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Letter

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Structure-Based Design and Synthesis of an HIV-1 Entry Inhibitor Exploiting X-ray and Thermodynamic Characterization

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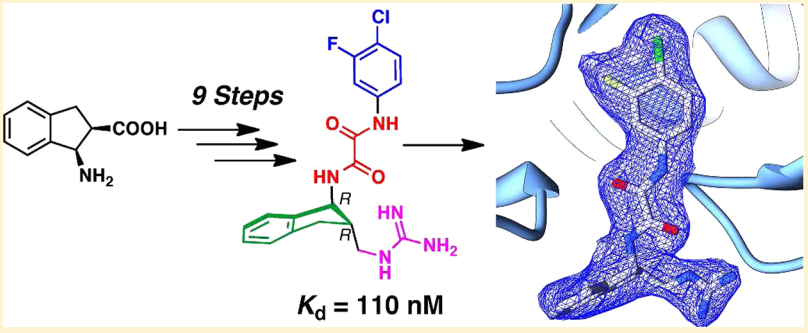
Supporting Information



ABSTRACT: The design, synthesis, thermodynamic and crystallographic characterization of a potent, broad spectrum, second-generation HIV-1 entry inhibitor that engages conserved carbonyl hydrogen bonds within gp120 has been achieved. The optimized antagonist exhibits a submicromolar binding affinity (110 nM) and inhibits viral entry of clade B and C viruses (IC_{50} geometric mean titer of 1.7 and 14.0 μM, respectively), without promoting CD4-independent viral entry. The thermodynamic signatures indicate a binding preference for the (*R*,*R*)- over the (*S*,*S*)-enantiomer. The crystal structure of the small-molecule/gp120 complex reveals the displacement of crystallographic water and the formation of a hydrogen bond with a backbone carbonyl of the bridging sheet. Thus, structure-based design and synthesis targeting the highly conserved and structurally characterized CD4−gp120 interface is an effective tactic to enhance the neutralization potency of small-molecule HIV-1 entry inhibitors.

KEYWORDS: *HIV, gp120, CD4, entry inhibitor, structure-based drug design, thermodynamics, X-ray crystallography, viral inhibition, protein−protein interactions*

The prevention of HIV-1 viral infection remains a worldwide public health objective.[1] Despite the continued reduction in AIDS-related deaths, an estimated 2.7 million adults were newly infected in 2010 with HIV-1.[2] Clinical trials of preventative approaches, including pre-exposure prophylaxis with oral antiretrovirals[3] and/or microbiocide formulations,[4] have demonstrated some success and, as such, continue to motivate the development of inhibitors that target alternative steps of the HIV-1 viral life cycle. Given that the initial step of viral infection comprises the attachment of gp120,[5] the major surface-exposed protein of the trimeric viral envelope spike,[6] to the human host cell receptor CD4,[7] inhibition of this protein−

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protein interaction is attractive and would appear to be a viable prophylactic strategy to prevent HIV-1 transmission. The viral attachment process consists of three stages: (**1**) gp120 binding to CD4; (**2**) a conformational change within gp120 induced by the protein−protein interaction that exposes the binding site for the secondary transmembrane chemokine receptors CCR5 or CXCR4; and (**3**) gp120−chemokine receptor binding, which promotes formation of a six-helix bundle within the gp41 component of the viral envelope spike.[5] This cascade of protein−protein interactions, triggered by conformational shifts,[8] culminates in fusion of the viral and host cell membranes to permit cellular infection and viral propagation. The development of small-molecule viral entry antagonists that target well conserved hot spots within the viral Env complex and block CD4−gp120 binding continues to hold significant potential for augmenting pre-exposure prophylactic approaches to prevent HIV-1 transmission.[4]

Mutagenesis studies have revealed key hot spots within the CD4−gp120 interface,[9] which were subsequently characterized structurally by a number of CD4/gp120 cocrystal structures.[10,11] One important hot spot, the internal Phe43 cavity, is formed at the interface of the three gp120 domains (inner, outer, and bridging sheet) and is capped by the aromatic side chain of CD4 residue Phe43. In fact, the crystal structure of the small molecule NBD-556[12] (**1**, Figure 1), first identified by

