Chicoric Acid Analogues as HIV-1 Integrase Inhibitors

Zhaiwei Lin,[‡] Nouri Neamati,[§] He Zhao,[‡] Yoshimitsu Kiryu,[‡] Jim A. Turpin,[||,⊥] Claudia Aberham,[◇] Klaus Strebel,[◇] Kurt Kohn,[§] Myriam Witvrouw,[†] Christophe Pannecouque,[†] Zeger Debyser,[†] Erik De Clercq,[†] William G. Rice,[||,▽] Yves Pommier,[§] and Terrence R. Burke, Jr.*[,‡]

Laboratories of Medicinal Chemistry and Molecular Pharmacology, Division of Basic Sciences, National Cancer Institute, Bethesda, Maryland 20892, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, Laboratory of Antiviral Drug Mechanisms, National Cancer Institute−Frederick Cancer Research and Development Center, SAIC Frederick, Frederick, Maryland 21702-1201, and Rega Institute for Medical Research, Minderbroedersstraat 10-B-3000, Leuven, Belgium

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The present study was undertaken to examine structural features of L-chicoric acid (**3**) which are important for potency against purified HIV-1 integrase and for reported cytoprotective effects in cell-based systems. Through a progressive series of analogues, it was shown that enantiomeric D-chicoric acid (**4**) retains inhibitory potency against purified integrase equal to its L-counterpart and further that removal of either one or both carboxylic functionalities results in essentially no loss of inhibitory potency. Additionally, while two caffeoyl moieties are required, attachment of caffeoyl groups to the central linking structure can be achieved via amide or mixed amide/ester linkages. More remarkable is the finding that blockage of the catechol functionality through conversion to tetraacetate esters results in almost no loss of potency, contingent on the presence of at least one carboxyl group on the central linker. Taken as a whole, the work has resulted in the identification of new integrase inhibitors which may be regarded as bis-caffeoyl derivatives of glycidic acid and amino acids such as serine and β-aminoalanine. The present study also examined the reported ability of chicoric acid to exert cytoprotective effects in HIV-infected cells. It was demonstrated in target and cell-based assays that the chicoric acids do not significantly inhibit other targets associated with HIV-1 replication, including reverse transcription, protease function, NCp7 zinc finger function, or replication of virus from latently infected cells. In CEM cells, for both the parent chicoric acid and selected analogues, antiviral activity was observable under specific assay conditions and with high dependence on the multiplicity of viral infection. However, against HIV-1- and HIV-2-infected MT-4 cells, the chicoric acids and their tetraacetylated esters exhibited antiviral activity (50% effective concentration (EC_{50}) ranging from 1.7 to 20 μM and 50% inhibitory concentration (IC_{50}) ranging from 40 to 60 μM).

Introduction

Treatment of AIDS using single agents often results in the development of tolerance. One way of potentially countering such tolerance is by employing combination therapies directed against different viral enzymes. To date, reverse transcriptase and protease inhibitors have been employed successfully in such a fashion; however, availability of inhibitors directed against alternate targets is also desirable. Integrase is a key enzyme in the viral life cycle which is required for replication.[1−4] The integrase enzyme therefore offers an attractive target for anti-AIDS drug design, with such inhibitors being potentially complementary to a multidrug treatment regime.[5] For these reasons, significant effort has been expended on the development of such agents.[6]

Integrase functions in a two-step manner by initially removing a dinucleotide unit from the 3′-ends of the viral DNA (termed "3′-processing"), with the 3′-processed strands then being transferred from the cytoplasm to the nucleus where they are introduced into the host DNA (termed "strand transfer" or "integration"). Radiolabeled oligonucleotide-based assays,[7,8] which allow the in vitro determination of IC_{50} values for inhibition of both 3′-processing and strand transfer, have been utilized by several groups to examine the inhibitory efficacy of large numbers of agents.[9] Using such assays, several classes of inhibitors have emerged, many of which contain multiple aromatic rings, with poly(arylhydroxylation), frequently in the 1,2-catechol arrangement. In many cases the aryl units of these inhibitors are separated by a central linker as typified by general structure **I** (Figure 1). Among such inhibitors are flavones,[10,11] arctigenins,[12] tyrphostins,[13] and bis-catechols such as β-conidendrol.[14] Another group of related inhibitors are based on caffeic acid phenethyl ester (CAPE, **1**)[10] and structural analogues such as **2**.[15,16] Even though catechols as a group exhibit favorable inhibitory profiles in cell-free integrase assays, often these compounds have failed to exhibit antiviral activity due to dose-limiting toxicities in in vitro assays. Recently, we have demonstrated that oxidation of CAPE-

* To whom correspondence should be addressed at Laboratory of Medicinal Chemistry, NCI, Bldg 37, Rm 5C06, NIH, Bethesda, MD 20892. Tel: 301-402-1717. Fax: 301-402-2275. E-mail: tburke@helix.nih.gov.

