Dicaffeoyltartaric Acid Analogues Inhibit Human Immunodeficiency Virus Type 1 (HIV-1) Integrase and HIV-1 Replication at Nontoxic Concentrations

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The human immunodeficiency virus type 1 (HIV-1) is a major health problem worldwide. In this study, 17 analogues of L-chicoric acid, a potent inhibitor of HIV integrase, were studied. Of these analogues, five submicromolar inhibitors of integrase were discovered and 13 compounds with activity against integrase at less than 10 μM were identified. Six demonstrated greater than 10-fold selectivity for HIV replication over cellular toxicity. Ten analogues inhibited HIV replication at nontoxic concentrations. Alteration of the linkages between the two bis-catechol rings, including the use of amides, mixed amide esters, cholate, and alkyl bridges, was explored. Amides were as active as esters but were more toxic in tissue culture. Alkyl and cholate bridges were significantly less potent against HIV-1 integrase in vitro and were inactive against HIV-1 replication. Two amino acid derivates and one digalloylderivative of L-chicoric acid (L-CA) showed improved selectivity over L-CA against integration in cell culture. These data suggest that in addition to the bis-catechols and free carboxylic acid groups reported previously, polar linkages are important constituents for optimal activity against HIV-1 integrase and that new derivatives can be developed with increased specificity for integration over HIV entry in vivo.

Introduction

The acquired immune deficiency syndrome (AIDS), caused by infection with the human immunodeficiency virus (HIV), remains a serious global health problem. Recent advances in treatment, including the use of combinations of both reverse transcriptase and protease inhibitors,[1−3] have resulted in significant increases in disease-free survival. However, expense and toxicity coupled with the emergence of single and multiple drug resistant viruses have made it clear that development of new inhibitors targeted toward other viral or cellular proteins is of paramount importance. One viral protein that is a potential target for therapy is HIV integrase (IN).

The IN protein is an attractive therapeutic target. First, integration is absolutely required for productive and stable infection by HIV.[4−8] Second, there is no known mammalian homologue of IN, although recent work suggests that inhibitors of IN may also inhibit the RAG1/RAG2 recombinase in human B and T lymphocytes.[9] Finally, integrase inhibitors in combination with either reverse transcriptase or protease inhibitors have been potently synergistic in vitro against both wild-type HIV and reverse transcriptase inhibitor resistant viruses.[10,11]

Integrase mediates the covalent linkage of the viral cDNA into the host chromosome, resulting in the formation of a stable provirus.[12−14] The integration reaction proceeds through a series of enzymatic "steps" beginning with the removal of two terminal nucleotides from each 3′ end of the viral DNA.[15−17] The resulting nucleophilic hydroxyl groups attack the host chromosome.[15,16] The resulting DNA gaps are repaired, and the provirus is formed, most likely by host DNA break repair machinery.[18−20] These biological reactions are illustrated in Figure 1. Purified recombinant integrase, synthesized in *Escherichia coli*, can mediate these reactions in vitro.[21] These integration reactions and the reverse of strand transfer, called disintegration,[22,23] have been used to determine the biochemical requirements for integration and to identify inhibitors of the reaction. The in vitro reactions[18,21,23−26] are described in detail in the Experimental Section.

While a large number of integrase inhibitors have been described {reviewed in refs 27 and 28}, few have met the criterion for "lead" compounds because of the lack of activity against replicating virus, cell toxicity, lack of selectivity, or a combination of these factors. The dicaffeoyltartaric acids (DCTAs) and dicaffeoylquinic acids (DCQAs), on the other hand, are excellent lead compounds against HIV integrase.[29,30] The most potent and selective of these agents to date is L-chicoric acid (L-CA), which inhibits type 1 HIV (HIV-1) replication at nontoxic concentrations. It is selective against HIV-1 integrase compared to other metalloenzymes including reverse transcriptase, restriction endonucleases, and other DNA binding proteins.[31,32] L-CA is one of the most potent inhibitors of integrase in biochemical assays with 50% inhibitory concentrations (IC_{50} values) of approximately 150 nM in the 3′-end-processing reaction.[29,30] Finally, it is an excellent template for structure−

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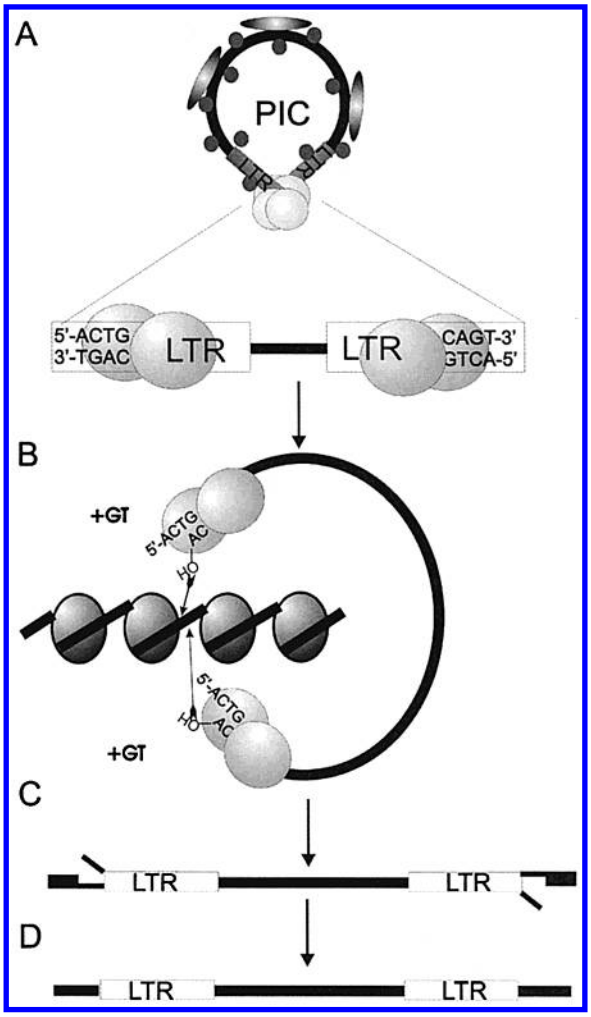


Figure 1. Integration reaction. (A**)** Viral cDNA enters the nucleus as part of the preintegration complex (PIC). (B) Integrase catalyzes the removal of two nucleotides from each 3′-end of the viral cDNA (3′-end-processing). Next, integrase catalyzes the nucleophilic attack by the free hydroxyls of the viral cDNA on the host chromosome. Favored sites are between nucleosomes in regions of DNA stress. (C) The subsequent covalent linkage of the viral 3′-ends to the host chromosome is termed strand transfer, generating unpaired DNA breaks at either end of the virus. (D) Finally, the overhangs and gaps are repaired, likely by host−DNA repair machinery (5′-end-joining), generating the integrated provirus.

activity relationship (SAR) studies.[33] Two previous rounds of SAR have identified key structural features involved in selectivity and potency against both integrase and HIV replication in tissue culture, i.e., a free carboxylic acid and two 3,4-catechol rings.[29,30,33,34] The latter group is common to many other polyhydroxylated inhibitors of integrase, but as revealed in our first SAR paper,[33] the former group is required for inhibition of HIV replication in tissue culture.

More recently, Sotriffer et al. utilized computational docking to demonstrate that several inhibitors of integrase fill an inhibitor binding cleft near the catalytic site. L-CA more completely fills the groove than other integrase inhibitors.[35] Such docking experiments suggested that L-CA interacts with lysines 156 and 159, as well as cysteine 65, threonine 66, histidine 67, glutamine 148, and glutamate 152. The most favorable contacts are with glutamate 152, a member of the catalytic triad, and glutamine 148.[35] These results are quite similar to results of previously reported docking experiments utilizing a different crystal structure and an alternative docking program,[30] which suggested that the DCTAs and DCQAs interacted with aspartate 64, histidine 67, glutamate 92, aspartate 116, asparagine 117, glutamine 148, and lysine 159. Furthermore, L-CA appeared to interact more tightly with the protein and to fill a putative drug binding cleft better than other, less potent DCQAs.[30] These interactions are virtually identical to the drug binding pocket identified by Sotriffer et al. as residues 64−67, 116, 148, 151−152, 155−156, 159, and, for larger molecules such as L-CA, residue 92.[35]

To identify more potent and selective inhibitors of HIV-1 integrase, the third round of SAR reported herein was performed. In particular, this paper examines variations in the nature of (1) the linkage between the catechol rings and the central acid core, the goal of which was to decrease susceptibility of the analogues to hydrolysis, (2) the carboxylic acid core, to ascertain whether blocking one carboxylic acid as an amide would increase cell entry thus leading to more effective inhibitors, and (3) possible heterocyclic substitutes for the catechol rings to potentially reduce toxicity of the molecules and decrease their chemical reactivity.

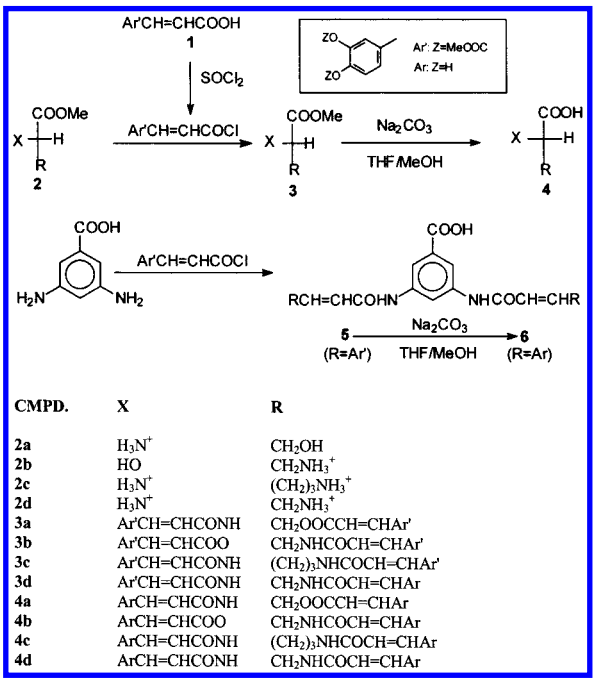
Results

Synthetic Goals and Methodology. Our prior SAR study [33] of L-CA analogues concluded that (i) at least one free carboxyl group was necessary for anti-HIV activity but not integrase inhibition, although maximal inhibition of integrase was observed only for molecules containing a free carboxyl group, (ii) the stereochemistry of the chicoric acid (D-, L-, or *meso*-) had modest effects on either activity, (iii) a minimum of two ortho phenolic OH groups in each aryl ring were necessary for activity, (iv) the catechol systems must be situated at the 3,4-, not the 2,3-positions, relative to the side chain, and (v) the nature and length of the link between the ester function and the catechol did not greatly affect the above activities. The present paper examines the effect of substituting the ester linkages between the central acid core and the two caffeoyl groups with amide and all carbon linkages. Several compounds in which the tartaric acid core was replaced with cholic acids and the catechols with heterocyclic rings were also prepared.

The dicaffeoyl derivatives of L-serine, D,L-isoserine, L-lysine, and D,L-2,3-diaminopropionic acid were prepared from the corresponding methyl ester hydrochlorides **2a**, **2b**, **2c**, and **2d** by reaction of the acid chloride of the blocked caffeic acid **1** to give the fully blocked ester amides **3a**, **3b**, **3c**, and **3d**, respectively. Simultaneous deblocking of the catechols and saponification of the methyl esters gave the target compounds **4a**, **4b**, **4c**, and **4d** (Scheme 1). The dicaffeoyl derivative of 3,5-diaminobenzoic acid was prepared directly from the acid by reaction with **1** to give **5** whose catechols were deblocked to give **6** (Scheme 1). All of these dicaffeoyl derivatives are new, although the racemate of **4a** and the acetoxy and methoxy derivatives of **4d** have been reported.[34]

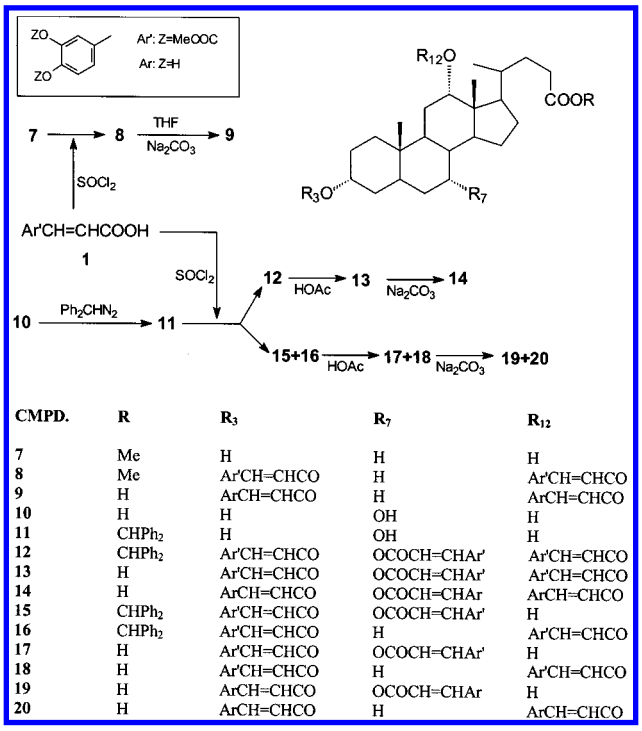
Reaction of methyl 7-deoxycholate (**7**) with the acid chloride of the blocked caffeic acid **1** gave the protected ester **8**, which was saponified and deblocked in one operation to give 3,12-dicaffeoyl-7-deoxycholic acid (**9**)

Scheme 1



CMPD.	X	R
2a	H_3N^+	CH_2OH
2b	HO	$CH_2NH_3^+$
2c	H_3N^+	$(CH_2)_3NH_3^+$
2d	H_3N^+	$CH_2NH_3^+$
3a	Ar'CH=CHCONH	$CH_2OOCCH=CHAr'$
3b	Ar'CH=CHCOO	$CH_2NHCOCH=CHAr'$
3c	Ar'CH=CHCONH	$(CH_2)_3NHCOCH=CHAr'$
3d	Ar'CH=CHCONH	$CH_2NHCOCH=CHAr$
4a	ArCH=CHCONH	$CH_2OOCCH=CHAr$
4b	ArCH=CHCOO	$CH_2NHCOCH=CHAr$
4c	ArCH=CHCONH	$(CH_2)_3NHCOCH=CHAr$
4d	ArCH=CHCONH	$CH_2NHCOCH=CHAr$