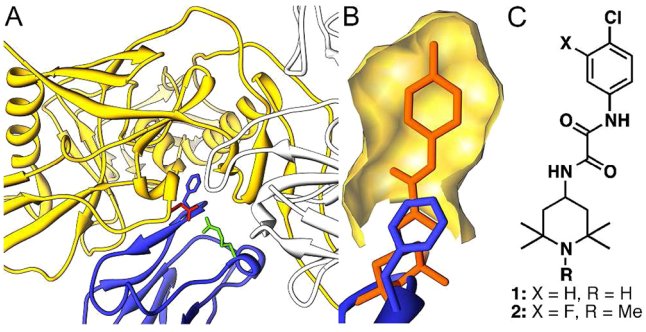

Figure 1. (A) CD4/gp120/17b structure (1GC1)[13] in blue, yellow, and light gray, respectively. The Phe43 side chain, blue, emanates from the CD4 β-turn. A second critical CD4−gp120 interaction between $Asp368_{gp120}$ and $Arg59_{CD4}$ is highlighted in red and green, respectively. (B) The NBD-556/gp120 structure (3TGS)[15] with similar orientation as part A. The surface of the Phe43 cavity is drawn in yellow with NBD-556 in orange. (C) NBD-556 (**1**) and TS-II-224 (**2**).

Debnath and co-workers,[13] reveals that **1** binds within the Phe43 cavity. Similarly, chemically derivatized CD4 constructs[14] and a miniprotein CD4 mimetic possessing a biphenyl side chain[15] form interactions deep within the Phe43 cavity. Subsequent synthesis and testing of NBD-based compounds by our group improved the interactions within the Phe43 cavity, affording the small molecule **2** (Figure 1).[16] However, **2** and other region III congeners did not appear to mimic the second hot spot, consisting of an electrostatic interaction between $Arg59_{CD4}$ and $Asp368_{gp120}$. Moreover, **1** and **2** functionally mimic CD4 binding and thus behave as agonists of CD4-independent viral entry, leading to the enhancement of HIV-1 entry into cells lacking the CD4 receptor ($CD4^-CCR5^+$ Cf2Th cells),[17] an undesired trait for the development of viral entry inhibitors.

Measurement of the binding thermodynamics of sCD4,[18,19] **1**, or **2**[20,21] to full-length, monomeric gp120 demonstrates that gp120 undergoes a large conformational change upon ligand binding, manifested by a thermodynamic signature reminiscent of protein folding (large unfavorable binding entropy and large favorable binding enthalpy). Not only do **1** and **2** competitively inhibit CD4 binding, but both compounds also mimic CD4 by structuring gp120 and triggering the allosteric signal that enhances coreceptor binding and undesired enhancement of viral infectivity. Interestingly, thermodynamic analysis of a large number of NBD-like small molecules revealed a wide distribution of enthalpic and entropic contributions (ΔH and $-T\Delta S$ terms vary by ±18.0 kcal/mol), even for compounds with similar binding affinities.[22] These results revealed the possibility of designing compounds that do not elicit the unwanted conformational structuring, and delineated a thermodynamic-based strategy for the optimization of those compounds. This strategy implies improving binding affinity (ΔG), while simultaneously minimizing the unfavorable binding entropy.[23] The optimization strategy essentially combines standard SAR with thermodynamic analysis by isothermal titration calorimetry (ITC). Importantly, thermodynamic-guided alanine scanning mutagenesis has recently demonstrated that gp120 residue contributions to allosteric structuring are not proportional to binding affinity, providing a structural foundation to the finding that not all NBD-like compounds elicit the unwanted allosteric structuring.[24]

We have previously described the design, synthesis, and characterization of guanidinium containing *trans*-1,2-indanes [(+)-**3** and (−)-**3**; Table 1].[21] Both (+)-**3** and (−)-**3** specifically inhibit viral infection of 42 Tier 2 clade B and C viruses, exhibiting geometric mean titers (GMT) IC_{50} of 7.9 μM and 8.9 μM, for the sensitive strains, respectively. We were pleased to observe that (+)-**3** also displays an improved thermodynamic signature, with a reduced entropic penalty ($-T\Delta S$) upon gp120 binding, relative to **1** (ΔG, ΔH, and $-T\Delta S$ values of −7.4, −24.5, and 17.1 kcal/mol, respectively).[20] Furthermore, the undesired property of enhancing CD4 independent viral entry was essentially eliminated (Table 1). The cocrystal structure of (+)-**3** bound to gp120 revealed specific interactions between the guanidinium moiety and a water mediated hydrogen-bonding network spanning both $Asp368_{gp120}$ and $Met426_{gp120}$. Thus, we concluded that incorporation of the guanidinium had converted the NBD congeners into functional antagonists. We therefore sought to optimize further the interactions between the guanidinium moiety of (+)-**3** based on the cocrystal structure with residues $Asp368_{gp120}$ and $Met426_{gp120}$, an "affinity hot spot"[24], in an effort to improve the functional antiviral potency.