⊥ Currently located at: Southern Research Institute, 431 Aviation Way, Frederick, MD 21701.

▽ Currently located at: Achillion Pharmaceuticals, 1003 West 7th St, Suite 401, Frederick, MD 21701.

‡ Laboratory of Medicinal Chemistry, NCI.

§ Laboratory of Molecular Pharmacology, NCI.

|| Laboratory of Antiviral Drug Mechanisms, FCRDC.

◇ Laboratory of Molecular Microbiology, NIAID.

† Rega Institute for Medical Research.

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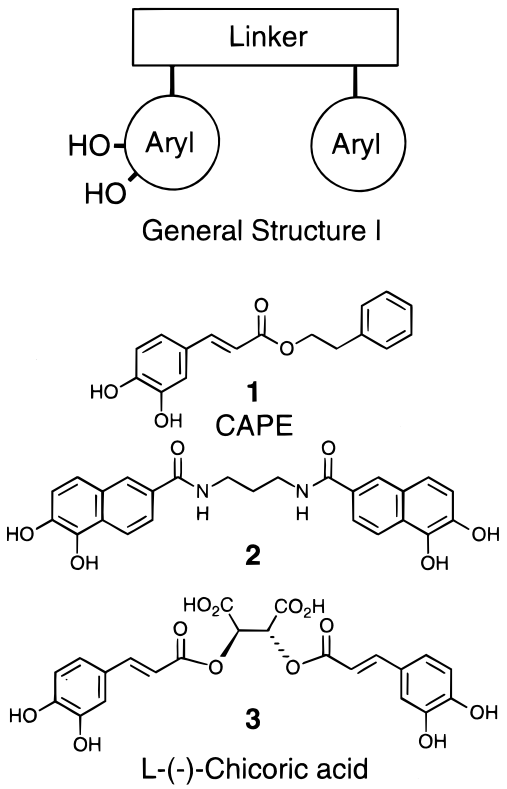


Figure 1. General structure **I** which summarizes features common to many agents that inhibit HIV integrase in isolated enzyme preparations, with compounds **1**−**3** representing specific examples.

like analogues leads to cross-linking of intracellular proteins.[17] Toxicities may reflect oxidation of the catechols to reactive quinone species, accounting for the observed losses in cell viability. Thus, development of integrase inhibitors either lacking the catechol moiety or modifications which overcome its toxic properties have been the focus of considerable work.[9]

It has been shown that compounds such as 4,5-di-*O*-caffeoylquinic acid, which has multiple caffeoyl groups appended to a carboxylic acid-containing framework, can inhibit HIV replication in cell-based systems, presumably by interference with gp120-mediated binding of virus to the CD4 receptor.[18] More recent work has reported that dicaffeoylquinic acids and the related compound, L-chicoric acid (dicaffeoyltartaric acid, **3**), not only inhibit HIV-1 replication but are also potent inhibitors of HIV-1 integrase.[19−21] Furthermore, it has been demonstrated that these agents are selective inhibitors of integrase when examined against a variety of other potential viral targets[22] and that they act on the catalytic core domain of the enzyme.[23] More recently, resistance to antiviral effects of L-chicoric acid in HIV-1-infected cells has been achieved by a single mutation of integrase amino acid 140, supporting previous evidence that L-chicoric acid acts by inhibiting integrase and that it does so by interacting near the enzyme catalytic triad.[24] The high potency of these bis-catechols in purified integrase is consistent with well-established SAR considerations. However, their reported efficacy in cell-based assays is remarkable in light of the failure of large numbers of structurally related compounds (for example, **1** and **2**). It was therefore of interest to examine structural features of L-chicoric acid (**3**) which are important for potency against the integrase, cellular toxicity, and antiviral activity.

