.2, 175.8. ESI-HRMS (m/z): $[M+H]^+$ calcd for $C_{36}H_{60}NO_4$, 570.4522; found, 570.4539.

Compound 7 Prepared from O-methylation serine (179 mg, 1.5 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **7** as a white solid (551.4 mg, 99%). m.p.: 139.7–140.8°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.69-1.16 (m, 7×CH_3), 1.16-2.00 (m, other aliphatic ring protons), 2.67 (d, J=11.24 Hz, 1H), 3.21 (s, J=10.64 Hz, 1H), 3.78 (s, 3H), 3.84 (d, J=11 Hz, 1H), 3.94 (brs, 1H), 4.12 (q, J=7.08 Hz, 1H), 4.54 (s, 1H), 5.46 (s, 1H), 6.87 (d, J=4.72 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.5, 15.7, 16.6, 18.4, 23.7, 23.8, 23.8, 25.9, 27.3, 27.6, 28.2, 30.8, 32.7, 33.1, 33.2, 34.2, 37.1, 38.7, 38.9, 39.6, 42.1, 42.2, 46.6, 46.7, 47.7, 52.7, 55.3, 55.9, 64.5, 79.1, 123.7, 143.6, 171.0, 179.4. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{34}H_{55}NO_5Na$, 580.3978; found, 580.3972.

Compound 7a Prepared from **7** (200 mg, 0.35 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **7a** as a white solid (179.5 mg, 92%). m.p.: 174.4–176.2°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.65-1.09 (m, 7×CH_3), 1.09-2.00 (m, other aliphatic ring protons), 2.73 (d, J=13.12 Hz, 1H), 2.99 (s, 1H), 3.55-3.72 (m, 2H), 4.16-4.24 (m, 1H) 7.19 (q, J=6.92 Hz, 1H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.2, 16.1, 16.5, 18.1, 22.6, 23.0, 23.6, 25.7, 27.0, 27.1, 28.3, 30.5, 32.5, 32.5, 33.0, 33.7, 36.6, 38.2, 38.5, 40.9, 41.4, 41.4, 45.4, 46.0, 47.2, 54.4, 54.9, 61.5, 76.9, 122.0, 143.8, 172.1, 176.3. ESI-HRMS (m/z): $[M]^+$ calcd for $C_{33}H_{53}NO_5$, 543.3924; found, 543.3917.

Compound 8 Prepared from O-methylation threonine (199 mg, 1.5 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **8** as a white solid (518 mg, 93%). m.p.: 139.8–140.5°C; ^{1}H NMR (400 MHz,

$CDCl_3$) δ: 0.70-1.16 (m, 7×CH_3), 1.16-2.00 (m, other aliphatic ring protons), 1.19 (d, J=6.16 Hz, 3H), 2.69 (d, J=9.04 Hz, 2H), 3.20 (d, J=7.32 Hz, 1H), 3.73 (s, 3H), 4.20 (brs, 1H), 4.51 (d, J=6.92 Hz, 1H), 5.42 (s, 1H), 6.61 (d, J=7.2 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.5, 15.7, 17.0, 18.4, 20.0, 23.7, 23.8, 24.0, 25.8, 27.3, 27.6, 28.3, 30.8, 32.9, 33.1, 33.4, 34.3, 37.2, 38.7, 38.9, 39.6, 42.2 (2C), 46.7, 46.9, 47.7, 52.5, 55.3, 57.7, 68.6, 79.1, 123.4, 143.7, 171.8, 179.0. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{34}H_{57}NO_5Na$, 594.4134; found, 594.4135.

Compound 8a Prepared from **8** (200 mg, 0.34 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **8a** as a white solid (190.5 mg, 93%). m.p.: 178.6–179.7°C; ^{1}H NMR (400 MHz, CD_3OD) δ: 0.77-1.19 (m, 7×CH_3), 1.19-2.20 (m, other aliphatic ring protons), 2.81 (d, J=10.6 Hz, 1H), 3.13-3.17 (m, 1H), 4.27-4.29 (m, 1H), 4.37 (s, 1H), 5.40 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ: 16.0, 16.3, 17.9, 19.5, 20.7, 24.0, 24.3, 24.6, 26.4, 27.9, 28.6, 28.8, 31.6, 33.5, 34.1, 34.5, 35.2, 38.1, 39.8, 39.9, 40.7, 43.0, 43.1, 47.7, 48.0, 49.1, 56.7, 59.0, 68.7, 79.7, 124.7, 144.5, 180.6. ESI-HRMS (m/z): $[M]^+$ calcd for $C_{34}H_{55}NO_5$, 557.4080; found, 557.4072.