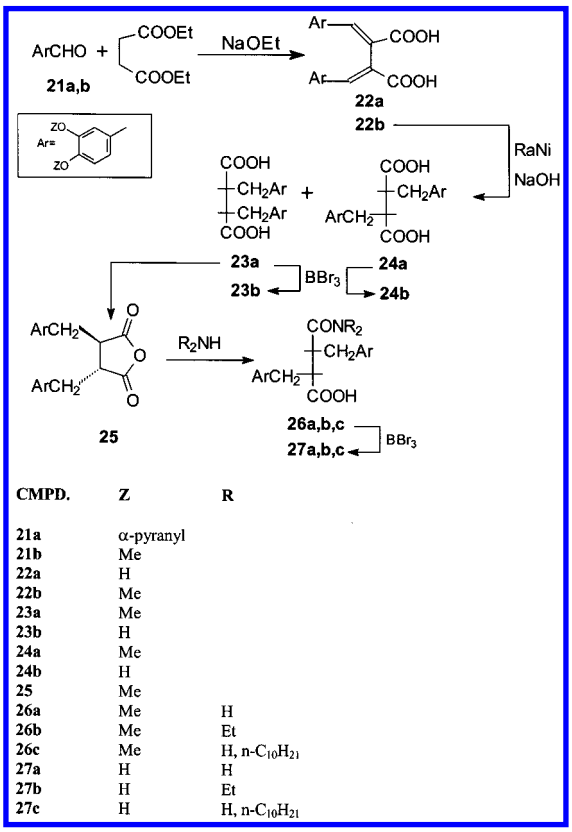
Scheme 2



CMPD.	R	R_3	R_7	R_{12}
7	Me	H	H	H
8	Me	Ar'CH=CHCO	H	Ar'CH=CHCO
9	H	ArCH=CHCO	H	ArCH=CHCO
10	H	H	OH	H
11	$CHPh_2$	H	OH	H
12	$CHPh_2$	Ar'CH=CHCO	OCOCH=CHAr'	Ar'CH=CHCO
13	H	Ar'CH=CHCO	OCOCH=CHAr'	Ar'CH=CHCO
14	H	ArCH=CHCO	OCOCH=CHAr	ArCH=CHCO
15	$CHPh_2$	Ar'CH=CHCO	OCOCH=CHAr'	H
16	$CHPh_2$	Ar'CH=CHCO	H	Ar'CH=CHCO
17	H	Ar'CH=CHCO	OCOCH=CHAr'	H
18	H	Ar'CH=CHCO	H	Ar'CH=CHCO
19	H	ArCH=CHCO	OCOCH=CHAr	H
20	H	ArCH=CHCO	H	ArCH=CHCO

(Scheme 2). Cholic acid (**10**) itself was converted to its diphenylmethyl ester **11**, which upon reaction with the acid chloride of **1** gave a mixture of the diphenyl methyl esters of the protected di- and tricaffeoylcholic acids. Chromatography separated the former as a 1:1 mixture (**15** + **16**) of the protected 3,7- and 3,12-dicaffeoyl compounds and the latter (**12**) as the 3,7,12-tricaffeoyl derivative. The diphenyl methyl ester group of each of these was removed with HOAc to give the free acids **17** + **18** and **13**, respectively. The methoxycarbonyl blocking groups of each were cleaved with mild base to give a 1:1 mixture of 3,7- and 3,12-dicaffeoylcholic acids (**19** + **20**) and 3,7,12-tricaffeoylcholic acid (**14**) (Scheme 2).

Scheme 3



CMPD.	Z	R
21a	α-pyranyl	
21b	Me	
22a	H	
22b	Me	
23a	Me	
23b	H	
24a	Me	
24b	H	
25	Me	
26a	Me	H
26b	Me	Et
26c	Me	H, n-$C_{10}H_{21}$
27a	H	H
27b	H	Et
27c	H	H, n-$C_{10}H_{21}$

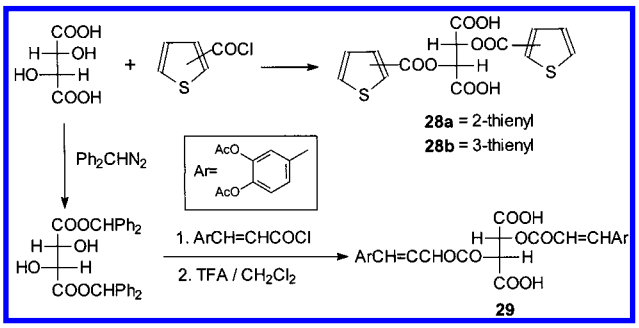
The identity and composition of the mixture **19** + **20** were determined from the position and relative areas of the carbinol hydrogens in the ^{1}H NMR and comparisons with the same peaks in **11**, **14**, and **9** (see Experimental Section).

Bis-catechol derivatives of a series of succinic acids were prepared by a double Stobbe condensation of diethyl succinate with the α-pyranyl-protected 3,4-dihydroxybenzaldehyde **21a**[36] or with veratraldehyde to give the bis(benzylidene)succinic acids **22a** (after deprotection) and **22b** (Scheme 3). The latter was reduced to give a mixture of the known[37] *meso*- and D,L-2,3-bis(3,4-dimethoxybenzyl)succinic acids **23a** and **24a**, respectively. Demethylation with BBr_3 led to the target bis-catechol acids **23b** and **24b**. Treatment of **23a** with acetic anhydride has been shown[38] to give the isomerized D,L-anhydride, **25**, which also was formed by heating either **23a** or **24a** in a vacuum without solvent. Reaction of **25** with ammonia, diethylamine, or *n*-decylamine gave the monoamides **26a**, **26b** and **26c**, respectively, which upon demethylation with BBr_3 led to the target bis-catechol monoamides **27a**, **27b**, and **27c** (Scheme 3).

The chicoric acid analogues in which the caffeoyl groups are replaced by 2-thenoyl (**28a**) and 3-thenoyl (**28b**) residues were prepared directly from L-tartaric acid and the corresponding acid chlorides. Tetraacetylchicoric acid (**29**)[33,34] was prepared from bisdiphenylmethyl tartrate and diacetylcaffeoyl chloride followed by selective hydrolysis of the diphenylmethyl esters (Scheme 4).

Biological Activities of Synthetic Compounds. Compounds were tested at multiple concentrations to determine the IC_{50} for both 3′-end-processing/strand

Scheme 4



transfer and disintegration reactions as described previously.[33] All compounds were diluted, and their toxicity to an established cell line, MT-2, and their anti-HIV effects were analyzed. Results for these assays are reported as the 5% cytotoxic dose (CT_5) and the 50% effective dose (ED_{50}), respectively. The CT_5 is 1 standard deviation from the cell control and represents a nontoxic dose. The anti-HIV assay described[39] and its modification[29,30,33] have been shown to correlate with other measures of viral replication, including viral RNA and protein synthesis, reverse transcriptase release, and numbers of infectious particles.[40]

Biological Activities of Amino Acid Derivatives of L-Chicoric Acid. Compounds **4a**, **4b**, and **4d** were all potent inhibitors of HIV-1 integrase in both the 3′-end-processing and strand transfer assays (Table 1). All four compounds, **4a**, **4b**, **4c**, and **4d**, were potent inhibitors of the disintegration reaction (Table 1). Potency for these molecules was similar to the potency for L-CA. Cell toxicity and anti-HIV activities of each of the four molecules are illustrated in Figure 2. The most potent and selective compound was **4b**. Interestingly, the toxicity of the dicaffeoyl derivative of isoserine (**4b**) is much reduced compared to those of the α-amino acid derivatives (**4a**, **4c**, and **4d**) and second only to L-CA itself. This leads to the therapeutic index (TI) of **4b** (88) exceeding that of L-CA (63) (Figure 2). Reproducibility in both antiviral activity and disintegration assays for compound **4b** is illustrated in Figure 3. This level of reproducibility is similar for all molecules reported herein and is merely representative. The relative potency of all the amino acid derivatives in the disintegration assay are illustrated in Figure 4A.

Biological Activities of Benzoic Acid Derivatives. We had previously reported that 3,5 dicaffeoylbenzoic acid (3,5-DCBA) was inactive against both integrase and HIV replication.[30] On the basis of docking experiments,[30] we had concluded that the linker between the two caffeic acid groups must be more flexible than allowed for by the planar benzene ring. Alternatively, since phenyl esters are more easily hydrolyzed than alkyl esters, 3,5-DCBA might not survive under the assay conditions, thus accounting for its lack of activity. Since amides are more stable to hydrolysis than esters, the dicaffeoyl derivative of 3,5-diaminobenzoic acid (**6**) was prepared and proved to be a potent inhibitor of HIV integrase and a selective inhibitor of HIV replication with an ED_{50} of 4.6 μM and a CT_5 of 47 μM (Table 1).

Biological Activities of Dicaffeoylcholates. The serum half-life of dicaffeoyltartaric acids is likely to be short. In an attempt to improve lipid solubility as well as to determine whether a large polycyclic group could replace tartaric acid, di- and tricaffeoylcholates were synthesized. A 1:1 mixture of 3,7- and 3,12-dicaffeoylcholic acids, **19** + **20**, and pure 3,12-dicaffeoyl-7-deoxycholic acid, **9**, were found to be fairly weak inhibitors of integrase but inactive against HIV at nontoxic concentrations in tissue culture (Table 1). A tricaffeoylcholic acid, **14**, was slightly less toxic with a CT_5 of 11 μM. Compound **14** was a potent inhibitor of integrase with an IC_{50} of 750−1000 nM in both the end-processing and disintegration assays (Figure 5) but did not inhibit HIV replication at concentrations as high as 140 μM. Figure 5 is representative of end-processing/strand transfer and disintegration assays for other molecules reported herein. The relative potencies of **19** + **20** and **14** are illustrated in Figure 4B.

Biological Activities of Succinate Derivatives. To determine if anti-HIV or anti-integrase activities required either ester or amide links between the catechol system and the central core, several succinic acid derivatives were prepared in which the catechols were connected to the central core by carbon−carbon bonds. The dimer of caffeic acid (**22a**) had an ED_{50} similar to that of L-CA. The same is true for both tetrahydro stereoisomers, **23b** and **24b**, which were active against HIV replication and inhibited HIV integrase (Table 1). The more potent of these molecules, **24b**, was active against HIV replication at concentrations below 2 μM. Both molecules were potent inhibitors of the end-processing reaction with an IC_{50} of 230−440 nM. Unlike other derivatives of the dicaffeoyltartaric acids, there was a significant difference between activities against end-processing and disintegration. The IC_{50} for the latter reaction was 2−4 μM (Figure 4C). Monoamides **27a**, **27b** and **27c** were weakly active against both viral replication and disintegration (Table 1). These derivatives also demonstrated significantly more potent inhibition of end processing than disintegration. Their activities in the disintegration reaction (Figure 4D) were similar to, although slightly less active than, those of other succinate derivatives (Figure 4C).

Replacement of Bis-Catechols. Two analogues in which the catechols were replaced with electron-rich thiophene rings were prepared. Compounds **28a** and **28b** were not active against HIV integrase or against HIV replication (Table 1). Acetylation of the catechols on L-CA to give **29** resulted in significantly attenuated inhibition of HIV integrase and little inhibition of HIV replication (Table 1). Indeed, unlike all other chicoric acid derivatives, the IC_{50} of **29** was higher against integrase than the ED_{50} against HIV replication, suggesting an alternative mechanism of action. One possibility is that the acetylated derivative acts to inhibit viral entry, as suggested by others.[41]

Select Analogues Demonstrate Improved Anti-Integrase Activity in Cells. Quantitative real-time polymerase chain reaction (PCR) was performed using primers that amplify HIV two LTR circle DNA and cDNA. Failure of integration results in two LTR circle formation; therefore, the ratio of two LTR circle DNA to cDNA is a measure of failed integration. Others have shown that this ratio increases in the presence of integrase inhibitors.[42,43] As illustrated in Figure 6, dicaffeoyl-L-lysine **4c**, dicaffeoyl-D,L-isoserine **4b**, and

Table 1. Biologic Activity of DCTA Analogues

compound	CT_5[a] (μM)	ED_{50}[b] (μM)	TI[c]	IC_{50} (μM)[d] end-processing	IC_{50}[e] (μM) disintegration
L-tartaric acid	>1000	>1000	<1	≫25	≫25
L-chicoric acid	264	4.2	63	0.53 (0.36−0.78)	0.18 (0.14−0.22)
4a	39	2.5	16	0.33 (0.23−0.46)	0.12 (0.1−0.14)
4b	175	2	88	0.37 (0.27−0.51)	0.19 (0.07−0.52)
4c	43	3.7	12	*f*	2.23 (1.63−3.05)
4d	35	35	1	0.83 (0.52−1.33)	0.57 (0.39−0.84)
6	47	4.6	10	*f*	0.73 (0.63−0.86)
9	6.4	>17	<1	*f*	1.97 (1.03−3.8)
14	11.4	>140	<1	0.97 (0.47−2.0)	0.75 (0.60−0.94)
19 + 20	4.6	>17	<1	*f*	>10
22a	55	4.4	12	0.83 (0.42−1.66)	2.19 (1.72−2.8)
23b	76	12.6	6	0.23 (0.12−0.44)	4.32 (3.01−6.22)
24b	71	1.9	37	0.44 (0.12−1.61)	2.78 (1.83−4.17)
27a	123	49	2.5	>10	2.84 (2.22−3.63)
27b	93	186	0.5	>10	5.27 (2.8−9.91)
27c	34	>37	<1	>10	>10
28a	190	>190	<1	≫10	≫10
28b	100	≫95	<1	≫10	≫10
29	18.6	4	4.5	>10	15.65 (9.97−24.55)

[a] The 5% cytotoxic dose is the concentration of compound that inhibits cell growth by 5% or less over a 72 h time period. This value is within 1 standard deviation of controls grown in the absence of any compound and is therefore nontoxic. [b] The 50% effective dose is the concentration of compound that inhibits HIV replication by 50% and was calculated using the CalcuSyn for Windows software package. [c] The therapeutic index is the ratio of the CT_5 to ED_{50} and thus indicates the relative selectivity of the compound for HIV over cells. [d] The 50% inhibitory concentration is the concentration of compound that inhibits by 50% the integrase-mediated catalysis of the V1/V2 substrate. This concentration was determined over a three log range in $^1/_2$ log 10 dilutions (see Figure 5A) and calculated using the CalcuSyn for Windows software package. Values in parentheses are 95% confidence intervals. [e] The 50% inhibitory concentration is the concentration of inhibitor that inhibits by 50% the integrase-mediated catalysis of the Y-oligo substrate. This concentration was determined over a 3 log range in $^1/_2$ log 10 dilutions (see Figure 5B) and calculated using the CalcuSyn for Windows software package. Values in parentheses are 95% confidence intervals. [f] Not determined.

digalloyltartaric acid[33] all showed 2- to 4-fold higher ratios of two LTR circle DNA to cDNA compared to virus control infections. In addition, both 4,5-dicaffeoylquinic acid and a succinate derivate (**24b**) showed much lower increases. Therefore, the amino acid derivatives and the digalloyl derivative show improved specificity for integrase over the previously described L-CA analogues. This selectivity for integrase in vivo exceeds that of L-CA (Reinke et al., unpublished results).