Synthesis

L-(−)-chicoric acid (**3**) and D-(+)-chicoric acid (**4**) were prepared from di-*tert*-butyl esters of L-(+)-tartaric acid and D-(−)-tartaric acid, respectively, using 3,4-diacetylcaffeoyl acid chloride (**5**) as previously reported.[25] The tetraacetylated L-chicoric acid (**6**) and D-chicoric acid (**7**) were similarly prepared.[25] Tetraacetylated bis-caffeoyl derivatives **8**−**12** were also synthesized by treatment of the corresponding diols or amino alcohol with acid chloride **5** in the presence of pyridine. Hydrolysis of the acetates to yield the free bis-caffeoyl analogues **13**−**17** was achieved by refluxing the tetraacetates in acetone containing aqueous HCl (Scheme 1).

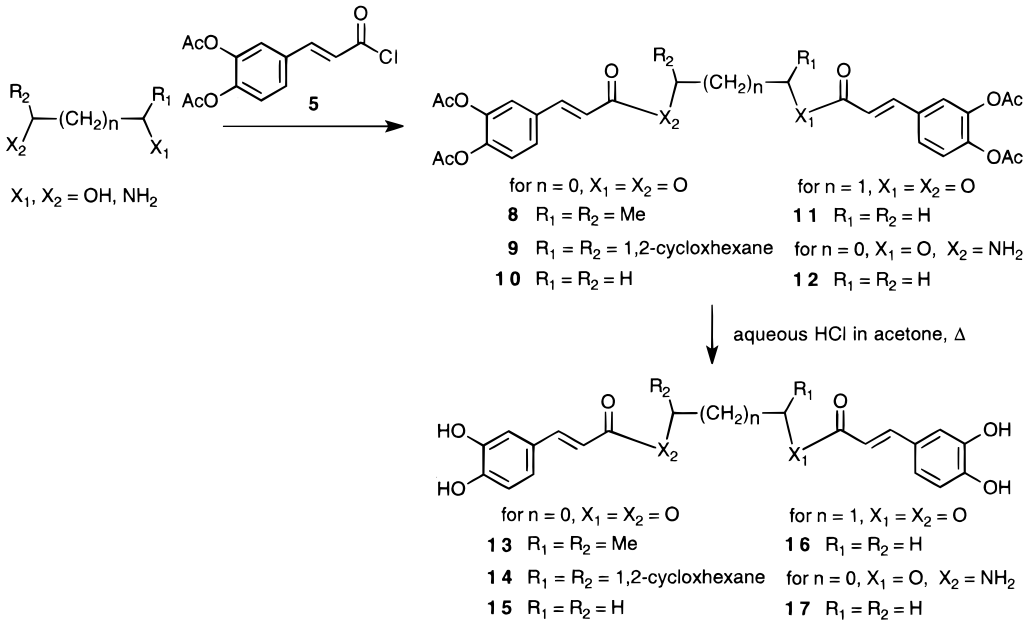
Monocarboxy analogues **18**−**29** were prepared in a similar but slightly altered fashion (Scheme 2). Central linkers were first esterified as their methyl esters (refluxing methanolic HCl) and then acylated with either diacetylcaffeoyl chloride (**5**) in pyridine (for compounds **18**−**21**) or diacetylcaffeic acid via HOBt mixed anhydride coupling (for compounds **22** and **23**). Using lithium iodide in refluxing pyridine, methyl esters **18**, **22**, and **23** were then selectively cleaved in the presence of acetoxy and methyl ether functionality, to afford free acids **24**−**26**, which retained caffeoyl protection. Alternatively, refluxing methyl esters **18** and **19** in acetone containing aqueous HCl provided globally deprotected free acids **27** and **28**, respectively, without cleavage of the caffeoyl linkages.

