Use of a Hexasubstituted Benzene Scaffold in the Development of Multivalent HIV-1 Integrase Inhibitors

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The highly directional hexasubstituted benzene moiety was used as the central scaffold to create new human immunodeficiency virus (HIV)-1 integrase inhibitors through the attachment of multiple active groups. A series of potential inhibitors having substituted polyhydroxylated mono, bis and tris-cinnamoyl derivatives connected on the scaffold were prepared through Claisen–Schmidt condensations with substituted benzaldehydes, followed by partial demethylation to uncover the active phenolic groups required for the interactions with the integrase enzyme active sites. Using a multiplate integration assay method, four compounds carrying at least two sets of interacting moieties were found to be relatively potent integrase inhibitors with IC50 values in the low micromolar range. The results confirmed that multiple polyhydroxylated groups were required on the platform in order to effectively interact with the enzyme. The results from molecular docking studies consistently complemented the experimental results and revealed the nature of the potential key binding interactions responsible for the apparent activity of the active compounds.

Key words multimvalent drug; hexasubstituted benzene; integrase inhibitor; cinnamoyl; polyhydroxylated aromatic

Multivalency, or polyvalency, the presence of multiple structural units connected to a central core that could induce simultaneous interactions with another entity or receptor, has become a ubiquitous strategy for the design of new biologically active agents.1–3) Multivalent compounds are potentially more effective than traditional one-site ligands due to the favorable multiple interactions with the desired targets. The design also allows one to diversify the interactive groups to suit the existing binding sites of the target, and facilitate the process of identifications of these binding units. The potential complementary binding, and diversity available on such a platform allows these molecules to closely resemble the actual biochemical interactions of the target enzymes with their substrates or inhibitors. Among many reported core platforms, hexasubstituted benzene derivatives are of particular interest.4,5) They are easy to prepare and can, in principle, hold up to six active groups. These groups are also uniquely arranged into two sets where three meta-arranged substituents point perpendicularly outward from one face of the central aromatic plane, while the other alternating arms are directed out from the opposite face (Fig. 1). This conformational rigidity potentially provides favorable synergistic interactions within the binding sites of the target enzyme. Such pre-organized control of directionality is quite unique and rarely found in other platforms.

One of the potential targets for this multivalent scaffold is the human immunodeficiency virus (HIV) integrase enzyme (IN). This enzyme has no counterpart in the human host cell and hence its inhibitors tend to show little or no side effect in humans. Currently there are only a few drugs of this class available.7,8) Among many agents investigated, the polyhydroxylated aromatics and their related derivatives repeatedly appear as one of the most potent classes of active IN inhibitors. Their activities were shown to inhibit both 3′-processing and strand transfer reactions of the enzyme in the submicromolar or nanomolar ranges. Compounds with two catechol units separated by a central linker have been shown to be more potent than compounds having only one such unit, indicating the apparent benefits of multivalent binding. Some of the leading active compounds in this class are divalent, including caffeic acid phenethyl ester (CAPE), 1-chicoric acid and its 3,5-dicaffeoyl derivatives,9–13) and a series of cinnamoyl or chalcone pharmacophores14–17)

Although current treatments for AIDS and HIV infected patients have shown significant improvements over those used previously, the eventual eradication of the virus has not yet been achieved.18,19) With the emergence of new strains of the virus showing resistance against the available drugs, the continued search for new therapeutic agents and targets at different stages of the viral life cycle is essential. It is widely accepted that the most effective strategy for HIV treatment requires several inhibitors combined in one dose, hereby known as highly active retroviral therapy (HAART). An addition of IN inhibitors as part of this strategy would expand the alternatives for new formulations and potentially raise their activities, especially against the resistant virus strains.

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In this report, we tested the feasibility of the hexasubstituted aromatic platform as a central linker between groups of polyhydroxylated aromatics as the basis for new inhibitors. Up to three groups were connected onto the platform as mono, bis and tris-cinnamoyl or chalcone-like analogs with various patterns of hydroxyl-, or methoxy substituents on the flanking aromatic rings. Without the ester linkages employed in many other scaffolds, the possible susceptibility toward hydrolytic enzymes was eliminated. The effect of the crowded rigid platform would be to stretch out the cinnamoyl groups and constrain them into the preferable syn disposition of the carbonyls with respect to their vinylic double bonds. To figure out the nature of the fundamental interactions and the multivalency effect of the binding groups, the positions and numbers of free hydroxyl groups, and the number of cinnamoyl moieties attached onto the core platform were varied over the series. The inhibitory activities of the synthesized compounds were evaluated against the integrase enzyme using a multiplate integration assay. Finally, computational docking studies of the potential enzyme-substrate complexes were performed in an attempt to determine the interactions between the active moieties of these potential inhibitors and the bound amino acid residues within the enzyme. Correlations between theory and the experimental bioactivity results are also discussed herein.