Discussion

The DCTAs and their derivatives have proven to be a rich source for biologically active inhibitors of HIV integrase. Since the original description of L-CA as an inhibitor of HIV integrase and HIV replication,[29] 74 analogues have been published.[30,33,34] Of those 74 analogues, 17 inhibit HIV integrase with an IC_{50} below 1 μM, 33 inhibit with an IC_{50} below 10 μM, and 13 inhibit HIV replication at nontoxic concentrations, having a therapeutic index of greater than 12. With the addition of the molecules described herein, the number of biologically active compounds has grown even further.

Whether the DCTAs and their analogues inhibit HIV replication through integrase or reverse transcriptase or at the level of viral entry is a subject of some debate. Although under certain conditions L-CA and several DCTA analogues inhibit HIV reverse transcriptase in vitro,[31,44] they do not appear to inhibit reverse transcription in vivo.[31] Likewise, several potent analogues have no inhibitory activity against reverse transcriptase yet are both potent inhibitors of integrase and block virus replication at nontoxic concentrations.[31] Furthermore, L-CA inhibits the replication of viruses resistant to both nucleoside analogue and nonnucleoside reverse transcriptase inhibitors as well as wild-type virus.[11] The strong correlation between inhibition of integrase and anti-HIV activity certainly suggested that the DCTAs and their analogues were acting to slow viral replication through inhibition of integrase. The isolation of a L-CA resistant variant that mapped to integrase further supported this interpretation.[45]

Recently it has been shown that L-CA also inhibits HIV entry into cells. This latter interpretation was based on a finding that resistance to L-CA and its esters was mapped to the viral envelope glycoprotein and that mutations were similar to those that conferred resistance to polyanions.[41] In addition, following selection in tissue culture, no mutations within the integrase gene were found. Concentrations of DCTAs used in this study were significantly higher than those used to raise resistance to L-CA previously,[45] raising the possibility that L-CA failed to enter cells and thereby failed to inhibit integrase. SAR studies were not performed to determine if analogues of L-CA display a similar propensity to inhibit viral entry.[41] We have recently confirmed that L-CA inhibits viral entry as well as integration (Reinke et al., manuscript submitted in 2002; Lee et al., unpublished results). To date, SAR of L-CA is consistent with inhibition of integrase as the predominant site of anti-HIV activity.[29,30,33] In particular, a requirement for two 3,4-catechol groups and the failure of other electron donor groups to replace the hydroxyl moiety[33] are consistent with those reported for other integrase inhibitors. Finally, in one study,[33] the resistance mutation described for integrase, glycine 140 to serine,[45] also led to resistance to several analogues.

This round of SAR identifies several novel core structures that promote both inhibition of integrase and viral replication. Most notably, compounds with amino acid cores are potent inhibitors of integrase, and perhaps more importantly, cells treated with dicaffeoyl-D,L-isoserine and dicaffeoyl-L-lysine show increased ratios of two LTR circle DNA to cDNA following HIV infection, consistent with failure of integration. This increased

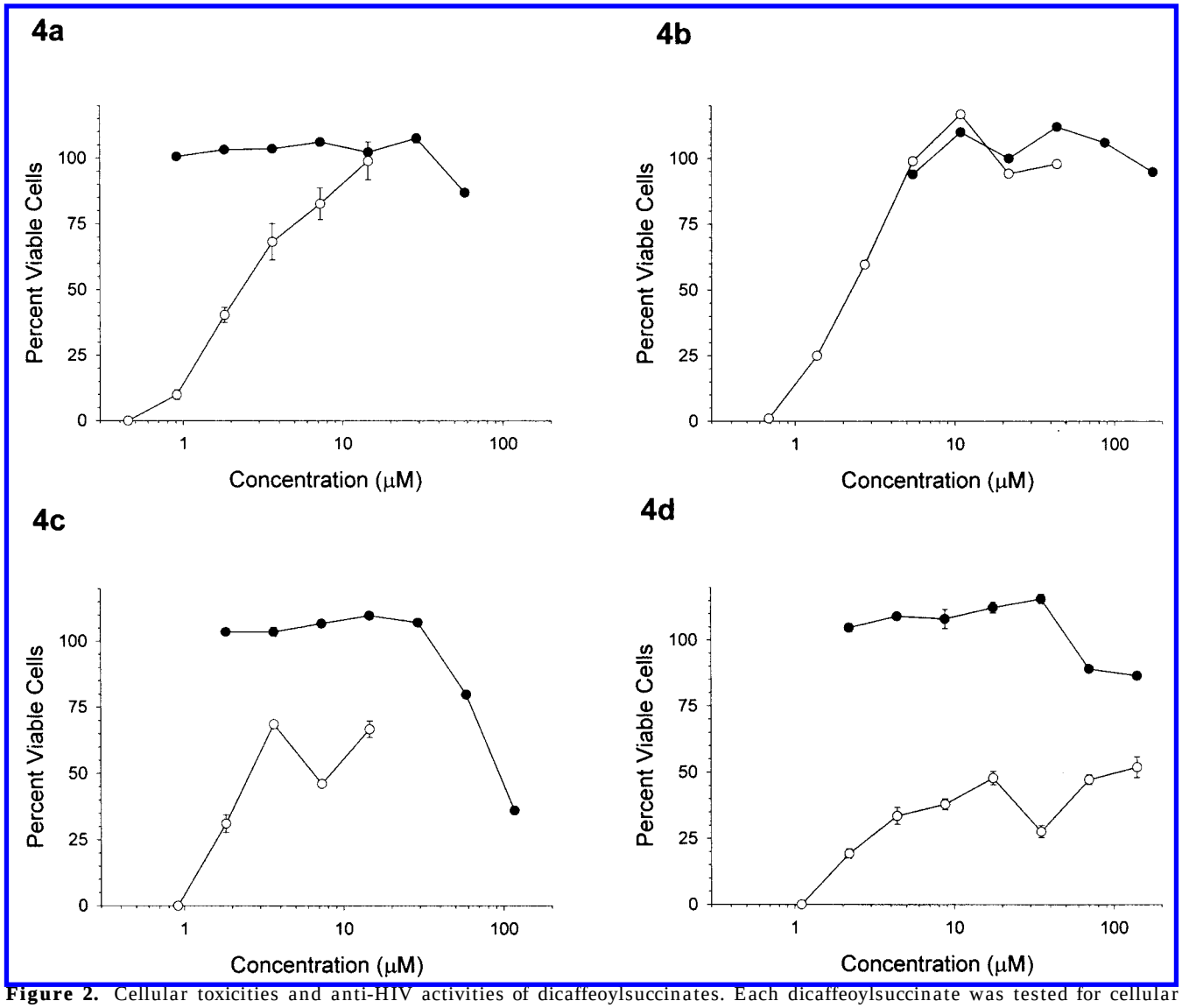


Figure 2. Cellular toxicities and anti-HIV activities of dicaffeoylsuccinates. Each dicaffeoylsuccinate was tested for cellular toxicity and anti-HIV activity using a vital dye assay. Closed circles are the results of cellular toxicity assays, while open circles are the results of anti-HIV assays. Each point is the mean of three replicates; error bars are 1 standard deviation. Results are for compounds **4a**−**4d**, as indicated on each panel. Other compounds reported herein were subject to identical testing.

ratio is even more apparent with digalloyltartaric acid,[33] raising the question of whether digalloyl-L-lysine or digalloyl-D,L-isoserine may display significant selectivity for integrase. The dicaffeoylquinic acids also show increased ratios of two LTR circle DNA to cDNA, indicating an effect on integration, but this effect is more modest (Figure 6). Also, compounds with several caffeoyl groups arranged around a large, lipophilic, multiring central core such as cholic acid still inhibit integrase in vitro but appear to be unable to enter cells, since antiviral activities were undetectable. Finally, π-electron-rich rings such as thiophene cannot substitute for catechols. Thus, this third round of SAR indicates that amino acid derivatives of L-CA are improved integrase inhibitors, demonstrating improved entry into cells.

Regardless of the debate about the in vivo mechanism, the potency of integrase inhibition by DCTAs in biochemical assays has been highly reproducible. This potency makes further SAR studies important for drug discovery. Docking experiments have been performed that suggested L-CA interacts with integrase in a manner similar to that of several other integrase inhibitors.[35] Interestingly, it appeared to fill the drug binding pocket more fully than other inhibitors, leading the authors to hypothesize that its potency, relative to the others, was based on its more complex interactions with the protein.[35] Further SAR studies based on these pharmacophores and docking studies should lead to the identification of an even larger group of integrase inhibitors to be studied.

To date, there are only two classes of molecule that meet the criteria necessary to be considered lead molecules in the search for clinically useful inhibitors of integrase. The first are the DCTAs, and the second are the diketo acids.[42] These two classes appear to act in a different manner on the integrase protein. L-CA and the DCTAs, like other bis-catechols, fail to inhibit integration mediated by a preintegration complex derived from infected cells.[44,46] This is not surprising, since bis-catechols appear to inhibit the assembly of integrase onto cDNA ends;[47] such an assembly has likely already occurred in isolated preintegration complexes. The DCTAs inhibit equally well the 3′-end-processing, strand transfer, and disintegration reactions.[30,33] The diketo

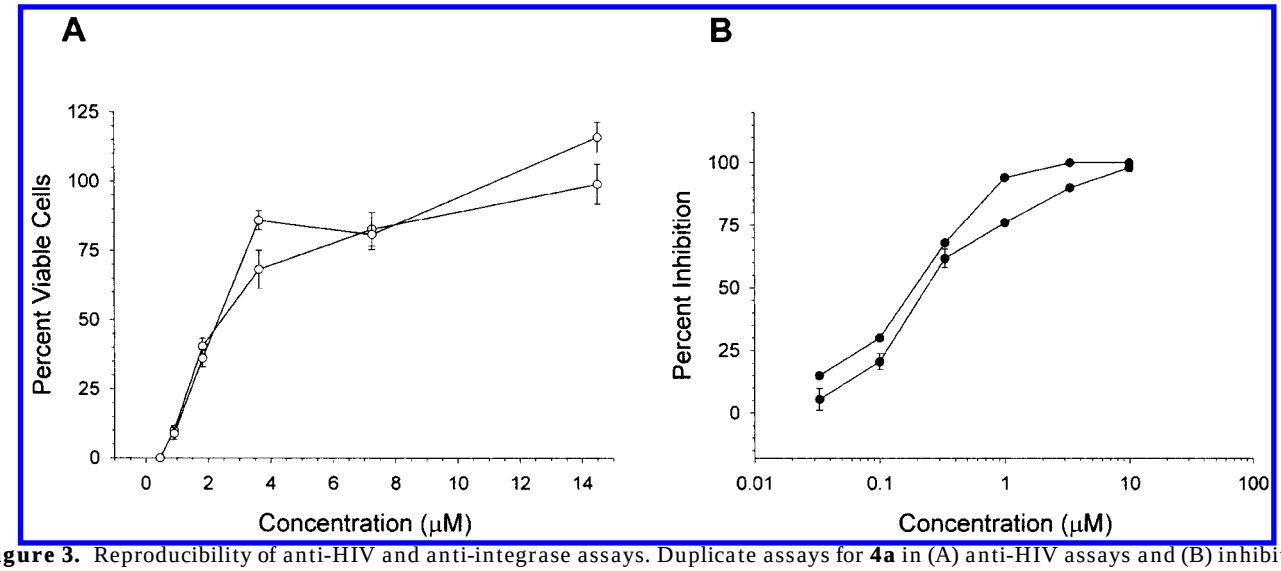


Figure 3. Reproducibility of anti-HIV and anti-integrase assays. Duplicate assays for **4a** in (A) anti-HIV assays and (B) inhibition of the disintegration reaction using HIV-1 integrase. Each point is the mean of triplicate infections (A) or reactions (B). Error bars are standard deviations.

