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Highly Suppressing Wild-Type HIV-1 and Y181C Mutant HIV-1 Strains by 10-Chloromethyl-11-demethyl-12-oxo-calanolide A with Druggable Profile

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We herein report a new compound: 10-chloromethyl-11-demethyl-12-oxo-calanolide A (**20**, EC_{50} = 7.4 nM, SI = 1417), which demonstrates a druggable profile with 32.7% oral bioavailability in rat, tolerated oral single dose toxicity in mice, and especially the feature of highly efficient suppression of the wild-type HIV-1 and Y181C mutant HIV-1 at an EC_{50} = 7.4 nM and EC_{50} = 0.46 nM, respectively.

Introduction

Over 20 antiretroviral medications have now been approved for the treatment of HIV-infected individuals.[1] Although highly active antiretroviral therapy (HAART[a]) has been very effective in suppressing HIV load, these medications present several limitations such as the rapid emergence of drug-resistant mutant strains due to the narrow range of chemical structure of the cocktail components. Because of viral resistance and issues related to drug side effects, there remains a great need to discover novel antivirals, especially ones that function as nonnucleoside reverse transcriptase inhibitors (NNRTIs). Today, four NNRTIs have been approved by the FDA,[2] therefore, the scarcity of unique inhibitors warrants the development of novel NNRTI compounds.

(+)-Calanolide A (Figure 1) was previously described as the first natural product that inhibits HIV-1 reverse transcriptase.[3] It inhibits reverse transcriptase by a mechanism involving at least two binding sites, which are distinguished as having competitive and uncompetitive components.[4] Therefore, this type of drug could inhibit diverse HIV-1 strains resistant to other nucleoside and nonnucleoside reverse transcriptase inhibitors.[5−7] In particular, (+)-calanolide A is unique in inhibiting HIV-1 isolates carrying the viral reverse transcriptase Y181C amino acid mutation, which is associated with high-level resistance to most current NNRTIs.[6−8] In addition, because (+)-calanolide A displays additive to synergistic anti-HIV activity with a range of nucleoside analogues, protease inhibitors, and NNRTIs,[4,5,7] this compound could be used in clinical settings in combination with other antiviral drugs to suppress HIV-1 mutants. Nevertheless, its low inhibitory potency against HIV-1 probably may account for the limitation in clinical trials although it was shown to be well tolerated in phase Ia/Ib studies.[9,10] Thus, targeted modification of (+)-calanolide A to find new analogues with high potency against HIV-1 has become crucial.

There are several papers documenting the chemical modifications of (+)-calanolide A and related natural species in an effort to find new analogues with higher anti-HIV-1 potency.[11−16] Zembower and co-workers illustrated for the first time that the 12-ketone form of calanolide A exhibits anti-HIV-1 activity; however, this molecule possesses a lower therapy index than the parent natural product (+)-calanolide A.[11,12] Through a systematic analysis of structure−activity relationships (SARs), we found that 10-bromomethyl-12-oxo-calanolide A (**3**, Figure 1) has significantly enhanced antiviral potency (EC_{50} = 2.85 nM).[16] To address the concern that compound **3** bearing a bromine atom at position C-10 of ring C may confer toxicity, in this article we describe the modifications of ring C involving a replacement of the bromine atom, with the aim of finding alternative antiviral drug candidate presenting similar or higher anti-HIV activity against both wild type and drug mutants with tolerated toxicity, and acceptable oral bioavailability.

Results and Discussion

Chemistry. To replace bromine atom, we carried out a nucleophilic substitution reaction of the three-rings bromated intermediate **4** with NaN_3 or $NaSCH_3$ in DMF to prepare the intermediates **5** and **10** followed by the construction of ring D with 1,1-diethoxy-3-methyl-2-butene to afford the analogues **6** and **11** (Scheme 1). Moreover, when compound **4** was treated with K_2CO_3 in anhydrous THF at room temperature, a three-membered cycle attached to ring C in compound **8** was formed through a intramolecular nucleophilic substitution. After ring closure utilizing 1,1-diethoxy-3-methyl-2-butene, a new analogue **9** was readily obtained, termed as 10,11-cyclopropyl-12-oxo-calanolide A. Oxidation

