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Sulfotyrosine dipeptide: Synthesis and evaluation as HIV-entry inhibitor

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) is responsible for the worldwide AIDS pandemic. Due to the lack of prophylactic HIV-1 vaccine, drug treatment of the infected patients becomes essential to reduce the viral load and to slow down progression of the disease. Because of drug resistance, finding new antiviral agents is necessary for AIDS drug therapies. The interaction of gp120 and co-receptor (CCR5/CXCR4) mediates the entry of HIV-1 into host cells, which has been increasingly exploited in recent years as the target for new antiviral agents. A conserved co-receptor binding site on gp120 that recognizes sulfotyrosine (sTyr) residues represents a structural target to design novel HIV entry inhibitors. In this work, we developed an efficient synthesis of sulfotyrosine dipeptide and evaluated it as an HIV-1 entry inhibitor.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) has evolved into one of the serious global public health crisis over the past thirty years. It is estimated that HIV/AIDS pandemic has already claimed AIDS-related deaths of 40 million people, and 35 million people are living with this virus, and approximately 2.0 million people are newly infected by HIV-1 each year according to UNAIDS [1]. There is currently no cure and no effective prevention methods available [2,3]. Drug treatment for controlling viral loads and for prolonging patients' lives is the main therapy for HIV-1 infections. A number of different antiviral agents are currently available, such as reverse transcriptase (RT) inhibitors, protease inhibitors, and integrase inhibitors [4]. However, HIV-1 drug resistance posts a serious problem in these antiviral therapies due to the high mutation rate of the virus. Therefore, searching for additional and especially new classes of antiviral drugs is still essential to fight against HIV/AIDS. To this end, efforts have been continuously made to develop new antiviral drugs including maturation inhibitors, entry inhibitors, and fusion inhibitors. Among them, antiviral agents that can block HIV-1 entry (entry inhibitors) are considered as an effective class of drug and has been increasingly exploited in recent years but is still scarce [5]. The entry inhibitors would have two major advantages over drugs targeting HIV enzymes: (1) limited cell toxicity due to their extracellular nature; and (2) beneficial effects upstream to damages that would occur to the cell later in the viral life cycle.

HIV-1 entry into the target cells requires two receptors: a primary receptor CD4 and a co-receptor CCR5 (or CXCR4). Upon binding to the primary receptor CD4, gp120 undergoes a large conformational change and exposes its binding site for co-receptor CCR5 or CXCR4 [6,7]. The binding of co-receptor causes further conformational change and leads to membrane fusion between the virus and the target cell, which allows the viral RNA genome to enter the target cell. Since viral entry involves a multi-stage process, such as viral attachment (CD4-engagement), co-receptor binding, membrane fusion (pre- or post-fusion), HIV-1 entry inhibitors can be classified into different stage inhibitors [8]. Currently, only two entry inhibition drugs are approved by FDA for clinical uses. One is a membrane fusion inhibitor, which is a 38-amino acid peptide drug derived from the HIV-1 gp41 glycoprotein sequence and is marketed under the trade name Fuzeon (T20, Enfuvirtide) [9–11]. This drug prevents membrane fusion through blocking the formation of the post-fusion structure [9–11]. The success of Fuzeon demonstrates that peptides can be used as drugs against HIV-1 infection. The other FDA-approved drug is Maraviroc (MVC; trade name Selzentry) [12–15], which targets the HIV-1 cellular factor, co-receptor CCR5, and inhibits HIV-1 entry by blocking gp120-CCR5 interaction. Besides the above two FDA-approved entry inhibitors, there are several small molecule drugs, such as BMS-663068 and cenicriviroc (TBR-652), and antibody-based

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drugs, such as Ibalizumab, and peptide-based drugs, such as Sifuvirtide, are currently under clinical trials. It is apparently that blocking viral entry is an effective strategy to prevent HIV-1 infections [5,8,16,17].