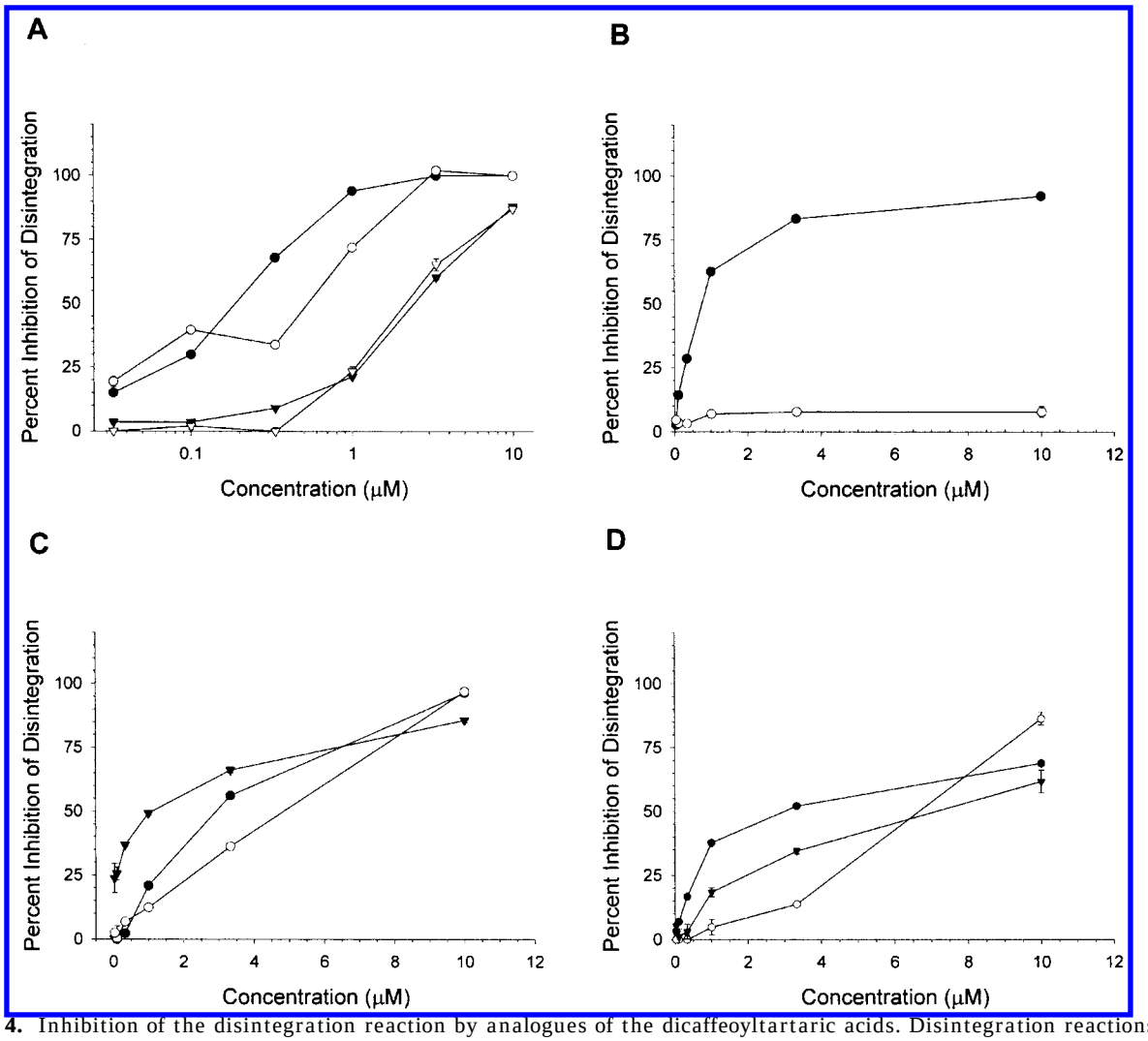


Figure 4. Inhibition of the disintegration reaction by analogues of the dicaffeoyltartaric acids. Disintegration reactions using 0.1 pmol of Y-oligo substrate and 1.5 pmol of HIV-1 integrase were performed in dilutions of DCTA analogues. (A) Amino acid derivatives: closed circles, **4a**; open circles, **4b**; closed inverted triangles, **4c**; open inverted triangles, **4d**. (B) cholic acid derivates: closed circles, **14**; open circles, **19** + **20**. (C) Succinate derivatives: closed circles, **22a**; open circles, **23b**; closed inverted triangles, **24b**. (D) Amide derivatives of succinates: closed circles, **27a**; open circles, **27b**; closed inverted triangles, **27c**. Each point is the mean of triplicate reactions; error bars are 1 standard deviation.

acids, on the other hand, preferentially interfere with strand transfer.[42] No structural studies or docking analyses of the interactions between the diketo acids and integrase have been reported to date, although a

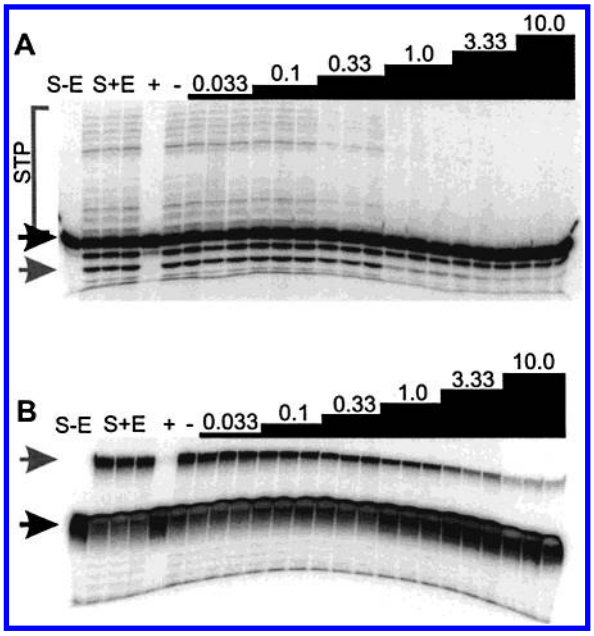


Figure 5. Representative integrase inhibition assays. Compound **14** was added to reactions containing HIV integrase and either (A) 3′-end-processing/strand transfer or (B) disintegration substrates. Concentrations of the compound ranged from 0.033 to 10.0 μM and are illustrated above each gel. Dark arrowheads indicate substrate; gray arrowheads indicate product; STP = strand transfer products. Lane 1: S − E contains substrate in the absence of enzyme. Lanes 2−4: S + E is substrate plus 1.5 pmol of HIV integrase. Lane 5: + indicates substrate plus 1.5 pmol of integrase plus 25 μM L-chicoric acid (positive control). Lane 6: − indicates substrate plus 1.5 pmol of integrase plus 25 μM L-tartaric acid (negative control). Lanes 7−23 indicate substrate plus 1.5 pmol of integrase plus **14** at the indicated concentrations. Reactions were allowed to proceed for 1 h at 37 °C and were stopped by the addition of EDTA. The reaction products were separated on a denaturing polyacrylamide gel as described in the Experimental Section. Products were visualized and quantified using a Molecular Dynamics Storm Phosphorimager.

similar diketotetrazole compound, 5-CITEP, formed the cocrystal on which the compounds were modeled.[48] More recently, drug resistance to the diketo acids has been reported to include amino acids T66, S153, and M154.[42] Such data suggest that the diketo acids interact with the same binding pocket as other integrase inhibitors[35] but in a slightly different manner. Perhaps interactions with amino acids 153 and 154 rather than with amino acids 151−152 and 155−156 are important in the inhibition of strand transfer rather than catalysis.

The requirement of integration to establish a stable HIV infection makes integrase an attractive target for anti-HIV drug development. Recent reports of increasing numbers of potent and selective integrase inhibitors that slow HIV replication at nontoxic concentrations suggest that clinically useful inhibitors of integrase will become available. The large number of such molecules that are DCTAs or derivatives of DCTAs suggests that additional SAR studies using this class of molecules have a high likelihood of success. Until additional classes of molecules with favorable profiles are developed, the DCTAs should be pursued as inhibitors of integrase with clinical potential.

Experimental Section

General. All melting points were measured on a Mel-Temp apparatus and are corrected. Elemental analyses were performed by M-H-W Laboratories of Phoenix, AZ. HRFABMS was provided by the Washington University Mass Spectrometry Resource, St. Louis, MO, an NIH Research Resource (Grant No. P41RR0954). NMR spectra were obtained on a Varian XL-300 instrument at 299.936 MHz (^{1}H) or 75.427 MHz (^{13}C) unless otherwise noted in the indicated solvent (D = DMSO-d_6, C = $CDCl_3$, M = CD_3OD) and are reported in order as the following: ppm downfield from TMS at $\delta = 0$, multiplicity (s, d, dd, m, bs), observed couplings J in hertz, and relative number of H atoms. APT spectral results are expressed as u = C or CH_2, d = CH or CH_3. HPLC used a C-18 10 μm, 250 mm × 4.6 mm analytical column or a C18 10 μm, 250 mm × 22 mm preparative column, with elution with either methanol/water or acetonitrile/water mixtures containing 1% HOAc and with UV detection at 254 nm. No attempt was made to optimize yields.

***N,O*-Dicaffeoyl-L-serine (4a).** A mixture of 2.96 g (10 mM) of 3,4-dimethoxycarbonylcaffeic acid (**1**)[33] and 8 mL of thionyl chloride (NaOH trap) was heated in an oil bath at 80−90 °C until no further evolution of HCl was observed. Removal of the thionyl chloride on a Rotovap gave a light-yellow solid, which was dissolved in dry benzene and added dropwise to a solution of 620 mg (4 mM) of L-serine methyl ester hydrochloride in 50 mL of anhydrous pyridine. After 60 min, the solvent was evaporated and the residue was separated on silica gel with $CHCl_3$ and $CHCl_3$/MeOH (500:1) to give 290 mg (11%) of *N,O*-bis(3,4-dimethoxycarbonylcaffeoyl)-L-serine methyl ester (**3a**) gum: ^{1}H NMR (C) 7.63 (d, 16.0, 1H), 7.61 (d, 15.6, 1H), 7.47 (s, 1H), 7.45 (d, 1.9, 1H), 7.38−7.44 (m, 2H), 7.33 (d, 7.3, 1H), 7.30 (d, 8.4, 1H), 6.59 (d, 7.5, 1H), 6.45 (d, 15.6, 1H), 6.38 (d, 15.9, 1H), 5.06 (m, 1H), 4.66 (dd, 11.6, 3.8, 1H), 4.58 (dd, 11.5, 3.3, 1H), 3.92 (s, 12H), 3.83 (d, 3H); ^{13}C NMR (C) 169.94u, 165.92u, 165.1u, 153.04u, 152.95u, 152.85u, 143.7d, 143.2u, 142.6u, 142.5u, 140.0d, 133.8u, 133.1u, 126.8d, 126.6d, 123.5d, 123.3d, 122.3d, 121.9d, 121.3d, 118.4d, 64.1u, 55.9d, 55.8d, 52.9d, 52.0d.

A solution of 85 mg (0.13 mM) of **3a** in 10 mL of THF, 10 mL of 2% aqueous Na_2CO_3, and 10 mL of MeOH under N_2 protection was stirred for 4.5 h at room temperature, at which time the pH was adjusted to 2−3 with 1 N HCl and the solution was extracted with 3 × 30 mL ether. Evaporation of the dried, combined extracts gave a residue that was purified on an LH-20 column with 1:1 MeOH/water to give 5.3 mg (10%) of *N,O*-dicaffeoyl-L-serine (**4a**): mp 122−123 °C; ^{1}H NMR (D) 9.67 (bs, 1H), 9.45 (bs, 1H), 9.20 (bs, 1H), 8.45 (d, 7.8, 1H), 7.50 (d, 15.8, 1H), 7.28 (d, 15.8, 1H), 7.04 (s, 1H), 7.00 (dd, 8.2, 1.7, 1H), 6.97 (d, 1.8, 1H), 6.86 (dd, 8.2, 1.7, 1H), 6.77 (d, 8.1, 1H), 6.75 (d, 8.1, 1H), 6.52 (d, 15.7, 1H), 6.24 (d, 15.9, 1H), 4.73 (m, 1H), 4.40 (d, 4.3, 2H); ^{13}C NMR (D) 170.9, 166.1, 165.4, 148.4, 147.4, 145.6, 145.5, 145.4, 139.9, 126.2, 125.3, 121.3, 120.5, 117.7, 115.7, 114.7, 113.8, 113.3, 63.3, 51.3. Anal. (HPLC purified) ($C_{21}H_{19}NO_9 \cdot 2H_2O$) C, H, N.

***N,O*-Dicaffeoyl-D,L-isoserine (4b).** A stirred solution of 450 mg (4.3 mM) of D,L-isoserine in 200 mL of MeOH was saturated with HCl gas for 4 h. Removal of the solvent gave 630 mg (95%) of methyl-D,L-isoserine hydrochloride (**2b**) gum: ^{1}H NMR (D) 8.31 (bs, 3H), 4.43(dd, 8.6, 3.6, 1H, 3.68 (s, 3H, 3.08 (m, 1H), 2.91 (m, 1H); ^{13}C NMR (D) 171.4, 66.8, 52.0, 41.4.

To a solution of 500 mg (3.2 mM) of **2b** in 7.5 mL of pyridine was added dropwise 75 mL of a benzene solution of the acid chloride prepared from 2.22 g (7.5 mM) of **1** and 8 mL of thionyl chloride as above, and the reaction was allowed to proceed for 30 min. The reaction mixture was washed with 100 mL of 6 N HCl and 200 mL of water, the dried (Na_2SO_4) benzene solution was evaporated, and the residue was separated on silica gel with $CHCl_3$/MeOH/HOAc (500:1:0, 250:1:0, and 100:1:0.1) to give 550 mg (26%) of *N,O*-bis(3,4-dimethoxycarbonylcaffeoyl)-D,L-isoserine methyl ester (**3b**): mp 44−45 °C; ^{1}H NMR (C) 7.69 (d, 15.9, 1H), 7.55 (d, 15.6, 1H), 7.48 (d, 1.9, 1H), 7.42 (dd, 8.4, 2.0, 1H), 7.41 (d, 2.0, 1H), 7.35 (dd, 8.5, 2.0, 1H), 7.32 (d, 8.5, 1H), 7.27 (d, 8.4, 1H), 6.47 (d, 16.0, 1H), 6.35 (d, 15.6,

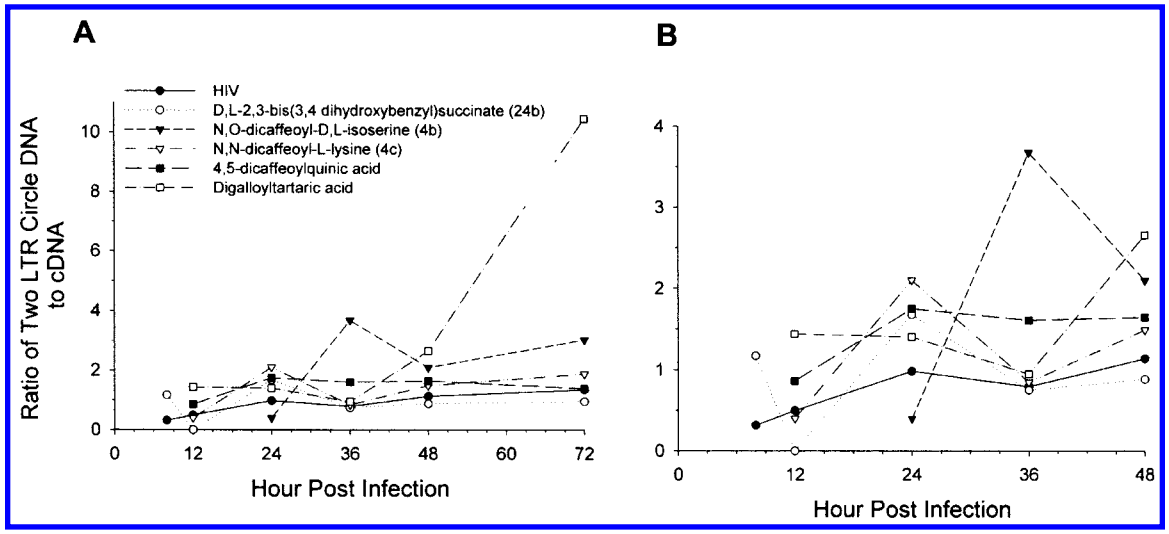


Figure 6. Ratio of HIV two LTR circle DNA to cDNA in the presence of representative L-CA analogues. HIV_{LAI} was preincubated with each of the indicated compounds for 1 h and then added to MT2 cells. Cells were lysed at 1, 2, 4, 8, 12, 24, 36, 48, and 72 h after infection. After lysis, real-time PCR was performed using primers that detect two LTR circle DNA[43] and complete cDNA synthesis.[52] The number of infected cell equivalents was determined from standard curves, and the ratio of two LTR circle DNA to cDNA in infected cell equivalents was determined. The total amount of two LTR circle DNA to cDNA based on copy numbers was approximately 1:200, similar to that reported by others.[43] Increases in two LTR circle DNA to cDNA are consistent with a failure to integrate.[42,43] (A) all timepoints. (B) time points through 48 h to expand the *y* axis.