Results and Discussion

Synthesis The triacetyl derivative of phloroglucinol 2 was selected as the hexasubstituted benzene core precursor due to its preparative simplicity and the possibility for further functionalization through both the acetyl- and hydroxyl groups as shown in Chart 1. The compound could be made in good yield from the acetylation of phloroglucinol 1 based on reported procedures or using our own developed methodology. In this report, we focused on the functionalization of 2 only through the acetyl groups. To obtain the alternating arrangements as shown in Fig. 1, compound 2 was subjected to excessive methylation of all phenolic hydroxyl groups to yield 1,3,5-triacetyl-2,4,6-trimethoxybenzene. Claisen–Schmidt condensations of the acetyl groups with selected benzaldehyde derivatives allowed up to three pharmacophores to be attached onto the central platform, giving rise to the multi-cinnamoyl analogs. Typical conditions as reported previously were attempted first with unsatisfactory results, providing only low yields of mixtures of incomplete condensed products, even at very long reaction times. It was later found that unusually high concentrations of hydroxide solution were needed to induce the effective condensations at room temperature to afford reasonable yields of mixtures of mono- (5), bis- (6) and tris-cinnamoyl (7) products, in various ratios. Because the main purpose is to obtain compounds having a range of substitution patterns in order to screen and compare their potential activities, the synthetic procedures developed here were

Reagents and conditions: (i) AcCl, AlCl₃; (ii) (CH₃)₂SO₄, K₂CO₃, CH₃CN; (iii) 80% KOH, MeOH and benzaldehyde derivatives 4 (4a: 3-hydroxybenzaldehyde, 4b: 4-hydroxybenzaldehyde, 4c: vanillyl); (iv) AlCl₃, pyridine/CH₂Cl₂.

Chart 1
not optimized to obtain the maximum yields of any particular products. Rather, we deliberately interrupted the reactions to obtain a mixture of the three desired products from each of the consecutive three steps of condensations, and subsequently separated each of them by column chromatography to allow the availability of all three condensed products for bioactivity tests. Longer reaction times and higher stoichiometric ratios of the substituted benzaldehyde derivatives would be expected to drive the reactions towards completion, and therefore provide higher yields of the tris-cinnamoyl products.

Under the conditions described above, mono-cinnamoyl compounds 5a–c were obtained as the major products from their corresponding condensations, along with sequentially lesser amounts of bis-cinnamoyl compounds 6a–c, and tris-cinnamoyl compounds 7a–c, respectively. The reactions of these benzaldehyde derivatives were rather sluggish and sometimes resulted in the formation of unknown byproducts that complicated the successive purification processes. Consequently, we prepared the multi-phenolic derivatives 5d, 6d, e and 7d–f from their methoxy precursors 5c, 6c and 7c respectively. Demethylations using AlCl$_3$ and pyridine could be controlled to remove some or all of the methyl groups to give both symmetric and non-symmetric compounds.$^{30}$ Interestingly, the inner three methoxy groups on the central aromatic core were not affected by this reaction and all remained intact in the products.

Because the presence of multiple phenolic groups in the molecule potentially raises the activity, complete demethylations including those methoxy groups on the hexasubstituted benzene core would generate another desirable group of active compounds. After our earlier failed attempts on core demethylation reactions, we decided to build these series from the 1,3,5-triacetyl-2,4,6-trihydroxybenzene precursor using a similar condensation strategy. Unfortunately, we once again encountered sluggish reactions, presumably resulting from the presence of the extra phenolic groups. Small amounts of cyclized flavonoid-like compounds derived from intramolecular additions of the free phenolic hydroxyl groups to the adjacent unsaturated ketones were unexpectedly obtained as products.$^{31-33}$

### IN Inhibition Assays

The studies on HIV-1 IN inhibitory activity were carried out using the reported multiplate integration assay (MIA) method.$^{21,22}$ Suramin was used as the positive control. Only the compounds showing relatively high activities were carried on to subsequent tests at lower concentrations. The results are shown in Table 1.

