Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 2807–2816

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis of Substituted Diarylmethylenepiperidines (DAMPs), a Novel Class of Anti-HIV Agents

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Received 25 October 2001; accepted 26 December 2001

Abstract—Substituted diarylmethylenepiperidines (DAMPs) were synthesized as conformationally restricted analogues of the alkenyldiarylmethane (ADAM) class of non-nucleoside reverse transcriptase inhibitors (NNRTIs). Although, like the ADAMs, the DAMPs were found to inhibit the cytopathic effect of HIV-1_{RF} in CEM-SS cells, they were completely inactive as inhibitors of HIV-1 reverse transcriptase. The DAMPs were assessed for inhibition of HIV attachment and fusion. DAMP **14** was active in both assays with IC_{50} values of 26.5 μM (TI 3.8) and 12.1 μM (TI: >8), respectively. DAMP **15** also inhibited HIV fusion with an IC_{50} 12.8 μM (TI: >6), but not virus attachment. However, attempts to verify inhibition of virus attachment and fusion as antiviral targets using time-of-addition experiments failed to confirm these observations, and instead identified an antiviral target occurring after completion of reverse transcription. DAMPs **11**, **12**, **14**, and **15** were found to inhibit virus replication if added 8 h post virus exposure, and DAMP **11** was equipotent at inhibition of virus replication if added 24 h after virus addition. DAMPs **11**, **12**, and **15** did not inhibit virus replication in TNF-α induced latently infected U1 cells, a model for post-integrative antiviral targets. When tested in both 3′ end-processing and strand-transfer assays in the presence of HIV-1 integrase, none of the DAMPs showed any inhibitory activity, indicating that HIV-1 integrase is not involved in the mechanism of the antiviral action. Thus, the DAMPs are novel conformationally restricted analogues of the previously published ADAM series with an unidentified antiviral target bounded by the completion of reverse transcription and virus integration.

Although combination chemotherapy of HIV infection with protease and reverse transcriptase inhibitors has been successful in delaying disease progression and improving the quality of life of AIDS patients, significant problems still remain, including drug toxicity and the emergence of resistant viral strains.[1–5] Consequently, there is a need for new anti-HIV agents with novel mechanisms of action. Recently, the appendage of a modified aurintricarboxylic acid (**1**) fragment to cholestane resulted in cosalane (**2**) and related analogues, which inhibit the binding of gp120 to CD4 and the fusion of the viral envelope with the cell membrane.[6–13] Work in the cosalane series led to the alkenyldiarylmethanes (e.g., **3**), which have proven to be effective non-nucleoside reverse transcriptase inhibitors.[14–18] Both **2** and **3** inhibit the cytopathic effect of HIV-1 in cell cultures.

The present investigation was undertaken in order to make and test conformationally restricted analogues of the ADAMs in which the alkenyl side chain is constrained in a piperidine ring. Another goal was to replace the terminal ester on the side chain with a carbamate, which would be expected to have enhanced metabolic stability. These considerations led to the design of substituted 4-(diarylmethylene)piperidines (DAMPs), represented by general structure **4**. As detailed in the present report, the DAMPs (**4**) have been successfully prepared and have been found to have significant anti-HIV activity, but they appear to act by a novel mechanism that is not related to either that of cosalane (**2**) or the ADAMs (e.g., **3**).

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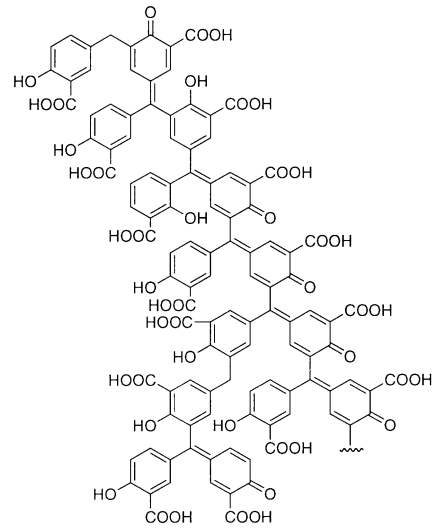
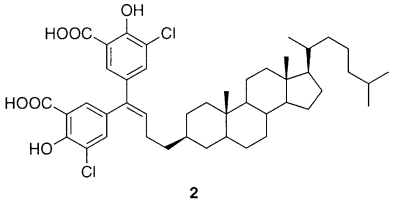
PII: S0968-0896(02)00095-0



1 (schematic representation)



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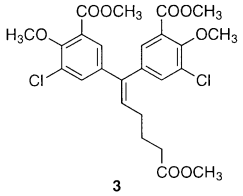

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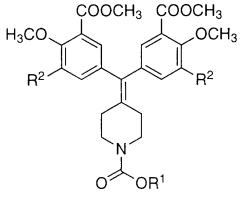

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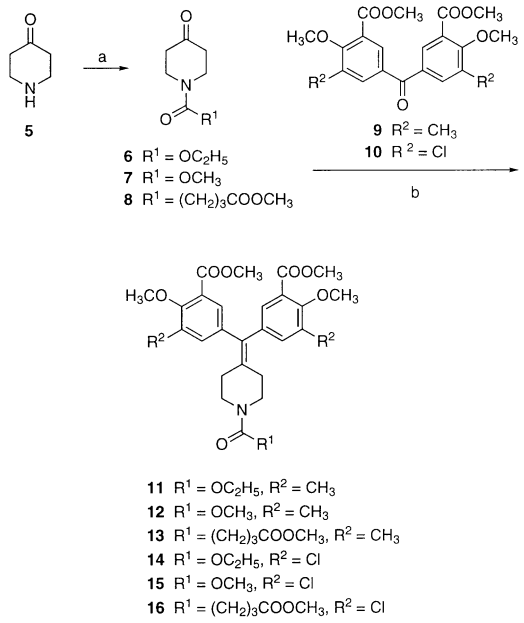
Chemistry

As shown in Scheme 1, treatment of 4-piperidone (**5**) with methyl chloroformate or 5-chloro-5-oxovalerate in aqueous sodium carbonate afforded the corresponding *N*-acylated 4-piperidones **7** and **8**. The related intermediate **6** is commercially available. The McMurry reaction of compounds **6**–**8** with the substituted benzophenones **9**[19] and **10**[6] afforded the desired DAMPs **11**–**16** in 78–98% yields. Similarly, reaction of the substituted piperidone **7** with dibenzosuberenone **17** or dibenzosuberone **18** produced the expected products **19** and **20** in 78 and 84% yield, respectively (Scheme 2).

In order to introduce more conformational flexibility into the DAMP system, the tetrasubstituted alkene was



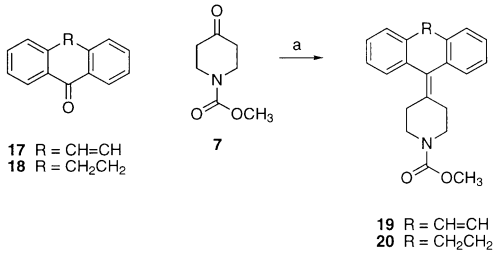
5

6 $R^1 = OC_2H_5$
7 $R^1 = OCH_3$
8 $R^1 = (CH_2)_3COOCH_3$

9 $R^2 = CH_3$
10 $R^2 = Cl$

11 $R^1 = OC_2H_5$, $R^2 = CH_3$
12 $R^1 = OCH_3$, $R^2 = CH_3$
13 $R^1 = (CH_2)_3COOCH_3$, $R^2 = CH_3$
14 $R^1 = OC_2H_5$, $R^2 = Cl$
15 $R^1 = OCH_3$, $R^2 = Cl$
16 $R^1 = (CH_2)_3COOCH_3$, $R^2 = Cl$

Scheme 1. Reagents and conditions: (a) R^1COCl, aq Na_2CO_3, 0 °C (2 h); (b)$TiCl_4$·THF, Zn dust, THF, reflux.

