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Design, synthesis, and anti-integrase activity of catechol–DKA hybrids

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Abstract—Following the discovery of diketoacid-containing compounds as HIV-1 integrase (IN) inhibitors, a plethora of new molecules have been published leading to four drugs under clinical trial. In an attempt to rationally design new dimeric diketoacids (DKAs) targeting two divalent metal ions on the active site of IN, potent inhibitors against purified IN were found with varied selectivity for strand transfer. In this context, we designed and synthesized a new series of catechol–DKA hybrids. These compounds presented micromolar anti-integrase activities with moderate antiviral properties.

1. Introduction

Since 1996, highly active antiretroviral therapy (HAART) was designed to rapidly control HIV replication. It has had a significant impact on patient health and progression of AIDS in developed countries but its success has not been complete.[1] HAART strategy still suffers from issues of patient compliance, cost, deleterious side effects, and emerging drug resistance.[2] Therefore, expansion of novel anti-HIV drugs and targets will be critical in the coming years. HIV-1 integrase (IN), one of the three constitutive viral enzymes required for replication, has emerged as an attracting target for chemotherapeutic intervention in the treatment of AIDS. IN cleaves the two terminal nucleotides from each 3′-end of the viral DNA (3′-processing, 3′-P) and in the second step, transfers viral DNA into host chromosomal DNA (strand transfer, ST). About a decade of research in the field of HIV-1 IN inhibitors has yielded four drugs in clinical trial:[3,4] S-1360 (Scheme 1) is a diketo derivative designed by Shionogi-GSK companies, L-870,810 and L-870,812 8-hydroxynaphthyridines designed by Merck Research Laboratories, and JTK-303 designed by Japan Tobacco and developed by Gilead.

The story of diketo derivatives started with the reports[5] of the selective inhibitory properties of L-708,906 (for the structure, see Table 1) and L-731,988 toward ST and the crystal structure of the catalytic domain of HIV-1 IN with 5-CITEP.[6] In spite of the fact that the crystal structure was obtained only for the central domain of a modified water-soluble IN with a modest IN inhibitor, several computational docking studies were undertaken. Since these pioneer works, numerous studies dealing with the design and the structure–activity relationships of DKAs have been published.[7–13] The other main interests in studying diketoacids now deal with mechanistic considerations (metal–DKA interactions, metal-dependent integrase inhibition, and competition with target DNA).[14–18]

Amongst the other classes of HIV-1 IN inhibitors, polyhydroxylated compounds represent the family with the largest number of members.[19] Although they are generally considered to be toxic to host cells, some of them present a high therapeutic index (chicoric acid, lithospermic acids).

In previous studies,[8,13] bis-DKAs have been designed and tested as IN inhibitors following the idea that two diketoacid moieties on the same molecule may interact with the putative two metal ions in the active site of

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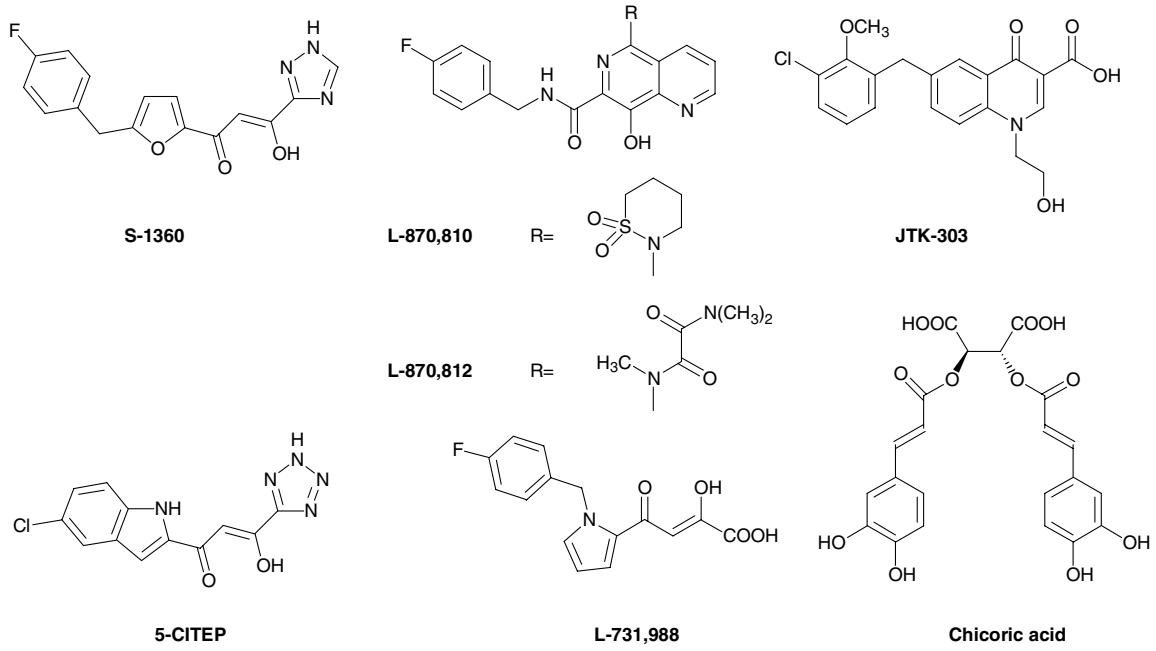