Compound 9 Prepared from O-methylation histidine (253 mg, 1.5 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **9** as a white solid (594 mg, 98%). m.p.: 238.6–239.4°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.63-1.15 (m, 7×CH_3), 1.15-2.00 (m, other aliphatic ring protons), 2.59 (d, J=12.4 Hz, 1H), 3.00-3.06 (m, 1H), 3.13-3.20 (m, 1H), 3.35 (s, 1H), 3.72 (s, 3H), 4.56-4.61 (m, 4H), 5.35 (brs, 1H), 6.80 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.0, 15.2, 16.2, 18.0, 23.0, 23.1, 23.2, 25.4, 26.4, 26.9, 27.6, 28.8, 30.3, 32.3, 32.5, 32.5, 33.8, 36.6, 38.3, 38.4, 39.1, 41.4, 41.6, 46.1 (2C), 47.3, 52.0, 52.5, 55.0, 78.3, 123.1, 134.8,

143.2, 171.9, 178.4. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{37}H_{57}N_3O4Na$, 630.4247; found, 630.4240.

Compound 9a Prepared from **9** (200 mg, 0.32 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **9a** as a white solid (163.6 mg, 80%). m.p.: 247.2–248.2°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) *δ:* 0.35-1.02 (m, 7×CH_3), 1.02-2.00 (m, other aliphatic ring protons), 2.71 (d, *J*=9.96 Hz, 1H), 2.96-3.09 (m, 3H), 4.33-4.38 (m, 1H), 5.11 (s, 1H), 7.16 (s, 1H), 7.61 (d, *J*=6.76 Hz, 1H), 8.43 (s, 1H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) *δ:* 15.2, 16.1, 16.3, 18.1, 22.3, 23.0, 23.6, 25.6, 26.8 (2C), 27.0, 28.3, 30.4, 32.3, 32.4, 33.0, 33.6, 36.6, 38.2, 38.5, 38.7, 40.5, 41.2, 45.3, 46.0, 47.2, 52.1, 54.9, 76.9, 116.7, 121.5, 131.8, 134.0, 143.9, 172.8, 176.5. ESI-HRMS (m/z): $[M+H]^+$ calcd for $C_{36}H_{56}NO_4$, 594.4271; found, 594.4263.

Compound 10 Prepared from O-methylation phenylalanine (268 mg, 1.5 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **10** as a white solid (549 mg, 89%). m.p.: 107.7–108.9°C; ^{1}H NMR (400 MHz, $CDCl_3$) *δ:* 0.62-1.12 (m, 7×CH_3), 1.12-2.00 (m, other aliphatic ring protons), 2.44 (d, *J*=9.4 Hz, 1H), 3.04 (dd, *J*=5.88, 13.8 Hz, 1H), 3.16-3.22 (m, 2H), 3.69 (s, 3H), 4.75 (q, *J*=5.92 Hz, 1H), 5.29 (s, 1H), 6.39 (d, *J*=6.12 Hz, 1H), 7.10 (d, *J*=7.2 Hz, 2H) 7.23-7.29 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ:* 15.5, 15.7, 16.6, 18.4, 23.6, 23.6, 23.9, 25.8, 27.3, 27.4, 28.2, 30.8, 32.7, 32.8, 33.1, 34.3, 37.1, 38.0, 38.6, 38.7, 38.9, 39.5, 42.1, 46.5, 46.7, 47.7, 52.3, 53.6, 55.3, 79.1, 123.5, 127.2, 128.6 (2C), 129.5 (2C), 136.3, 143.8, 172.2, 177.7. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{40}H_{59}NO_4Na$, 640.4342; found, 640.4352.