The results showed that the core structure compounds 2 and 3 without the extending cinnamoyl arms appeared inactive. The rather compact size of 2 and the lack of hydroxyl binding groups in 3 were assumed to be possible reasons for their inactivity. These results eliminated the possibility that the core structure alone might be involved in the interactions with the enzyme binding sites. The series of mono-cinnamoyl analogs 5a–d also showed no activity against the integrase enzyme, suggesting that the presence of only one binding group was not sufficient to show any detectable effect, emphasizing the necessity of building in multivalent active groups. Adding one more cinnamoyl side chain (compound 6 series) showed improvement, with the derivative carrying two catechol moieties (6e) becoming highly active, in comparison to 6d, which has one group and was almost inactive. These results confirmed that a polyhydroxylated aromatic compound bearing more than one catecholic group was preferred, or even required, to become an effective integrase inhibitor.

The series of tris-cinnamoyl analogs 7a–f further underlined the above observations. While compound 7d with only one catecholic group was inactive, both bis-catechol 7e and tris-catechol 7f appeared to be quite potent inhibitors. Although the differences of the IC$_{50}$ values among 7e, f and that of bis-catechol 6e previously discussed were not substantial, with only slightly higher potency for 7f than 7e, this implied that at least two catecholic groups were needed for the inhibition potency. The concept of multivalency in inhibitor design was then justified, but these results indicated that there was no

| Compound | % Inhibition at concentration | IC$_{50}$ (µM) |
|----------|-------------------------------|---------------|
|          | 100 µM | 30 µM | 10 µM |          |
| 2        | 21.02±3.73 | —     | —     | >100     |
| 3        | 11.74±3.80 | —     | —     | >100     |
| 5a       | 17.91±1.61 | —     | —     | >100     |
| 5b       | NP$^{a}$ | —     | —     | >100     |
| 5c       | 25.63±4.89 | —     | —     | >100     |
| 5d       | 24.94±3.16 | —     | —     | >100     |
| 5e       | 36.12±4.08 | —     | —     | >100     |
| 6a       | 26.24±2.56 | —     | —     | >100     |
| 6c       | 33.07±0.05 | —     | —     | >100     |
| 6d       | 55.95±1.02 | 16.25±1.20 | 2.74±0.32 | 89.4 |
| 6e       | 90.36±0.03 | 86.88±1.60 | 59.46±1.91 | 3.5  |
| 6f       | 79.36±2.13 | 68.4±1.09  | 49.15±2.33 | 9.5  |
| 6g       | 40.45±1.27 | —     | —     | >100     |
| 6h       | 35.75±4.98 | —     | —     | >100     |
| 7a       | 44.36±3.30 | —     | —     | >100     |
| 7d       | 74.78±2.61 | 56.58±1.39 | 44.01±2.70 | 16.5 |
| 7e       | 93.60±0.94 | 81.03±0.13 | 75.75±1.65 | 7.0  |
| 7f       | 99.89±0.41 | 89.40±1.73 | 69.72±1.56 | 2.7  |

$^a$ NP=No positive result.
significant additive effect from adding more than two active pendant groups onto the molecules, at least for this particular platform.

An interesting result was observed with the unexpected activity of compound 7a, which lacks the catechol moiety but has three meta-hydroxyl groups on the flanking cinnamoyl appendages, showing a potent IC$_{50}$ value of 9.5 \mu M. The result indicated that the main interactions of the hydroxyl groups from either phenol or catechol side chains of the compounds with the enzyme relied heavily on those groups at the meta-positions, relative to the connections to the core linker. In this case, only the cinnamoyl derivative with three meta-hydroxyl groups could induce such interactions sufficiently. All derivatives prepared from the condensations with vanillin, in which the meta-hydroxyl group was masked as a methoxy group, showed significant activity. The fact that compound 6c having both hydroxyl and methoxy groups could potentially form more. Although compound 6d, e showed significant activity. The fact that 6c formed more