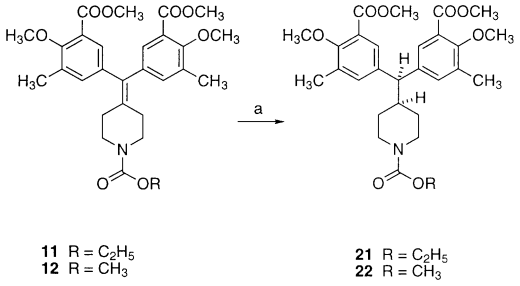


17 R = CH=CH
18 $R = CH_2CH_2$

7

19 R = CH=CH
20 $R = CH_2CH_2$

Scheme 2. Reagents and conditions: (a) $TiCl_4$·THF, Zn dust, THF, reflux (14 h).



11 $R = C_2H_5$
12 $R = CH_3$

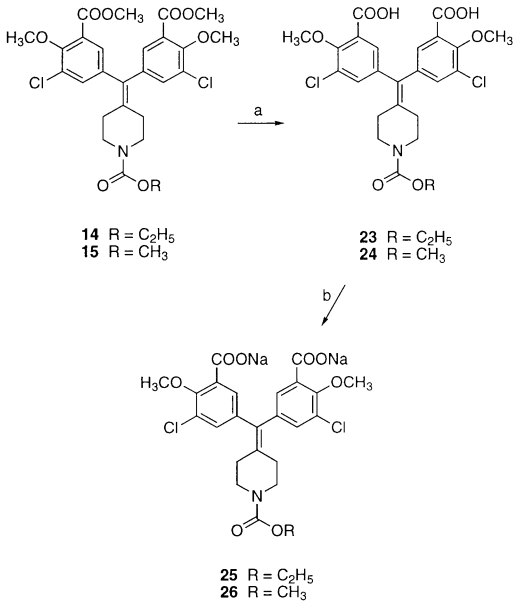
21 $R = C_2H_5$
22 $R = CH_3$

Scheme 3. Reagents and conditions: (a) H_2, Pd/C (30%), MeOH.

hydrogenated. As displayed in Scheme 3, hydrogenation of DAMPs **11** and **12**, using 30% palladium on charcoal as the catalyst, afforded the expected dihydro products **21** and **22**.

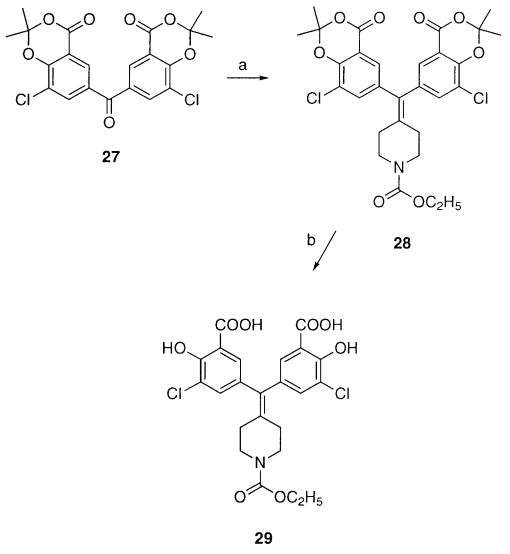
Hydrolysis of the two ester groups of DAMPs **14** and **15** in ethanolic sodium hydroxide led to the carboxylic acids **23** and **24** (Scheme 4). The disodium salts **25** and **26** were prepared by adding two equivalents of sodium hydroxide to the acids **23** and **24**.

In order to demethylate both the ester and ether groups of **14** while leaving the carbamate intact, the bis(acetonide) **27** was employed as the starting material.



Scheme 4. Reagents and conditions: (a) (1) NaOH, EtOH; (2) HCl; (b) NaOH, EtOH.

McMurry reaction of **27** with **6** yielded intermediate **28**, which was deprotected with sodium hydroxide in methanol to afford the desired compound **29** (Scheme 5).



Scheme 5. Reagents and conditions: (a) **6**, $TiCl_4$·THF, Zn dust, THF, reflux (14 h); (b) (1) NaOH, MeOH, 50–55 °C (3 h), (2) HCl.

Biological Results and Discussion

All of the new DAMPs were evaluated for inhibition of the cytopathic effect of HIV-1_{RF} in CEM-SS cell culture, and the results are listed in Table 1. The DAMPs **11**–**14** inhibited HIV-1 replication with a 50% reduction in virus-mediated cytopathicity (EC_{50}) in the 1 μM range. DAMPs **15**, **21**, **23**, and **25** were less potent, while the remaining DAMPs were without detectable antiviral activity. The cytotoxicities of the compounds in uninfected CEM-SS cells were also determined, and the resulting CC_{50} values are also listed in Table 1. The therapeutic index (TI) listed for each compound is the ratio of the cytotoxic concentration (CC_{50}) divided by the concentration required for antiviral efficacy (EC_{50}). The most active compound proved to be DAMP **12**, and it was also the compound with the highest therapeutic index.

Comparison of the activities of **11** and **12** with those of the dihydro derivatives **21** and **22** suggests that the greater conformational restriction provided by the double bond does contribute to the anti-HIV activity. The activities of the esters **14** and **15** versus the less active compounds **23** and **25**, or the inactive compounds **24**, **26**, and **29**, indicates that the four methyl groups of the

Table 1. Anti-HIV-1 activities of 6,6-diarylmethylenepiperidines

Compd	RT (IC_{50} μM)[a]	EC_{50} (μM)[b]	CC_{50} (μM)[c]	TI[d,e]
ADAM (2)	0.3	0.013	31.6	2430
Cosalane (4)		5.1	200	39.2
11	> 100	1.16	4.34	3.76
12	> 100	0.40	5.00	30
13	> 100	1.04	8.62	8.29
14	> 100	1.26	4.87	3.88
15	> 100	8.40	48.00	5.71
16	> 100	NA	12.70	
19	> 100	NA	11.90	
20		NA	12.20	
21	> 100	5.23	12.30	2.35
22	> 100	NA	8.58	
23		64.9	200	3.08
24	> 100	NA	200	
25		88.5	100	1.13
26	> 100	NA	200	
28		NA	100	
29	> 100	NA	10.5	

[a]Inhibitory activity versus HIV-1 reverse transcriptase with rCdG as the template primer.
[b]The EC_{50} is the inhibitory concentration for cytopathicity of HIV-1_{RF} in CEM-SS cells.
[c]The CC_{50} is the 50% cytotoxic concentration for mock-infected CEM cells.
[d]The TI is the therapeutic index, which is the CC_{50} divided by the EC_{50}.
[e]NA means there was no observed inhibition of HIV-1 cytopathicity up to the cytotoxic concentration in uninfected cells.

Table 2. Effects of selected 6,6-diarylmethylenepiperidines on viral attachment

Compd	IC_{50} (μM)[a]	CC_{50} (μM)[b]
Chicago Sky Blue	1.8[c]	> 10
Cosalane (**4**)	21	
11	> 100	> 100
12	> 100	> 100
13	> 100	> 100
14	26.5	100
15	> 100	> 100
23	52.7	100
25	48.8	100

[a]Attachment of HIV-1_{RF} to human PBLs, as quantitated by the association of virion p24 with the cells.
[b]Cytotoxic concentration in PBLs.
[c]The concentration of Chigago Sky Blue is reported in μg/mL.