Compound 10a Prepared from **10** (200 mg, 0.31 mmol) according to general procedure C. The crude product was washed with water three

times without further purification to afford **10a** as a white solid (182 mg, 89%). m.p.: >250°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) *δ:* 0.66-0.98 (m, 7×CH_3), 0.98-2.00 (m, other aliphatic ring protons), 2.62 (d, *J*=10.28 Hz, 1H) 2.88-2.96 (m, 2H), 3.06 (dd, *J*=3.92, 13.44 Hz 1H), 4.28-4.32 (m, 2H), 5.04 (s, 1H), 7.14-7.18 (m, 1H), 7.23 (d, *J*=4.16 Hz, 4H) 7.33 (d, *J*=7.48 Hz, 1H) 12.67 (s, 1H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) *δ:* 15.2, 16.0, 16.1, 17.9, 22.9, 23.5, 25.6, 26.8, 26.8, 28.3, 30.4, 31.9, 32.1, 33.0, 36.2, 36.6, 38.5, 38.7, 39.3, 40.1, 40.4, 41.2, 45.2, 46.0, 47.2, 54.1, 54.1, 54.9, 76.9, 121.3, 126.3, 128.2 (2C), 129.3 (2C), 138.2, 144.1, 173.4, 176.4. ESI-HRMS (m/z): $[M+H]^+$ calcd for $C_{39}H_{58}NO_4$, 604.4366; found, 604.4323.

Compound 11 Prepared from O-methylation tryptophan (327 mg, 1.5 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **11** as a white solid (632 mg, 95%). m.p.: >250°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) *δ:* 0.67-0.93 (m, 7×CH_3), 0.93-2.00 (m, other aliphatic ring protons), 2.62 (d, *J*=10.36 Hz, 1H), 2.92-2.97 (m, 1H), 3.02-3.08 (m, 1H), 3.15-3.20 (m, 1H), 3.62 (s, 3H), 4.26 (d, *J*=5.04 Hz, 1H), 4.37-4.42 (m, 1H), 4.94 (s, 1H), 6.96 (t, *J*=7.6 Hz, 1H), 7.04 (t, *J*=7.36 Hz, 1H), 7.19 (s, 1H), 7.28-7.34 (m, 2H), 7.49 (d, *J*=7.8 Hz, 1H), 10.83 (s, 1H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) *δ:* 15.0, 15.1, 16.0, 18.0, 22.1, 22.8, 23.5, 25.5, 26.4, 26.5, 26.9, 28.3, 30.3, 31.7, 31.9, 32.9, 33.5, 36.5, 38.0, 38.3, 38.3, 40.2, 40.9, 44.9, 45.7, 47.0, 51.7, 53.3, 54.8, 76.8, 109.6, 111.5, 117.8, 118.3, 120.7, 121.0, 124.0, 127.0, 136.3, 144.0, 172.7. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{42}H_{60}N_2O_4Na$, 679.4451; found, 679.4441.

Compound 11a Prepared from **11** (200 mg, 0.28 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **11a** as a white solid (178.4 mg, 91%). m.p.: >250°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) *δ:* 0.67-0.93 (m,

7×CH_3), 0.93-2.00 (m, other aliphatic ring protons), 2.6 (d, *J*=10.4 Hz, 1H), 2.92-2.97 (m, 1H), 3.02-3.08 (m, 1H), 3.19 (dd, *J*=4.04, 14.28 Hz, 1H) 4.26 (d, *J*=4.72 Hz, 1H), 4.34 (dd, *J*=4.12, 9.76 Hz, 1H), 4.95 (brs, 1H), 6.95 (t, *J*=7.52 Hz, 1H), 7.04 (t, *J*=7.32 Hz, 1H), 7.17 (s, 1H), 7.29 (d, *J*=8.08 Hz, 1H), 7.52 (d, *J*=7.84 Hz, 1H), 10.81 (s, 1H), 12.51 (s, 1H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) *δ:* 15.0, 15.1, 16.0, 18.0, 22.2, 22.8, 23.4, 25.5, 26.4, 26.9, 28.3, 30.3, 31.7, 32.0, 32.9, 33.5, 36.5, 38.0, 38.3, 40.3, 40.9, 44.9, 45.8, 47.0, 53.1, 53.2, 54.8, 76.8, 109.9, 111.4, 118.0, 118.2, 120.6, 121.1, 123.9, 127.1, 136.3, 144.0, 173.8, 176.1, 176.2. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{41}H_{58}NO_4Na$, 665.4294; found, 665.4287.