Scheme 1.

IN. In these two reports, molecules bearing two 4-oxobutenoic acid moieties significantly lost ST/3′-P selectivity. In order to study the possible interaction between the two main classes of inhibitors (polyphenols and DKAs), we synthesize new compounds that possess the two functions, that is, 2-hydroxy-4-oxobutenoic acid and catechol. For this purpose, we design 4-(3,4-dihydroxyphenyl)-2-hydroxy-4-oxobutenoic acid **1** and 4-(6,7-dihydroxynaphthyl)-2-hydroxy-4-oxobutenoic acid **2** as new catechol–DKA hybrids (Scheme 2). 4-Phenyl-2-hydroxy-4-oxobutenoic acid **3** and 4-(2-naphthyl)-2-hydroxy-4-oxobutenoic acid **4** were tested in order to evaluate the influence of the catechol function on the activity. Since 2-alkoxyaryldioxobutanoic acids have been reported to possess a submicromolar antiviral activity[7], 4-(2-methoxyphenyl)-2-hydroxy-4-oxobutenoic acid **5** was also prepared. 4-(2-Hydroxyphenyl)-2-hydroxy-4-oxobutenoic acid **6** has been synthesized due to its structural similitude to polyhydroxylated anthrones and anthraquinones.[20,21] Additionally, the intermediate esters **7** and **8** and the dimethyl ether analogue of **1** were tested. 4-(3,5-Dibenzyloxyphenyl)-2-hydroxy-4-oxobutenoic acid (L-708,906) was used as reference.

In this paper, we present the synthesis, the anti-integrase and antiviral activities of these new methoxylated and hydroxylated DKAs.

2. Synthesis

4-Aryl-2-hydroxy-4-oxobutenoic acid **3**–**5** and L-708,906 were obtained according to our previously published procedure[22] (Scheme 3). Compounds **1** and **2** were synthesized from the methylated precursors **10** and **11**[22] in a two-step procedure involving a demethylation of the ether function with boron tribromide followed by a hydrolysis of the ester group using 1 M HCl in refluxed dioxane (Scheme 2). Methyl 2-hydroxy-4-oxo-4-(3,4-dimethoxyphenyl)butenoate **10** and methyl 2-hydroxy-4-oxo-4-(6,7-dimethoxynaphthyl)butenoate **11** were treated with boron tribromide for 1 h. The precipitates obtained after 15 min of hydrolysis were almost exclusively the esters **7** and **8**. Prolonging the hydrolysis time gives a mixture of the ester **7** or **8** and the acid **1** or **2** but satisfactory yields in **1** or **2** cannot be obtained by this method. Compounds **7** and **8** were therefore treated in a refluxed mixture of 1 M HCl and dioxane to give **1** and **2** in 82% and 84% yields, respectively. The intermediate esters **7** and **8** were isolated, purified, and also tested as IN inhibitors. Compound **6** was obtained from methyl 2-hydroxy-4-oxo-4-(2-methoxyphenyl)butenoate by an exhaustive demethylation using boron tribromide in 45% yield (Scheme 2). In this case, even short time hydrolysis afforded a mixture of ester and acid **6**. After 1 h of hydrolysis, **6** precipitated and was purified by crystallization (45% yield). Compound **10** was treated with a refluxed mixture of 1 M HCl and dioxane to give **9** in 45% yield.