### Table 2. The Docking Fitness Scores of the Ligands with Their Interacting Residues within IN

| Compound | Score | Ligand | Amino acid residues | Distance (Å) |
|----------|-------|--------|---------------------|--------------|
| 5a       | 47.30 | 1-Ph-m-OH | ASP64              | 1.99         |
| 5b       | 51.38 | 1-Ph-p-OH | ASN155             | 2.44         |
| 5c       | 51.59 | 1-Ph-p-OH | ASP64              | 2.09         |
| 5d       | 51.12 | 1-Ph-m,p-di-OH | ASP64, ASN155 | 1.73, 1.64 |
| 6a       | 64.47 | 1-Ph-m-OH | GLN148             | 2.11         |
| 6b       | 60.54 | 3-Ph-p-OH | LYS159             | 2.06         |
| 6c       | 59.54 | 1-Ph-m-OMe | GLN148 | 1.64, 2.41 |
| 6d       | 61.15 | 1-Ph-m-OH | LYS159             | 1.93, 2.29 |
| 6e       | 58.15 | 1-Ph-p-OH | LYS159             | 2.00         |
| 6f       | 58.15 | 1-Ph-p-OH | LYS159             | 2.49         |
| 7a       | 73.49 | 1-Ph-m-OH | LYS159             | 2.09         |
| 7b       | 67.26 | 1-Ph-p-OH | LYS159             | 2.06         |
| 7c       | 70.62 | 3-Ph-p-OH | GLN148             | 1.64         |
| 7d       | 72.85 | 3-Ph-p-OMe | PHE139 | 2.00         |
| 7e       | 70.95 | 1-Ph-m-OH | LYS159             | 2.06         |
| 7f       | 69.32 | 1-Ph-p-OH | LYS159             | 2.08         |
| NPTH     | 73.18 | 1-NPTH-1-SO$_2$H | GLU 152 | 1.66         |

NPTH = naphthalene.
H-bonding interactions than 6d due to more catechol groups was reflected in its higher activity.

In the case of tris-cinnamoyl analogs, most compounds formed more than one H-bonding interaction with IN. The reason just discussed for the apparent activity of the bis-cinnamoyl 6d and e, i.e., the binding of the ligand to both LYS156 and LYS159, could as well be used to explain the greater activity of the tris-cinnamoyl 7e and f. The surprisingly active tris-m-hydroxycinnamoyl analog 7a was found to form H-bonding interactions with PHE139, and LYS159 as shown for 6e, the most active compound. This result underlined the leading importance of having free hydroxyl groups at meta-positions of the flanking cinnamoyl arms as a requirement to enhance binding to the IN active site.

In order to investigate the effect of the number of active groups on the binding efficiency, compounds 5d, 6e and 7f, which contained one, two, and three catechol groups respectively, were selected as a visual comparison (Fig. 2). It is clearly seen that the mono-cinnamoyl molecule 5d is located quite far from the IN amino acid residues LYS156 and LYS159, thus explaining its inactivity. On the other hand, the bis-cinnamoyl derivative 6e is bound to IN in very similar fashion to the tris-cinnamoyl compound 7f. Both compounds appear to have comparable activities. In addition, this result validated our earlier speculation that the third additional catechol group in 7f compared to 6e is not necessary for the enzyme binding and its addition does not enhance the associated biological activity.

The three most active compounds from the bioassays as reported in Table 1 (6e, 7a, f) shared similar binding patterns (Fig. 3), in which all compounds formed H-bonding interactions with the LYS159 residue of IN. Although the orientation upon binding of 7a was partly different from those of 6e and 7f, it could also form H-bonding interactions with PHE139, similar to 6e. In addition, the binding of these three compounds are also similar to that of Suramin, which forms H-bonding interactions with GLU152, LYS156, LYS159, ASP116 and SER119, reflecting in their comparable activities.

Conclusion

We have successfully prepared a series of cinnamoyl analogs based on the hexasubstituted benzene platform through
the Claisen–Schmidt condensations of 1,3,5-triacetyl-2,4,6-trimethoxybenzene and substituted benzaldehydes. The synthesized mono-, bis- and tris-cinnamoyl compounds displayed various patterns of hydroxyl and methoxy substituents on the flanking aromatic rings. These compounds became the first group of hexa-substituted benzene derivatives to be tested as potential biological inhibitors. The bioassays of these compounds indicated that two catecholic groups were needed to be present on the platform in order for the molecules to become active against the HIV-1 integrase enzyme. An additional binding group would just slightly improved the activity and may not be necessary to be included on the platform. However, an exception was noted when each of the flanking aromatic ring carried only one hydroxyl group at its meta-position relative to the connection to the core, as demonstrated by the activity of tris-cinnamoyl derivatives 7a. These results support the earlier observations regarding the requirement of multivalency for potential inhibitors. Molecular docking studies of these compounds supported the above results and suggested the importance of LYS156 and LYS159 of the IN being the key residues that participate in forming binding interactions. The importance of LYS156 and LYS159 of the IN being the key residues that participate in forming binding interactions with the active inhibitors. Now that the active functional key residues that participate in forming binding interactions with the active inhibitors. An additional compound 155.4, 127.3, 64.6, 32.6; HR-MS (ESI–methanol) m/z: (M+Na)⁺ 317.1019 (Calcd for C₁₃H₁₁NaO₆; 317.0996).