Sulfotyrosine (sTyr) residues on the N-terminal region of cell surface co-receptor (CCR5 or CXCR4) are the key recognition element of gp120 [18,19]. It was reported previously that sulfopeptides exhibited activity to block the association of gp120 with CCR5 co-receptor and inhibited the viral entry [20]. An antibody (412d) that binds to the co-receptor CCR5 binding site was found to be sulfated in its heavy chain CDR3 loop [21]. A recent report in *Nature* showed that an AAV-expressed eCD4-Ig, a fusion of CD4-Ig with a small CCR5-mimetic sulfopeptide, could provide durable protection against multiple SHIV infections in the non-human primate model [22]. These results indicated that the CCR5-mimetic sulfopeptide could inhibit HIV entry by competing for the co-receptor binding site. In the present work, we seek to synthesize and evaluate a sulfotyrosine dipeptide as an HIV-1 entry inhibitor by targeting viral envelope glycoprotein gp120. According to our computational analysis, sulfotyrosine dipeptide is likely a minimum active peptide sequence to bind to gp120. Sulfotyrosine dipeptide or its derivatives can be potentially converted into small drug-like organic molecules for therapeutic applications. Comparing to tyrosine-sulfated gp120 antibodies (e.g., 412d [23]) and peptides as HIV entry inhibitors, sulfopeptide-derived drug-like organic molecules should possess advantages in cost-effectiveness, conformational restriction, metabolic stability, and oral bioavailability.

2. Results and discussion

2.1. Computational analysis and rationale

Recently acquired structural information on the interaction between co-receptor CCR5 and gp120 [19] and the interaction between CD4-induced antibody [23] and gp120 has revealed several important sites for drug discovery. One of them is a conserved CCR5-binding site of gp120 [24], which is mainly located at the base of the gp120 V3-loop (Fig. S1). This site features a small, deep, and positively charged pocket for the binding of negatively charged sTyr residues on either the N-terminus of CCR5 [19] or the 412d antibody. The V3-loop of gp120 represents a very attractive site for drug development (Fig. S1). A sequence alignment (Fig. S1B) suggests that an acidic and tyrosine-rich peptide sequence, especially in the presence of sTyr residues, is the critical recognition element of the gp120 V3-loop.

In the present study, we sought to identify CCR5 N-terminus-like or 412d CDR3-loop-like short sulfopeptides that have similar or better binding affinity towards the gp120 V3-loop. Based on the sequences of the N-terminus and the CDR3 loop of 412d antibody, a series of peptides were designed and their docking energies towards the gp120 V3-loop were shown in Table 1. Our calculations indicated that the docking (Cdoc) and the binding (Potential energy) energies of sulfotyrosine dipeptide were not significantly reduced although this dipeptide has a largely reduced size. Therefore, we decided to examine sulfotyrosine dipeptide as an HIV-1 entry inhibitor.

Table 1
Computational analyses of sulfopeptides.[a]

Entry	Peptides	Cdoc energy	Initial energy	CHARMm energy	Potential energy
1	sTyr-Asp-Ile-Asn-sTyr	58.42	21446.60	−276.15	−276.15
2	sTyr-Asp-Ile-Asp-sTyr	70.94	22083.00	−186.74	−186.74
3	sTyr-Ile-Asp-Asn-sTyr	62.13	21390.70	−225.04	−225.04
4	sTyr-Asn-Ile-Asn-sTyr	58.84	20507.00	−255.83	−255.83
5	sTyr-Asp-Asn-sTyr	61.05	20935.60	−203.78	−203.78
6	sTyr-Asp-Asp-sTyr	69.17	21557.30	−188.33	−188.33
7	sTyr-Asn-Asp-sTyr-	54.93	20557.50	−200.32	−200.32
8	sTyr-Ile-Asp-sTyr	53.41	20753.50	−141.32	−141.32
9	sTyr-Asp-Ile-sTyr	51.33	20945.30	−157.43	−157.43
10	sTyr-Ser-Ser-sTyr	40.15	20089.90	−144.26	−144.26
11	sTyr-Asn-Asn-sTyr	48.65	20010.40	−234.48	−234.48
12	sTyr-Ile-Asn-sTyr	41.27	20160.80	−180.89	−180.89
13	sTyr-Asn-Ile-sTyr	39.27	19943.20	−194.14	−194.14
14	sTyr-Ser-Asn-sTyr	41.81	19891.70	−180.58	−180.58
15	sTyr-Ile-Ile-sTyr	33.75	20083.30	−138.22	−138.22
16	sTyr-Asp-sTyr	47.04	20747.20	−203.13	−203.13
17	sTyr-Asn-sTyr	41.56	20133.50	−186.00	−186.00
18	V-Ile-sTyr	41.02	20360.70	−135.52	−135.52
19	sTyr-Ser-sTyr	35.79	20681.20	−139.00	−139.00
20	sTyr-sTyr	33.28	22192.50	−123.73	−123.73

[a] A full set of data can be found in Table S1.