1H), 6.34 (t, 5.7, 1H), 5.32 (t, 4.5, 1H), 3.92 (s, 8H, 2 × OMe + CH_2), 3.91 (s, 6H), 3.80 (s, 3H); ^{13}C NMR (C) 168.8, 165.5, 165.4, 153.14, 153.10, 153.05, 152.9, 144.5, 143.9, 143.2, 142.7, 142.6, 139.6, 133.9, 133.1, 126.9, 126.7, 123.6, 123.4, 122.5, 121.8, 121.6, 118.1, 71.3, 56.0, 55.9, 52.8, 40.2; ^{1}H NMR (D) 8.50 (t, 5.9, 1H), 7.92 (d, 1.9, 1H), 7.78 (d, 16.2, 1H), 7.76 (dd, 8.5, 2.0, 1H), 7.69 (d, 1.9, 1H), 7.57 (dd, 8.5, 1.9, 1H), 7.52 (d, 8.5, 1H), 7.48 (d, 8.5, 1H), 7.47 (d, 15.6, 1H), 6.75 (d, 16.1, 1H), 6.69 (d, 15.8, 1H), 5.32 (t, 4.5, 1H), 3.86 (s, 3H), 3.85 (s, 3H), 3.74 (m, 2H), 3.70 (s, 3H); ^{13}C NMR (D) 168.5, 165.2, 165.0, 152.5, 152.4, 152.2, 143.5, 143.4, 142.5, 142.2, 142.1, 137.4, 134.0, 133.0, 127.5, 126.4 123.8, 123.7, 123.1, 122.9, 122.0, 118.5, 71.1, 56.01, 55.96, 52.4, 39.7 (overlaps solvent, detected by HMQC).

A solution of 250 mg (0.37 mM) of **3b** in 25 mL of THF, 25 mL of 2% aqueous Na_2CO_3, and 25 mL of MeOH under N_2 protection was stirred for 5 h at room temperature, at which time the pH was adjusted to 2−3 with 1 N HCl and the solvent was removed at reduced pressure to give a residue that was purified on LH-20 with 1:1 MeOH/water to give 51 mg (32%) of *N*,*O*-dicaffeoyl-D,L-isoserine (**4b**): mp 127−128 °C; ^{1}H NMR (M) 7.65 (d, 16.0, 1H), 7.42 (d, 15.7, 1H), 7.07 (s, 1H), 7.01 (s, 1H), 6.97 (dd, 8.2, 1.6, 1H), 6.91 (dd, 8.1, 1.6, 1H), 6.78 (d, 7.8, 1H), 6.76 (d, 7.8, 1H), 5.23 (bs, 1H), 3.91 (dd, 14.1, 3.6, 1H), 3.80 (dd, 17.7, 7.0, 1H); ^{13}C NMR (M) 172.4, 169.6, 169.4, 149.8, 148.9, 148.0, 146.8, 146.7, 142.9, 128.3, 127.8, 123.2, 122.3, 118.0, 116.54, 116.50, 115.3, 115.1, 114.4, 73.1, 41.4. Anal. ($C_{21}H_{19}NO_9 \cdot 0.8H_2O$) C, H, N.

***N*,*N*-Dicaffeoyl-L-lysine (4c).** A stirred solution of 1 g (5.9 mM) of L-lysine hydrochloride in 200 mL of MeOH was saturated with HCl gas for 4.5 h. Removal of the solvent gave 1.261 g (99%) of methyl-L-lysine dihydrochloride (**2c**): mp 189−192 °C; ^{1}H NMR (D) 8.79 (bs, 2H), 8.25 (bs 2H), 3.97 (bs, 1H), 3.75 (s, 3H), 2.74 (bs 2H), 1.84 (dd, 13.6, 6.6, 2H), 1.61 (m, 2H), 1.44 (m, 2H); ^{13}C NMR (D) 169.7, 52.7, 51.5, 38.0, 29.1, 26.0, 21.1.

To a heterogeneous mixture of 2.368 g (8 mM) of **1**, 928 mg (4 mM) of **2c**, and 1.2 mL of triethylamine (8.61 mM) in 200 mL of CH_2Cl_2 was added 1.687 g (8.8 mM) of 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride with vigorous stirring. After 48 h, the resulting solution was washed with 3 × 200 mL each of 2 N HCl and water and dried (Na_2SO_4), the solvent was evaporated, and the residue was separated on silica gel with $CHCl_3$/MeOH (20:0.35) to give 791 mg (28%) of bis(3,4-dimethoxycarbonylcaffeoyl)-L-lysine (**3c**): mp 45−46 °C; ^{1}H NMR (D) 8.56 (d, 7.2, 1H), 8.18 (t, 5.5, 1H), 7.67 (s, 1H), 7.65 (s, 1H), 7.57 (dd, 8.5, 1.7, 1H), 7.55 (dd, 8.5, 1.7, 1H), 7.47 (d, 8.4, 1H), 7.46 (d, 9.1, 1H), 7.45 (d, 15.0, 1H), 7.42 (d, 16.4, 1H), 6.74 (d, 15.7, 1H), 6.63 (d, 15.9, 1H), 4.37 (dd, 13.1, 7.6, 1H), 3.85 (s, 12H), 3.64 (s, 3H), 3.18 (dd, 12.2, 6.2, 2H), 1.75 (m, 2H), 1.47 (m, 2H), 1.38 (m, 2H); ^{13}C NMR (D) 172.5, 164.6, 164.4, 152.5, 152.4, 142.4, 142.3, 142.1(×2), 137.4, 136.4, 134.3, 134.1, 126.2, 126.1, 123.9, 123.7(×2), 123.1, 122.0, 121.8, 56.0, 52.1, 51.8, 38.4, 30.6, 28.6, 22.8.

A solution of 254 mg (0.43 mM) of **3c** in 20 mL of THF, 20 mL of 5% aqueous Na_2CO_3, and 20 mL of MeCN under N_2 protection was stirred for 12 h at room temperature, at which time an additional 15 mL of 5% aqueous Na_2CO_3 was added. After an additional 10 h, the pH was adjusted to 2−3 with 1 N HCl, the solvent was removed at reduced pressure, and the EtOH-soluble portion of the residue was separated on polyamide with EtOH to give 48 mg (8%) of *N*,*N*-dicaffeoyl-L-lysine (**4c**): mp 124−125 °C; ^{1}H NMR (D) 8.20 (d, 7.5, 1H), 8.02 (t, 5.4, 1H), 7.25 (d, 15.6, 1H), 7.22 (d, 15.6, 1H), 6.96 (d, 1.7, 1H), 6.94 (d, 1.7, 1H), 6.85 (dd, 8.0. 1.7, 1H), 6.82 (dd, 8.0, 1.7, 1H), 6.75 (d, 8.1, 1H), 6.74 (d, 8.1, 1H), 6.47 (d, 15.8, 1H), 6.32 (d, 15.8, 1H), 4.27 (m, 1H), 3.14 (m, 2H), 1.75 (m, 1H), 1.66 (m, 1H), 1.45 (m, 2H), 1.38 (m, 2H); ^{13}C NMR (D) 173.9, 165.3, 165.2, 147.2, 147.1, 145.4 (×2), 139.4, 138.8, 126.31, 126.25, 120.3, 120.2, 118.5, 118.1, 115.7, 113.8, 113.7, 52.0, 38.3, 31.0, 28.8, 22.9. Anal. ($C_{24}H_{26}N_2O_8$) C, H, N.

***N*,*N*-Dicaffeoyl-D,L-2,3-diaminopropionic Acid (4d).** A stirred solution of 500 mg of D,L-2,3-diaminopropionic acid hydrochloride in 200 mL of MeOH was saturated with HCl gas for 2 h. Removal of the solvent gave crude methyl D,L-2,3-diaminopropionate dihydrochloride (**2d**): ^{1}H NMR (M) 4.51 (bs, 1H), 3.95 (s, 3H), 3.52 (bs, 2H); ^{13}C NMR (M) 168.0, 55.2, 51.4, 39.9. To a solution of 478 mg (2.5 mM) of **2d** in 50 mL of pyridine was added dropwise 50 mL of a benzene solution of the acid chloride prepared from 1.68 g (5.68 mM) of **1** and 5 mL of thionyl chloride as above, and the reaction was allowed to proceed for 30 min. The combined solution and precipitate (2.157 g) was separated on silica gel with $CHCl_3$ and $CHCl_3$/MeOH/HOAc (125:1:0.1 and 65:1:0.1) to give 229 mg (14%) of *N*,*N*-bis(3,4-dimethoxycarbonylcaffeoyl)-D,L-2,3-diaminopropionic acid methyl ester (**3d**): mp 87−89 °C; ^{1}H NMR (M) 7.47−7.55 (m, 6H), 7.34 (d, 8.5, 1H), 7.33 (d, 8.5, 1H), 6.68 (d, 15.7, 1H), 6.59 (d, 15.8), 4.75 (t, 5.8, 1H), 3.86 (dd, 13.8, 5.4, 1H), 3.70 (dd, 13.8, 6.7, 1H), 3.88 (s, 12H), 3.77 (s, 3H); ^{13}C NMR (M) 172.0, 168.8, 168.1, 154.7, 154.5, 144.83, 144.77, 144.2,

140.5, 140.2, 135.5, 135.4, 127.7, 127.6, 124.7, 123.3, 123.2, 123.1, 123.0, 56.6, 54.3, 53.1, 41.7.

A solution of 135 mg (0.2 mM) of **3d** in 25 mL of THF, 25 mL of 2% aqueous Na_2CO_3, and 10 mL of MeOH under N_2 protection was stirred for 8 h at room temperature, at which time the pH was adjusted to 2−3 with 1 N HCl and the solvent removed at reduced pressure to give a residue that was purified on an LH-20 column with 3:1 MeOH/water to give 43 mg (50%) of *N*,*N*-dicaffeoyl-D,L-2,3-diaminopropionic acid (**4d**): mp 118−119 °C; ^{1}H NMR (M) 7.41 (bd, 16.1, 2H), 7.02 (bs, 1H), 7.00 (bs, 1H), 6.92 (d, 7.6, 1H), 6.90 (m, 2H), 6.76 (1H, d, 8.1), 6.75 (1H, dd, 8.1, 2.1), 6.45 (d, 15.6, 1H), 6.37 (d, 15.6, 1H), 4.71 (dd, 7.1, 4.1, 1H), 3.86 (dd, 14.0, 4.8, 1H), 3.68 (dd, 13.8, 7.5 1H); ^{13}C NMR (M) 172.3, 170.1, 169.3, 149.04, 148.98, 146.8, 143.3, 143.2, 143.0, 128.2, 122.4, 122.3, 117.9, 117.8, 117.7, 116.5, 115.2, 54.4, 41.8. Anal. (HPLC purified) ($C_{21}H_{20}N_2O_8 \cdot 1.1H_2O$) C, H, N.

3,5-Biscaffeoyldiaminobenzoic Acid (6). A mixture of 3.78 g (12.8 mM) of 3,4-dimethoxycarbonylcaffeic acid (**1**) and 10 mL of thionyl chloride (NaOH trap) was heated in an oil bath at 80−90 °C until no further evolution of HCl was observed. Removal of the thionyl chloride on a Rotovap gave a light-yellow solid, which was dissolved in dry benzene and added dropwise to a solution of 760 mg (5 mM) of 3,5-diaminobenzoic acid in 10 mL of pyridine and 40 mL of anhydrous benzene. After 60 min, the green gum that formed (4.675 g) was separated on a silica gel column with $CHCl_3$/MeOH/HOAc [20:(0.5−0.85):(0.15−0.3)] to give 3,5-bis(3,4-dimethoxycarbonylcaffeoyl)diaminobenzoic acid (**5**): 1.171 g (33%); mp 178−179 °C; ^{1}H NMR (D) 10.55 (s, 2H), 8.40 (s, 1H), 8.13 (d, 1.8, 2H), 7.76 (d, 1.8, 2H), 7.66 (dd, 8.5, 1.8, 2H), 7.63 (d, 15.6, 2H), 7.53 (d, 8.5, 2H), 6.88 (d, 15.7, 2H), 3.88 (s, 6H), 3.87 (s, 6H); ^{13}C NMR (D) 167.0, 163.3, 152.5, 152.4, 142.6, 142.2, 139.6, 138.4, 133.9, 131.8, 126.4, 123.8, 123.7, 122.2, 115.2, 113.7, 56.0.

A solution of 400 mg (0.56 mM) of **5** in 50 mL of THF, 50 mL of 1% aqueous Na_2CO_3, and 50 mL of MeOH under N_2 protection was stirred for 30 min at room temperature, at which time the pH was adjusted to 2−3 with 1 N HCl and the solution extracted with 3 × 100 mL of ether. Evaporation of the dried, combined extracts gave 320 mg of residue, which was purified on silica gel with $CHCl_3$/MeOH/HOAc (20:2.5:0.5) to give 153 mg (74%) of 3,5-biscaffeoyldiaminobenzoic acid (**6**): mp 191−193 °C dec; ^{1}H NMR (D) 10.14 (bs, 2H), 8.28 (s, 1H), 7.92 (s, 2H), 7.40 (d, 15.5, 2H), 7.02 (s, 2H), 6.90 (d, 8.2, 2H), 6.77 (d, 8.0, 2H), 6.60 (d, 15.6, 2H); ^{13}C NMR (D) 170.0, 164.0, 148.3, 146.0, 140.6, 139.0, 138.1, 125.9, 120.7, 118.3, 116.1, 115.3, 114.2, 111.8. A sample was purified through an LH-20 column; mp 255−257 °C. Anal. ($C_{25}H_{20}N_2O_8 \cdot 1.33H_2O$) C, H, N.