General Procedure A: Synthesis of Multi-cinnamoyl Analog (5a–c, 6a–c and 7a–c)\(^{14,28,29}\) A mixture of compound (588 mg, 4.0 mmol) and one of the corresponding benzaldehyde derivatives (4.0 mmol) in methanol (20–30 mL) was added slowly into a well stirred aqueous suspension of 80% w/v KOH (6.0 mL). The mixture was stirred at room temperature for 1–2 d and worked up with 10% HCl and extracted with ethyl acetate (3×50 mL). The organic extracts were collected and washed with brine (3×50 mL), dried with anhydrous Na₂SO₄ and the solvent was evaporated. The crude product was purified by gradient chromatography on silica gel column (hexane/ethyl acetate, 70:30, 60:40 and 20:80) to yield the corresponding pure products 5a–c, 6a–c or 7a–c.

(E)-1-[3,5-Diacetyl-2,4,6-trimethoxyphenyl]-3-(3-hydroxyphenyl) prop-2-enone (5a): Yellow solid (242 mg, 28% yield), mp 107–110°C; \(^{1}H\)-NMR (acetone-\(\delta\)): 7.46 (1H, d, \(J=16.0\) Hz, H-\(\beta\)), 7.29 (1H, t, \(J=8.0\) Hz, ArH), 7.20 (1H, d, \(J=8.0\) Hz, ArH), 7.17 (1H, s, ArH), 7.07 (1H, d, \(J=16.0\) Hz, H-\(\alpha\)), 6.96 (1H, d, \(J=8.0\) Hz, ArH), 3.79 (3H, s, –OCH₃), 3.73 (6H, s, –OCH₃), 2.53 (6H, s, –COCH₃); \(^{13}C\)-NMR (acetone-\(\delta\)): 7.96 (1H, d, \(J=8.0\) Hz, ArH), 7.39 (3H, s, –OCH₃), 3.73 (6H, s, –OCH₃), 2.53 (6H, s, –COCH₃); \(^{1}C\)-NMR (acetone-\(\delta\)): 200.6, 192.9, 158.7, 156.8, 146.9, 136.8, 130.9, 128.9, 127.5, 125.5, 121.2, 118.9, 115.8, 64.5, 64.2, 32.6; HR-MS (ESI–methanol) m/z: (M+Na)⁺ 421.1275 (Calcd for C₁₆H₁₅NaO₆; 421.1258).

(E)-1-[3-Acetyl-5-[\(-\)3-hydroxyphenyl]prop-2-enoyl]-2,4,6-trimethoxyphenyl]-3-(3-hydroxyphenyl) prop-2-enone (6a): Yellow solid (148 mg, 14% yield), mp 164–166°C; \(^{1}H\)-NMR (acetone-\(\delta\)): 7.51 (2H, d, \(J=16.0\) Hz, H-\(\beta\)), 7.28 (2H, t, \(J=8.0\) Hz, ArH), 7.20 (2H, d, \(J=8.0\) Hz, ArH), 7.19 (2H, s, ArH), 7.08 (2H, d, \(J=16.0\) Hz, H-\(\alpha\)), 6.95 (2H, d, \(J=8.0\) Hz, ArH), 3.77 (6H, s, –OCH₃), 2.55 (6H, s, –OCH₃), 3.70 (3H, s, –OCH₃); \(^{1}C\)-NMR (acetone-\(\delta\)): 200.7, 193.1, 158.8, 157.5, 156.9, 146.9, 136.8, 130.9, 128.9, 127.5, 125.5, 121.2, 118.9, 115.7, 64.2, 63.9, 32.6; HR-MS (ESI–methanol) m/z: (M+Na)⁺ 525.1540 (Calcd for C₂₀H₂₁NaO₆; 525.1520).

(E)-1-[3,5-Bis\((-\)3-hydroxyphenyl)prop-2-enoyl]-2,4,6-trimethoxyphenyl]-3-(3-hydroxyphenyl) prop-2-enone (7a): Yellow solid (43 mg, 3% yield), mp 244–246°C; \(^{1}H\)-NMR (acetone-\(\delta\)): 7.56 (3H, d, \(J=16.0\) Hz, H-\(\beta\)), 7.28 (3H, t, \(J=8.0\) Hz, ArH), 3.74 (9H, s, –OCH₃), 2.71 (3H, s, –OCH₃); \(^{13}C\)-NMR (acetone-\(\delta\)): 193.2, 158.8, 157.9, 146.8, 136.8, 130.9, 129.0, 125.4, 121.3, 118.9, 115.6, 63.9; HR-MS (ESI–methanol) m/z: (M+Na)⁺ 629.1782 (Calcd for C₂₆H₂₆NaO₆; 629.1782).