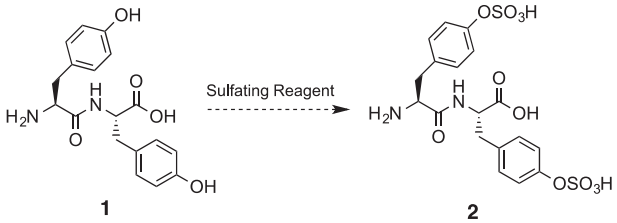
2.2. Synthesis of sulfotyrosine dipeptide

Sulfopeptides can be chemically synthesized either by direct incorporation of sTyr residues into a growing peptide chain, or by sulfating Tyr residues in a peptide post-assembly [25–28]. Regardless of recent progress [29,30], the preparation of sTyr-containing peptides is still challenging. Direct incorporation of sTyr residues during solid-phase peptide syntheses often suffers low yield. This is mainly due to the extensive sTyr hydrolysis under the reaction conditions, the incomplete deprotection of protected sTyr precursors, and/or the incomplete hydrolysis of peptide product from the resin under mild conditions [25–28]. To overcome this difficulty, a global sulfation approach was developed. In this approach, sulfation of tyrosine residues was conducted after the peptide was fully assembled. Different types of protection/deprotection strategy [31,32] or new protecting groups [33] have been developed to minimize the desulfation or achieve the selective sulfation of certain tyrosine residues in the peptide sequence.

In this study, we focused on the solution-based global sulfation approach to synthesize the desirable sulfotyrosine dipeptide. Our first synthetic scheme involved the direct sulfation of unprotected tyrosine dipeptide (NH_2-Tyr-Tyr-COOH) using either concentrated sulfuric acid [34] or chlorosulfuric acid [35]. We have shown that both reagents could be used to synthesize sTyr with excellent yield [36]. Unfortunately, incomplete sulfation of the unprotected NH_2-Tyr-Tyr-COOH was observed. Only mono-sulfated dipeptide was obtained with yields in the range of 20–47% (Table 2). This result indicated that concentrated sulfuric acid or chlorosulfuric acid only have limited sulfating power. The formation of a small amount (<5%; entries 3 and 4, Table 1) of sulfotyrosine dipeptide (NH_2-sTyr-sTyr-COOH) could be observed in two cases when chlorosulfuric acid was used as sulfating reagent. Longer reaction time, higher temperature, and more equivalences of sulfating agents did not improve the yield of NH_2-sTyr-sTyr-COOH (data not shown). The very low yield of di-sulfated product might be resulted from a potential strong repulsion of the two negatively charged sulfate groups.

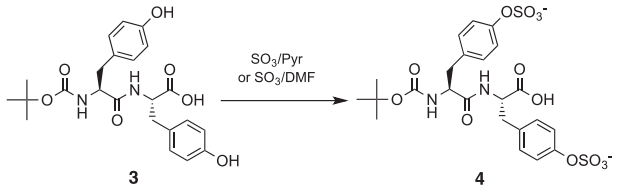
Next, a couple of stronger sulfating agents, SO_3/pyridine and SO_3/DMF, were examined. Since these two reagents are reactive towards amine [34], the free amino group of the substrate, NH_2-Tyr-Tyr-COOH, was protected with Boc group to yield Boc-Tyr-Tyr-COOH. As shown in Table 3, the desired Boc-sTyr-sTyr-COOH product could be obtained by using either SO_3/Pyr or SO_3/DMF as sulfating reagent. SO_3/DMF was apparently a more powerful sulfating reagent than SO_3/Pyr, which is consistent with the literature [37]. After an optimization of reaction time and solvent, the desired product could be obtained in 71% yield when the best set of reaction conditions was used (entry 4, Table 3).

Table 2
Sulfation of NH_2-Tyr-Tyr-COOH dipeptide using sulfuric acid or chlorosulfuric acid.