3,12-Dicaffeoyl-7-deoxycholic Acid (9). A mixture of 1.04 g (3.5 mM) of **1** and 15 mL of thionyl chloride (NaOH trap) was heated in an oil bath at 80−90 °C for 20 min. Removal of the thionyl chloride on a Rotovap gave a light-yellow solid, which was dissolved in 20 mL of dry benzene and added dropwise to a solution of 630 mg (1.5 mM) of methyl 7-deoxycholate (**7**) in 0.5 mL of pyridine and 20 mL of anhydrous benzene. After 2 h, the precipitate was removed by filtration, the filtrate was evaporated, and the residue was separated on successive silica gel columns with $CHCl_3$/MeOH (20:0.3) and then with Et_2O/petroleum ether 40:60 to give 356 mg (28%) of crude methyl 3,12-bis(3,4-dimethoxycarbonylcaffeoyl)-7-deoxycholate (**8**).

A solution of 356 mg (0.42 mM) of **8** in 20 mL of THF, 20 mL of 4% aqueous Na_2CO_3, and 20 mL of MeOH under N_2 protection was stirred for 96 h at room temperature, at which time the pH was adjusted to 2−3 with 6 M HCl and the solution extracted with 3 × 100 mL of ether. Evaporation of the dried, combined extracts gave 257 mg of residue, which was purified on LH-20 with H_2O/MeOH (1:1) to give 23 mg (8%) of 3,12-dicaffeoyl-7-deoxycholic acid (**9**): mp 154−155 °C; ^{1}H NMR (M) 7.62 (d, 15.8, 1H), 7.40 (d, 15.9, 1H), 7.13 (d, 1.7, 1H), 7.03 (dd, 8.1, 1.9, 1H), 6.97 (s, 1H), 6.82−6.77 (m, 3H), 6.37 (d, 15.9, 1H), 6.07 (d, 15.9, 1H), 5.19 (s, 1H), 4.83−4.72 (m, 1H), 2.31−1.00 (m, 36H), 0.96 (s, 3H), 0.85 (d, 6.5, 3H), 0.80 (s, 3H); ^{13}C NMR (M) 178.1, 168.9, 168.7, 149.6, 149.4, 147.0, 146.8, 146.6, 127.8, 127.7, 123.2, 123.1, 116.7, 116.6, 115.7, 115.5, 115.4, 115.0, 77.3, 75.4, 51.1, 49.2, 46.5, 43.2, 37.0, 36.0, 35.9, 35.7, 35.1, 33.5, 32.1, 32.0, 28.4, 28.0, 27.7, 27.3, 26.6, 24.6, 23.5, 18.1, 12.9. Anal. (HPLC purified) ($C_{42}H_{52}O_{10}$) C, H.

3,7,12-Tricaffeoylcholic Acid (14). To a solution of 2.0 g (4.9 mM) of cholic acid (**10**) in 20 mL of 1:1 MeOH/$CHCl_3$ was added 2.50 g (12.9 mM) of diphenyldiazomethane in 20 mL of $CHCl_3$, and the mixture was heated at 45 °C for 18 h. The solvent was evaporated, and the residue was separated on silica gel with $CHCl_3$/MeOH (20:0.5) to give 2.88 g (99%) of diphenylmethyl cholate (**11**): mp 73−74 °C; ^{1}H NMR (M) 7.73−7.23 (m, 10H), 6.83 (s, 1H), 3.91 (s, 1H), 3.78 (s, 1H), 2.49−1.05 (m, 36H), 0.99 (d, 5.4, 3H), 0.92 (s, 3H), 0.61 (s, 3H); ^{13}C NMR (M) 174.7, 141.8 (×2), 129.5 (×2), 128.91, 128.85, 128.1, 128.0, 78.2, 74.0, 72.8, 69.0, 48.1, 47.5, 43.2, 42.9, 41.0, 40.4, 36.51, 36.48, 35.9, 35.8, 32.5, 32.3, 31.2, 29.5, 28.6, 27,8, 24.2, 23.2, 17.5, 13.0.

A mixture of 2.50 g (8.7 mM) of **1** and 5 mL of thionyl chloride (NaOH trap) was heated in an oil bath at 80−90 °C for 15 min. Removal of the thionyl chloride on a Rotovap gave a light-yellow solid, which was dissolved in 20 mL of dry benzene and added dropwise to a solution of 1.00 g (1.7 mM) of **11** in 20 mL of anhydrous benzene and 0.4 mL of pyridine. After 4 h, the precipitate was removed by filtration, the filtrate was evaporated, and the residue was separated on successive silica gel columns with $CHCl_3$/MeOH/HOAc (500:1:0.5) to remove recovered **1** and then with Et_2O/cyclohexane 40:60 to give 115 mg (5%) of diphenylmethyl 3,7,12-tris(3,4-dimethoxycarbonylcaffeoyl)cholate (**12**) and 482 mg (25%) of a 1:1 isomeric mixture of diphenylmethyl 3,7- and 3,12-bis(3,4-dimethoxycarbonylcaffeoyl)cholates (**15** + **16**) gum: ^{1}H NMR (C) 7.70−7.26 (m, 36H), 6.872 (s, 1H), 6.867 (s, 1H), 6.51 (d, 16.1, 1H), 6.46 (d, 16.6, 1H), 6.35 (d, 15.8, 1H), 6.30 (d, 15.8, 1H), 5.24 (bs, 1H), 5.04 (bs, 1H), 4.69 (m, 2H), 4.01 (bs, 1H), 3.91 (s, 15H), 3.90 (s, 6H), 3.88 (s, 4H), 2.48−1.03 (m, 48H), 0.97 (bs, 6H), 0.93 (s, 3H), 0.82 (d, 6.2, 3H), 0.72 (s, 3H), 0.65 (s, 3H); ^{13}C NMR (C) 173.0, 166.2, 166.1, 166.04, 165.99, 153.1, 153.0, 152.93, 152.88, 143.61, 143.59, 143.47, 143.4, 142.7, 142.61, 142.57, 142.3, 142.1, 140.3, 133.7, 133.61, 133.57, 128.5, 127.9, 127.8, 127.2, 127.0, 126.83, 126.80, 126.6, 126.5, 123.5, 123.4, 122.34, 122.30, 120.3, 120.2, 120.14, 120.07, 76.6, 75.9, 74.7, 74.6, 72.8, 71.3, 68.1, 55.9, 47.8, 47.3, 46.6, 45.3, 43.6, 42.1, 41.3, 41.1, 39.3, 38.3, 35.5, 34.8, 34.7, 34.6, 34.5, 31.6, 31.5, 30.8, 29.7, 28.6, 28.3, 27.8, 27.3, 27.2, 26.9, 26.8, 25.7, 23.0, 22.62, 22.58, 17.4, 17.3, 12.5, 12.3.

Data for the **12** gum are as follows: ^{1}H NMR (C) 7.88−7.26 (m, 22H), 6.85 (s, 1H), 6.52 (d, 15.9, 1H), 6.46 (d, 17.0, 1H), 6.31 (d, 15.9, 1H), 5.23 (bs, 1H), 5.13 (bs, 1H), 4.72 (m, 1H), 3.91 (s, 6H), 3.90 (s, 3H), 3.89 (s, 3H), 3.88 (s, 3H), 3.85 (s, 3H), 2.44−1.04 (m, 26H), 1.26 (s, 3H), 0.98 (d, 5.3, 3H), 0.72 (s, 3H); ^{13}C NMR (C) 173.0, 166.2, 166.1, 165.9, 153.1 (×3), 152.9, 152.8 (×2), 143.7, 143.5, 143.4, 142.93, 142.86, 142.7, 142.6, 142.5, 142.4, 140.2 (×2), 133.61, 133.58, 133.4, 128.5 (×4), 127.9, 127.8, 127.2 (×2), 127.0 (×2), 126.9 (×2), 126.5, 123.6, 123.4, 122.4, 122.3, 122.2, 120.1, 120.0 (×3), 76.7, 76.0, 74.8, 71.1, 55.9 (×6), 47.7, 45.4, 43.6, 41.1, 38.0, 35.0, 34.8, 34.6, 34.5, 31.5, 30.7, 29.7, 29.2, 27.1, 26.9, 25.8, 23.0, 22.6, 18.0, 12.3.

A mixture of 400 mg (0.36 mM) of **15** + **16**, 30 mL of 70% HOAc, and 5 mL of $CHCl_3$ was heated on a steam bath for 3.7 h. The solvent was removed by lyophilization, and the 420 mg residue combined with 62 mg from another run was purified on silica gel with $CHCl_3$/MeOH/HOAc (20:0.1:0.1) to give 255 mg (64%) of a 1:1 isomeric mixture of 3,7- and 3,12-bis(3,4-dimethoxycarbonylcaffeoyl)cholic acids (**17** + **18**) gum: ^{1}H NMR (C) 7.71−7.26 (m, 16H), 6.53 (d, 15.8, 1H), 6.47 (d, 15.9, 1H), 6.34 (d, 16.0, 1H), 6.30 (d, 16.1, 1H), 5.25 (bs, 1H), 5.05 (bs, 1H), 4.71 (m, 2H), 4.06 (bs, 1H), 3.91 (s, 15H), 3.90 (s, 6H), 3.88 (s, 4H), 2.38−1.08 (m, 48H), 0.98 (d, 5.9, 3H), 0.97 (s, H), 0.94 (s, 3H), 0.83 (d, 6.3, 3H), 0.79 (s, 3H), 0.71 (s, 3H); ^{13}C NMR (C) 180.05, 180.00, 177.1, 166.3, 166.2, 166.13,

166.08, 153.1, 153.0, 152.93, 152.87, 143.6, 143.5, 143.48, 143.39, 142.72, 142.69, 142.56, 142.4, 142.2, 133.63, 133.56, 126.9, 126.8, 126.6, 126.5, 123.5, 123.4, 122.4, 122.3, 120.2, 120.1, 120.0, 119.9, 77.3, 75.9, 74.74, 74.65, 73.0, 71.4, 68.2, 55.9, 47.7, 47.1, 46.6, 45.3, 43.6, 42.1, 41.2, 41.0, 39.3, 38.3, 35.4, 34.9, 34.8, 34.7, 34.5, 31.4, 31.1, 31.0, 30.6, 29.7, 28.4, 28.3, 27.8, 27.3, 26.8, 25.7, 23.0, 22.6, 22.5, 20.8, 17.5, 17.3, 12.5, 12.3.

A solution of 165 mg (0.17 mM) of **17** + **18** in 20 mL of THF, 20 mL of 4% aqueous Na_2CO_3, and 20 mL of MeOH under N_2 protection was stirred for 2 h at room temperature, at which time the pH was adjusted to 2−3 with 6 M HCl and the solution extracted with 3 × 100 mL of ether. Evaporation of the dried, combined extracts gave 125 mg of residue, which was purified on LH-20 with MeOH to give 95 mg (75%), a portion of which was purified by HPLC to give a 1:1 mixture of 3,7- and 3,12-dicaffeoylcholic acids (**19** + **20**): mp 173−177 °C; ^{1}H NMR (M) 7.68 (d, 13.5, 1H), 7.63 (d, 15.6, 1H), 7.41 (d, 16.1, 1H), 7.37 (d, 15.8, 1H), 7.14−6.69 (m, 12H), 6.38 (d, 16.0, 1H), 6.30 (d, 16.1, 1H), 6.13 (d, 15.9, 1H), 6.05 (d, 16.1, 1H), 5.21 (bs, 1H, H-12, ester), 5.01 (bs, 1H, H-7, ester), 4.62 (m, 2H, H-3, ester), 4.03 (bs, 1H, H-12, alcohol), 3.88 (bs, 1H, H-7, alcohol), 2.49−1.07 (m, 48H), 1.02 (d, 7.0, 3H), 1.01 (s, 3H), 0.97 (s, 3H), 0.88 (d, 6.5, 3H), 0.84 (s, 3H), 0.72 (s, 3H); ^{13}C NMR (C) 176.4, 169.0, 168.9, 168.8, 149.6, 149.51, 149.47, 147.1, 146.9, 146.72, 146.69, 146.6, 146.5, 128.0, 127.7, 123.3, 123.2, 123.1, 116.6, 115.8, 115.7, 115.3, 114.9, 77.2, 75.7, 73.6, 72.7, 68.7, 48.1, 47.7, 46.4, 44.8, 43.5, 42.9, 42.5, 40.7, 39.7, 36.8, 36.6, 36.3, 36.2, 36.0, 35.9, 35.8, 35.6, 32.6, 32.4, 32.24, 32.16, 32.11, 29.9, 29.6, 29.0, 28.53, 28.46, 27.9, 26.5, 24.2, 24.0, 23.05, 22.99, 18.1, 17.6, 13.0, 12.7; HRFABMS calcd *m/z* for $C_{42}H_{52}O_{11}Na$ 755.3408, found 755.3414.

A mixture of 115 mg (0.08 mM) of **12** and 10 mL of 70% HOAc was heated on a steam bath for 4 h, the solvent was removed by lyophilization, and the 123 mg of crude 3,7,12-tris(3,4-dimethoxycarbonylcaffeoyl)cholic acid (**13**) was dissolved directly in 10 mL of THF, 10 mL of 4% aqueous Na_2CO_3, and 10 mL of MeOH under N_2 protection. After 3 h at room temperature, the pH was adjusted to 2−3 with 6 M HCl and the solution extracted with 3 × 100 mL of ether. The ether was evaporated, and the residue was purified on LH-20 with MeOH/H_2O (1:1) and by HPLC to give 7 mg (10%) of 3,7,12-tricaffeoylcholic acid (**14**): mp 165−166 °C; ^{1}H NMR (M) 7.64 (d, 15.7, 1H), 7.59 (d, 15.8, 1H), 7.31 (d, 16.1, 1H), 7.28−6.57 (m, 9H), 6.35 (d, 16.3, 1H), 6.30 (d, 16.4, 1H), 6.00 (d, 15.9, 1H), 5.24 (bs, 1H), 4.63 (m, 1H), 2.38−1.14 (m, 24H), 1.03 (s, 3H), 0.86 (d, 8.8, 3H), 0.85 (s, 3H); ^{13}C NMR (C) 178.3, 168.9, 168.7, 168.3, 149.69, 149.66, 149.4, 147.3, 147.1, 146.8, 146.6 (×2), 146.5, 129.5, 127.63, 127.55, 123.24 (×2), 123.2, 116.84, 116.78, 116.70, 116.65, 115.62, 115.5, 115.3, 115.2, 115.0, 77.0, 75.2, 72.4, 48.1, 47.9, 46.6, 45.0, 42.3, 39.5, 36.4, 36.1, 35.8, 35.5, 32.5, 32.2, 30.3, 28.2, 27.9, 26.5, 24.0, 22.9, 18.1, 12.6; HRFABMS calcd *m/z* for $C_{51}H_{48}O_{14}Na$ 917.3725, found 917.3726.