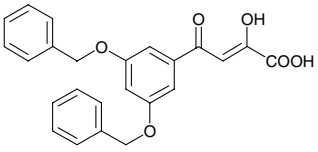
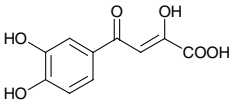
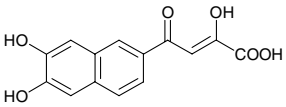
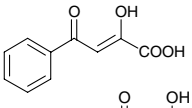
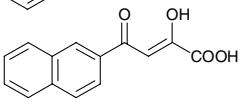
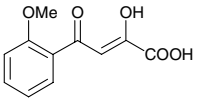
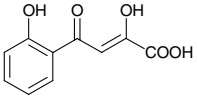
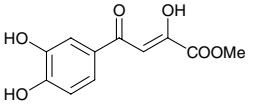
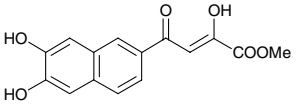
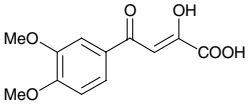
3. Anti-integrase activity

3′-P and ST reaction inhibitions were measured using wild-type IN and Mg^{2+}. Compounds **1**–**9** and L-708,906 were tested against purified IN and the data are summarized in Table 1.

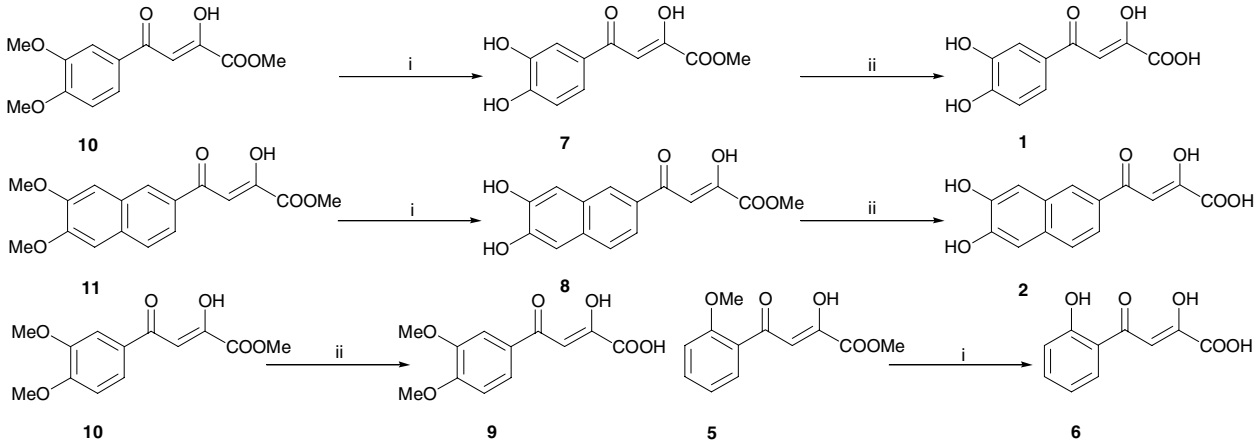
4. Antiviral activity

Compounds **1**, **2**, and **8** were evaluated ex vivo for their antiviral activity against HIV-1 replication in CEM

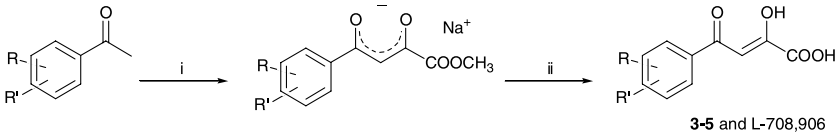
Table 1. Inhibition of HIV-1 integrase catalytic activities (3′-P, 3′-processing and ST, strand transfer) of a series of catechol–DKA hybrids and related compounds

Compound	Structure	In vitro IC_{50} values (μM)		3′-P/ST[a]
		3′-P	ST	
L-708,906		0.96	0.14	≈7
1		3.9	1.1	≈4
2		1.9	0.9	≈2
3		>100[18]	>100[18]	
4		>100	>100	
5		>100	>100	
6		>100	>100	
7		>100	84.4	
8		11.3	3.7	≈3
9		>100	29.9	>3

[a] 3′-P/ST, IC_{50} (3′-P)/IC_{50} (ST).