Entry	NH_2-Tyr-Tyr-COOH (eq.)	Sulfating agent	Reaction condition	Outcomes
1	1	Con. H_2SO_4 (6 eq.)	−5 °C, 40 min	20% mono-sulfated
2	1	HSO_3Cl (1.25 eq.)	−10 °C, 5 min	30% mono-sulfated
3	1	HSO_3Cl (1.25 eq.)	−5 °C, 30 min	45% mono-sulfated <5% disulfated
4	1	HSO_3Cl (3 eq.)	0 °C, 1 h	47% mono-sulfated <5% disulfated
5	1	HSO_3Cl (5 eq.)	0 °C, 2 h	47% mono-sulfated

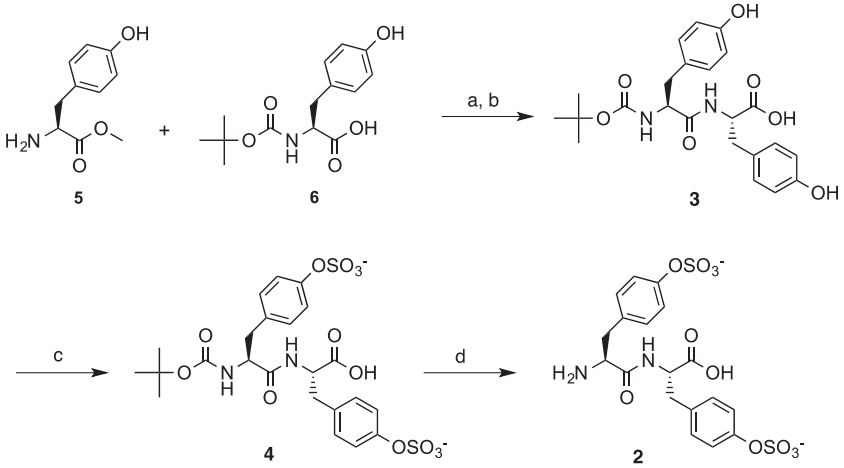
Table 3
Sulfation of NH_2-Tyr-Tyr-COOH dipeptide using SO_3/Pyr or SO_3/DMF.



Entry	Sulfating agent	Time (h)	Temperature (°C)	Solvent	Isolated yield (%)
1	SO_3/Pyr (10 eq.)	12	30	DMF	20
2	SO_3/Pyr (24 eq.)	24	30	DMF/Pyr (4:1)	37
3	SO_3/DMF (24 eq.)	12	70	DMF	25
4	SO_3/DMF (24 eq.)	24	25	DMF/Pyr (4:1)	71
5	SO_3/DMF (24 eq.)	48	25	DMF/Pyr (4:1)	67
6	SO_3/DMF (24 eq.)	24	70	DMF/Pyr (4:1)	65

In the final step of synthesis, the Boc protecting group was removed by using TFA/H_2O (90%) at 0 °C [38]. The reaction was monitored by HPLC using an analytical C18 RP column. The deprotection could be complete in 40 min with 95% yield. The overall synthetic scheme was shown in Scheme 1.

However, severe degradation of the final product (NH_2-sTyr-sTyr-COOH) was observed during a large-scale purification using a prep-RP column. Three additional peaks were found and identified to contain NH_2-sTyr-Tyr-COOH, NH_2-Tyr-sTyr-COOH, and NH_2-Tyr-Tyr-COOH, respectively (Fig. S2). We next examined a



Scheme 1. (a) DCC, TEA, 4:1 CH_3CN:DMF; (b) LiOH, r.t.; (c) SO_3/DMF, 4:1 DMF:pyridine; (d) 9:1 TFA:water, 0 °C.

purification procedure using anion-exchange chromatography. We were able to obtain NH_2-sTyr-sTyr-COOH in 90% yield with higher than 96% purity (estimated by analytical RP-HPLC). The final product was confirmed by both NMR (Figs. S3 and S4) and mass spectrometry (Fig. S5 and Table S2).

2.3. Biological evaluation

To test if sulfotyrosine dipeptide could act as an HIV-entry inhibition, we performed *in vitro* neutralization assay by using our previously reported method [39]. This assay measures the neutralization as a function of the reduction in Tat-regulated Luc reporter gene expression after a single round infection of TZM-bl cell (a HeLa cell clone expressing both CD4 and CCR5) [40]. The expression of the two reporter genes, firefly luciferase and *Escherichia coli* β-galactosidase, were under the control of an HIV long-terminal repeat sequence. Env-pseudotyped viruses, which are deficient of *env* gene in the genome of the mature virions, were used to infect TZM-bl cells. The infection by Env-pseudotyped viruses activated the expression of luciferase reporter gene. The luciferase activity should be directly proportional to the number of infectious virus particles present in the initial inoculum. In the presence of an HIV-entry inhibitor, a lower infection rate would lead to a decrease in the relative luminescence unit (RLU) of the emitted light from the luciferase reaction.