2,3-Bis(3,4-dihydroxybenzylidene)succinic Acid (22a). To a stirred suspension of 4.28 g (79.2 mM) of NaOMe in 100 mL of of dry ether in an ice bath was added over 30 min a solution of 4.84 g (27.6 mM) of diethyl succinate and 16.96 g (55.4 mM) of 3,4-bis(tetrahydropyran-2-yloxy)benzaldehyde (**21a**)[36] in 100 mL of ether. The reaction continued for another hour in the ice bath and at room temperature for 65 h, the ether was allowed to evaporate, 200 mL of 1 N HCl was added, and the mixture was extracted with 3 × 200 mL of ether. The combined ether extracts were evaporated, and the MeOH soluble portion of the residue was combined with the EtOH soluble portion of the residue from lyophilization of the aqueous layer to give a crude product, which was separated on silica gel with $CHCl_3$/MeOH/HOAc (20:1.7:0.6) to give 1.48 g (15%) of bis(3,4-dihydroxybenzylidene)succinic acid (**22a**), which after purification on LH-20 with 1:1 MeCN/H_2O had a melting point of 135−136 °C: ^{1}H NMR (M) 7.75(s, 2H), 7.09 (d, 2.0, 2H), 6.93 (dd, 8.3, 2.0, 2H), 6.68 (d, 8.4, 2H); ^{13}C NMR (M) 171.0, 148.8, 146.3, 144.3, 128.4, 125.5, 124.8, 117.6, 116.2. Anal. ($C_{18}H_{14}O_8$) C, H.

***meso*- and D,L-2,3-Bis(3,4-dihydroxybenzyl)succinic Acid (23b and 24b).** The data for diveratrylidenesuccinic acid (**22b**) are as follows: ^{1}H NMR (C) 8.00 (s, 2H), 7.17 (s, 2H), 7.16 (d, 8.7, 2H), 6.80 (d, 8.2, 2H), 3.87 (s, 6H), 3.86 (s, 6H); ^{13}C NMR (C) 172.5, 151.0, 148.8, 144.5, 127.1, 125.1, 123.2, 112.2, 111.0, 55.9, 55.7. **22b** was prepared, and 7.333 g (17.7 mM) of the sample was then reduced by the methods of Schrecker[37] to give a mixture of diveratrylsuccinic acids, which were separated on silica gel with $CHCl_3$/MeOH/HOAc (20:0.5:0.2) into less and more $CHCl_3$-soluble fractions **23a** and **24a**, respectively.

***meso*-2,3-Bis(3,4-dimethoxybenzyl)succinic acid (23a):** 3.092 g (42%); mp 217−218 °C, lit.[37] 222−223 °C; ^{1}H NMR (D) 6.84 (d, 8.1, 2H), 6.73 (s, 2H), 6.68 (d, 8.6, 2H), 3.71 (s, 12H), 2.79 (m, 6H); ^{13}C NMR (D) 174.2, 148.4, 147.2, 131.3, 120.6, 112.5, 111.6, 55.4, 55.2, 49.2, 34.8.

D,L-2,3-Bis(3,4-dimethoxybenzyl)succinic acid (24a): 2.085 g (28%); mp 111−113 °C, lit.[38] 95−105 °C (hydrate), 110−112 °C (anhydride); ^{1}H NMR (D) 6.83 (d, 8.1, 2H), 6.69 (s, 2H), 6.65 (d, 8.2, 2H), 3.71 (s, 6H), 3.68 (s, 6H), 2.89 (m, 4H), 2.72 (m, 2H); ^{13}C NMR (D) 174.5, 148.3, 147.2, 131.4, 120.8, 112.5, 111.5, 55.3, 55.2, 47.7, 34.6.

To a suspension of 300 mg (0.72 mM) of **23a** in 30 mL of CH_2Cl_2 at −78 °C was added 4.5 mL of 1 M BBr_3 in CH_2Cl_2, and the reaction was allowed to proceed for 2 h at −78 °C and 2 h at room temperature. An amount of 30 mL of H_2O was added, the CH_2Cl_2 was evaporated, the precipitate was removed by filtration, and the filtrate was extracted with 4 × 60 mL of ether to give a residue, which was purified on silica gel with $CHCl_3$/MeOH/HOAc (20:1.8:0.5) to give 86 mg (33%) of *meso*-2,3-bis(3,4-dihydroxybenzyl)succinic acid (**23b**): mp 77−79 °C; ^{1}H NMR (400 MHz) (M) 6.62 (d, 8.1, 2H), 6.59 (d, 2.0, 2H), 6.46 (dd, 8.0, 2.0, 2H), 2.68−2.80 (m, 4H), 2.63 (dd, 13.4, 3.4); ^{13}C NMR (M) 176.0, 146.0, 144.7, 132.2, 121.4, 117.2, 116.3, 52.4, 37.2. Anal. ($C_{18}H_{18}O_8 \cdot H_2O$) C, H.

To a suspension of 350 mg (0.84 mM) of **24a** in 30 mL of CH_2Cl_2 at −78 °C was added 6.5 mL of 1 M BBr_3 in CH_2Cl_2, and the reaction was allowed to proceed for 2 h at −78 °C and 2 h at room temperature. An amount of 30 mL of H_2O was added, the CH_2Cl_2 was evaporated, and the filtrate was extracted with with 4 × 80 mL of ether to give a residue, which was purified on silica gel with $CHCl_3$/MeOH/HOAc (20:1.5:0.6) to give 69 mg (23%) of D,L-2,3-bis(3,4-dihydroxybenzyl)succinic acid (**24b**): mp 63−66 °C; ^{1}H NMR (D) 6.55 (d, 8.0, 2H), 6.48 (bs, 2H), 6.32 (bd, 7.5, 2H), 2.71 (dd, 13.4, 5.5, 2H), 2.50 (m, 2H), 2.32 (bm, 2H); ^{13}C NMR (D) 176.4, 144.6, 143.2, 130.6, 119.4, 116.1, 115.1, 51.3, 36.9. Anal. ($C_{18}H_{18}O_8$) C, H.

***threo*-2,3-Bis(3,4-dihydroxybenzyl)succinic Acid Monoamide (27a).** Heating 395 mg of **23a** under vacuum in an oil bath at 245−250 °C for 20 min gave D,L-2,3-bis(3,4-dimethoxybenzyl)succinic acid anhydride (**25**). The same anhydride was obtained by heating **24a**: ^{1}H NMR (C) 6.77 (d, 8.7, 2H), 6.55 (d, 6.5, 2H), 6.54 (s, 2H), 3.87 (s, 6H), 3.83 (s, 6H), 3.09 (dd, 7.9, 4.2), 2.98 (dd, 14.0, 6.3, 2H), 2.82 (dd, 14.0, 4.2, 2H); ^{13}C NMR (C) 172.6, 149.3, 148.4, 128.2, 121.4, 112.2, 111.3, 55.9, 46.0, 34.7. This sample was stirred with 15 mL of NH_4OH at room temperature for 2 h and at 60 °C for 1 h. The solution was acidified to pH 1−2 with 1 N HCl and extracted with 3 × 50 mL of $CHCl_3$, and the solvent was evaporated to give 397 mg (100%) of *threo*-2,3-bis(3,4-dimethoxybenzyl)succinic acid monoamide (**26a**): mp 150−153 °C; ^{1}H NMR (C) 6.63−6.78 (m, 6H), 6.40 (bs, 1H), 5.77 (bs, 1H), 3.82 (s, 6H), 3.79 (s, 6H), 2.83−3.04 (m, 5H), 2.57 (m, 1H); ^{13}C NMR (C) 178.2, 176.4, 148.9 (×2), 147.8 (×2), 130.6, 130.4, 121.02, 120.96, 112.2, 112.1, 111.3, 111.2, 55.8 (×4), 49.8, 49.4, 36.5, 35.8.

To a solution of 312 mg (0.73 mM) of **26a** in 20 mL of CH_2Cl_2 at −78 °C was added 12.0 mL of 1 M BBr_3 in CH_2Cl_2, and the reaction was allowed to proceed for 2 h at −78 °C and 2 h at room temperature, at which time 1 mL of H_2O was added, the CH_2Cl_2 was evaporated, and the residue was purified on LH-20 with 1:1 MeOH/H_2O to give 216 mg (63%) of *threo*-2,3-bis(3,4-dihydroxybenzyl)succinic acid monoamide (**27a**): mp 171−173 °C; ^{1}H NMR (M) 6.51−6.66 (m, 4H), 6.48−6.51 (m, 2H), 2.72−2.90 (m, 6H); ^{13}C NMR (M) 179.5, 178.1, 146.11,

146.07, 144.9 (×2), 131.8 (×2), 121.5, 121.4, 117.3, 117.2, 116.3 (×2), 51.6, 51.2, 36.9, 36.5. Anal. (HPLC purified) ($C_{18}H_{19}O_7N$) C, H, N.

***threo*-2,3-Bis(3,4-dihydroxybenzyl)succinic Acid Mono-*N*,*N*-diethylamide (27b).** The anhydride **25** prepared from 325 mg (0.78 mM) of **23a** as above was stirred with 5 mL of diethylamine at room temperature for 2 h. The solution was acidified to pH 1−2 with 1 N HCl and extracted with 3 × 50 mL of $CHCl_3$, and the solvent was evaporated to give 343 mg of crude *threo*-2,3-bis(3,4-dimethoxybenzyl)succinic acid mono-*N*,*N*-diethylamide (**26b**): mp 137−140 °C; ^{1}H NMR (C) 6.67−6.80 (m, 6H), 3.84 (s, 3H), 3.83 (s, 6H), 3.81 (s, 3H), 2.82−3.13 (m, 10H), 0.97 (t, 7.1, 3H), 0.71 (t, 7.1, 3H); ^{13}C NMR (C) 178.3, 174.0, 148.6 (×2), 147.5, 147.2, 133.4, 132.4, 121.2, 120.9, 112.6, 112.5, 111.1, 110.9, 55.9 (×2), 55.8 (×2), 52.9, 46.1, 42.0, 40.3, 36.3, 35.7, 13.7, 12.9. To a solution of 320 mg (0.73 mM) of **26b** in 20 mL of CH_2Cl_2 at −78 °C was added 10.0 mL of 1 M BBr_3 in CH_2Cl_2, and the reaction was allowed to proceed for 2 h at −78 °C and 2 h at room temperature, at which time 2 mL of H_2O was added, the CH_2Cl_2 was evaporated, and the residue was purified on LH-20 with 1:1 MeOH/H_2O to give 270 mg (89%) of *threo*-2,3-bis(3,4-dihydroxybenzyl)succinic acid mono *N*,*N*-diethylamide (**27b**): mp 85−89 °C; ^{1}H NMR (M) 6.47−6.70 (m, 6H), 3.21 (q, 7.3, 2H), 2.65−3.05 (m, 8H), 0.99 (t, 7.0, 3H), 0.70 (t, 7.0, 3H); ^{13}C NMR (M) 178.0, 175.8, 146.2 (×2), 145.1, 144.9, 131.6, 131.5, 121.7, 121.5, 117.5, 117.3, 116.4 (×2), 52.2, 46.4, 43.4, 41.7, 37.6, 36.5, 13.6, 13.0. Anal. (HPLC purified) ($C_{22}H_{27}O_7N\cdot0.5H_2O$) C, H, N.

***threo*-2,3-Bis(3,4-dihydroxybenzyl)succinic Acid Mono-*n*-decylamide (27c).** The anhydride **25** prepared from 401 mg (0.96 mM) of **23a** as above was stirred with a solution of 5 mL of *n*-decylamine in 30 mL of $CHCl_3$ at 60 °C for 1 h. The solution was extracted with 2 × 50 mL of concentrated HCl and washed with H_2O, and the solvent was evaporated to give a residue, which was suspended in acetone and filtered. The filtrate was then evaporated. The residue was purified on silica gel with $CHCl_3$/MeOH/HOAc (20:0.3:0.1) to give 414 mg (89%) of *threo*-2,3-bis(3,4-dimethoxybenzyl)succinic acid mono-*n*-decylamide (**26c**) oil: ^{1}H NMR (C) 6.75 (d, 8.2, 2H), 6.65 (dd, 8.7, 1.9, 2H), 6.59 (d, 1.9, 2H), 5.46 (t, 5.7, 1H), 3.85 (s, 6H), 3.83 (s, 6H), 3.02−3.18 (m, 4H), 2.92 (d, 8.1, 2H), 2.78 (dd, 13.3, 9.5, 1H), 2.34 (dd, 13.0, 8.0, 1H), 1.14−1.35 (m, 16H), 0.88 (t, 6.7, 3H); ^{13}C NMR (C) 175.6, 174.6, 149.12, 149.05, 148.05, 147.98, 130.33, 130.25, 120.9, 120.8, 112.1, 111.7, 111.4, 111.1, 56.0, 55.93, 55.87 (×2), 51.5, 49.2, 40.1, 37.5, 36.2, 31.9, 29.52, 29.48, 29.3, 29.2, 29.0, 26.8, 22.6, 14.1.

To a solution of 390 mg (0.70 mM) of **26c** in 20 mL of CH_2Cl_2 at −78 °C was added 14.0 mL of 1 M BBr_3 in CH_2Cl_2. The reaction was allowed to proceed for 2 h at −78 °C and 2 h at room temperature, at which time 2 mL of H_2O was added, the solvents were evaporated, and the residue was purified on LH-20 with 1:1 MeOH/H_2O to give 258 mg (74%) of *threo*-2,3-bis-(3,4-dihydroxybenzyl)succinic acid mono-*n*-decylamide (**27c**): mp 65−66 °C; ^{1}H NMR (M) 6.63−6.68 (m, 3H), 6.59 (d, 1.9, 1H), 6.50 (dd, 7.8, 1.9, 1H), 6.48 (dd, 7.9, 1.9, 1H), 3.07−2.62 (m, 8H), 1.33−1.12 (m, 16H), 0.89 (t, 6.7, 3H); ^{13}C NMR (M) 177.8, 176.2, 146.2, 146.1, 144.95, 144.91, 131.6, 131.5, 121.4 (×2), 117.3, 117.1, 116.3, 116.2, 51.6, 51.5, 40.5, 37.2, 36.6, 33.1, 30.8, 30.7, 30.5, 30.4, 30.2, 28.0, 23.8, 14.5. Anal. ($C_{28}H_{39}O_7N\cdot0.5H_2O$) C, H, N.