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[a]Abbreviations: HIV, human immunodeficiency virus; HAART, highly active antiretroviral therapy; NNRTI, nonnucleoside reverse transcriptase inhibitor; EC50, half maximal effective concentration; SI, selectivity index; FDA, Food and Drug Administration (USA); DMF, dimethylformamide; THF, tetrahydrofuran; DCM, dichloromethane; DCE, 1,2-dichloroethane; m-CPBA, m-chloroperbenzoic acid; $TCID_{50}$, 50% tissue culture infective dose; MRT, mean residence time.

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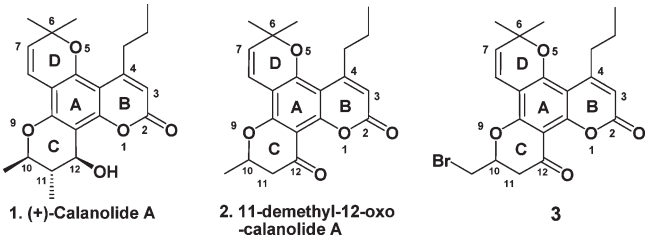
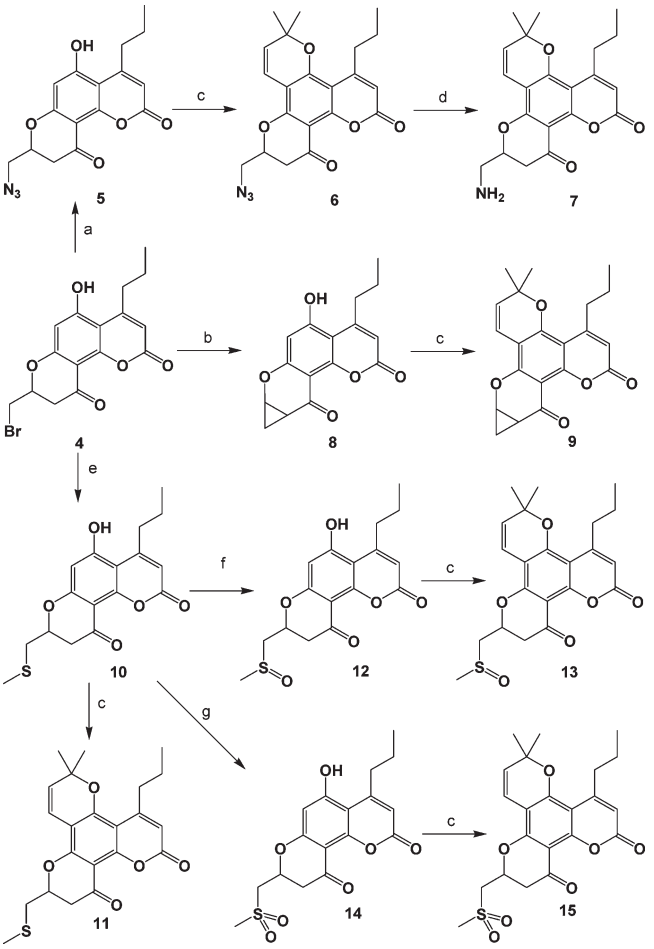


Figure 1. Chemical structure of the biologically active dipyranocoumarins.

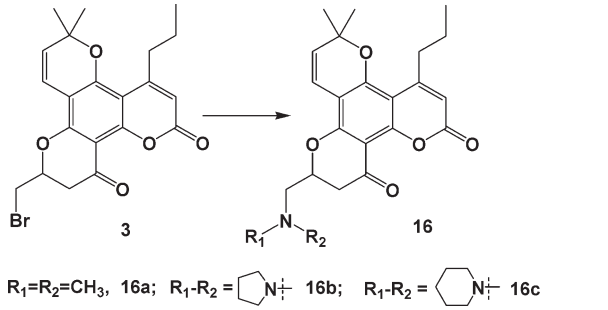
Scheme 1[a]



[a] Reagents and conditions: (a) NaN_3, DMF, 75 °C, 1 h; (b) K_2CO_3, ethanol, reflux, 3 h; (c) 1,1-diethoxy-3-methyl-2-butene, pyridine, toluene, 1.5 h; (d) $SnCl_2 \cdot H_2O$, ethanol, reflux,3 h; (e) $NaSCH_3$, DMF, 75 °C, 0.5 h; (f) *m*-CPBA, THF, 0 °C, 1 h; (g) *m*-CPBA, THF, reflux, 3 h.