In the neutralization assay, the pseudotyped viruses were mixed with each of the serial diluted compounds (ranging from 0 μM to 960 μM), including tyrosine dipeptide (NH_2-Tyr-Tyr-COOH), sTyr, mono-sulfated tyrosine didpeptide (a mixture of NH_2-sTyr-Tyr-COOH and NH_2-Tyr-sTyr-COOH), and sulfotyrosine dipeptide (NH_2-sTyr-sTyr-COOH). As shown in Fig. 1, no obvious inhibition was observed for the sulfotyrosine dipeptide. Moderate neutralization effects were observed for sTyr and mono-sulfated tyrosine didpeptide at a very high concentration. As shown in Fig. S1, the CCR5-binding pocket of gp120 contains two sTyr binding sites for sTyr10 and sTyr14 of CCR5 [19]. At very high concentrations, two sTyr (or mono-sulfated tyrosine dipeptides) might bind with gp120, which impaired the function of gp120 and led to the observed inhibitory effects. The inhibition assay with sulfotyrosine dipeptide showed large standard deviations. It is not uncommon for an inactive drug to display large variations in biological assays. This observation might also be a result of the low stability of sulfotyrosine dipeptide. Unpredictable hydrolysis of the sulfotyrosine dipeptide could happen during the assay (48 h) at 37 °C.

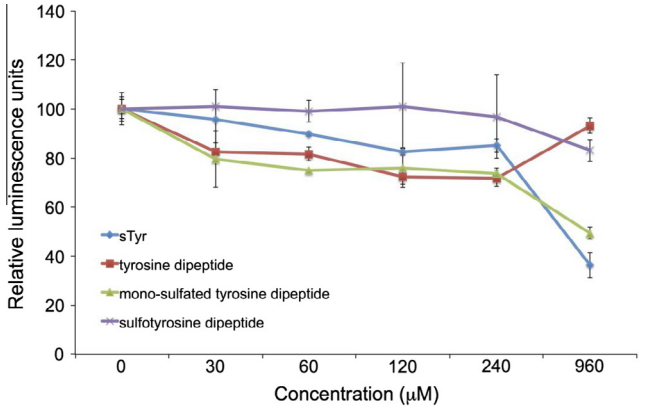


Fig. 1. Neutralization assays. Four compounds were examined in the assay, including sTyr, tyrosine dipeptide (NH_2-Tyr-Tyr-COOH), mono-sulfated tyrosine dipeptide (a mixture of NH_2-sTyr-Tyr-COOH and NH_2-Tyr-sTyr-COOH), and sulfotyrosine dipeptide (NH_2-sTyr-sTyr-COOH). The relative luminescence unit (RLU) of the emitted light from the luciferase reaction was measured in an Illuminometer. Each data point is the average of multiple measurements with standard deviation.

3. Conclusions

We developed a facile procedure to efficiently synthesize sulfotyrosine dipeptide by using N^{α}-protected tyrosine dipeptide as the starting material and SO_3/DMF complex as the sulfating agent. To our best knowledge, this is the first report for the synthesis of this compound. Although sulfotyrosine dipeptide did not show any inhibitory effect against HIV-1 entry, our optimized synthetic procedure could be applied to the synthesis of longer peptides that contain multiple sTyr residues. This will facilitate the synthesis and screening of a large library of sulfopeptides as HIV-1 entry inhibitors.