To improve these interactions, we chose to vary the distance between the *trans* indane ring system (region III) and the guanidinium functionality (region IV; Table 1). Hence, the binding properties of the methylene and ethylene congeners of (+)-**3** were evaluated by docking (see Supporting Information). These results led to selection of **4** as an initial synthetic target (Table 1). Initially, (±)-**4** was constructed (see Supporting Information). When assessed in a single-round viral infection assay, (±)-**4** demonstrated a 2-fold improvement of the IC_{50} value (10.3 ± 3.2 μM) relative to (+)-**3** (22.9 ± 2.4 μM). Titration of gp120 with (±)-**4**, employing ITC, resulted in a complex binding curve that suggested more than one binding event (Figure 2). We reasoned that this observation was related to one enantiomer having a higher affinity within the mixture of (±)-**4**.

We turned next to X-ray crystallography to investigate the interactions between antagonist (±)-**4** and gp120 and to define

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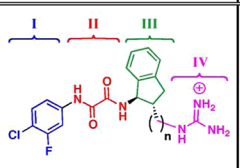
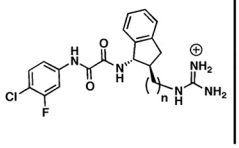
Table 1. Antagonists of CD4-gp120 Binding and HIV-1 Entry

Compound		HIV-1, YU-2 IC_{50} (μM)[a]	A-MLV IC_{50} (μM)[b]	Activation of Viral Infectivity[c]	K_D (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)
(+)-**3**, (n=0)		22.9 ± 2.4	> 100	0.1 ± 0.0 (19) (relative to **2**)	0.25	–9.0	–14.9	+5.6
(+)-**4**, (n=1)		2.3 +/- 0.05 (11)	> 100	0.0 ± 0.0 (4)	0.11 +/- 0.03	-9.50 +/- 0.1	-17.9 +/- 1.0	+8.4 +/- 0.6
(+)-**5**, (n=2)		28.2 +/-5.9 (3)	> 100	0.17 ± 0.15 (2)	0.34 +/- 0.03	-8.8 +/- 0.06	-11.4 +/- 0.5	+2.6 +/- 0.2
(–)-**3**, (n=0)		21.3 ± 5.0	> 100	0.0 ± 0.0 (4) (relative to **2**)	0.3	–9.0	–19.4	+10.4
(–)-**4**, (n=1)		37.9 +/- 22.7 (4)	> 100	0.11 ± 0.11 (2)	6.2 +/- 1.0	-7.1 +/- 0.03	-19.7 +/- 1.5	+12.6 +/- 1.0
(–)-**5**, (n=2)		68.5 +/-31.5 (3)	> 100	0.0 ± 0.0 (2)	2 +/- 0.3	-7.8 +/- 0.09	-14.7 +/- 0.5	+6.9 +/- 1.0

[a]The IC_{50} was determined in Cf2Th-CD4/CCR5 cells infected with HIV-1 YU2 virus. [b]The IC_{50} in cells infected with amphotropic murine leukemia virus (A-MLV). [c]The relative activation of viral infectivity in CD4 negative Cf2Th-CCR5 cells infected with HIV-1 YU2 virus normalized to that of **1**. Data for (+)-**3** and (–)-**3** have been published.[22] See experimental details in the Supporting Information.