Compound 12 Prepared from O-methylation methionine (245 mg, 1.5 mmol) according to general procedure B. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **12** as a white solid (571 mg, 95%). m.p.: 196.1–198.5°C; ^{1}H NMR (400 MHz, $CDCl_3$) *δ:* 0.68-1.15 (m, 7×CH_3), 1.15-2.00 (m, other aliphatic ring protons), 2.41-2.55 (m, 2H), 2.64 (d, *J*=9.44 Hz, 1H), 3.21 (dd, *J*=4.04, 6.8 Hz, 1H), 3.30 (s, 3H), 3.73 (s, 3H), 4.12 (q, *J*=7.68 Hz, 1H), 4.59 (q, *J*=6.04 Hz, 1H) 5.44 (t, *J*=3.84 Hz, 1H) 6.63 (d, *J*=6.08 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ:* 15.5, 15.7 (2C), 16.7, 18.4, 23.7, 23.8, 25.9, 27.4, 27.6, 28.3, 30.1, 30.9, 32.0, 32.8, 33.1, 33.2, 34.3, 37.1, 38.7, 38.9, 39.6, 42.1, 42.2, 46.6, 46.7, 47.8, 51.9, 52.6, 55.3, 77.4, 79.1, 123.6, 143.8, 172.7, 178.0. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{36}H_{59}NO_4Na$, 624.4063; found, 624.4055.

Compound 12a Prepared from **12** (200 mg, 0.32 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **12a** as a white solid (185.7 mg, 95%). m.p.: 168.9–171.7°C; ^{1}H NMR (400 MHz, CD_3OD) *δ:* 0.76-1.18 (m, 7×CH_3), 1.18-2.20 (m, other aliphatic ring protons), 2.06 (s, 3H), 2.49 (t, *J*=7.68 Hz, 2H), 2.76 (d, *J*=11.08 Hz, 1H), 3.04 (s, 1H), 3.12-3.06 (m,

1H), 4.31 (t, *J*=5.4 Hz, 2H), 5.41 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD) *δ:* 15.4, 15.9, 16.3, 17.7, 19.5, 24.0, 24.2, 24.5, 24.6, 26.4, 28.0, 28.7, 28.8, 31.1, 31.6, 32.7, 33.5, 34.0, 34.1, 35.1, 38.1, 39.8, 39.9, 40.7, 43.0 (2C), 47.6, 47.7, 49.1 (2C), 54.4, 56.7, 79.7, 124.9, 144.5, 179.8. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{35}H_{57}NO_4S$, 587.4008; found, 587.4005.

Compound 13 Prepared from diO-methylation asparticacid (242 mg, 1.5 mmol) according to general procedure B. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **13** as a white solid (543 mg, 88%). m.p.: 87–88.7°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) *δ:* 0.71-1.16 (m, 7×CH_3), 1.16-2.00 (m, other aliphatic ring protons), 2.67 (d, *J*=10.24 Hz, 1H) 2.95 (d, *J*=4.68 Hz, 1H), 3.21 (d, *J*=7.64 Hz, 1H), 3.67 (s, 3H), 3.76 (s, 3H), 4.69 (dd, *J*=4.68, 5.88 Hz, 1H), 5.43 (s, 1H), 6.81 (d, *J*=6.24 Hz, 1H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) *δ:* 15.5, 15.7, 16.8, 18.4, 23.7, 23.7, 25.9, 27.4, 27.5, 28.2, 30.9, 32.9, 33.2, 33.2, 34.2, 36.2, 37.1, 38.7, 38.9, 39.6, 42.0, 42.1, 46.5, 46.6, 47.7, 49.2, 52.0, 52.9, 55.3, 77.4, 79.1, 123.4, 143.6, 171.5, 171.5, 177.8. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{36}H_{57}NO_6Na$, 622.4084; found, 622.4092.