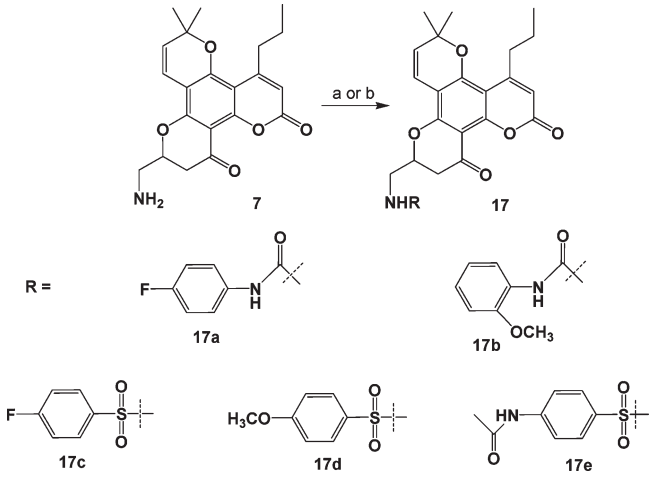
of **10** in THF with *m*-CPBA at different temperature could produce the intermediate **12** (0 °C) and **14** (reflux), which were further converted into the corresponding final sulfoxide analogue **13** and the sulfone analogue **15**, respectively. The amine analogue **7** was also synthesized through reduction of the azide analogue **6** with $SnCl_2$ in the presence of hydrochloride acid. When compound **3** was reacted with secondary amines in THF at room temperature, the classical nucleophilic substitution products (**16a**, **16b**, and **16c**) were obtained (Scheme 2) in which hydrophilic groups were introduced at the C-10 position of 12-oxo-calanolide A. The amine analogue **7** reacted with 4-fluorophenyl isocyanate in THF at 65 °C to afford compound 17a. Similarly, treatment of compound **7** with 2-methoxyphenyl isothiocyanate in THF, and 4-fluorobenzenesulfonyl

Scheme 2. Nucleophilic Substitution with the Secondary Amine[a]



[a] Reagents and conditions: pyrrolidine (or dimethylamine, piperidine), THF, 12−36 h.

Scheme 3[a]

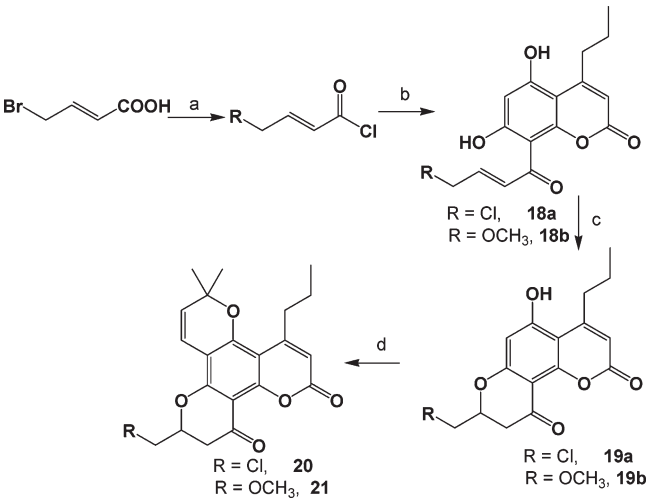


[a] Reagents and conditions: (a) 4-fluorophenyl isocyanate (or 2-methoxyphenyl isothiocyanate), THF, reflux, 3 h; (b) 4-fluorobenzenesulfonyl chloride (or 4-methoxybenzenesulfonyl chloride, *N*-acetylsulfanilyl chloride), DCM, reflux, 3 h.

chloride, 4-methoxybenzenesulfonyl chloride, and *N*-acetylsulfanilyl chloride in dicloromethane, respectively, in the presence of pyridine provided the corresponding amino derivatives **17b**, 17c, **17d**, and **17e** (Scheme 3).