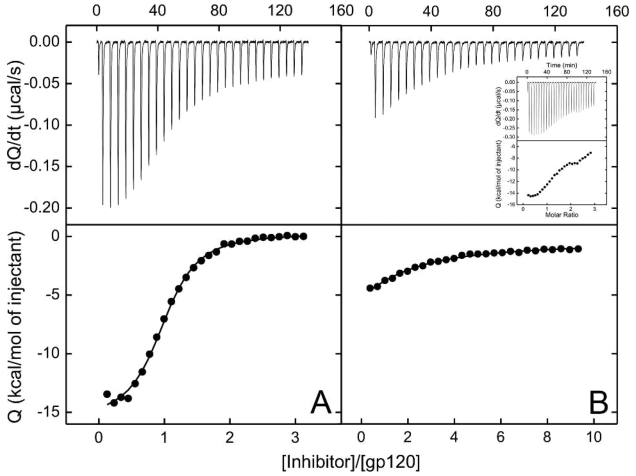
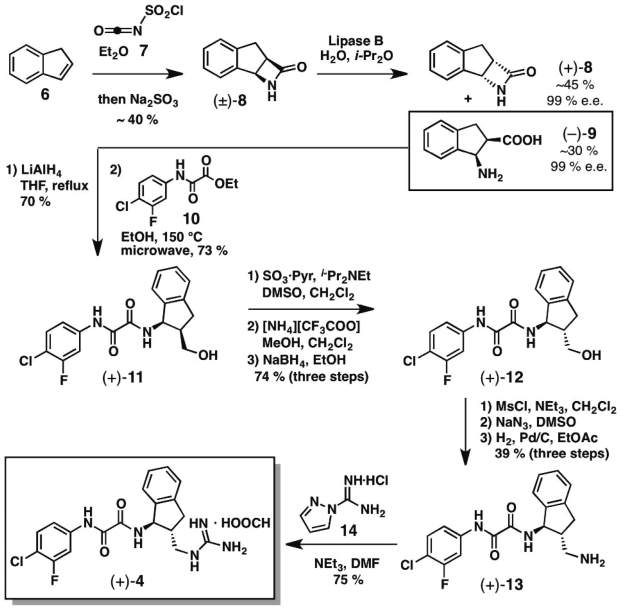


Figure 2. ITC titrations of gp120 with (A) (+)-**4** and (B) (–)-**4** at 25 °C. The titration with (±)-**4** (inset) resulted in a complex binding curve (see text).

Scheme 1. Synthetic Route to Single Enantiomer (+)-4



the enantiomer that preferentially binds to the gp120 core. The formate salt of (±)-**4** was soaked into preformed crystals of gp120 from Clade C1086,[12] and diffraction data were obtained to 2.5 Å Bragg spacings (Supporting Information Table S1). The observed electron density for each of the two **4**/gp120 complexes in the asymmetric unit clearly revealed preferential binding of the (*R*,*R*)-**4** enantiomer to gp120 during the soaking process (Supporting Information Figure S1). Interestingly, the (*R*,*R*)-**4**/gp120 crystal structure revealed that the guanidinium moiety did not directly interact with $Asp368_{gp120}$.

With the crystal structure suggesting that the (*R*,*R*)-**4** enantiomer possesses higher affinity for gp120, a synthetic route to the single (*R*,*R*)-enantiomer was developed (Scheme 1). Toward this end, [2 + 2] cycloaddition of indene (**6**) and chlorosulfonyl isocyanate (**7**) furnished the racemic β-lactam (±)-**8**. A kinetic resolution employing lipase B was carried out to yield a separable mixture of β-amino acid (–)-**9** and β-lactam (+)-**8**, both in 99% ee.[25−27] The enantiomeric excess was determined by supercritical fluid chromatography (SFC), as described in the Supporting Information. The β-amino acid (–)-**9** was next converted to the *cis* indanol (+)-**11** in two steps.[21] Although the initial synthetic plan to incorporate a primary amine via oxidation to the aldehyde, followed by reductive animation, proved unsuccessful, we were pleased to find that mildly acidic conditions led to epimerization of the α stereocenter. Subsequent reduction with sodium borohydride established the desired *trans* stereochemical relationship [cf. (+)-**12**]. A three-step sequence involving mesylation, displacement of the mesylate with sodium azide, and reduction of the azide led to amine (+)-**13**. Finally, installation of the guanidinium functionality employing 1*H*-pyrazol-1-carboxamidine monohydrochloride (**14**) furnished (+)-**4** (99% ee by chiral SFC).[21] An identical synthetic sequence was employed to furnish (–)-**4** following opening of the β-lactam (+)-**8**.