Table 3. Effects of selected 6,6-diarylmethylenepiperidines on cell fusion

Compd	IC_{50} (μM)[a]	TC_{50} (μM)[b]
Chicago Sky Blue	2.3[e]	>10[e]
Cosalane (**4**)	33	
11	19.7	23.0
12	12.4	21.0
13	22.8	30.5
14	12.1	>100
15	12.8	>81.7

[a]Fusion of HeLaCD4/β-gal cells with HL2/3 cells expressing *env* and *tat* proteins.
[b]The TC_{50} is the 50% cytotoxic concentration for the combined population of HeLaCD4/β-gal cells and HL 2/3 cells.

aromatic esters and ethers are important for maximal antiviral activity.

Since the ADAMs (e.g., **2**), which function as non-nucleoside reverse transcriptase inhibitors, are structural analogues of the DAMPs, we expected that the biologically active DAMPs would be effective inhibitors of HIV-1 reverse transcriptase (RT). However, when tested for inhibition of RT with rCdG as the template primer, none of the DAMPs were active (Table 1).

Because cosalane (**2**) and related compounds are inhibitors of virus attachment and fusion, the DAMPs **11**, **12**, **13**, **14**, **15**, **23**, and **25** were also tested for those activities. In p24 ELISA assays designed to quantitate virus attachment to cells, compounds **11**, **12**, **13**, and **15** failed to inhibit attachment (Table 2). DAMP **14** was an inhibitor of virus attachment, but the IC_{50} of 26.5 μM was considerably higher than the EC_{50} of 1.26 μM necessary for inhibition of the cytopathic effect of the virus, which casts doubt on the idea that attachment inhibition is related to antiviral activity. The corresponding acid **23** (IC_{50} 52.7 μM) and the salt **25** (IC_{50} 48.8 μM) were less active attachment inhibitors than **14**, but their potencies as attachment inhibitors did approximate their antiviral activities (EC_{50} values of 74.9 and 88.5, respectively), suggesting that their anti-HIV-1 effects may be due to attachment inhibition.

To assess inhibition of fusion, HL2/3 cells expressing gp120 and intracellular Tat were mixed with HeLa CD4 LTR β-gal cells. Following cytoplasmic mixing after the gp120/CD4 mediates membrane fusion, Tat *trans* activates the β-galactosidase reporter, with inhibition of fusion correlating with decreased β-galactosidase production. The results of these assays involving DAMPs **11**–**15** are reported in Table 3. Although all five compounds yielded IC_{50} values, only **14** (IC_{50} 12.1 μM) and **15** (IC_{50} 12.8 μM) were sufficiently non-cytotoxic to be graded as active. These two DAMPs were essentially equipotent as fusion inhibitors, but they were less potent in the fusion assay than they were in the assay for anti-HIV-1 activity.

In order to gain further insight into the mechanism of action of the DAMPs, 'time-of-addition' (TOA) experiments were performed in order to identify the stage(s) of the HIV-1 replication cycle at which the compounds

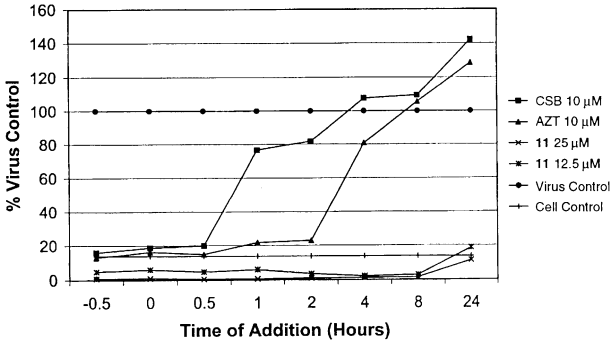


Figure 1. Time of addition experiment for DAMP **11**.

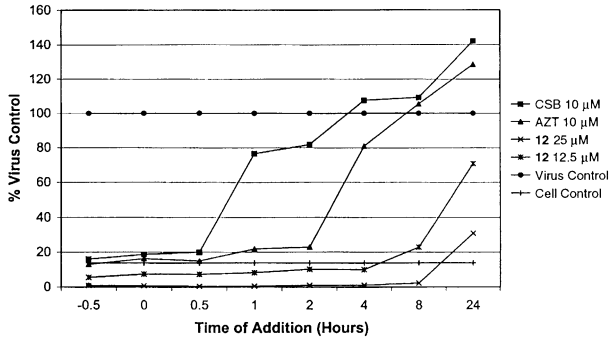


Figure 2. Time of addition experiment for DAMP **12**.

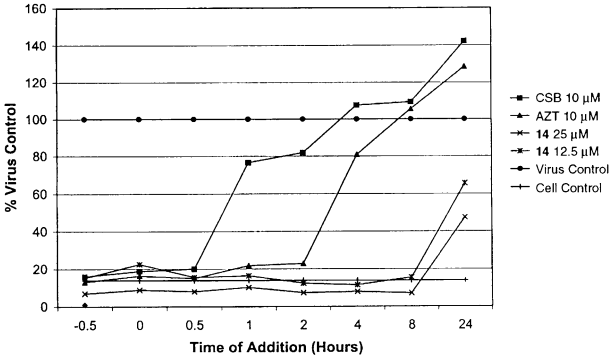


Figure 3. Time of addition experiment for DAMP **14**.

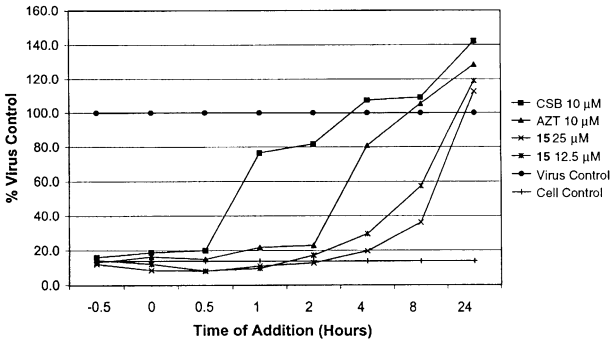


Figure 4. Time of addition experiment for DAMP **15**.

were acting. To accomplish this, a single round of infection assay using HeLa CD4 LTR β-gal cells was used. Virus replication was assessed at 48 h post-infection by β-galactosidase expression following the integration of the provirus, production of new Tat protein and transactivation of the LTR β-galactosidase enzyme reporter. Thus the time-of-addition assay models antiviral targets from initial virus-cell interaction (gp120-CD4) to the initiation of new virus transcription following synthesis of Tat. Timed addition of known inhibitors, such as zidovudine (AZT, reverse transcriptase) and Chicago Sky Blue[20] (CSB, attachment and fusion), are used to monitor the initiation and completion of these key processes during the pre-integrative phases of virus replication. The results of the TOA experiments are shown in Figure 1–4 for DAMPs **11**, **12**, **14**, and **15**, along with the control compounds AZT and CSB. These experiments show that the processes of virus attachment and fusion and reverse transcription are completed within the first 1 and 4 h of infection, respectively. In contrast, all four DAMPs retained significant antiviral activity (>60% inhibition of virus replication) if added 8 h post-infection. After 8 h, the potencies of the compounds vary, with compound **15** loosing all antiviral activity and **11** maintaining complete suppression, even if added 24 h post-infection. The DAMPs **12** and **14** show intermediate patterns of virus inhibition at 24 h with 70 and 35% inhibition of virus replication, respectively.