General Procedure A: Synthesis of Multi-cinnamoyl Analog (5a–c, 6a–c and 7a–c) The reaction was stirred under reflux for 1 h and quenched with 10% HCl (10 mL), water (150 mL) was added and the mixture was stirred under reflux for 13 h. The solvent was then removed and water (150 mL) was added. The resulted precipitate was filtered, washed with cool water and recrystallized from ethanol to give a white solid of 3: 6.50 g, yield 93%; mp 124–126°C (lit.\(^{23}\)) mp 122–124°C; \(^{1}H\)-NMR (CDCl₃-\(\delta\)): 3.75 (9H, s, –OH), 2.72 (9H, s, –COCH₃); \(^{13}C\)-NMR (CDCl₃-\(\delta\)): 205.1, 175.9, 103.3, 33.0; high resolution (HR)-MS (electrospray ionization (ESI)/methanol) m/z: (M+Na)⁺ 275.0539 (Calcd for C₁₃H₁₁NaO₆; 275.0532).

Synthesis of 1-(3,5-Diacetyl-2,4,6-trimethoxyphenyl)ethanone (1,3,5-Triacetyl-2,4,6-trimethoxybenzene, 3)\(^{20}\) Compound 2 (5.99 g, 23.8 mmol) was dissolved in acetonitrile (250 mL). Dimethyl sulfate (13.5 mL, 142.8 mmol) and K₂CO₃ (49.26 g, 357.0 mmol) were added and the mixture was stirred under reflux for 13 h. The solvent was then removed and water (150 mL) was added. The resulted precipitate was filtered, washed with cool water and recrystallized from ethanol to give a white solid of 3: 6.50 g, yield 93%; mp 124–126°C (lit.\(^{23}\)) mp 122–124°C; \(^{1}H\)-NMR (CDCl₃-\(\delta\)): 3.75 (9H, s, –OH), 2.72 (9H, s, –COCH₃); \(^{13}C\)-NMR (CDCl₃-\(\delta\)): 205.1, 175.9, 103.3, 33.0; high resolution (HR)-MS (electrospray ionization (ESI)/methanol) m/z: (M+Na)⁺ 275.0539 (Calcd for C₁₃H₁₁NaO₆; 275.0532).

Synthesis of 1-(3,5-Diacetyl-2,4,6-trimethoxyphenyl)ethanone (1,3,5-Triacetyl-2,4,6-trimethoxybenzene, 3)\(^{20}\)
for 10min and pyridine (1.0mL) was added. The reaction was then stirred under reflux for 30h and worked up with 10% HCl. The separated water layer was extracted with ethyl acetate (3×50mL). The organic extracts were combined with the previous dichloromethane layer and washed with brine (3×50mL) and dried with anhydrous Na2SO4. Evaporation of the solvent and purification of the crude product by gradient chromatography on silica gel column (hexane–ethyl acetate = 80:30, 70:40) afforded unreacted 5c: 60mg (29% recovered) and pure product 5d.

(E)-1-(3,5-Diacetyl-2,4,6-trimethoxyphenyl)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]-2,4,6-trimethoxyphenyl]-3-(4-hydroxy-3-prop-2-enoyl]-2,4,6-trimethoxyphenyl]-3-(3,4-di-TLC for C29H20O10Na: 571.1575.

(E)-1-[3-Acetyl-5-{(E)-3-(3,4-dihydroxy-4-methoxyphenyl)prop-2-enoyl}-2,4,6-trimethoxyphenyl]-3-(3,4-dihydroxyphenyl)prop-2-enoyl]-2,4,6-trimethoxyphenyl]-3-(3,4-dihydroxyphenyl)prop-2-enoyl]-2,4,6-trimethoxyphenyl]-3-(3,4-di-
(CH2Cl2–methanol=99:1) to recover the unreacted 7c: 37 mg (9% recovered) and pure products 7d, 7e and 7f.

(E)-1-[3,5-Bis[(E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]-2,4,6-trimethoxy-phenyl]-3-(3,4-dihydroxyphenyl)prop-2-ene (7d): Orange solid (31 mg, 7.4% yield), mp 152–154°C; 1H-NMR (acetone-d6) δ: 7.49 (2H, d, J=16.0Hz, H-β), 7.44 (1H, d, J=16.0Hz, H-α), 7.38 (2H, s, ArH), 7.28 (2H, d, J=8.0Hz, ArH), 7.21 (1H, s, ArH), 7.06 (1H, d, J=8.0Hz, ArH), 6.99 (2H, d, J=16.0Hz, H-β), 6.88 (2H, d, J=4.0Hz, ArH), 6.86 (1H, d, J=16.0Hz, H-α), 6.83 (1H, d, J=4.0Hz, ArH), 3.91 (6H, s, –OCH3); 13C-NMR (acetone-d6) δ: 193.4, 193.1, 157.1, 157.0, 151.6, 150.8, 150.7, 148.9, 147.7, 147.4, 127.3, 126.5, 125.9, 125.8, 125.5, 124.8, 123.6, 116.6, 116.3, 115.1, 111.8, 63.9, 63.8, 56.4; HR-MS (ESI–methanol) m/z: (M+Na)+ 705.1965 (Calcd for C38H36O12Na: 705.1952).