Antiviral assays revealed that (+)-**4** inhibits viral entry of the YU-2 primary HIV-1 isolate with an IC_{50} value of 3.1 ± 0.6 μM, while the (–)-**4** antipode exhibits a 10-fold reduction in antiviral activity, with an observed IC_{50} = 37.9 ± 22.7 μM (Table 1). To assess further the HIV-1 neutralization breadth and potency, we assayed **1**, (+)-**3**, and (+)-**4** against 42 diverse strains of clades B and C Env-pseudoviruses (see Table S2 in

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the Supporting Information). As previously observed for **1** and (+)-**3**,[21] (+)-**4** also neutralized clade B viruses better than clade C viruses, with 100% breadth and an IC_{50} GMT of 1.7 μM against clade B viruses, compared to 59% breadth and an IC_{50} GMT of 14.0 μM against the sensitive clade C viruses. Moreover, (+)-**4** demonstrated a 60% improvement over (+)-**3** based on IC_{50} titers in clade B viruses and a 1.5-fold improvement based on IC_{80} titers. In addition, ITC measurements found that (+)-**4** binds full-length gp120 with K_d = 110 nM (Table 1 and Figure 2). In contrast, (−)-**4** has a significantly reduced binding affinity of 6200 nM. These results are consistent with the gp120-bound cocrystal structure derived from (±)-**4**, suggesting the (*R*,*R*)-enantiomer preferentially binds to the monomer gp120 core.

Given that inclusion of the methylene spacer between regions III and IV led to significant improvements in both binding affinity and functional antagonism of HIV-1 viral entry into target cells, we constructed (±)-**5**, containing an additional methylene spacer between the indane scaffold and the guanidinium moiety (see Supporting Information). Semipreparative chiral SFC furnished samples of (+)-**5** and (−)-**5** for biological evaluation. Assessment of the functional antagonist activity of (+)-**5** and (−)-**5** revealed that both were less potent than (+)-**4** (Table 1). Evaluation of compounds (+)-**4**, (−)-**4**, (+)-**5**, and (−)-**5** by ITC (Table 1 and Figure 2) when compared to (+)-**3** and (−)-**3** demonstrates that (+)-**4** exhibits the best submicromolar binding affinity observed thus far for a small-molecule/gp120 complex. Moreover, (+)-**3**, (+)-**4**, and (+)-**5** all exhibit improved binding affinities when compared to the corresponding (−)-antipodes. Examination of the thermodynamic signatures (Table 1) reveals that (−)-**3**, (−)-**4**, and (−)-**5** exhibit a larger favorable enthalpy coupled to a larger compensatory unfavorable entropy, suggesting that these compounds trigger a more significant structuring in gp120 than the (+)-**3**, (+)-**4**, and (+)-**5** compounds. The smaller entropic penalty for (+)-**3**, (+)-**4**, and (+)-**5** indicates that the (*R*,*R*)-configuration possesses the greatest potential for improving enthalpic contributions to binding that are not related to gp120 structuring. In general, improving inhibitor−gp120 interactions should be reflected in better enthalpic interactions not correlated to the structuring of gp120.[22]

Crystallography was once more employed to ascertain the specific binding interactions between gp120 and (+)-**4**. Cocrystallization of (+)-**4** with the clade A/E93TH057 extended $gp120_{(H375S)}$ core[12] produced crystals that diffracted to 2.5 Å spacings (crystallographic data are summarized in Table S1 in the Supporting Information). There are two complexes in the asymmetric unit of these crystals, and each (+)-**4** molecule in both complexes has similar conformations that closely resemble those observed in the (*R*,*R*)-**4**/gp120 structure obtained from (±)-**4** (Supporting Information Figure S1 and Table S3). As expected, the previously observed hydrogen bonds between the oxalamide linker and the $Asn425_{gp120}$ and $Gly473_{gp120}$ amide nitrogen atoms[12,21] are preserved in the (+)-**4**/gp120 complex (Figure 3). Surprisingly, as noted for (*R*,*R*)-**4**/gp120 (*vide supra*), the guanidinium moiety did not directly interact with $Asp368_{gp120}$. Instead, a hydrogen bond is formed between one guanidinium nitrogen and the bridging sheet backbone carbonyl of $Met426_{gp120}$. Importantly, the crystallographic water molecules necessary for the indirect interaction with $Met426_{gp120}$ in the (+)-**3**/gp120 structure are now displaced by the extended guanidinium of the