We have previously used latently HIV-1 infected TNF-α induced U1 cells to identify inhibitors with specificity for antiviral targets occurring after proviral integration.[21] DAMPs **11**, **12**, and **15** failed to inhibit virus replication in TNF-α induced U1 cells. This lack of activity for post-integrative antiviral targets was confirmed in chronically HIV-1 infected cells. CEM-SS cells chronically infected with the clinical HIV-1 isolate SK-1 were treated for 6 days with DAMPs **11**–**15**, and virus replication measured by supernatant reverse transcriptase activity. All five DAMPs failed to inhibit HIV replication in chronically infected cells. These results strongly suggest that the antiviral mechanism of action for the DAMPs is restricted to cellular or viral targets occurring at or prior to provirus integration. When tested in both 3′ end-processing and strand-transfer assays in the presence of HIV-1 integrase, none of the DAMPs showed any inhibitory activity up to a concentration of 110 μM, indicating that HIV-1 integrase is not involved in the mechanism of the antiviral action.

Therefore, these data indicate that despite the structural similarities of the DAMPs to the cosalanes and ADAMs, these compounds do not mediate their antiviral activity by inhibition of HIV attachment (cosalane) or reverse transcription (ADAM). Rather, the data supports interaction with a novel cellular or antiviral target present during the interval between the completion of reverse transcription and the initiation of new virus transcription. Potential antiviral targets in this interval include cytoplasmic transport of the pre-integration complex and the nuclear import of the complex.

Experimental

General

Melting points were determined in capillary tubes on a Mel-Temp apparatus and were uncorrected. Spectra were obtained as follows: CI mass spectra on a Finnegan 4000 spectrometer; FAB mass spectra and EI mass spectra on a Kratos MS50 spectrometer; and NMR spectra on a Bruker ARX-300 spectrometer. Microanalyses were performed at the Purdue Microanalysis Laboratory, and all values were within ±0.4% of the calculated compositions. Silica gel used for column chromatography was 230–400 mesh. 1-Carboethoxy-4-piperidone (**6**) was obtained from Aldrich.

1-Carbomethoxy-4-piperidone (7). 4-Piperidone monohydrate hydrochloride (3.07 g, 20.0 mmol) was dissolved in water (12 mL). The solution was stirred and cooled on an ice bath, and an ice-cold solution of potassium carbonate (6.97 g, 50.4 mmol) in water (40 mL) was added, followed by addition of methyl chloroformate (2.80 g, 29.6 mmol). Stirring was continued at 0 °C for 2 h, and the reaction mixture was then extracted with CH_2Cl_2 (3×40 mL). The CH_2Cl_2 extracts were dried (Na_2SO_4), evaporated and the crude residue purified by flash chromatography on silica gel (ethyl acetate/hexanes 1:1, v/v) to afford a colorless liquid (3.10 g, 98.7%). ^{1}H NMR ($CDCl_3$) δ 3.76 (t, J=6.04 Hz, 4H), 3.75 (s, 3H), 2.45 (t, J=6.18 Hz, 4H); ^{13}C NMR ($CDCl_3$) δ 207.22, 155.80, 53.00, 43.10, 41.06; CIMS m/z 158 (MH^+).

1-(4-Carbomethoxybutanoyl)-piperidin-4-one (8). 4-Piperidone monohydrate hydrochloride (1.53 g, 9.96 mmol) was dissolved in water (8 mL). The solution was stirred and cooled on an ice bath, and an ice-cold solution of potassium carbonate (3.48 g, 25.2 mmol) in water (20 mL) was added at 0 °C, followed by addition of methyl 5-chloro-5-oxovalerate (2.44 g, 14.8 mmol). Stirring was continued at 0 °C for 2 h, and the reaction mixture was extracted with CH_2Cl_2 (3 40 mL). The CH_2Cl_2 extracts were dried (Na_2SO_4), evaporated and the crude residue purified by flash chromatography on silica gel (ethyl acetate/hexanes 1:1, v/v) to afford a colorless liquid (1.77 g, 78.2%). ^{1}H NMR ($CDCl_3$) δ 3.86 (t, J=5.79 Hz, 2H), 3.75 (t, J=5.81 Hz, 2H), 3.66 (s, 3H), 2.45 (m, 8H), 1.98 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 206.72, 173.66, 170.91, 51.54, 43.97, 41.17, 40.77, 32.95, 32.06, 20.25; CIMS m/z (relative intensity) 228 (MH^+, 100), 196 (M^+ - OCH_3, 43).

Ethyl 4-(4′,4″-dimethoxy-3′,3″-di(methoxycarbonyl)-5′,5″-dimethyl-diphenylmethylene)piperidinecarboxylate (11). A slurry of $TiCl_4$·THF 1:2 complex (0.70 g, 2.1 mmol) and Zn dust (0.28 g, 4.2 mmol) in THF (25 mL) was heated under reflux and kept stirring for 2 h. A solution of benzophenone **9** (0.27 g, 0.70 mmol) and 1-carbethoxy-4-piperidone (**6**) (0.18 g, 1.1 mmol) in THF (10 mL) was added. The black mixture was heated under reflux for 15 h, and it was then cooled to room temperature and water (25 mL) was added. The mixture was stirred at room temperature 6 h. Then it was filtered through a pad of Celite and washed with ethyl acetate

(3×20 mL). The layers were separated and the aqueous one was extracted with ethyl acetate (2×20 mL). The combined organic extracts were washed with brine, dried over Na_2SO_4, filtered, and the solvent removed to give a brown residue. The crude residue was purified by flash chromatography (ethyl acetate/hexanes 1:2, v/v) to afford the product **11** (227 mg, 61.7%): mp 47–50 °C. ^{1}H NMR ($CDCl_3$) δ 7.32 (d, J = 3.0 Hz, 2H), 7.00 (d, J = 3.0 Hz, 2H), 4.09 (q, J = 7.0 Hz, 2H), 3.78 (s, 6H), 4H), 3.75 (s, 3H), 3.45 (t, J = 6.0 Hz, 4H), 2.26 (t, J = 6.0 Hz, 4H), 2.22 (s, 6H),1.20 (t, J = 7.5 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 166.77, 157.03, 155.42, 136.82, 136.10, 135.33, 134.90, 132.56, 129.96, 124.12, 61.39, 61.24, 52.11, 44.98, 31.37, 16.07, 14.59; CIMS m/z 526 (MH^+); HRMS calcd for $C_{29}H_{36}NO_8$ (MH^+) 526.2441, found 526.2431. Anal. calcd for $C_{29}H_{35}NO_8$: C, 66.21; H, 6.66; N, 2.66. Found C, 65.88; H, 6.90; N, 2.96.