Compound 13a Prepared from **13** (200 mg, 0.32 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **13a** as a white solid (164.2 mg, 86%). m.p.: >250°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) *δ:* 0.67-1.08 (m, 7×CH_3), 1.08-2.00 (m, other aliphatic ring protons) 2.50-2.56 (m, 4H) 2.72 (dd, *J*=6.08, 12.28 Hz, 1H), 2.95-3.03 (m, 1H), 4.27 (d, *J*=5.28 Hz, 1H), 4.42 (q, *J*=6.52 Hz, 1H), 5.19 (brs, 1H), 7.45 (d, *J*=7.24 Hz, 1H), 12.48 (s, 2H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) *δ:* 15.2, 16.1, 16.8, 18.1, 22.4, 23.0, 23.5, 25.6, 26.9, 27.0, 28.3, 30.5, 32.4, 32.5, 32.9, 33.6, 35.8, 36.6, 38.2, 38.5, 40.1, 40.7, 41.4, 45.3, 46.0, 47.2, 48.9, 54.9, 76.9, 121.7,

143.8, 171.9, 172.6, 176.2. ESI-HRMS (m/z): $[M]^+$ calcd for $C_{34}H_{53}NO_6$, 571.3873; found, 571.3882.

Compound 14 Prepared from diO-methylation a glutamicacid (242 mg, 1.5 mmol) according to general procedure B. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **14** as a white solid (543 mg, 88%). m.p.: 166.5–167.5°C; ^{1}H NMR (400 MHz, $CDCl_3$) *δ:* 0.66-1.15 (m, 7×CH_3), 1.15-2.50 (m, other aliphatic ring protons), 2.63 (d, *J*=11.56 Hz, 1H), 3.20 (dd, *J*=4.00, 10.88 Hz, 1H), 3.67 (s, 3H), 3.72 (s, 3H), 4.50 (q, *J*=6.24 Hz, 1H), 5.43 (t, *J*=3.56 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ:* 15.5, 15.7, 16.7, 18.4, 23.7, 23.8, 25.9, 27.4, 27.6, 27.7, 28.2, 30.1, 30.9, 32.8, 33.2, 33.2, 34.2, 37.1, 38.7, 38.9, 39.6, 42.1, 42.2, 46.6, 46.6, 47.7, 51.9, 52.0, 52.5, 55.3, 79.1, 123.5, 143.8, 172.6, 173.4, 178.1. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{37}H_{59}NO_6Na$, 636.4240; found, 636.4248.

Compound 14a Prepared from **14** (200 mg, 0.31 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **14a** as a white solid (166.2 mg, 87%). m.p.: 248.3–248.7°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) *δ:* 0.64-1.08 (m, 7×CH_3), 1.08-2.00 (m, other aliphatic ring protons) 2.76 (d, *J*=9.76 Hz, 1H), 2.95-3.03 (m, 1H) 3.56 (dd, *J*=5.36, 17.4 Hz, 1H), 3.74 (dd, *J*=5.96, 17.24 Hz, 1H), 4.28 (d, *J*=4.36 Hz, 1H), 5.19 (brs, 1H), 7.56 (t, *J*=5.2 Hz, 1H), 12.37 (s, 1H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) *δ:* 15.1, 16.0, 16.6, 18.0, 22.5, 22.9, 23.5 (2C), 25.6, 26.8, 27.0, 28.2 (2C), 30.4, 32.4, 32.5, 32.9, 33.6, 36.6, 38.1, 38.4, 39.6, 40.5, 40.9, 41.2, 45.1, 46.0, 47.1, 54.8, 76.8, 121.5, 143.9, 171.4, 176.7. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{32}H_{51}NO_4Na$, 536.3716; found, 536.3711.

Compound 15 Prepared from O-methylation arginine (282 mg, 1.5 mmol) according to general procedure B. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **15** as a

white solid (502 mg, 78%). m.p.: 119.3–121.8°C; ^{1}H NMR (400 MHz, $CDCl_3$) *δ:* 0.73-1.18 (m, 7×CH_3), 1.18-2.00 (m, other aliphatic ring protons), 2.14 (s, 1H) 2.32 (d, *J*=13.53 Hz, 1H), 2, 51 (d, *J*=13. 68 Hz, 1H), 2.84 (d, *J*=13.36 Hz, 1H), 3.32 (s, 1H), 3.74 (s, 3H), 4.68-4.72 (m, 2H), 5.29 (s, 1H) 6.58 (d, *J*=8.28 Hz, 1H) 7.94 (s, 1H), 8.89 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ:* 16.5, 16.6, 18.1, 18.3, 21.3, 21.6, 23.3, 23.8, 24.9, 25.6, 26.4, 28.0, 32.5, 33.1, 33.6, 34.4, 36.1, 37.1, 37.3, 38.2, 38.5, 40.3, 40.9, 41.7, 44.5, 49.0, 50.4, 52.8, 55.3, 77.4, 86.3, 113.4, 116.2, 127.1, 143.6, 172.2, 177.9. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{37}H_{62}N_4O_4Na$, 649.4669; found, 649.4664.