Scheme 2. Reagents and conditions: (i) BBr_3/CH_2Cl_2, rt 1 h, then H_2O, 15 min (1 h for **6**); (ii) 1 N HCl, dioxane, reflux 4 h.



Scheme 3. Reagents and conditions: (i) Dimethyl oxalate (1 equiv) in Et_2O, then 2 M MeONa in MeOH, 1 h and 30 min, rt, 75–92% yield; (ii) 1,4-dioxane/1 M HCl, 4 h, reflux, 55–68% yield.

Table 2. Antiviral activities of compounds **1**, **2**, and **8** compared to that of L-708,906

Compound	CC_{50}[a]	EC_{50}[b]	TI[c]
L-708,906	>100	4	>25
1	160	46	3.5
2	74	31	2.4
8	43	30	1.4

[a] Cytotoxic concentration 50%.
[b] Effective concentration 50%.
[c] Therapeutic index = CC_{50}/EC_{50}.

cells. They were tested for their ability to lower the viral charge in culture supernatants. CEM cells were infected with HIV-1 and subsequently treated with increasing concentrations of drugs. The amount of virus was assayed by β-galactosidase assay, with HeLa-CD4-β-gal cells as reporting cells. Toxicity was estimated by MTT transformation assay. Antiviral properties are reported in Table 2.

5. Discussion

The selectivity of integrase inhibitors for the strand transfer reaction is of particular importance since it seems to be a prerequisite for an antiviral activity correlated with the integrase inhibition.[5] For example, zintevir (a non-selective inhibitor of IN)[23,24] and chicoric acid (a selective inhibitor of the 3′-processing reaction)[25] owe their antiviral activity to an interaction with the viral envelope glycoprotein gp120. Since the comparative study of Marchand et al.[17] (wild-type vs double-mutant IN and Mn^{2+} vs Mg^{2+}), it is clear that assays performed using wild-type enzyme and Mg^{2+} represent the most stringent conditions with respect to drug inhibition. Whereas it was found that DKAs inhibit HIV-1 IN independently of the order of addition with wild-type enzyme and Mn^{2+}, it was not true when we used Mg^{2+}. The so-called selectivity toward the strand transfer reaction versus the 3′-processing of DKAs (that can be illustrated by the ratio of IC_{50}s for each reaction) in the case of L-708,906 was found to be >2400 (double-mutant IN and Mn^{2+}), 375 ± 139 (wild-type IN and Mn^{2+}), and 41 ± 15 (wild-type IN and Mg^{2+}). In our hand, when the IN–DNA complex was not preformed (addition of the drug at the same time as IN and DNA fragment) the selectivity dropped to about 7. The inhibition of strand-transfer is decreased by 2.3-fold (IC_{50} = 0.14 μM vs 0.06 μM[17]) and the inhibition of 3′-processing is increased by 2.5-fold (IC_{50} = 0.96 μM vs 2.45 μM[17]). Since divalent metal cofactor stimulates and stabilizes IN-viral DNA complexes and decreases binding of IN to target DNA,[26] these results are consistent with the fact that L-708,906 interacts with Mg^{2+}. As previously mentioned,[18] the unsubstituted DKA **3** is inactive in this assay. Its naphthalene analogue **4**, the *ortho*-methoxy-DKA **5**, and the *ortho*-hydroxy-DKA **6** were also found to be inactive on both 3′-processing and strand transfer reactions. It is well known that the ortho-substitution has a direct effect on the keto–enol equilibrium. In the case of the diketo–ketoenol equilibrium, a steric effect of the methoxy group should have a negative effect on the ketoenol form and the possibility to form hydrogen bond between a carbonyl oxygen atom and the hydrogen of a hydroxyl group a positive effect. Indeed, the diketo–ketoenol ratios measured in DMSO-d_6 (that gives comparable results to D_2O solution) were 9:91, 17:83, and 0:100 for **3**, **5**, and **6**, respectively. Due to possible multi-binding sites, **6** was expected to inhibit IN. Unfortunately, **6** was inactive emphasizing the role of aromatic substituents in the inhibition process.