4. Experimental section

4.1. Materials and general methods

All starting materials and reagents unless otherwise noted were obtained from Sigma-Aldrich Chemicals Company (St. Louis, MO). All reactions were carried out under argon with freshly distilled solvents. CH_2Cl_2 was distilled from calcium hydride under argon. DMF was dried and distilled over calcium hydride under vacuum and stored over 4 Å sieves under argon. Flash chromatography was performed using silica gel 60 Å (234–400 mesh) obtained from Sigma-Aldrich. ^{1}H NMR spectra were obtained using a Bruker Avance III 300 MHz or a Bruker Avance III 400 MHz spectrometer. Chemical shifts (δ) for ^{1}H NMR spectra run in $CDCl_3$ were reported in parts per million (ppm) relative to the internal standard tetramethylsilane (TMS). Chemical shifts (δ) for ^{1}H NMR spectra run in CD_3OD were reported in ppm relative to residual solvent protons (δ 3.30). Chemical shifts (δ) for ^{1}H NMR spectra run in D_2O were reported in ppm relative to sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 (TSP). TLC was performed by using Analtech TLC Uniplates™ developed in a solvent system containing *n*-butanol: acetic acid:water = 3.5:0.5:1. TLC plates were stained by Ninhydrin stain solution (0.1% Ninhydrin with 0.5% acetic acid in acetone) or visualized under 254 nm UV light. Analytical RP-HPLC was performed with a Poroshell 120 EC-C18 column (2.7 μm, 4.6 mm × 50 mm) by using Agilent 1260 Infinity Quaternary pump equipped with a 1260 Infinity Variable Wavelength Detector (VWD). Positive and negative ion electrospray (ESI) experiments were performed with a Waters Q-TOF Ultima mass spectrometer. 1:1 CH_3CN/H_2O is used as a solvent for +ve ion analysis. Analytical HPLC was performed using a linear gradient of CH_3CN (0–25%; in 0.1% TFA) at 1 mL/min over 15 min with the UV detector set to 260 nm. Prep-HPLC was conducted with YMC-AA1205 C18 column (3 μm, 4.6 mm × 250 mm) using a linear gradient of CH_3CN (5–35%; in 0.1% TFA) at 1 mL/min over 60 min, with UV detector set to 230 nm and 260 nm.

4.2. Molecular docking

The docking was preformed using Discovery Studio Client version 4.0 (BIOVIA, San Diego, CA). The ligand docking program CDOCKER [41] was used for all the docking simulations with the CHARMm force field. CDOCKER is a grid-based program in which the receptor is held rigid but the ligand is allowed the flexibility for binding. The structure of gp120 (PDB: 3QAD) in complex with CD4 (two domains) and a CD4-induced antibody 412d was used as the receptor, which was cleaned and prepared by adding hydrogens, and typed with the CHARMm force field. The CCR5-N-termus structure (PDB: 2RLL) was used as a reference. All peptide ligands

were constructed using Sketch and typed with the same force field. The ligand-biding site was defined based on the binding site of 412d antibody (or the CCR5 N-terminus) (19). Docking parameters were set up as the following: top hits numbers 10, conformation dynamics steps 1000, annealing heating steps 2000, target temperature cooling 5000. The best poses from each ligand were selected based on the Cdocking energy scores and listed in Table 1.

4.3. L-Tyrosine methyl ester (**5**)

This compound was synthesized according to a procedure by Rudolf [42]. Thionyl chloride (4 mL, 55.0 mmol) was added dropwise to a suspension of L-tyrosine (5.45 g, 30.1 mmol) in anhydrous MeOH (100 mL, 247 mmol) at 0 °C under argon protection. When half amount of thionyl chloride was added, the solution turned clear. The reaction mixture was allowed to reach room temperature and stirred overnight. After removing the solvent by rotary evaporation, the residue was washed with diethyl ether (2 × 50 mL). Compound **5** was obtained as a white solid (5.8 g, 29.2 mmol, 97%). ^{1}H NMR (300 MHz, D_2O): δ 7.13 (d, J = 8.7 Hz, 2H), 6.87 (d, J = 8.7 Hz, 2H), 4.36 (dd, J = 5.7, 7.2 Hz, 1H), 3.82 (s, 3H), 3.24 (dd, J = 5.7, 14.7 Hz, 1H), 3.13 (dd, J = 7.2, 14.7 Hz, 1H).

4.4. N^{α}-[tert-butoxycarbonyl]-L-tyrosine (**6**) [43]

L-Tyrosine (2 g, 11 mmol) was dissolved in a mixture of dioxane/water (50 mL/25 mL), followed by the addition of 25 mL of NaOH (1 M). Di-*tert*-butyl dicarbonate (2.64 g, 12.1 mmol) was then added and the reaction mixture was allowed to stir at room temperature for 2 h. The reaction mixture was extracted by EtOAc (3 × 20 mL). The combined extraction was dried over Na_2SO_4. After removing the solvent by rotary evaporation, the compound **6** was obtained as a white solid (3 g, 10 mmol, 90%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.10 (d, J = 8.3 Hz, 2H), 6.71 (d, J = 8.4 Hz, 2H), 5.25 (d, J = 6.9 Hz, 1H), 4.52 (s, 1H, OH), 4.32–4.30 (m, 1H), 3.00–2.96 (m, 2H), 1.38 (s, 9H).