Compound 15a Prepared from **15** (200 mg, 0.31 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **15a** as a white solid (175.5 mg, 87%). m.p.: 222.6–224.4°C; ^{1}H NMR (600 MHz, $(CD_3)_2SO$) *δ:* 0.66-1.13 (m, 7×CH_3), 1.13-2.00 (m, other aliphatic ring protons), 2.21-2.23 (m, 1H), 2.43 (d, *J*=13.68 Hz, 1H) 2.78 (d, *J*=11.7 Hz, 1H) 2.99-3.02 (m, 1H) 4.22-4.26 (m, 1H) 6.60 (d, *J*=7.56 Hz, 1H) 7.79 (s, 1H) 8.56 (s, 1H) 12.70 (s, 1H). ^{13}C NMR (150 MHz, $(CD_3)_2SO$) *δ:* 16.3, 16.7, 17.9, 18.4, 21.4, 21.7, 24.3 (2C), 25.4, 26.4, 27.6 (2C), 28.6, 28.7, 32.5, 33.1, 33.3, 34.6, 35.7, 36.8, 37.2, 38.9, 39.0, 40.5, 41.1, 44.4, 48.5, 50.3, 52.3, 55.3, 77.3, 128.0, 141.2, 152.8, 173.7, 175.2. ESI-HRMS (m/z): $[M]^+$ calcd for $C_{36}H_{60}N_4O_4$, 612.4615; found, 612.4600.

Compound 16 Prepared from O-methylation tyrosine (293 mg, 1.5 mmol) according to general procedure B. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **16** as a white solid (436 mg, 67%). m.p.: 137.7–139.0°C; ^{1}H NMR (400 MHz, $CDCl_3$) *δ:* 0.62-1.12 (m, 7×CH_3), 1.12-2.00 (m, other aliphatic ring protons), 2.45 (d, *J*=8.72 Hz, 1H), 2.94 (dd, *J*=5.68, 13.8 Hz, 1H), 3.08 (dd, *J*=5.8, 14.04 Hz, 1H), 3.21 (dd, *J*=3.84, 10.28 Hz, 1H) 3.68 (s, 3H),

4.11 (q, *J*=7.12 Hz, 1H), 4.71 (dd, *J*=5.88, 14.04 Hz, 1H), 5.30 (s, 1H), 6.45 (d, *J*=6.16 Hz, 1H), 6.73 (d, *J*=5.36 Hz, 2H), 6.94 (d, *J*=7.68 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 15.5, 15.7, 16.6, 18.4, 23.6, 23.6, 23.9, 25.8, 27.3, 27.4, 28.2, 30.8, 32.7, 32.8, 33.1, 34.3, 37.1, 37.4, 38.7, 38.9, 39.6, 42.1, 42.2, 46.6, 46.7, 47.7, 52.3, 53.8, 55.3, 79.2, 115.6 (2C), 123.6, 127.6, 130.5 (2C), 143.7, 155.6, 172.2, 178.1. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{40}H_{59}NO_5Na$, 656.4291; found, 656.4281.