Methyl 4-(4′,4″-dimethoxy-3′,3″-di(methoxycarbonyl)-5′,5″-dimethyl-diphenylmethylene)piperidinecarboxylate (12). $TiCl_4$·THF (1:2 complex) (0.93 g, 2.8 mmol) was added to a stirred suspension of zinc powder (0.37 g, 5.6 mmol) in dry THF (15 mL) under argon. The resulting dark mixture was heated under reflux for 2 h. The suspension was cooled to room temperature and a mixture of benzophenone **9** (348 mg, 0.901 mmol) and 1-carbomethoxy-4-piperdione **7** (152 mg, 0.967 mmol) in dry THF (10 mL) was added. The mixture was stirred at room temperature for 1.5 h and then heated at reflux for 15 h. The resulting solution was poured into 10% aqueous potassium carbonate (15 mL), followed by filtration through a pad of Celite. The filtrate was evaporated and the crude residue was purified by flash chromatography on silica gel (18 g, column: 2.5×31 cm), eluting with EtOAc–hexanes (1:2, v/v) to afford compound **12** (260 mg, 56.4%): mp 45–46 °C. ^{1}H NMR ($CDCl_3$) δ 7.34 (d, J = 2.08 Hz, 2H), 7.01 (d, J = 2.09 Hz, 2H), 3.87 (s, 6H), 3.83 (s, 6H), 3.67 (s, 3H), 3.46 (t, J = 5.66 Hz, 4H), 2.27 (t, J = 5.66 Hz, 4H), 2.24 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 166.80, 157.04, 155.84, 136.79, 136.10, 135.20, 135.02, 132.59, 129.96, 124.17, 61.44, 52.53, 52.15, 45.06, 32.54, 31.37; CIMS m/z (relative intensity) 512 (MH^+, 28), 480 ($M^+ - OCH_3$, 100); HRMS calcd for $C_{28}H_{33}NO_8$ 511.2206, found 511.2210. Anal. calcd for $C_{28}H_{33}NO_8$: C, 65.74; H, 6.50; N, 2.74. Found C, 65.36; H, 6.61; N, 2.50.

Methyl 5-[4-{4′,4″-dimethoxy-4′,4″-bis(methoxycarbonyl)-5′,5″-(dimethyl)diphenylmethylene}piperidyl]-5-oxopentanoate (13). A slurry of $TiCl_4$·THF (1:2 complex) (1.28 g, 3.84 mmol) and Zn dust (0.50 g, 7.6 mmol) in THF (20 mL) was heated under reflux for 2 h. A mixture of benzophenone **9** (0.46 g, 1.2 mmol) and ketone **8** (0.38 g, 1.7 mmol) in dry THF (10 mL) was added. The dark mixture was stirred at room temperature for 1.5 h and then heated under reflux for 12 h. It was cooled to room temperature and an aqueous 10% K_2CO_3 solution (10 mL) was added. The mixture was stirred overnight. The precipitate was filtered off and washed with chloroform. The filtrate was concentrated and the residue was chromatographed on silica gel (13 g, column: 2.5 cm × 31 cm), eluting with EtOAc– hexanes (1:1.2, v/v) to provide **13** as a light-yellow liquid (100 mg, 14.4%). ^{1}H NMR ($CDCl_3$) δ 7.35 (d, J = 2.23 Hz, 2H), 7.02 (d, J = 2.23 Hz, 2H), 3.90 (s, 6H), 3.81 (s, 6H), 3.65 (s, 3H), 3.59 (br, 2H), 3.45 (br, 2H), 2.40 (t, J = 7.03 Hz, 2H), 2.39 (t, J = 7.18 Hz, 2H), 2.31 (m, 4H), 2.26 (s, 6H), 1.94 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 173.75, 170.60, 166.80, 157.12, 136.60, 136.50, 136.06, 135.29, 134.78, 132.68, 129.93, 124.20, 61.43, 52.17, 51.48, 47.00, 43.57, 33.11, 32.27, 31.99, 31.26, 20.40, 16.10; CIMS m/z 582 (MH^+); HRMS calcd for $C_{32}H_{39}NO_9$ 581.2625, found 581.2625. Anal. calcd for $C_{32}H_{39}NO_9$: C, 66.08; H, 6.76; N, 2.41. Found C, 65.87; H, 6.70; N, 2.32.

Ethyl 4-(3′,3″-dichloro-4′,4″-dimethoxy-5,5-di(methoxycarbonyl)-diphenylmethylene)piperidinecarboxylate (14). $TiCl_4$·THF (1:2 complex) (1.55 g, 4.65 mmol) was added to a stirred suspension of zinc powder (608 mg, 9.30 mmol) in THF (20 mL) under argon. The resulting dark mixture was heated under reflux for 1 h. The suspension was cooled to room temperature and a mixture of benzophenone **10** (639 mg, 1.50 mmol) and 1-carboethoxy-4-piperidone (**6**) (0.28 g, 1.6 mmol) in THF (10 mL) was added. The mixture was heated at reflux and stirred for 15 h, cooled, and poured into 10% aqueous potassium carbonate (20 mL). Then it was filtrated through a pad of Celite and the filtrate was evaporated to give a light-yellow residue, which was purified by flash chromatography (EtOAc–hexanes 1:2, v/v) to afford compound **14** (495 mg, 58.3%): mp 51–53 °C. ^{1}H NMR ($CDCl_3$) δ 7.40 (d, J = 2.37 Hz, 2H), 7.24 (d, J = 2.03 Hz, 2H), 4.12 (q, J = 7.13 Hz, 2H), 3.92 (s, 6H), 3.89 (s, 6H), 3.49 (t, J = 5.62 Hz, 4H), 2.29 (t, J = 5.57 Hz, 4H), 1.23 (t, J = 7.16 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 165.38, 155.36, 154.64, 137.93, 137.29, 134.84, 132.60, 130.73, 129.57, 126.66, 61.94, 61.38, 52.50, 44.76, 31.42, 14.59; CIMS m/z 566 (MH^+); HRMS calcd for $C_{27}H_{29}NCl_2O_8$ 566.1348, found 566.1331. Anal. calcd for $C_{27}H_{29}NCl_2O_8$: C, 57.25; H, 5.16; N, 2.54. Found C, 57.01; H, 5.19; N, 2.14.

Methyl 4-(3′,3″-dichloro-4′,4″-dimethoxy-5′,5″-di(methoxycarbonyl)-diphenylmethylene)piperidinecarboxylate (15). A mixture of $TiCl_4$·THF (1:2 complex) (1.07 g, 3.20 mmol) and Zn powder (419 mg, 6.41 mmol) in dry THF (25 mL) was heated under reflux for 2 h under nitrogen. A solution of benzophenone **10** (426 mg, 0.997 mmol) and 1-carbomethoxy-4-piperidone **7** (199 mg, 1.27 mmol) in dry THF (15 mL) was added, the reaction mixture was stirred at room temperature for 1 h, and then heated under reflux for 15 h. The mixture was allowed to cool and 10% aqueous potassium carbonate (20 mL) was added. Then it was filtrated through a pad of Celite, and washed with ethyl acetate (3×15 mL). The organic solvents were evaporated and the crude residue was purified by silica gel flash chromatography (hexanes/EtOAc 2:1, v/v) to afford a white solid (269 mg, 48.8%): mp 48–50 °C. ^{1}H NMR ($CDCl_3$) δ 7.39 (d, J = 2.29 Hz, 2H), 7.23 (d, J = 2.31 Hz, 2H), 3.91 (s, 6H), 3.89 (s, 6H), 3.67 (s, 3H), 3.47 (t, J = 5.45 Hz, 4H), 2.28 (t, J = 5.45 Hz, 4H); ^{13}C NMR ($CDCl_3$) δ 165.54, 155.78, 154.67, 137.71, 137.23, 134.81, 132.68, 130.68, 129.57, 126.66, 61.93, 52.49, 44.81, 31.37; EIMS m/z 551 (M^+); HRMS calcd for $C_{26}H_{27}Cl_2NO_8$ 551.1114, found 551.1094. Anal. calcd for $C_{26}H_{27}NCl_2O_8$: C, 56.53; H, 4.93; N, 2.54. Found C, 56.43; H, 5.17; N, 2.44.