Replacing 3,5-dibenzyloxyphenyl group by a 3,4-dihydroxyphenyl or 6,7-dihydroxynaphthyl moiety moderately affected the 3′-processing catalysis. Conversely ST was more sensitive to the structural change and the 3′-P/ST ratio decreased to about 4 and 2 for **1** and **2**, respectively. More surprising is the difference between **7** and **8**. Whereas the ester became inactive in the phenyl series **8**, the ester of **2**, is only about 4-fold less active than **2**. Since diketoesters are known to be generally inactive, we considered that the IN inhibitory activity of **8** is probably due to the presence of the 6,7-dihydroxynaphthyl moiety. Conversely **9**, a diketoacid bearing two methoxy groups on the phenyl ring was found to be only modestly active on the ST reaction.

Comparison of IN inhibitory activities of **1**, **7**, and **9** exemplifies the concept of multi-active site approach developed by Dayam and Neamati.[27]

Comparison of CC_{50} and EC_{50} of compounds **2** and **8** shows that their antiviral properties are quite similar but the acid **2** is twice less toxic than the ester **8**. Compound **1** inhibits the HIV-1 cytopathic effect in cell-based assays at non cytotoxic concentrations (EC_{50} = 46 μM, CC_{50} = 160 μM), whereas **2** and **8** are antiviral at cytotoxic concentrations. Compared to L-708,906, **1** is 10-fold less active and therefore its therapeutic index is very modest. This result exemplifies the correlation between selectivity toward ST and antiviral activity.

In summary, we demonstrated that catechol–DKA hybrids are active against IN with slight decrease in the 3′-P/ST selectivity. Using wild-type enzyme, Mg^{2+}, DNA fragment and no preincubation of the enzyme, the 3′-P/ST selectivity of L-708,906 was found to be

below 7 and is probably due to difference in binding of IN to viral and target DNA. As previously demonstrated,[8,13] the presence of an acidic function (carboxylic or phenolic) on the left side of the diketoacid (using the schematic structure of Ref. 8) strongly affects the selectivity toward ST reaction and the antiviral properties.

6. Experimental

All solvents were of commercial quality used from freshly opened containers and were dried and purified by conventional methods. Mps were determined on a Reichert Thermopan apparatus, equipped with a microscope and are uncorrected. NMR spectra were obtained on an AC 300 Bruker spectrometer in DMSO-d_6 with TMS as internal reference (^{1}H 300 MHz, ^{13}C 75 MHz). Mass spectra were recorded on a Thermo-Finnigan Polaris Q mass spectrometer (70 eV, Electronic Impact) or on a Voyager DE STR mass spectrometer (Applied Biosystems) (MALDI-TOF). Elemental analyses were performed by CNRS laboratories (Vernaison). Known 4-aryl-2-hydroxy-4-oxobutenoic acids and esters were obtained according to our previously published procedure.[22]

6.1. Ester hydrolysis—synthesis of 1, 2, and 9—general procedure

Ketoenol ester **7**, **8** or **10** (1 mmol) was dissolved in freshly distilled 1,4-dioxane (25 mL) and 1 M HCl (25 mL) was added. The mixture was refluxed for 4 h. Removal of the solvents under reduced pressure gave a solid, which was washed with water, CH_2Cl_2 and dried. The resulting ketoenol acid **1**, **2** or **9** was recrystallized from the appropriate solvent.

6.2. 2-Hydroxy-4-oxo-4-(3,4-dihydroxyphenyl)butenoic acid (1)

Yellow powder; yield 82%; mp = 180–182 °C (acetone); ^{1}H NMR: 6.86 (d, 1H, $^3J_{H5'\text{-}H6'} = 8.4$, $H_{5'}$), 6.94 (s, 1H, H_3), 7.43 (d, 1H, $^4J_{H2'\text{-}H6'} = 2.2$, $H_{2'}$), 7.48 (dd, 1H, $^3J_{H5'\text{-}H6'} = 8.4$, $^4J_{H2'\text{-}H6'} = 2.2$, $H_{6'}$); 9.55 (br s, 1H, OH), 10.21 (br s, 1H, OH); ^{13}C NMR: 97.5 (CH), 114.5 (CH), 115.7 (CH), 121.8 (CH), 126.2 (C), 145.7 (C), 152.1 (C), 163.4 (C), 167.4 (C), 190.6 (C); MS (MALDI-TOF): *m/z* (%) = 226 (21), 225 ([MH$^+$], 100).