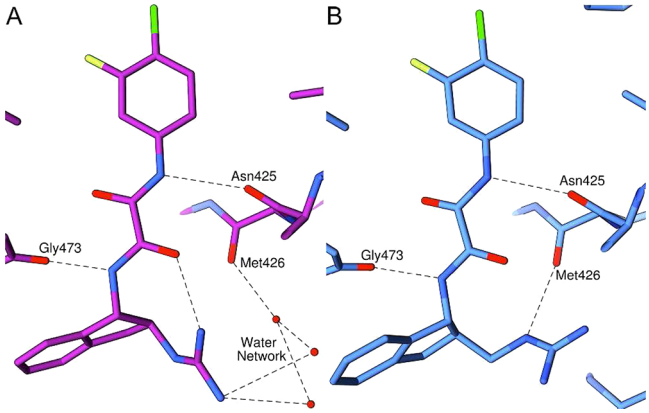

Figure 3. Comparison of the structures of (A) (+)-**3**/gp120 (4DKQ)[21] and (B) (+)-**4**/gp120 (Clade E, copy A) indicates that (+)-**3** interacts with $Met426_{gp120}$ via a network of water molecules whereas the guanidinium group of (+)-**4** hydrogen bonds directly to the backbone carbonyl of $Met426_{gp120}$. Coloring is by atom type: nitrogen (blue), oxygen (red), chlorine (neon-green), fluorine (neon-yellow-green), and carbon [magenta for (+)-**3** and sky blue for (+)-**4**]. Hydrogen bonds are represented by dashed lines.

(+)-**4**/gp120 (Figure 3), allowing for direct hydrogen bonding to the carbonyl of $Met426_{gp120}$. The direct hydrogen bond and the displacement of crystallographic water molecules, as well as the improved binding affinity of (+)-**4** over the previous described (+)-**3**, provide additional motivation to modify region III moieties. Further modification of the guanidinium-$Asp368_{gp120}$ contact, as previously observed in the (+)-**3**/gp120 complex with the newly revealed guanidinum−$Met426_{gp120}$ interaction exhibited in the (+)-**4**/gp120 structure, will in the future yield even more potent inhibitors of viral entry.

In summary, we have employed the crystal structures of antagonist/gp120 complexes to achieve an increase of both binding affinity and antiviral potency for HIV-1 entry inhibitors. The crystal structure of this new compound, (+)-**4**, bound to gp120, reveals a newly formed hydrogen bond to $Met426_{gp120}$ and the displacement of crystallographic water derived from the extension of the guanidinium group via a methylene spacer. Whereas the parent compounds (−)-**3** and (+)-**3** had comparable potency and bound alternatively to gp120, the (*R*,*R*)-enantiomer (+)-**4** is strongly preferred and binds uniquely. On the basis of the antiviral potency and binding affinity, the methylene spacer between regions III and IV of (+)-**4** is favored compared to the ethylene spacer of (+)-**5**. Furthermore, the thermodynamic signatures indicate that the (*R*,*R*)-stereochemical configuration encounters less entropic penalty than the (*S*,*S*) one when binding to gp120. The design, development, and synthesis of small-molecule viral entry antagonist (+)-**4** thus validates the application of static structures of ligands bound to the conserved monomeric core of gp120 to improve antagonist potency. Structural information from small-molecule/gp120 complexes, however, must be combined with thermodynamic information, as allosteric structuring and solvation effects are not revealed in the X-ray structures. Moreover, the improved antiviral potency and structural characterization of (+)-**4** provides an important contribution to small-molecule viral entry antagonists for prophylactic strategies for the prevention of HIV-1 transmission.

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■ ASSOCIATED CONTENT

Supporting Information

Synthesis, experimental methods, and crystallographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Coordinates and structure factors have been deposited in the Protein Data Bank with the following accession numbers: 4I53 and 4I54.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

(HIV-1), Human immunodeficiency virus type 1; (SIV), simian immunodeficiency virus; (sCD4), soluble CD4; (ITC), isothermal titration calorimetry; (A-MLV), amphotropic murine leukemia virus; (GMT), geometric mean titer; (GA), genetic algorithm; (HRMS), high-resolution mass spectroscopy; (DMEM), Dulbecco's Modified Eagle Medium; (TsCl), tosyl chloride; (DMAP), 4-dimethylaminopyridine

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