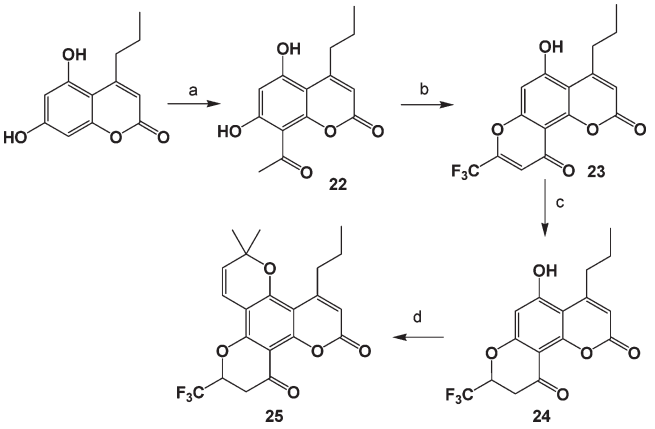
To obtain the Cl-mimic analogue of compound **3**, 4-bromo-substituted crotonic acid was converted into the corresponding 4-hydyoxy species,[17] which was then treated with $SOCl_2$. The resulting acyl chloride was reacted with 4-propyl-5,7-dihydroxy coumarin through Friedel−Crafts acylation, using anhydrous $AlCl_3$ as catalyst. The subsequent ring closure using sodium acetate afforded the three-ringed chloro-intermediate **19a**. The target compound **20** was obtained after the construction of ring D, according to the synthetic method described in Scheme 4. Similarly, the analogue **21** containing an CH_3O-substituent at position C-10 was produced using 4-methoxy-crotonic acid as the starting material.[18] Attempts to prepare the CF_3-mimic analogue of **3** with this strategy were unsuccessful. An alternative approach was developed which consists of first acylation of coumarin at C-8 position to afford **22**, the subsequent classic condensation with trifluoro anhydride in the presence of pyridine, to produce the unsaturated species **23** followed by reduction of 10,11-double bond with trimethylsilane catalyzed by $Pd[P(Ph)_3]_3Cl$, and the ultimate ring closure of the above resulted intermediate **24** treated with 1,1-diethoxy-3-methyl-2-butene to obtain the title compound **25** (Scheme 5).

Scheme 4. Synthetic Route for the Cl-Mimic Analogue **20**[a]



[a] Reagents and conditions: (a) (i) KOH or $NaOCH_3$, H^+, (ii) $SOCl_2$; (b) 4-propyl-5,7-dihydroxy coumarin, anhydrous $AlCl_3$, $PhNO_2$/DCE, 75 °C, 36 h; (c) anhydrous sodium acetate, ethanol, 5 h; (d) 1,1-diethoxy-3-methyl-2-butene, pyridine, toluene, 1.5 h.

Scheme 5[a]

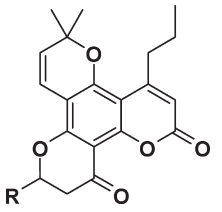


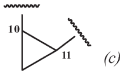
[a] Reagents and conditions: (a) acetic anhydride, $AlCl_3$, DCE/CH_3NO_2, 80 °C, 16 h; (b) (i) trifluoroacetic anhydride, pyridine, rt, (ii) HCl (2N), reflux; (c) triethylsilane, $Rh[P(Ph)_3]_3Cl$, THF, reflux, 6 h; (d) 1,1-diethoxy-3-methyl-2-butene, pyridine, toluene, 1.5 h.

Antiviral Activity in Vitro. All synthesized compounds were assayed for inhibition of HIV-1 replication against 100 $TCID_{50}$ HIVADA infections in a pseudovirus-based assay, as previously described.[19] Clearly, aminomethylation at position C-10 or further various acylation of aminomethylated-12-oxo calanolide A were useless for improving the activity against HIV-1. For instance, compounds **6**, **16a**−**16c** and **17a**−**17e** became inactive (Table 1). These compounds were also observed with their higher toxicity to the virus host cells (data not show); in particular, the azide analogue **6** exhibited extreme cellular toxicity. The results imply that positive charge groups are inappropriate at C-10 of position.