Elemental analysis: Calcd for $C_{10}H_8O_6$: C, 53.58; H, 3.60. Found: C, 53.67; H, 3.58.

Detectable signals of the diketo form (7%): ^{1}H NMR: 4.39 (s, 2H, CH_2), 6.81 (d, 1H, $^3J_{H5'\text{-}H6'} = 8.6$, $H_{5'}$), 7.31 (s, 1H, $H_{2'}$), 7.34 (d, 1H, $H_{6'}$), 9.55 (br s, 1H, OH), 10.21 (br s, 1H, OH).

6.3. 2-Hydroxy-4-oxo-4-(6,7-dihydroxynaphthyl)butenoic acid (2)

Green powder; yield 84%; mp > 250 °C (dec.) (acetone); ^{1}H NMR: 7.20 (s, 1H, H_3, $H_{5'}$ or $H_{8'}$), 7.24 (s, 1H, H_3, $H_{5'}$ or $H_{8'}$), 7.39 (s, 1H, H_3, $H_{5'}$ or $H_{8'}$), 7.68–7.76 (m, 2H, $H_{3'}$ and $H_{4'}$), 8.51 (br s, 1H, $H_{1'}$), 9.96 (br s, 1H, OH), 10.18 (br s, 1H, OH); ^{13}C NMR: 98.1 (CH), 109.7 (CH), 111.7 (CH), 120.6 (CH), 126.5 (CH), 128.1 (C), 128.3 (CH), 129.3 (C), 132.3 (C), 147.9 (C), 150.3 (C), 163.6 (C), 169.0 (C), 191.3 (C); MS (EI): *m/z* (%) = 274 ([M$^+$], 41), 229 (50), 187 (27), 161 (100), 115 (17), 86 (24), 69 (30).

Elemental analysis: Calcd for $C_{14}H_{10}O_6$: C, 61.32; H, 3.68. Found: C, 61.20; H, 3.60.

Detectable signals of the diketo form (7%): ^{1}H NMR: 4.61 (s, 2H, CH_2), 8.36 (br s, 1H, $H_{1'}$).

6.4. 2-Hydroxy-4-oxo-4-(3,4-dimethoxyphenyl)butenoic acid (9)

Yellow powder; yield 45%; mp = 188–189 °C (acetone) (lit.[28] 179–180 °C dec.); ^{1}H NMR: 3.84 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 7.08 (s, 1H, H_3), 7.10 (d, 1H, $^3J_{H5'\text{-}H6'} = 8.6$, $H_{5'}$), 7.53 (d, 1H, $^4J_{H2'\text{-}H6'} = 1.5$, $H_{2'}$), 7.76 (dd, 1H, $^3J_{H6'\text{-}H5'} = 8.6$, $^4J_{H6'\text{-}H2'} = 1.5$, $H_{6'}$); ^{13}C NMR: 55.6 (CH_3), 55.9 (CH_3), 97.8 (CH), 109.9 (CH), 111.3 (CH), 123.0 (CH), 127.3 (C), 148.9 (C), 154.0 (C), 163.4 (C), 168.3 (C), 190.4 (C).

Detectable signals of the diketo form (10%): ^{1}H NMR: 3.81 (s, 3H, OCH_3), 4.50 (s, 2H, H_3), 7.43 (br s, 1H, $H_{2'}$), 7.63 (br d, 1H, $^3J_{H6'\text{-}H5'} = 8.8$, $H_{6'}$).

6.5. Ether cleavage—synthesis of 6, 7, and 8—general procedure

To a solution of methyl 4-aryl-2-hydroxy-4-oxobutenoate[22] (1 mmol in 20 mL) boron tribromide (1 M in dichloromethane, 3 mL for **6** and 4 mL for **7** and **8**) was added dropwise. The mixture was stirred at room temperature for 1 h.

6.6. Purification of 6

Twenty milliliters of distilled water was slowly added and after stirring for 1 h, the precipitate was filtered, washed with water then with dichloromethane, and dried over P_2O_5. Compound **6** was crystallized in acetone (yield 45%).