Di-2-thenoyltartaric Acid (28a). A mixture of (500 mg, 3.9 mM) thiophene-2-carboxylic acid and 6.2 mL (85 mmol) of thionyl chloride was heated at reflux for 2 h. The excess thionyl chloride was removed at reduced pressure, and the residue was heated with 98 mg (0.65 mM) of L-tartaric acid at 140−160 °C for 30 min and then with 5 mL of 80% HOAc on a steam bath for another 30 min. Removal of the solvent at reduced pressure and purification of the residue on silica gel with 10:1 to 1:1 $CHCl_3$/CH_3OH gave 235 mg (97%) of di-2-thenoyltartaric acid (**28a**): mp 186−187 °C; ^{1}H NMR (M) 7.92 (d, 3.2, 2H), 7.79 (d, 4.5, 2H), 7.13 (t, 4.3, 2H), 5.86 (s, 2H); ^{13}C NMR (M) 174.1, 163.5, 135.2, 134.9, 134.3, 128.8, 76.4. Anal. ($C_{14}H_{10}O_8S_2\cdot0.5H_2O$) C, H.

Di-3-thenoyltartaric Acid (28b). A mixture of 350 mg (2.73 mM) of thiophene-3-carboxylic acid and 4.2 mL (58 mmol) of thionyl chloride was heated at reflux for 2 h. The excess thionyl chloride was removed at reduced pressure, and the residue was heated with 82 mg (0.55 mM) of L-tartaric acid at 140−160 °C for 30 min and then with 5.0 mL of 80% HOAc on a steam bath for another 30 min. Removal of the solvent at reduced pressure and purification of the residue on silica gel with 8:1 to 1:1 $CHCl_3$/CH_3OH gave 192.8 mg (95%) of di-3-thenoyltartaric acid (**28b**): mp 194−195 °C; ^{1}H NMR (M) 8.37 (d, 2.9, 2H), 7.58 (d, 5.2, 2H), 7.47 (dd, 5.0, 3.0, 2H), 5.84 (s, 2H); ^{13}C NMR (M) 174.4, 163.9, 134.9, 134.5, 129.0, 127.1, 76.0. Anal. ($C_{14}H_{10}O_8S_2\cdot1.5H_2O$) C, H.

Tetraacetyl-L-chicoric Acid (29). A mixture of 400 mg (10 mM) of diacetylcaffeic acid[49] (^{1}H NMR (C), 7.73 (d, 15.9), 7.44 (dd, 8.4, 1.9), 7.395 (d, 1.9), 7.25 (d, 8.4), 6.40 (d, 16.0), 2.32 (s, 3H), 2.31 (s, 3H); ^{13}C NMR (C) 171.3, 168.1, 167.9, 145.1, 143.9, 142.5, 132.9, 126.7, 124.0, 123.0, 118.4, 20.7, 20.6), 2.4 mL of thionyl chloride, and 0.5 mL of benzene was heated at reflux for 2 h. The solvent and excess thionyl chloride were removed on a Rotovap, and the residue was dissolved in 4.5 mL of dry toluene. To this solution was slowly added 293 mg of diphenylmethyl tartrate[33] dissolved in 4 mL of toluene and 1.0 mL of pyridine. The resulting mixture was stirred overnight, and the solvents were removed on a Rotovap to give a pale powder, which was purified on a silica gel column with hexane/EtOAc (2.5:1) to give 490 mg (82%) of bisdiphenylmethyl tetraacetyl-L-chicorate: mp 200.5−202 °C; ^{1}H NMR (C) 7.50 (d, 16.0, 2H), 7.17−7.33 (m, 26H), 6.93 (s, 2H), 6.15 (d, 16.0, 2H), 6.02 (s, 2H), 2.33 (s, 6H), 2.32 (s, 6H); ^{13}C NMR (C) 168.0, 167.9, 164.9, 164.8, 144.6, 143.8, 142.5, 138.8, 138.7, 132.8, 128.64, 128.57, 128.3, 128.2, 127.3, 127.1, 126.6, 123.9, 123.1, 117.2, 79.1, 71.1, 20.7, 20.6.

A solution of 97.4 mg of the above compound in 0.2 mL of trifluoroacetic acid and 0.6 mL of CH_2Cl_2 was stirred overnight at room temperature, and the residue after removal of the solvent passed through a silica gel column with hexane/EtOAc/MeOH/HOAc to give 63 mg (98%) of tetraacetyl-L-chicoric acid (**29**): mp 200−202 °C, lit.[50] 186−188 °C; ^{1}H NMR (D) 7.77 (d, ∼17.0, overlap, 2H), 7.74 (bs, overlap, 2H), 7.70 (bd, ∼7.5, overlap, 2H), 7.33 (d, 8.5, 2H), 6.76 (d, 16.1, 2H), 5.68 (s, 2H), 2.50 (s, 6H), 2.29 (s, 6H) (C/M, 1:1), 7.78 (d, 16.0, 2H), 7.65 (bs, 2H), 7.55 (dd, 8.5, 1.5, 2H), 7.30 (d, 8.3, 2H), 6.61 (d, 16.0, 2H), 5.88 (s, 2H), 2.35 (s, 6H), 2.34 (s, 6H); ^{13}C NMR (D) 168.0, 167.9, 167.2, 164.9, 144.2, 143.7, 142.3, 132.4, 127.1, 124.1, 123.4, 117.8, 70.8, 20.3, 20.2.

Biological Assays. Cells and Virus. All cell lines were grown in RPMI-1640 containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and supplemented with 11.5% fetal bovine serum and 2 mM L-glutamine. HIV_{LAI} is the LAI isolate of HIV-1. HIV_{LAI} was propagated in H9 cells and obtained from supernatant fluid clarified of cells by low-speed centrifugation followed by filtration through 0.45 μm nitrocellulose filters. HIV-1 completely lyses MT-2 cells, a $CD4^+$ lymphoblastoid cell line, within 2.5−3 days of inoculation with HIV_{LAI} at a multiplicity of infection greater than 1.

Cell Toxicity Assays. Cell toxicity was determined as reported previously.[29,30] Briefly, lyophilized compounds were solubilized in H_2O, 95% ethanol, or an ethanol/dimethyl sulfoxide mixture. Compounds were diluted in growth medium, filter-sterilized, and 2-fold serially diluted in triplicate wells of a microtiter plate. To each diluted compound, MT-2 cell suspension (2 × 10^5 cells) was added. The cells were incubated with drug for 72 h at 37 °C, then harvested for cell viability in a neutral red dye assay as described previously.[33,39] The cytostatic/cytotoxic dose was defined as 5% inhibition of MT-2 cell growth in 72 h (CT_5). The dose was calculated across the linear portion of the dose−response curve using the CalcuSyn for Windows software package. Several of the compounds were not available in sufficient quantity or demonstrated a solubility profile that precluded determination of a true CT_5. For these compounds, cell toxicity is defined as greater than the maximum concentration of the compound tested. The CT_5 is a better measure of toxicity than CT_{50}, since 5% inhibition of cell

growth is within 1 SD of the cell controls. Thus, this is a truly nontoxic concentration of the compound.

Anti-HIV Assay. Anti-HIV assays were performed as described.[29,30,33,39] On the basis of cell toxicity data, compounds were diluted in growth medium such that a final 1:4 dilution of the sample would result in a concentration of sample equal to the CT_5. The compounds were then 2-fold serially diluted in triplicate. To each diluted compound, HIV_{LAI} was added and the virus/compound mixture was incubated for 1 h at 37 °C. Next, MT-2 cell suspension (2×10^5 cells) was added to each well and cells were incubated for 72 h at 37 °C. Final multiplicity of infection (MOI) was 1−5. Cells were harvested to quantitate the cytopathic effect using a neutral red dye assay as described. The antiviral concentration reported is the concentration of sample necessary to protect 50% of MT-2 cells from viral-induced cell death; this is referred to as the 50% effective dose (ED_{50}). The ED_{50} was calculated across the linear portion of the dose−response curve using the CalcuSyn for Windows software package. Inhibition of virus-induced cell death has correlated well with virus replication, as measured by synthesis of antigens, reverse transcriptase release, and production of infectious progeny virions.[40]

Quantitative Real-Time PCR Measurements of HIV Two LTR Circle DNA and cDNA. a. Primers. Primers are the following: M661-5′-CCTGCGTCGAGAGAGCTCCTCTGG-3′ (HIV-1_{LAI} antisense 695-672); M667−5′-GGCTAACTAGG-GAACCCACTG-3′ (HIV-1_{LAI} sense 496-516); MH535, 5′-AACTAGGGAACCCACTGCTTAAG-3′ (HIV-1_{LAI} sense 9683-9697); MH536, 5′-TCCACAGATCAAGGATATCTTGTC-3′ (HIV-1_{LAI} antisense 58-21)

b. Cell Lysis and Sample Preparation. At each time point, 1.0×10^6 cells were lysed in 100 μL of a lysis solution composed of equal parts of solution A (100 mM KCl, 10 mM Tris-HCl, pH8.3, 2.5 mM $MgCl_2$) and solution B (10 mM Tris-HCl, pH8.3, 2.5 mM $MgCl_2$, 1% Tween-20, 1% Nonidet 40, and 20 μg of proteinase K per 1.0×10^6 cells) as described previously.[51] Cells were lysed at 65 °C for 1 h, followed by heat inactivation of proteinase K at 95 °C for 15 min.

c. Real-Time PCR for HIV cDNA. Real-time PCR was performed using the Smart Cycler (Cepheid). PCR conditions to detect HIV cDNA were the following: initial denaturation at 95 °C for 150 s, followed by 40 rounds of cycling at 95 °C for 15 s, then 61 °C for 30 s. Following amplification, melt curve analysis between 60 and 95 °C (0.2 °C/s) was performed to determine the T_m of the amplified product. Each PCR reaction mixture contained 0.2 μM each of dATP, dCTP, dGTP, and dTTP, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.625 U of Taq DNA polymerase (GIBCO/BRL), 0.2 μM of M661 and M667, HIV-1 specific primers pairs that detect complete cDNA,[52] 0.25X SYBR-I Green (Sigma-Aldrich or Molecular Probes), and 2 μL of cell lysate (equivalent to the DNA from 2×10^4 cells) in a 25 μL reaction volume. For two LTR circle DNA, MH535/MH536,[43] PCR conditions were the following: initial denaturation at 95 °C for 150 s, followed by 40 rounds of cycling at 95 °C for 15 s, then 59 °C for 30 s. Following amplification, melt curve analysis between 60 and 95 °C (0.2 °C/s) was performed to determine the T_m of the amplified product. Each PCR reaction mixture contained 0.2 μM each of dATP, dCTP, dGTP, and dTTP, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, 5 μg of non-acetylated BSA, 0.15 M trehalose, 0.2% Tween-20, 0.625 U of Taq DNA polymerase (GIBCO-BRL), 0.2 μM of each primer, MH535 and MH536,[43] 0.25X SYBR-I Green (Molecular Probes), and 2 μL of cell lysate in a 25 μL reaction volume. For both primer pairs, products were quantified using standard curves ($n > 15$, $r^2 > 0.99$). Standard curves were generated using dilutions of 20000−20 chronically HIV_{LAI}-infected H9 cells. While ratios were calculated using infected cell equivalents, there was approximately 200-fold more cDNA than two LTR circle DNA by copy number, consistent with previously published results.[43]

Biochemical Assays of Integrase Activity. The 3′-end-processing, strand transfer, and disintegration activities of IN in the presence and absence of inhibitors were assayed in vitro[29,30,33] as modified from Chow et al.[22] The following oligonucleotides (GenoSys, Inc.) were used as DNA substrates:

T1 (16 mer), 5′-CAGCAACGCAAGCTTG-3′; T3 (30 mer), 5′-GTCGACCTGCAGCCCAAGCTTGCGTTGCTG-3′; V2 (21mer), 5′-ACTGCTAGAGATTTTCCACAT-3′; V1/*T*2 (33 mer), 5′-ATGTGGAAAATCTCTAGCAGGCTGCAGGTCGAC-3′; V1 (21-mer), 5′-ATGTGGAAAATCTCTAGCAGT-3′; db-Y1 (38mer), 5′-TGCTAGTTCTAGCAGGCCCTTGGGCCGGCGCTTGCGCC-3′.

The oligonucleotides were gel-purified. Oligonucleotides T1, V1, and db-Y1 were labeled at the 5′-ends using T4 polynucleotide kinase and [γ-^{32}P] ATP (3000 Ci/mmol, Amersham). Labeled substrate was purified using a p6 spin column. The substrate for assaying disintegration activity, the Y oligomer, was prepared by annealing the labeled T1 strand with oligonucleotides T3, V2, and V1/T2. The end-processing/strand transfer substrate was prepared by annealing the labeled V1 strand with V2. The dumbbell substrate was prepared by heating labeled db-Y1 to 95 °C followed by slow cooling. In a 20 μL volume, the DNA substrate (0.1 pmol of db-Y1 and "y"-oligo; 0.2 pmol of V1/V2) was incubated with 1.5 pmol of recombinant IN for 60 min at 37 °C in a buffer containing a final concentration of 20 mM HEPES, pH 7.5, 10 mM DTT, 0.05% Nonidet P-40, and 10 mM $MnCl_2$. To each 19 μL of reaction mixture, 1 μL of inhibitor at various concentrations in solvent or solvent alone was added. The reaction was stopped by the addition of EDTA to a final 18 mM concentration. Reaction products were heated at 90 °C for 3 min before analysis by electrophoresis on a 15% polyacrylamide gel with 7 M urea in Tris-borate/EDTA buffer. All reactions were performed at enzyme excess, and reactions were stopped within the linear range of the reaction. Reactions were performed in the presence of $MnCl_2$ rather than $MgCl_2$ because this class of molecules inhibits HIV IN whether Mg^{2+} or Mn^{2+} is the source of divalent cation, and for the interaction of IN with L-CA, the metal ion is not required.[32] These data are more consistent with the inhibitory activity of bis-catechols against avian sarcoma virus IN. IC_{50} analysis was determined from a median effect plot using CalcuSyn software (Biosoft, Cambridge, U.K.) on 0.5 log dilutions of inhibitor in triplicate experiments.

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