(E)-3-((3,4-Dihydroxyphenyl)-1-[(E)-3-((3,4-dihydroxyphenyl)prop-2-enoyl)-2,4,6-trimethoxyphenyl]prop-2-ene (7e): Orange solid (27 mg, 6.5% yield), mp 168–170°C; 1H-NMR (acetone-d6) δ: 7.48 (1H, d, J=16.0Hz, H-β), 7.42 (1H, s, ArH), 7.39 (2H, (J=20Hz, H-α), 7.23 (2H, s, ArH), 7.22 (1H, d, J=8.0Hz, ArH), 7.08 (2H, d, J=8.0Hz, ArH), 6.99 (1H, d, J=16.0Hz, H-β), 6.88 (1H, d, J=4.0Hz, ArH), 3.90 (3H, s, –OCH3), 3.71 (9H, s, –OCH3); 13C-NMR (acetone-d6) δ: 191.9, 155.6, 149.3, 147.4, 147.1, 147.6, 147.0, 148.9, 148.0, 147.6, 147.1, 127.4, 126.9, 126.5, 125.9, 125.8, 124.8, 123.3, 116.5, 116.3, 115.5, 111.8, 63.9, 56.4; HR-MS (ESI–methanol) m/z: (M+Na)+ 691.1801 (Calcd for C37H34O11Na: 691.1786).

(E)-1-[3,5-Bis[(E)-3-((3,4-dihydroxyphenyl)prop-2-enoyl)-2,4,6-trimethoxyphenyl]-3-(3,4-dihydroxyphenyl)prop-2-ene (7f): Orange solid (10 mg, 2.4% yield), mp 166–170°C; 1H-NMR (acetone-d6) δ: 7.49 (2H, d, J=16.0Hz, H-β), 7.42 (1H, d, J=16.0Hz, H-α), 7.39 (2H, (J=20Hz, H-α), 7.23 (2H, s, ArH), 7.22 (1H, d, J=8.0Hz, ArH), 7.08 (2H, d, J=8.0Hz, ArH), 6.99 (1H, d, J=16.0Hz, H-β), 6.88 (1H, d, J=4.0Hz, ArH), 3.90 (3H, s, –OCH3), 3.71 (9H, s, –OCH3); 13C-NMR (acetone-d6) δ: 193.4, 193.2, 157.2, 157.1, 150.8, 150.7, 148.9, 148.0, 147.6, 147.1, 147.6, 147.0, 148.9, 148.0, 147.6, 147.1, 127.4, 126.9, 126.5, 125.9, 125.8, 124.8, 123.3, 116.5, 116.3, 115.5, 111.8, 63.9, 56.4; HR-MS (ESI–methanol) m/z: (M+Na)+ 677.1630 (Calcd for C36H32O12Na: 677.1629).

Oligonucleotide Substrates Oligonucleotides of long terminal repeated bases from donor DNA (LTRD) and from target substrate DNA (TS) were purchased from QIAGEN Operon, U.S.A., and stored at −80°C before use.

Annealing of the Substrate DNA LTR-D1 and LTR-D2, and TS-1 and TS-2 were mixed separately and then the former solution was diluted to a concentration of 2 pmol/mL, while the latter one was made to 5 pmol/mL by diluting with KTE (a buffer solution containing 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 100 mM KCl). Both solutions were heated at 85°C for 15 min in water bath. Each sample solution was then cooled gradually to room temperature in water bath for 3 h and stored at −20°C.