6.7. Purification of 7 and 8

Twenty milliliters of distilled water was slowly added and after stirring for additional 15 minutes, the precipitate was filtered, washed with water then with dichloromethane, and dried over P_2O_5. Methyl 2-hydroxy-4-oxo-4-(3,4-dihydroxyphenyl)butenoate **7** and methyl 2-hydroxy-4-oxo-4-(6,7-dihydroxynaphthyl) butenoate **8** were obtained after crystallization in acetone in 87% and 95% yield, respectively.

6.8. 2-Hydroxy-4-oxo-4-(2-hydroxyphenyl)butenoic acid (6)

White powder; mp > 250 °C; ^{1}H NMR: 6.91 (s, 1H, H_3), 7.53 (td, 1H, $^3J_{H5'\text{-}H4'} = {}^3J_{H5'\text{-}H6'} = 8.05$, $^4J_{H5'\text{-}H3'} = 1.2$, $H_{5'}$), 7.73 (dd, 1H, $^3J_{H3'\text{-}H4'} = 8.05$, $^4J_{H3'\text{-}H5'} = 1.2$, $H_{3'}$),

7.88 (td, 1H, $^3J_{H4'\text{-}H3'} = {}^3J_{H4'\text{-}H5'} = 8.05$, $^4J_{H4'\text{-}H6'} = 1.7$, $H_{4'}$), 8.04 (dd, 1H, $^3J_{H6'\text{-}H5'} = 8.05$, $^4J_{H6'\text{-}H4'} = 1.7$, $H_{6'}$); ^{13}C NMR: 113.2 (CH), 118.9 (CH), 123.7 (C), 124.9 (CH), 126.0 (CH), 135.1 (CH), 154.1 (C), 155.5 (C), 161.5 (C), 177.7 (C); MS (MALDI-TOF): *m/z* (%) = 209 (100), 208 ([M^+], 23).

Elemental analysis: Calcd for $C_{10}H_8O_5$: C, 57.70; H, 3.87. Found: C, 57.52; H, 3.91.

6.9. Methyl 2-hydroxy-4-oxo-4-(3,4-dihydroxyphenyl)butenoate (7)

Pale green powder; mp = 170–174 °C; ^1H NMR: 3.83 (s, 3H, CH_3), 6.86 (d, 1H, $^3J_{H5'\text{-}H6'} = 8.3$, $H_{5'}$), 6.96 (s, 1H, H_3), 7.44 (d, 1H, $^4J_{H2'\text{-}H6'} = 2.2$, $H_{2'}$), 7.50 (dd, 1H, $^3J_{H6'\text{-}H5'} = 8.3$, $^4J_{H6'\text{-}H2'} = 2.2$, $H_{6'}$), 9.57 (br s, 1H, OH), 10.26 (br s, 1H, OH); ^{13}C NMR: 52.9 (CH_3), 97.8 (CH), 114.6 (CH), 115.7 (CH), 121.9 (CH), 125.9 (C), 145.8 (C), 152.3 (C), 162.4 (C), 166.1 (C), 190.3 (C); MS (EI): *m/z* (%) = 239 (23), 238 ([M^+], 18), 179 (80), 137 (45), 111 (31), 69 (100).

Elemental analysis: Calcd for $C_{11}H_{10}O_6$: C, 55.47; H, 4.23. Found: C, 55.21; H, 4.12.

Detectable signals of the diketo form (8%): ^1H NMR: 3.75 (s, 3H, CH_3), 4.43 (s, 2H, CH_2), 6.83 (d, 1H, $^3J_{H5'\text{-}H6'} = 8.1$, $H_{5'}$), 7.30 (d, 1H, $^4J_{H2'\text{-}H6'} = 2.0$, $H_{2'}$), 7.35 (dd, 1H, $^3J_{H6'\text{-}H5'} = 8.1$, $^4J_{H6'\text{-}H2'} = 2.0$, $H_{6'}$), 9.57 (br s, 1H, OH), 10.26 (br s, 1H, OH).