Compound 16a Prepared from **16** (200 mg, 0.30 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **16a** as a white solid (142.8 mg, 73%). m.p.: >250°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) *δ:* 0.67-1.00 (m, 7×CH_3), 1.00-2.00 (m, other aliphatic ring protons), 2.62 (d, *J*=10.4 Hz, 1H) 2.79 (dd *J*=10.04, 13.52 Hz, 1H), 2.92-2.98 (m, 2H) 4.22 (dd *J*=5.52, 7.72 Hz, 1H) 4.28 (d, *J*=4.88 Hz, 1H) 5.05 (s, 1H), 6.61 (d, *J*=8.28 Hz, 2H) 7.00 (d, *J*=8.28 Hz, 2H) 7.22 (d, *J*=7.24 Hz, 1H), 9.13 (s, 1H) 12.58 (s, 1H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) *δ:* 15.1, 15.9, 16.1, 18.0, 22.9, 23.4, 25.6, 26.8, 27.0, 28.3, 30.3, 31.9, 32.0, 32.9, 33.6, 35.4, 36.5, 38.4, 38.6, 40.4, 41.2, 45.1, 45.9, 47.2, 54.2, 54.9, 76.8, 76.9, 114.8, 114.9, 121.3, 127.8, 130.0, (2C), 144.0, 156.1, 173.4, 176.2, 176.3. ESI-HRMS (m/z): $[M+H]^+$ calcd for $C_{39}H_{28}NO_5$, 620.4315; found, 620.4306.

Compound 17 Prepared from O-methylation proline (193 mg, 1.5 mmol) according to general procedure B. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 3/1 v/v) to afford **16** as a white solid (503 mg, 86%). m.p.: >250°C; ^{1}H NMR (600 MHz, $(CD_3)_2SO$) *δ*: 0.63-1.08 (m, 7×CH_3), 1.08-2.05 (m, other aliphatic ring protons), 2.92 (d, *J*=12.06 Hz, 1H), 2.97-3.0 (m, 1H), 3.43 (dd, *J*=9.12 *J*=16.26 Hz, 1H), 3.57 (s, 3H), 3.82 (brs, 1H), 4.13 (d, *J*=7.38 Hz, 1H), 4.29 (d, *J*=5.16 Hz, 1H), 5.06 (s, 1H). ^{13}C NMR (150 MHz, $(CD_3)_2SO$) *δ*: 15.6, 16.5, 16.8, 18.4, 20.8, 23.3, 24.2, 25.9, 26.2, 27.3, 27.4, 27.5, 28.7,

30.6, 32.9, 33.4, 33.7, 37.1, 38.5, 38.9, 39.2, 40.5, 42.0, 42.7, 46.0, 46.7, 47.7, 47.9, 51.9, 55.4, 61.4, 77.3, 121.6, 144.9, 173.2, 174.6. ESI-HRMS (m/z): $[M+Na]^+$ calcd for $C_{36}H_{57}NO_4Na$, 590.4185; found, 590.4187.

Compound 17a Prepared from **17** (200 mg, 0.34 mmol) according to general procedure C. The crude product was washed with water three times without further purification to afford **17a** as a white solid (138.6 mg, 71%). m.p.: 188.8–191.3°C; ^{1}H NMR (400 MHz, CD_3OD) *δ:* 0.75-1.16 (m, 7×CH_3), 1.16-2.20 (m, other aliphatic ring protons) 3.03 (d, *J*=13.08 Hz, 1H), 3.14 (dd, *J*=4.8, 11.12 Hz, 1H), 3.52-3.60 (m, 1H), 3.93 (t, *J*=7.96 Hz, 1H), 4.34 (d, *J*=6.24 Hz, 1H), 5.19 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD) *δ:* 15.9, 16.3, 17.5, 19.5, 22.0, 24.4, 24.5, 26.6, (2C), 27.9, 28.6, 28.8, 30.1, 31.4, 33.7, 34.0, 34.8, 38.2, 39.8, 39.8, 40.5, 43.1, 44.2, 47.2, 48.5, 49.2, 49.2, 56.8, 63.3, 63.3, 79.7, 123.5, 145.5, 176.6, 177.5. ESI-HRMS (m/z): $[M+H]^+$ calcd for $C_{35}H_{56}NO_4$, 554.4209 ; found, 554.4181.

4.2. Anti-influenza virus assay

MDCK cells at 5,000 cells/well were cultured in 96-well plates for a day before IAV infection. The infection medium used was DMEM (High Glucose) containing 1% FBS and 0.2% trypsin (1 μg/mL). Various concentrations of compounds were added to the cell culture. The cells were then infected with IAV at a multiplicity of infection (MOI) of 0.1, unless otherwise indicated. After 45 h of incubation, Promega CellTiter-Glo® reagent was added to each well following the protocol provided by the supplier. The luminescence (RLU) emitted from each well was quantified with a Molecular Device SpectraMax M2 plate reader. IC_{50}, the concentration required to inhibit 50% of A/WSN/33 was

calculated using the software GraphPad Prism 7.