Methylthioylation at C-10 position (**11**) did also not give an improved inhibitory potency against HIV-1 as compared to (+)-calanolide A. This included its gradually oxidated products of sulfoxide (**13**) and sulfone (**15**). Replacement of bromo atom of compound **3** (EC_{50} = 0.18 μM) with methoxyl group (**21**) resulted in a similar inhibitory activity to (+)-calanolide A, however, with lower selectivity index (Table 1).

Table 1. Inhibitory Activities of 12-Oxo-calanolide A Analogues against 100 $TCID_{50}$ HIVADA Infection



Compd.	R	EC_{50} (μM)	SI [b]
(+)-1	CH_3	0.1	16-279
6		*a*	*d*
7		*a*	*d*
9	 *(c)*	0.0141	709
11		0.456	*d*
13		14.4	*d*
15		33.9	*d*
16a-16c	*see scheme 2*	*a*	*d*
17a-17e	*see scheme 3*	*a*	*d*
20		0.0074	1417
21		0.18	39.7
25	CF_3	14.9	*d*
Nevirapine		0.0082	3300

[a] No suppression. [b] Selectivity index(SI) was calculated by the formula: IC_{50}/EC_{50} (cytotoxicity data IC_{50} were supplemented in Supporting Information). [c] The substituent at position C10,C11. [d] Not determined.

Significant improvement of activity compared to the natural product (+)-calanolide A was observed with compounds *cis*-10,11-cyclopropyl-12-oxo-calanolide A (**9**) and 10-chloromethyl-12-oxo-calanolide A (**20**). Compound **9** yielded an EC_{50} value of 14.1 nM (SI = 709) and compound **20** yielded 7.4 nM (SI = 1417), respectively. C-10 halogenmethylation of 12-oxo-calanolide A gave the variable results with their atomic order. Trifluoromethylated compound **25** offered the antiviral activity to 14.9 μM of EC_{50} value, however, chloromethylated **20** gave a remarkably higher potency of 7.4 nM. With the observation of previous result of bromomethylation of 12-oxo-calanolide A (**3**, EC_{50} = 2.85 nM), it is likely that the penetration ability of cell membrane is the key factor because, in principle, the fluorine atom is able to improve the binding ability of small molecule through the mimic of hydrogen atom, however, the chlorine atom could help a small molecule passing through a cell membrane with its hydrophobic property. Furthermore, the

Table 2. Biological Activity of **20** in Drug Resistant HIV-1 Strains

EC_{50} (nM)	mutants					
	PNL4−3[a]	K103N	Y181C	Y188L	G190A	T139I
20	7.4	316	0.46	6127	43.9	103.5
(+)-calanolide A	97.5	13416	42.6	10475	385	6151
neverapine	11.4	464	10591	7166	159.4	2.2

[a] Wild-type HIV-1.

Table 3. Pharmacokinetic Parameters of **20** in Rats after a Single Oral and Intravenous Dosing ($n = 3$)

	AUC (μg/mL·h)	MRT (h)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (μg/mL)
intravenous	1.5	1.7	1.9	0.03	0.93
oral	4.7	9.2	3.8	8.00	0.34

much stronger electron-withdrawing ability of CF_3 group than that of CH_2Br or CH_2Cl might be another reason that it significantly decreased the electronic interaction between **25** and HIV-1 reverse transcriptase.