4.5. N^{α}-(tert-butoxycarbonyl)-L-tyrosyl-L-tyrosine (**3**) [44]

To a mixture of **5** (0.7 g, 3.55 mmol) and **6** (1 g, 3.55 mmol) in CH_3CN/DMF (40 mL/10 mL), triethylamine (0.48 mL, 3.6 mmol) was added while the reaction vessel was cooled on an ice bath. DCC (0.95 g, 4.6 mmol) was subsequently added and the reaction mixture was allowed to stir at room temperature for 3 h. The precipitated DCU was removed by filtration and the solvent from the filtrate was evaporated. The residue was partitioned between EtOAc (100 mL) and 1 M HCl (100 mL). The aqueous phase was extracted with EtOAc (3 × 100 mL). The combined organic fractions were washed with saturated $NaHCO_3$ (100 mL), brine (100 mL), and dried ($MgSO_4$). The solvent was removed under reduced pressure and the residue was purified by flash chromatography (2:1 EtOAc:hexane) to afford Boc-Tyr-Tyr-OMe as a white solid (1.3 g, 3.0 mmol, 84%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.37 (br, 2 H), 6.91 (d, J = 8.0 Hz, 2H), 6.80 (d, J = 8.0 Hz, 2H), 6.68 (d, J = 8.0 Hz, 4H), 6.55 (1H, buried amide NH), 5.27 (br, 1H), 4.72 (m, 1H), 4.29 (m, 1H), 3.64 (s, 3H), 2.96–2.89 (m, 4H), 1.40 (s, 9H).

To a solution of Boc-Tyr-Tyr-OMe (500 mg, 1.1 mmol) in THF (12.5 mL), an aqueous solution (12.5 mL) of $LiOH{\cdot}H_2O$ (92 mg, 2.2 mmol) was added dropwise while the reaction vessel was cooled on an ice bath. The resulting reaction mixture was subsequently stirred at room temperature for 4 h until all the starting material was consumed (monitored by TLC). Acetic Acid was added to adjust pH to 5. The reaction mixture was then extracted by EtOAc (3 × 15 mL). The combined organic phase was dried over $MgSO_4$. Evaporation of the solvent afforded **3** as a white solid, which was directly used in the next synthetic step without further purification.

4.6. N^{α}-[tert-butoxycarbonyl]-L-(O-sulfate)-tyrosyl-L-(O-sulfate)-tyrosine di-ammonium salt (**4**)

To a solution of **3** (450 mg, 1 mmol) in a mixture of anhydrous DMF (20 mL) and anhydrous pyridine (5 mL), SO_3/DMF complex (3.7 g, 24 mmol) was added. Under the protection of argon, the reaction was allowed to stir at 25 °C for 24 h. After the removal of solvent, a colorless residue was obtained. While being cooled on an ice-water bath, 30 mL of water was added on to the reaction vessel, followed by the addition of 20 mL of saturated $NaHCO_3$ until pH reached 4–5. Precipitation was then filtered out, and the filtrate was reduced to an appropriate volume. The product was purified on the diethylaminoethyl cellulose column by a gradient elution of 1 L NH_4HCO_3 buffer (0–0.8 M, pH ≈ 7.0, pH was adjusted by dry ice) in chromatography chamber at 4 °C. The collected fractions (8 mL in each tube) were analyzed by UV–vis spectroscopy. The fractions with strong UV absorbance at 265 nm (corresponding to 0.3–0.6 M NH_4HCO_3 fractions) were combined. The solvent of the combined pool was evaporated under reduced pressure. To the residues, 10–20 mL of frozen-dry MeOH was added to extract the desired compound. Evaporation of MeOH afforded a colorless solid of diammonium salt of **4** (453 mg, 0.71 mmol, 71%). ^{1}H NMR (MeOD, 300 MHz): δ 7.22–7.15 (m, 8H), 4.68–4.63 (dd, J = 8.0 Hz, 1H), 4.02–3.99 (dd, J = 8.0 Hz, 1H), 3.20–3.18 (m, 2H), 3.00–2.91 (m, 2H), 1.40 (s, 9H). ESI MS(+): $C_{23}H_{28}N_2O_{13}S_2$, theoretical = 604.10, $[M+H]^+$ = 605.10.