Methyl 5-[4-{3′,3″-dichloro-4′,4″-dimethoxy-5,5-di(methoxycarbonyl)diphenylmethylene}piperidyl]-5-oxopentanoate (16). A mixture of $TiCl_4$·THF (1:2 complex) (1.10 g, 3.29 mmol) and Zn powder (431 mg, 6.59 mmol) in dry THF (20 mL) was heated under reflux for 2 h under nitrogen. A solution of benzophenone **10** (426 mg, 0.997 mmol) and ketone **8** (269 mg, 1.18 mmol) in dry THF (15 mL) was added. The reaction mixture was stirred at room temperature for 0.5 h, and then heated under reflux for 14 h. The mixture was allowed to cool and 10% aqueous potassium carbonate (20 mL) was added. The resulting mixture was stirred at room temperature overnight, then filtrated through a Celite pad, and washed with ethyl acetate (3×15 mL). The organic solvents were evaporated and the crude residue was purified by silica gel flash chromatography (hexanes/EtOAc 1:2, v/v) to afford a colorless liquid (80.8 mg, 13.0%). ^{1}H NMR ($CDCl_3$) δ 7.40 (d, J = 2.28 Hz, 2H), 7.25 (d, J = 2.28 Hz, 2H), 3.94 (s, 6H), 3.91 (s, 6H), 3.66 (s, 3H), 3.63 (br, 2H), 3.49 (br, 2H), 2.41 (t, J = 6.98 Hz, 2H), 2.40 (t, J = 7.15 Hz, 2H), 2.33 (m, 4H), 1.95 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 173.72, 170.65, 165.54, 154.71, 137.30, 137.20, 137.02, 134.78, 132.93, 130.66, 129.66, 129.63, 126.69, 61.97, 52.53, 51.50, 46.14, 42.58, 33.05, 32.24, 31.97, 31.10, 20.35; CIMS m/z 523 (M^+). Anal. calcd for $C_{30}H_{33}NCl_2O_9$: C, 57.89; H, 5.34; N, 2.25. Found C, 57.77; H, 5.47; N, 2.22.

Methyl 4-dibenzo[*b*,*f*][7]annulen-5-ylidenepiperidinecarboxylate (19). $TiCl_4$·THF (1:2) complex (2.24 g, 6.71 mmol) was added to a stirred suspension of zinc dust (879 g, 13.44 mmol) in dry THF (10 mL). The resulting black mixture was heated at reflux for 1 h under argon. The suspension was cooled to room temperature and a solution of dibenzosuberenone **17** (433 mg, 2.10 mmol) and 1-carbomethoxy-4-piperidone **7** (363 mg, 2.31 mmol) in dry THF (12 mL) was added. The mixture was heated at reflux for 14 h and then concentrated. The concentrated residue was passed through a short column (silica gel: 5 g, eluent: ethyl acetate, 20 mL) to remove the black solid. The combined fractions were evaporated and the yellow residue was purified by flash chromatography on silica gel (60 g), eluting with hexanes-ethyl acetate (3:1, v/v), to give a light-yellow solid (542 mg, 77.9%): mp 63–65 °C. ^{1}H NMR ($CDCl_3$) δ 7.22–7.41 (m, 8H), 6.97 (s, 2H), 3.66–3.80 (br, 2H), 3.73 (s, 3H), 3.12 (ddd, J = 12.89, 8.98, and 3.88 Hz, 2H), 2.31 (ddd, J = 12.89, 8.89, and 4.40 Hz, 2H), 2.18 (dt, J = 19.0 and 4.77 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 155.74, 138.64, 134.80, 134.59, 134.20, 130.85, 128.18, 128.12, 127.79, 126.35, 52.44, 45.22, 29.84; CIMS m/z 332 $(MH)^+$. Anal. calcd for $C_{22}H_{21}NO_2$: C, 79.76; H, 6.34; N, 4.23. Found C, 79.63; H, 6.60; N, 3.97.

Methyl 4-(5,6-dihydrodibenzo[*b*,*f*][7]annulen-11-ylidene)piperidinecarboxylate (20). $TiCl_4$·THF (1:2) complex (4.27 g, 12.8 mmol) was added to a stirred suspension of zinc dust (1.67 g, 25.6 mmol) in dry THF (12 mL). The resulting black mixture was heated at reflux for 1 h under argon. The suspension was cooled to room temperature and a solution of dibenzosuberone **18** (833 mg, 4.00 mmol) and 1-carbomethoxy-4-piperidone **7** (691 mg, 4.40 mmol) in dry THF (18 mL) was added. The mixture was heated at reflux for 14 h and then concentrated. The concentrated residue was passed through a short column (silica gel: 10 g, eluent: ethyl acetate 40 mL) to remove the solid. The combined fractions were evaporated and the yellow residue was purified by flash chromatography on silica gel (80 g), eluting with hexanes–ethyl acetate (3:1, v/v), to give a light-yellow solid (1.07 g, 80.2%): mp 59–61 °C. ^{1}H NMR ($CDCl_3$) δ 7.09–7.30 (m, 8H), 3.76 (m, 2H), 3.76 (s, 3H), 3.45 (ddd, J = 15.07, 8.36, and 5.45 Hz, 2H), 3.21 (m, 2H), 2.88 (ddd, J = 15.20, 8.49, and 4.18 Hz, 2H), 2.42 (t, J = 5.71 Hz, 4H); ^{13}C NMR ($CDCl_3$) δ 155.81, 140.28, 137.80, 136.18, 132.73, 129.26, 128.51, 126.92, 125.47, 52.47, 45.11, 32.32, 30.60; CIMS m/z 334 $(MH)^+$. Anal. calcd for $C_{22}H_{23}NO_2$: C, 79.25; H, 6.95; N, 4.20. Found C, 79.52; H, 7.09; N, 4.10.

Ethyl 4-(4′,4″-dimethoxy-3′,3″-di(methoxycarbonyl)-5′,5″-dimethyl-diphenylmethyl)piperidinecarboxylate (21). The piperidinecarboxylate **11** (42 mg, 0.08 mmol) was hydrogenated at 60 psi over palladium (30%) on activated carbon (40 mg) in MeOH (15 mL). After TLC had shown that all the starting material was consumed, the catalyst was filtered and the solvent was evaporated. The crude residue was flash chromatographed on silica gel (15 g) using hexanes/EtOAc (1:1 v/v) to give product **21** as a colorless oil (41.8 mg, 99.1%). ^{1}H NMR ($CDCl_3$) δ 7.51 (d, J = 1.87 Hz, 2H), 7.20 (d, J = 1.87 Hz, 2H), 4.10 (q, J = 7.08 Hz, 2H), 3.91 (s, 6H), 3.82 (d, J = 2.83 Hz, 1H), 3.77 (s, 6H), 3.70 (m, 1H), 3.39 (m, 1H), 2.72 (t, J = 12.63 Hz, 2H), 2.28 (s, 3H), 2.19 (m, 1H), 1.51 (m, 2H), 1.23 (t, J = 7.08 Hz, 3H), 1.04 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 166.83, 156.79, 155.38, 138.03, 134.41, 132.97, 128.18, 124.38, 61.32, 61.04, 57.15, 52.10, 43.75, 39.47, 30.90, 16.16, 14.55. Anal. calcd for $C_{29}H_{37}NO_8$: C, 66.03; H, 7.02; N, 2.66. Found C, 65.75; H, 7.31; N, 2.54.