4.3. HI assay

Compound **15a** from a 2-fold serial dilution in saline was mixed with an equal volume of influenza virus (2 HA units) in the V-bottomed 96-well microplates. Subsequently, 50 μL of freshly prepared chicken red blood cells (cRBC) (1% v/v in saline) was added to each well. The mixture was incubated for 30 min at room temperature before observing cRBC aggregation on the plate.

4.4. Docking Simulation

Compound **15a** were docked into the HA protein using AutoDock 4.2.4. The structural template of influenza HA (Protein Data Bank: 1RVT) was obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). To carry out blind docking experiments, grids of points covering the whole HA head and partial stem were generated with ADT.46 (Box size: 100 × 100 × 100 points with a standard space of 0.375 Å). Docking simulations of the compound were carried out using the Lamarckian genetic algorithm and through a protocol with a number of 50 GA runs, an initial population of 100 randomly placed individuals, a maximum number of 25 million energy evaluations, a mutation rate of 0.02, and a crossover rate of 0.80. The resulting conformations that differed by less than 2.0 Å in positional root-mean-square deviation (rmsd) were clustered together. Other

parameters were set as default. All the relevant torsion angles were treated as rotatable during the docking process, thus allowing a search of the conformational space. After running autogrid and autodock, the possible poses of compounds in HA were obtained.

Conflict of interest

The authors declared that they have no conflicts of interest to this work.

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Figure 1. Inhibitory effects of natural amino acids-oleanolic acid conjugates against influenza A/WSN/33 (H1N1) virus. (A) The cytotoxic effect-based screen of natural amino acids-oleanolic acid conjugates (100 μM) using CellTiter-Glo® Assay. DMSO acted as negative control. Error bars indicate standard deviations of triplicate experiments. (B) The cytopathic effect-based screen of natural amino acids-oleanolic acid conjugates (100 μM). MDCK was utilized as the host cell to test A/WSN/33 virus infection; DMSO acted as negative control. Error bars indicate standard deviations of triplicate experiments.

Figure 2. Comparisons of the behaviors of compound **15a** vs. monoclonal antibody against HA protein in inhibition of influenza virus-induced aggregation of chicken erythrocytes. **15a** exerted identical capability as anti-HA antibody in hemagglutination inhibition in a dose-dependent manner.

Figure 3. Structural representative of compound **15a** binding within HA protein (Protein Data Bank: 1RVT) according to blind docking calculation. (A) Overview of HA protein. The inhibitor pocket is highlighted in yellow square. (B) Closer

view of the inhibitor pocket. Compound **15a** is shown as green sticks. (C) 2D interaction plot green dashes and red curves indicate hydrogen bonds and hydrophobic interactions correspondingly.

Scheme 1. Reagents and conditions: (a)TBTU, DIEA, THF, rt, overnight;(b) amino acid methyl esters, Na_2CO_3, rt, 20min, DMF;(c) NaOH, rt, 3h, THF/CH_3OH (1:1).

Scheme 2. Reagents and conditions: (a) amino acid methyl esters, TEA, EDC, rt, overnight, THF; (a') proline methyl ester, TEA, EDC, rt, overnight, THF; (b) NaOH, rt, 3h, THF/CH_3OH (1:1).

Table 1. Comparisons of the anti-influenza virus activity of compound **15a** with that of OSV-P.

Compd	CC_{50} (μM)[a]	IC_{50} (μM)[b]			
		A/WSN/33[c]	A/Texas/50/2012	BX-35	BX-51B
15a	>100	6.64	14.05	61.58	45.8
OSV-P	>100	1.82	>100	>100	>100

[a]CC_{50}, the concentration required to reduced normal, non-infected cell viability by 50%. Values represent the mean of duplicate samples from three independent experiments.

[b]IC_{50}, the concentration required to reduced inhibition of viral infection-induced cytopathogenicity by 50%. Values represent the mean of duplicate samples from three independent experiments.

[c]A/WSN/33 (H1N1); A/Texas/50/2012 (H3N2); BX-35, B/Brisbane/60/2008 (Influenza B Victoria lineage); BX-51B, B/Massachusetts/2/2012 (Influenza B Yamagata lineage).