4.7. L-(O-sulfate)-tyrosyl-L-(O-sulfate)-tyrosine di-ammonium salt (**2**)

Compound **4** (453 mg, 0.71 mmol) was dissolved in the cold solution of TFA/water (9:1; 16 mL). The resulting reaction mixture was stirred at 0 °C for about 40 min, until all the starting material was completely consumed (monitored by TLC). Upon removal of TFA by rotary evaporation, the residue, as a foaming white solid, was washed twice by Et_2O and evaporated again to further remove TFA. To the residue, 4 mL of 0.05 M NH_4HCO_3 buffer (pH = 7) was added, and a diluted ammonium hydroxide solution was applied to adjust pH to 6–7. The product was purified by DEAE column with a gradient elution of NH_4HCO_3 buffer (0–0.8 M; 1 L). The desired compound was found in the 0.3–0.6 M NH_4HCO_3 fractions. The combined fractions were evaporated by under reduced pressure. To the residue, 10–20 mL of ice-cold dry MeOH was added to extract the desired compound. Evaporation of MeOH afforded the diammonium salt of **2** (343.8 mg, 0.64 mmol, 90%) as a colorless solid. ^{1}H NMR (D_2O, 400 MHz): δ 7.35–7.12 (m, 8H), 4.56–4.47 (dd, J = 8.0 Hz, 1H), 4.30–4.20 (dd, J = 8.0 Hz, 1H), 3.34–3.23 (m, 2H), 3.12–2.89 (m, 2H); ^{13}C NMR (D_2O, 175 MHz): δ 177.10, 168.05, 150.66, 149.81, 135.53, 131.63, 130.78, 130.39, 122.08, 121.46, 56.74, 54.02, 36.79, 36.01. HR-ESI MS(+): $C_{18}H_{20}N_2O_{11}S_2$, theoretical = 504.0508, $[M+H]^+$ = 505.0599.

4.8. Generation and titration of viral stocks

293T cells were grown at 37 °C with 5% CO_2, and maintained in DMEM medium with 4.5 g/L D-glucose and 584 mg/L L-Glutamine (Gibco), supplemented with 10% FBS and 1× penicillin-streptomycin. Twenty-four hours prior to the transfection, 1.5×10^6 cells were plated in 10 cm dishes coated with 0.01% Poly-L-lysine (Sigma-Aldrich). Cells were transfected by adding 6 μg of replication-deficient plasmid that encodes the genome of the pseudotyped HIV (pCMV-Gag-Pol), 600 ng of HIV envelope construct (pSVIIIenv), and 18 μL of FuGENE 6 (Promega) diluted in un-supplemented DMEM. Supernatants of cell cultures were

collected 48 h post-transfection, and passed through a 0.45 μM syringe tip filter. To perform infectivity assays using TZM-bl cells, viral stocks were diluted in four replicate 4-fold dilution series and added to 1.5×10^4 cells per well in 96-well plates. Tat-inducible β-galactosidase was measured at 48 h post-infection, and the Reed-Muench method was used to calculate $TCID_{50}$ based on endpoint staining.

4.9. Inhibition assay

TZM-bl cells were grown at 37 °C with 5% CO_2, and maintained in DMEM medium with 4.5 g/L D-glucose and 584 mg/L L-Glutamine, supplemented with 10% FBS and 1× penicillin-streptomycin. The day preceding infection, cells were seeded at 1.5×10^4 per well in 96-well, flat-bottom, black-sided plates (Grenier Bio-One). In a separate 96-well plate, the four compounds were serially diluted to a final volume of 150 μL with cell growth media, mixed with 50 μL cell growth media containing 200 $TCID_{50}$ pseudotyped-HIV, and incubated for 1 h at 37 °C with 5% CO_2. During this incubation, TZM-bl growth media was replaced with 50 μL media containing 160 μg/mL DEAE-Dextran (Sigma-Aldrich) (40 μg/mL final concentration). The compound-virus mixture was added to cells and incubated for 48 h. The luciferase assay kit (Promega) was used to detect Tat-inducible luciferase activity. Luminescence was measured on a Veritas luminometer (Turner Biosystems).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2016.07.012.

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