Methyl 4-(4′,4″-dimethoxy-3′,3″-di(methoxycarbonyl)-5′,5″-dimethyl-diphenylmethyl)piperidinecarboxylate (22). The piperidinecarboxylate **12** (82 mg, 0.16 mmol) was hydrogenated at 60 psi over palladium (30%) on activated carbon (50 mg) in MeOH (20 mL). After TLC had shown that all the starting material was consumed, the catalyst was filtered and the solvent was evaporated. The crude residue was flash chromatographed on silica gel (15 g) using hexanes/EtOAc (1:1 v/v) to give product **22** as a colorless oil (60.4 mg, 73.4%). ^{1}H NMR ($CDCl_3$) δ 7.50 (d, J = 2.22 Hz, 2H), 7.19 (d, J = 2.02 Hz, 2H), 3.91 (s, 6H), 3.84 (d, J = 3.14 Hz, 1H), 3.76 (s, 6H), 3.69 (m, 1H), 3.67 (s, 3H), 3.38 (m, 1H), 2.73 (t, J = 12.63 Hz, 2H), 2.27 (s, 6H), 2.18 (m, 1H), 1.51 (m, 2H), 1.02 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 166.79, 156.79, 155.76, 137.98, 134.38, 132.97, 128.15, 124.38, 61.31, 57.12, 52.34, 52.09, 43.84, 39.40, 30.88, 16.15. Anal. calcd for $C_{28}H_{35}NO_8$: C, 65.50; H, 6.82; N, 2.73. Found C, 65.75; H, 6.30; N, 2.46.

Ethyl 4-(3′,3″-dicarboxy-5′,5″-dichloro-4′,4″-dimethoxydiphenylmethylene)piperidinecarboxylate (23). Diester **14** (200 mg, 0.353 mmol) and NaOH (1.2 mL of 1 N NaOH solution) were added to aqueous ethanol (3:2, v/v, 6 mL). The mixture was heated on a water bath at 65–70 °C for 3 h. The reaction mixture was concentrated to remove ethanol, and then acidified with concd HCl.

The acidified suspension was extracted with ethyl acetate (3×10 mL). The organic layer was separated, dried over Na_2SO_4 and crystallized in hexanes–ethyl acetate (2:1, v/v) to afford white crystals (180 mg, 94.7%): mp 116–118 °C. ^{1}H NMR ($CDCl_3$) δ 7.71 (d, J = 2.15 Hz, 2H), 7.36 (d, J = 2.34 Hz, 2H), 4.15 (d, J = 7.14 Hz, 2H), 4.03 (s, 6H), 3.56 (t, J = 4.99 Hz, 4H), 2.35 (t, J = 5.12 Hz, 4H), 1.29 (t, J = 7.07 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 163.06, 151.74, 150.87, 134.80, 134.03, 132.38, 128.19, 125.58, 125.19, 125.08, 120.70, 58.64, 57.85, 40.87, 27.56, 10.72; CIMS m/z 538 $(MH)^+$. Anal. calcd for $C_{25}H_{25}NCl_2O_8$: C, 55.76; H, 4.65; N, 2.60. Found C, 54.63; H, 4.75; N, 2.22.

Methyl 4-(3′,3′-dicarboxy-5′,5″-dichloro-4′,4″-dimethoxydiphenylmethylene)piperidinecarboxylate (24). Diester **15** (410 mg, 0.742 mmol) and NaOH (2.23 mL of 1 N NaOH solution) were added to aqueous ethanol (3:2, v/v, 10 mL). The mixture was heated on a water bath at 50–55 °C for 3 h. The reaction mixture was concentrated to remove ethanol, and then acidified with concd HCl. The acidified suspension was extracted with ethyl acetate (3×15 mL). The organic layer was separated, dried over Na_2SO_4 and crystallized in hexanes–ethyl acetate (2:1, v/v) to afford white crystals (356 mg, 91.5%): mp 143–145 °C. ^{1}H NMR ($CDCl_3$) δ 7.65 (d, J = 2.28 Hz, 2H), 7.30 (d, J = 2.13 Hz, 2H), 4.02 (s, 6H), 3.66 (s, 3H), 3.51 (t, J = 5.35 Hz, 4H), 2.31 (t, J = 5.35 Hz, 4H); ^{13}C NMR ($CDCl_3$) δ 167.25, 155.98, 154.81, 138.39, 137.78, 136.15, 132.19, 131.94, 129.17, 124.70, 62.43, 52.87, 44.78, 31.36; ESI m/z 524 $(MH)^+$. Anal. calcd for $C_{24}H_{23}NCl_2O_8$: C, 54.96; H, 4.39; N, 2.67. Found C, 54.14; H, 4.41; N, 2.52.

Ethyl 4-(3′,3″-dicarboxy-5′,5″-dichloro-4′,4″-dimethoxydiphenylmethylene)piperidinecarboxylate disodium salt (25). The diacid **23** (107 mg, 0.199 mmol) was dissolved in a mixture of ethanol (2 mL) and a 1 N solution of NaOH (0.4 mL). The resulting solution was concentrated and dried in vacuo to yield a white solid (116 mg, 100%): mp 300 °C. ^{1}H NMR (D_2O) δ 7.26 (d, J = 2.12 Hz, 2H), 7.15 (d, J = 2.15 Hz, 2H), 4.09 (q, J = 7.20 Hz, 2H), 3.85 (s, 6H), 3.46 (t, J = 5.06 Hz, 4H), 2.31 (t, J = 5.06 Hz, 4H), 1.21 (t, J = 7.01 Hz, 3H). Anal. calcd for $C_{24}H_{21}NNa_2Cl_2O_8$: C, 50.70; H, 3.70; N, 2.46. Found C, 50.32; H, 3.92; N, 2.15.

Methyl 4-(3′,3″-di(carboxy)-5′,5″-dichloro-4′,4″-dimethoxy-diphenylmethylene)piperidinecarboxylate disodium salt (26). The diacid **24** (104 mg, 0.198 mmol) was dissolved in a mixture of ethanol (2 mL) and a 1 N solution of NaOH (0.4 mL). The resulting solution was concentrated and dried in vacuo to yield a white solid (113 mg, 100%): mp 300 °C. ^{1}H NMR (D_2O) δ 7.22 (d, J = 1.83 Hz, 2H), 7.15 (d, J = 2.12 Hz, 2H), 3.86 (s, 6H), 3.68 (s, 3H), 3.48 (t, J = 5.35 Hz, 4H), 2.33 (t, J = 5.35 Hz, 4H). Anal. calcd for $C_{25}H_{23}NNa_2Cl_2O_8$: C, 51.55; H, 3.95; N, 2.41. Found C, 51.21; H, 3.78; N, 2.32.

Ethyl 4-[bis(8-chloro-2,2-dimethyl-4-oxobenzo[3,4-*E*]1,3-dioxin-6-yl)methylene]piperidinecarboxylate (28). $TiCl_4$·THF (1:2 complex) (1.15 g, 3.44 mmol) was added to a stirred suspension of zinc powder (452 mg, 6.91 mmol) in THF (25 mL) under argon. The resulting dark mixture was heated under reflux for 1 h. The suspension was cooled to room temperature and a mixture of benzophenone **27** (485 mg, 1.08 mmol) and 1-carbethoxy-4-piperidone (**6**) (216 mg, 1.29 mmol) in THF (15 mL) was added. The mixture was heated at reflux and stirred for 14 h, cooled, and poured into 10% aqueous potassium carbonate (20 mL). Then it was filtrated through a pad of Celite and the filtrate was evaporated to give a light yellow residue, which was purified by flash chromatography (EtOAc–hexanes 1:1, v/v) to afford compound **28** as a white powder (204 mg, 32.1%): mp 154–156 °C. ^{1}H NMR ($CDCl_3$) δ 7.58 (d, J = Hz, 2H), 7.30 (d, J = Hz, 2H), 4.12 (q, J = Hz, 2H), 3.50 (t, J = Hz, 4H), 2.29 (t, J = Hz, 4H), 1.77 (s, 12H), 1.23 (t, J = Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 159.93, 155.32, 151.01, 138.54, 137.34, 135.55, 132.0, 128.83, 122.39, 114.59, 107.39, 61.43, 44.69, 31.45, 25.90, 14.58; EIMS m/z 590 $(MH)^+$. Anal. calcd for $C_{29}H_{29}NCl_2O_8$: C, 58.98; H, 4.92; N, 2.37. Found C, 58.91; H, 5.15; N, 2.14.