Anti-HIV Drug Mutants Profile of Compound 20. Resistance to chemotherapy can develop in a significant number of patients during the long-term HAART that combines three or more different drugs. Efavirenz and nevirapine are the most widely used NNRTIs in combination regimens. Many studies demonstrated that each of these NNRTIs is usually associated with a single point mutation that triggers the development of clinical resistance. For efavirenz, it is the K103N mutation. For nevirapine, it is the Y181C mutation.[20] The Y181C mutation can even cause multidrug resistance to NNRTIs.[21] More importantly, a dual mutation of Y181C and K103N has occurred, which can cause high-level resistance to each of the available NNRTIs. Studies indicated that compound **20** presents the similar potency as nevirapine against the K103N mutant, but at a much higher level than that of (+)-calanolide A (Table 2). The compound **20** was also proved with a moderate potency against G190A single mutation of HIV-1 ($EC_{50} = 43.9$ nM) but not for (+)-calanolide A ($EC_{50} = 385$ nM) and nevirapine ($EC_{50} = 159.4$ nM). Compound **20** was demonstrated an in vitro potency against wild-type HIV-1 approximately 10-fold higher than the natural product (+)-calanolide A, however, exceeding much higher ability (~90-fold) to inhibit the Y181C mutation than (+)-calanolide A while the nevirapine was completely inactive. The compound **20** presents less potency against T139I, which was indicated as a mutant site point of (+)-calanolide A treatment.[8] The suppression was not observed for the Y188L viral strain in the assays of compound **20**, (+)-calanolide A, and neverapine (Table 3). Accordingly, it is of particular interest in the inclusion of **20** in a combination regimen to treat patients who have failed other NNRTIs and have developed the Y181C mutation or the Y181C/K103N dual mutations. Therefore, compound **20** deserved to be selected and developed as a new drug candidate against the resistant HIV-1 infection.

Pharmacokinetic Study and Safety Assessment of 20. As indicated in Table 3, when administered at 50 mg/kg orally as a suspension in 0.5% CMC, the compound **20** was rapidly absorbed with a T_{max} of 8 h, a favorable half-life of 3.8 h, and mean residence time (MRT) of 9.2 h. The C_{max} of **20** was 0.34 μg/mL (860 nM) that is more than 100-fold the EC_{50} value of anti-HIV-1 activity in vitro (Table 1). The measured oral bioavailability in this experiment was moderately high (= 32.7%). Single dose toxicity test of compound **20** was carried out in mice. After intragastric administration of **20** with a dose of 4640 mg/kg, no death of the mice was observed and there was no abnormity of the body weight increase for the animals in two weeks (seeing Supporting Information). A long-term toxicity study is underway that will be reported elsewhere when it is finished.

Conclusion

This study provided one new HIV-1 inhibitor of compound **20** with low EC_{50} value (7.4 nM) and high SI (1417). Compound **20** has presented a druggable profile for its 32.7% oral bioavailability in rat, tolerated oral acute toxicity in mice, and extremely high potency against the clinical Y181C single mutation of HIV-1.

Experimental Section

Preparation of the Cl-Mimic Analogue 20. In a 50 mL round-bottom flask, 4-hydroxy-crotonic acid (250 mg) was treated in $SOCl_2$ (3 mL) at reflux for 2 h and then the resulting solution of acyl chloride was condensed to an yellow oil. The mixture of anhydrous $AlCl_3$ (500 mg) and 5,7-dihydroxy-coumarin (330 mg) in 3 mL of $PhNO_2$ and 15 mL of DCE were added to the flask and were simultaneously stirred at 75 °C for 36 h. After hydrolysis with 100 g of ice and 8 mL of hydrochloric acid, the mixture was extracted with EtOAc for 3 times and then the organic phase was dried and filtered. Chromatography on silica gel of the filtrate eluting first with petroleum and then with petroleum EtOAc (2:1) afforded 228 mg compound **18a** as a yellow powder with the yield of 47.2%; mp 174.5−176.1 °C. ^{1}H NMR (300 M Hz, DMSO-d_6, ppm): 11.290 (s, 1H, OH), 11.196 (s, 1H, OH), 6.635−6.798 (m, 2H, alkene-H), 6.376 (s, 1H, Ar−H), 5.911 (s, 1H, 3-H), 4.442 (d, 2H, $J = 8.4$ Hz, Cl-CH_2,), 2.861 (t, 2H, $J = 7.2$ Hz, 4-CH_2CH2CH3), 1.592 (sex, 2H, $J = 7.2$ Hz, 4-CH_2C$H_2$$CH_3$), 0.939 (t, 3H, $J = 7.5$ Hz, 4-CH_2CH_2CH_3). ESI-MS (m/z): 323.14/325.16 $[M + H]^+$ (MW = 322.74). HRESIMS obsd m/z 323.0689, calcd for $C_{16}H_{16}ClO_5^+$ 323.0681.