Results and Discussion

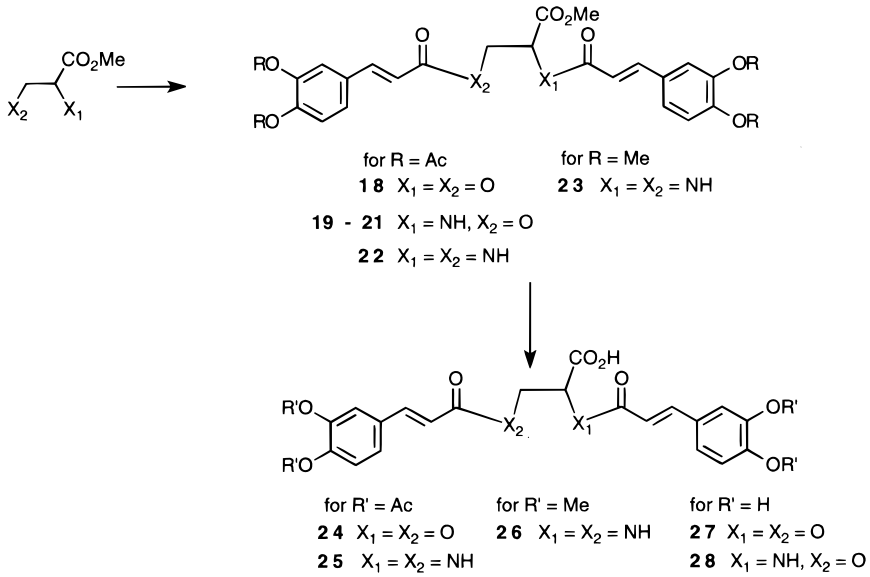
Catechol-containing bis-aryl moieties are a significant structural component in many potent HIV integrase inhibitors. Frequently such compounds are characterized by two aryl units (at least one of which bears 1,2-bis-hydroxylation) separated by a central linker.[16] Although such analogues exhibit good inhibition against isolated HIV integrase, often corresponding protective effects in HIV-infected cells are not observed,[13,20,21,26] perhaps at least partially as a result of limiting collateral cytotoxicity. The revelation that L-chicoric acid (**3**) both exhibits potent integrase inhibition in isolated enzyme preparations and provides protective effects in HIV-infected cells is therefore worthy of note, since its structure falls within the parameters of prior analogues which lack antiviral activity due to cellular toxicity. The present study was undertaken to examine features of L-chicoric acid responsible for this differential effect and to develop analogues which mimic their reported activities.

Chirality. One striking feature of L-chicoric acid is the presence of chiral centers in the central linker. Biological systems often discriminate between D- and L-isomers because the interactions of enantiomeric ligands with chiral host proteins are diastereomeric in nature. To our knowledge, previous reports have detailed the HIV integrase inhibitory potency of only L-chicoric acid (**3**).[19−24] To examine the inhibitory profile of D-(+)-chicoric acid (**4**), we recently prepared this material from D-(−)-tartrate.[25] (It should be noted that although L-chicoric acid exhibits a (−)-sign of optical rotation, it bears the (2*R*,2*R*)-absolute configuration of

Scheme 1



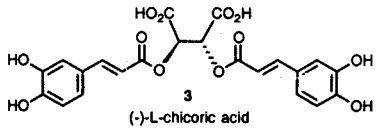
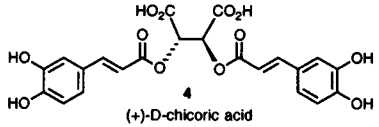
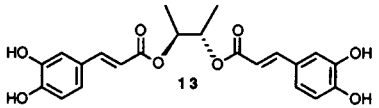
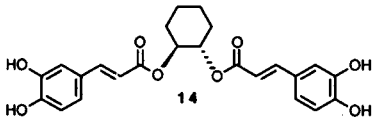
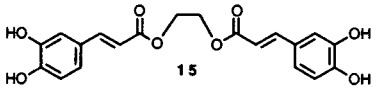
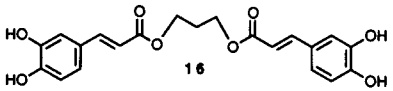
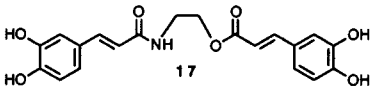
Scheme 2