Ethyl 4-(3′,3″-di(carboxy)-5′,5″-dichloro-4′,4″-dihdroxydiphenylmethylene)piperidinecarboxylate (29). Diester **28** (150 mg, 0.254 mmol) and NaOH (3 mL of 1 N NaOH solution) were added into aqueous methanol (3:2, v/v, 5 mL). The mixture was heated on a water bath at 50–55 °C for 3 h, the reaction mixture was concentrated to remove methanol, and then acidified with 1 N HCl (5.4 mL). The acidified suspension was extracted with ethyl acetate (3×10 mL). The organic layer was separated, dried over Na_2SO_4 and crystallized in hexanes/ethyl acetate (2:1, v/v) to afford white crystals (94 mg, 72%): mp 270–272 °C. ^{1}H NMR ($CDCl_3$) δ 7.70 (d, J = 2.11 Hz, 2H), 7.51 (d, J = 2.10 Hz, 2H), 4.10 (q, J = 7.12 Hz, 2H), 3.56 (t, J = 5.55 Hz, 4H), 2.38 (t, J = 5.77 Hz, 4H), 1.22 (t, J = 7.09 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 172.64, 157.88, 156.30, 138.64, 138.10, 134.27, 133.88, 131.28, 122.71, 114.83, 62.03, 45.93, 32.65, 15.39; CIMS m/z 510 $(MH)^+$. Anal. calcd for $C_{29}H_{29}NCl_2O_8$: C, 54.13; H, 4.15; N, 2.75. Found C, 54.42; H, 4.55; N, 2.37.

Cells and viruses

U1, CEM-SS, HL2/3 and HeLa CD4 LTR β-gal cell lines were obtained from the NIAID AIDS Research and Reference Reagent Program (Bethesda, MD, USA). The U1 and CEM-SS cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL), and the HeLa CD4 LTR β-gal cells were maintained in DMEM 10% FBS, penicillin, streptomycin and glutamine. HeLa CD4 LTR β-gal cell cultures were also supplemented with G418 (200 μg/mL) and hygromycin (100 μg/mL), which was removed prior to assays by a 24 h pre-culture in selection antibiotic-free medium. Human immunodeficiency virus type 1 (HIV-1) strains RF and IIIB were obtained from the NIAID AIDS Research and Reference Reagent Program. CEM-SS cells chronically infected with the clinical isolate SK-1[22] of HIV-1 were derived from an acute infection, and maintained as described above.

HIV cytoprotection assay

The inhibitory activities of the compounds were evaluated as previously described with the cytopathic RF strain of HIV-1 and CEM-SS cells.[23] These are microtiter assays, which quantitate the ability of a compound to inhibit HIV-1 induced cell killing via syncytium formation. Cytoprotection and compound cytotoxicity are measured with the CellTiter 96® Reagent (Promega, Madison, WI, USA) 6 days post infection. This reagent contains the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) and the electron coupling agent phenazine ethosulfate in a colorless stable solution, which upon reduction by viable cells forms a colored solution with absorbance at 490 nm. Antiviral and toxicity data are reported as the concentration of compound required to inhibit 50% virus-induced cell killing (50% effective concentration [EC_{50}]) and the concentration of compound required to reduce cell viability by 50% (cytotoxicity). All data are derived from triplicate tests with the variation of the mean averaging 10%.

RT inhibition assay

Analysis of the effects of the compounds on recombinant HIV-1 RT enzyme (p66/51 dimer) were performed as previously described.[24] Briefly, inhibition of purified recombinant reverse transcriptase enzyme was measured by the incorporation of [^{32}P] GTP into poly(rC)-oligo(dG) (rCdG) homopolymer template primers.

HIV attachment and fusion assays

Attachment and fusion assays were performed as previously described with minor modifications.[22] Briefly, for the attachment assay, compound and cells (1×10^4) were preincubated for 30 min and a pre-titered amount of HIV-1 IIIB added. Cells and virus were incubated for 1 h and unbound virus removed by washing. The fusion assay was performed by coculture of 5×10^3 HL2/3 and HeLa CD4 LTR β-gal cells, following pre-exposure of both cell lines to the compounds for 30 min. Virus replication in both assays was detected by chemiluminescence using a single step lysis and detection method (Tropix Gal-screen™, Bedford, MA, USA). Virus binding to HeLa CD4 LTR β-gal cells was quantitated by determination of cell-associated p24 antigen (ELISA, Coulter Electronics Hialeah, Fl, USA) to confirm lack of inhibitory activity on virus-cell interaction, following a 1 h adsorption of virus and vigorous washing to remove unbound virus.

Time-of-addition assay

Time-of-addition assays (TOA) were performed using a pre-titered concentration of the IIIB strain of HIV and HeLa CD4 LTR β-gal cells. Test materials were added 30 min before, at the time of infection or at 30 min, 1, 2, 4, 8, or 24 h post infection. At 2 h post infection unbound virus was removed by washing and cultures continued with fresh compound. At 48 h post infection β-galactosidase enzyme expression was determined by chemiluminescence. The compounds CSB and AZT were used as positive controls.

Integration enzyme assay

The integration reaction mixtures contained MOPS (250 mM), pH 7.2, $MnCl_2$ (75 mM), β-mercaptoethanol (14.3 mM), BSA (0.1 mg/mL) and the 21 mer duplex (10 nM) of the HIV-1 U5 LTR. The sequence of the top strand of the U5 LTR was 5′ GTGTGGAAAATCTCTAGCAGT3′, and the bottom strand was the complementary sequence. The 5′ end of the top strand was labeled with ^{32}P, by T4 polynucleotide kinase according to the manufacturer's recommendations (New England BioLabs, Beverly, MA). Unincorporated nucleotide was removed by applying the labeling mixture to a BioSpin6 column (BioRad, Hercules, CA). The top and bottom strands were annealed by heating the mixture at 95 °C for 5 min and cooling to room temperature. The DAMPs **11**, **12**, **14**, and **15** were dissolved in DMSO at a concentration equal to 10 times the final concentration, and the resulting solutions (1 μL) were added to reaction mixtures without DNA (8.5 μL). The final DAMP concentrations were 111, 37, and 12.3 μM. After a 10 min preincubation of the drug and integrase at room temperature, the DNA was added (0.5 μL) to allow the reaction to occur. The mixtures were incubated at 37 °C for 30 min. Gel loading dye (98% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 10 mM EDTA) was added to each tube to stop the reactions, and the reactions were heated at 95 °C. The 21mer substrate was separated from 19mer and strand transfer products on a 20% denaturing gel. The gels were exposed to a Molecular Dynamics phosphoimager screen and quantified by ImageQNT.[25]

Acknowledgements

This investigation was made possible by Grant RO1-AI-43637, awarded by the National Institutes of Health, DHHS.

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