Anhydrous sodium acetate (10 mg) was added into the solution of **18a** in 20 mL of ethanol and 1 mL of H_2O and then stirred for 5 h at reflux. After condensation to about 3 mL, the resulted mixture was extracted with EtOAc for 3 times. Purification of the dried organic phase through chromatography on silica gel eluting with petroleum−EtOAc(1:1) produced 20 mg **19a** as white powder with the yield of 75%, mp 216.5−218.2 °C. ^{1}H NMR (300 M Hz, DMSO-d_6, ppm): 11.814 (s, 1H, OH), 6.315 (s, 1H, Ar−H), 6.046 (s, 1H, 3-H), 4.655−4.737 (m, 1H, 8-H), 3.602 (d, 2H, $J = 7.2$ Hz, $ClCH_2$), 2.871 (t, 2H, $J = 7.5$ Hz, 4-C$H_2CH_2CH_3$), 2.752 (m, 1H, 9-CH_2), 2.562 (d, 1H, $J = 3.3$ Hz, 9-CH_2), 1.550 (m, 2H, 4-CH_2CH_2CH_3), 0.930 (t, 3H, $J = 7.5$ Hz, 4-CH_2CH_2CH_3). ESI-MS (m/z) $[M + H]^+$: 323.09/325.08 (MW = 322.74). HRESIMS obsd m/z 323.0685, calcd for $C_{16}H_{16}ClO_5^+$ 323.0681.

6,6,-Dimethyl-10-chloromethyl-4-propyl-2*H*,6*H*,12*H*-benzo-[1,2-*b*:3,4-*b*′:5,6-*b*″]tripyran-2,12-dione (20). Compound **20** (white-off powder) was prepared from **19a** in the procedure described as the literature (ref 16) with the yield of 79%; mp 159.2−160.0 °C. ^{1}H NMR (300 M Hz, DMSO-d_6, ppm): 6.624 (d, 1H, $J = 10.2$ Hz, 8-H), 6.133 (s, 1H, 3-H), 5.852 (d, 1H, $J = 9.9$ Hz, 7-H), 4.857−4.918 (m, 1H, 10-H), 3.941−4.100 (m, 2H, $ClCH_2$), 2.842−2.939 (m, 3H, 11-CH_2, 4-C$H_2CH_2CH_3$), 2.683 (dd, 1H, J = 3.0 Hz, 16.2 Hz, 11-CH_2), 1.604 (m, 2H, 4-CH_2CH_2CH_3), 1.514, 1.472 (2s, 6H, 6-CH_3), 0.972 (t, 3H, $J = 7.2$ Hz, 4-CH_2CH_2CH_3). ESI-MS (m/z): 389.78 $[M + H]^+$ (MW = 388.84). HRESIMS obsd m/z389.1152, calcd for $C_{21}H_{22}ClO_5^+$ 389.1150.

Cell-Based Anti-HIV-1 Assay. The inhibitory activities of the compounds against HIV are evaluated as described previously by anti-HIV assays using TZM-b1 cells.[19] TZM cells contain the HIV primary receptor CD4 and coreceptor CCR5 as well as

a reporter luciferase gene driven by the HIV promoter. These assays quantify the activity of a drug to inhibit HIV-induced reporter luciferase activity. Briefly, a serial diluted drug was tested against 100 $TCID_{50}$ HIV infection. The viral infection was determined on day 3 by measuring the reporter luciferase activity in TZM-b1 cells postinfection using commercially available kits. Antiviral data are reported as the quantity of drug required to inhibit production by 50% (EC_{50}).

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Supporting Information Available: Experimental details of the synthesis of **6**−**17**, **21**−**25**; biological methods and detailed data of cytotoxicity and single dose acute toxicity test. This material is available free of charge via the Internet at http://pubs.acs.org.

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