6.10. Methyl 2-hydroxy-4-oxo-4-(6,7-dihydroxynaphthyl) butenoate (8)

Dark green powder; mp = 180 °C; ^1H NMR: 3.87 (s, 3H, CH_3), 7.19 (s, 1H, H_3, $H_{5'}$ or $H_{8'}$), 7.26 (s, 1H, H_3, $H_{5'}$ or $H_{8'}$), 7.37 (s, 1H, H_3, $H_{5'}$ or $H_{8'}$), 7.69–7.76 (m, 2H, $H_{3'}$ and $H_{4'}$), 8.52 (s, 1H, $H_{1'}$), 10.02 (br s, 2H, 2 OH); ^{13}C NMR: 53.0 (CH_3), 98.2 (CH), 109.6 (CH), 111.5 (CH), 120.4 (CH), 126.4 (CH), 127.9 (C), 128.3 (CH), 128.8 (C), 132.2 (C), 147.8 (C), 150.3 (C), 162.4 (C), 167.4 (C), 191.1 (C); MS (EI): *m/z* (%) = 290 (20); 289 ([MH^+], 100).

Elemental analysis: Calcd for $C_{15}H_{12}O_6$: C, 62.50; H, 4.20. Found: C, 62.32; H, 4.16.

Detectable signals of the diketo form (10%): ^1H NMR: 3.77 (s, 3H, CH_3); 4.65 (s, 2H, CH_2); 8.35 (s, 1H, $H_{1'}$).

6.11. HIV-1 integrase inhibitory assay: oligonucleotides

Oligonucleotides were purchased from Eurogentec and further purified on 18% acrylamide/urea denaturing gel. U5B: GTGTGGAAAATCTCTAGCA; U5B-2: GTGTGGAAAATCTCTAG; U5A: 59-ACTGCTAG AGATTTTCCACAC; ST1: AGTGAATTAGCCCTT GGTCA-biotin; ST2: 59-TGACCAAGGGCTAATTC ACT-biotin; U5B and U5B-2 were radio labeled using T4 polynucleotide kinase for, respectively, 3′-processing and strand transfer reactions.

6.12. HIV-1 integrase assays

Wild-type HIV-1 integrase was purified as described previously.[29] 3′-Processing assay was performed in a reaction volume of 20 mL containing 0.025 pmol of labeled U5A/U5B double-stranded DNA substrate and 1 pmol of integrase in buffer A [20 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 25 mM NaCl, and 1 mM DTT]. Products were separated on an 18% acrylamide/urea denaturing gel and quantified on a phosphoimager using ImageQuant software (Amersham Pharmacia Biotech). Strand transfer reactions were performed in triplicate in 96-well plates using 0.25 pmol of labeled U5A/U5B-2 double-stranded DNA substrate, 12 pmol of ST1/ST2 39-biotinylated target DNA and 2 pmol of integrase in buffer A in a final volume of 40 mL. Radiolabeled reaction products were bound to streptavidin-coated magnetic beads (DynaL), washed twice in buffer B (PBS buffer supplemented with 0.025% Tween 20 and 10 mg/mL BSA), and quantified on a beta radiation counter. Inhibition in the presence of drugs is expressed as the fractional product in percent of the control without drug.

6.13. Antiviral assays

The lymphocytes cell line CEM4fx was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum. HeLa-CD4+-βGal (P4) cells were grown in DMEM with 10% fetal calf serum and 0.5 mg/mL geneticin. Cell-free viral supernatants were obtained by transfection of P4 cells with HIV-1 PLN4-3 genomic clone.[30] CEM4fx cells were plated in triplicate on a 96-well plate (100 μL) and infected with cell-free virus. Viral supernatants were removed 2 h after infection and drugs dissolved in DMSO were added in fresh medium. Infected cells were grown in the presence of drugs for 3 days. Supernatants were then collected at $t = 72$ h and used to infect P4 cells. P4 cultures were incubated for 24 h and subsequently lysed in a phosphate buffer containing 50 mM 2-mercaptoethanol, 10 mM $MgSO_4$, 25 mM EDTA, and 0.125% NP40. 20 μL of lysate was incubated with 100 μL of CPRG-containing buffer. The red staining intensity was quantified on a multiscan photometer at 570 nm. CEM4fx cell viability was estimated by the MTT (Sigma) assay after 3 day treatment with drugs (20 μL). A solution of (7.5 mg/mL) in phosphate buffer was added to each well of microtiter trays. Plates were further incubated at 37 °C in a CO_2 incubator for 4 h. Solubilization of formazan crystals was achieved by adding 100 mL of 10% SDS, 10 mM HCI. Absorbance was read in a multiscan photometer at 570 nm. Experiments were performed in triplicate and averaged.

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