Pretreatment of the Multiplex To a 96-well plate was added 50 µL of a steptavidin solution containing 40 µg/mL steptavidin, 90 mM Na2CO3 and 10 mM KCl. The microplate was covered with plastic seal and allowed to stand gently overnight at 4°C (for coating). After discarding the steptavidin coating solution, the coated microplate was washed with phosphate buffer saline (PBS) solution (300 µL) twice. The blocking buffer (300 µL) containing 1% skim milk in PBS was added into each well and the plate was kept at room temperature for 30 min (for blocking). After discarding blocking buffer, each well was washed with PBS solution (300 µL) twice and the PBS solution was completely removed. A biotinylated LTR donor DNA (50 µL) solution containing 10 mM Tris–HCl (pH 8.0), 1 mM NaCl and 40 µmol/mL of LTR donor DNA was added into each well and mixed gently at room temperature for 30 min (for adsorption). After discarding the LTR donor solution, the microplate was washed with PBS solution (300 µL) twice and each well was filled with 300 µL of PBS (if necessary, stored at 4°C). Just before the integration reaction, the PBS solution was discarded and each well was rinsed with 300 µL distilled water.

Multiplex Integration Assay (MIA) The integration reaction was evaluated using the method previously described.21,22 A mixture (45 µL) composed of 12 µL of IN buffer [containing 150 mM 3-(N-morpholino)propane sulfonic acid pH 7.2 (MOPS), 75 mM MnCl2, 5 mM dithiothreitol (DTT), 25% glycerol and 500 µg/mL bovine serum albumin], 1 µL of 500 µmol/mL digoxigenin-labelled target DNA and 32 µL of sterilized water were added into each well of a 96-well plate. Subsequently, 6 µL of a sample solution and 9 µL of 1/5 dilution of integrase enzyme was added to the mixture in the designated well and incubated at 37°C for 80 min. The content in each well was washed with PBS four times, and then 100 µL of 500 mM/L alkaline phosphatase (AP) labelled anti-digoxigenin antibody was added prior to further incubation at 37°C for 1 h. The mixture was washed again with washing buffer containing 0.05% Tween 20 in PBS four times and then with plain PBS four times. Then, AP buffer (150 µL) containing 100 mM Tris–HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl2 and 10 mM p-nitrophenyl phosphate was added to each well followed by incubation at 37°C for 1 h. Finally, the absorbance of each solution on the plate was measured with a microplate reader at the wavelength of 405 nm. A control composed of the similar reaction mixture with 50% DMSO in place of the solution, while a blank was buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM ethylenediaminetetraacetate disodium salt (EDTA·2Na), 0.1% Nonidet-P 40 (NP-40), 20% glycerol, 1 mM DTT and 4 mM urea without the integrase enzyme. Suramin, a polyanionic HIV-1 IN inhibitor was used as a positive control. Percentage Inhibition against HIV-1 IN could be calculated from the equation below:

% inhibition against HIV-1 IN = \[
\frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100
\]
mean±S.D. from four determinations. The IC<sub>50</sub> values were calculated using the Microsoft Excel<sup>3</sup> program.

Molecular Modeling, Structure of Inhibitor The 3D structures of the multi-cinnamoyl compounds 5a−a, 6a−e and 7a−f were generated using Hyperchem professional 8.0. Since the hexasubstituted benzene could have their substituents arranged into eight possible conformations based on their positions relative to the benzene ring whether they were “above” or “below” the benzene plane,<sup>39</sup> compound 7a was selected as a model to identify the lowest energy conformation by quantum mechanical method at B3LYP/6-31G(d) level of theory using the Gaussian 03 program. The obtained lowest energy conformation of 7a was then used as a guideline to build the appropriate conformers for all other compounds. Subsequently, geometry optimization at the B3LYP/6-31G(d) level was performed for each compound. Finally, the resulting structures were applied for docking calculations.

Structure of Enzyme As it is known that an inhibitor binds to HIV-1 IN at the catalytic core domain, the only available X-ray structure of IN core domain in the complex with 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone (5CITEP) was used in our studies.<sup>31</sup> The structure was obtained from the protein data bank (PDB) code 1QS4. Since 5CITEP is bound to chain A, only chain A was selected for the current study. All water molecules and 5CITEP were removed while a magnesium ion at the active site was maintained. Finally, missing residues and hydrogen atoms were added. The protonation state of Lys156 and Lys159 were set to protonated forms as identified by Nuthaboot et al.<sup>30</sup>

Molecular Docking Molecular docking calculations were performed using version 4.1.1 of the GOLD software package. To define the binding site, all atoms within 10 Å around the Mg<sup>2+</sup> ion were chosen. The CHEMPLP scoring function was used. The GA run was 50. All other parameters were maintained at their default GOLD software settings. All conformers of the compounds were docked into the binding site of the IN. Based on the GOLD fitness score for each molecule, a bound structure with the highest fitness score was considered as the best bound conformation. In order to validate the docking procedure, 5CITEP was re-docked back into the IN. The docked structure agrees well with the X-ray complex structure (1QS4).

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