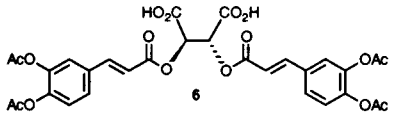
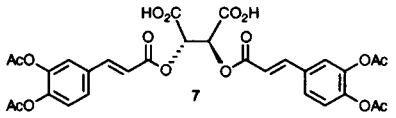
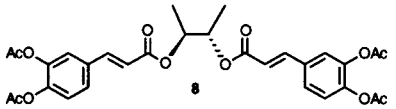
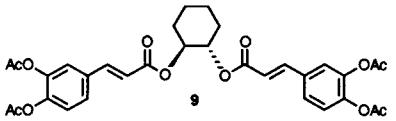
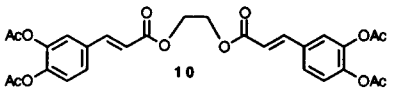
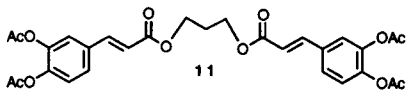
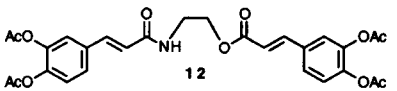


(+)-tartaric acid from which it is derived.) As shown in Table 1, both L- and D-chicoric acids (**3** and **4**, respectively) are nearly identical in potency against integrase (IC_{50} values of approximately 1 μM). Both D- and L-chicoric acid were assessed by the AIDS Drug Screening Program of the NCI in a standardized XTT cytoprotection assay, which measures the ability of compounds to protect CEM-SS cells from the cytopathic effects of the HIV-1_{RF} virus strain.[27] Neither compound significantly inhibited the replication of HIV-1 in this system. In both cases, either cytoprotection did not reach a 50% value (EC_{50}) or the antiviral effect was observed just prior to doses approaching the IC_{50} values (dose exhibiting 50% cell toxicity on uninfected cells). Since observed protection occurred in parallel with loss of cell viability, its significance is unknown.

Central Linker Carboxylic Acid Functionality. The chicoric acids are set apart from many other bis-aryl integrase inhibitors by having both a center of chirality and a carboxylic acid functionality on their central linkers. To examine the importance of carboxyl groups, analogue **13** was prepared which lacks the carboxylic oxygens yet maintains the chirality of L-chicoric acid (**3**). As shown in Table 1, while integrase strand transfer inhibitory potency of **13** ($IC_{50} = 8$ μM) is moderately reduced relative to L-chicoric acid, significant potency is retained. As a further examination of **13**, conformational constraint was introduced by incorporation of its central linker into a cyclohexane ring (compound **14**). No apparent effect was observed on integrase strand transfer inhibitory potency. Finally, substituents were removed from the central linker (compound **15**), effectively eliminating chirality as well as any conformation constraints originally induced by such groups. This modification reinstated the high integrase inhibitory potency ($IC_{50} = 0.5$ μM) originally displayed by the parent chicoric acids **3** and **4**. Even though modifications of the bis-carboxylic acid linker resulted in significant changes in the ability of purified integrase enzyme to mediate 3′-processing and integra-

Table 1. Results of Radionucleotide-Based Integrase Assays and Cytoprotection in the NCI AIDS Screen

Structure	Integrase Assay[a] (IC_{50} μM) 3′-processing	Integration	Cell Data[b] (μM) IC_{50}	EC_{50}	TI_{50}
 3 (-)-L-chicoric acid	1.1 (2.3)	0.8 (1.0)	20.1 14.2	NR[c] NR	
 4 (+)-D-chicoric acid	1.1	0.5	16.0 35.5	16.7 13.6	1.0 2.6
 13	38.4 (>10)	7.8 (7.4)	3.30 3.64	NR NR	
 14	24.8 (>10)	7.6 (6.1)	5.67	NR	
 15	0.4 (0.9)	0.4 (0.3)	9.89 10.0	NR NR	
 16	27.5 (16)	7.5 (6.4)	13.5 13.9	NR NR	
 17	27.5 (>10)	6.5 (6.1)	9.22 7.71	NR NR	

Structure	Integrase Assay[a] (IC_{50} μM) 3′-processing	Integration	Cell Data[b] (μM) IC_{50}	EC_{50}	TI_{50}
 6	9.8 (10)	6.2 (7)	50 ± 4	16 ± 3	3.1
 7	33.3 (10)	4.6 (5.2)	23 ± 8	21 ± 9	1.1
 8	>333	>333	5.45 5.71	NR NR	
 9	>333	>333	5.74 5.76	NR NR	
 10	>100	>100	6.7 ± 2.5	NR	
 11	>333	>333			
 12	>333	100	14.8 15.6	>14.8 >15.6	

[a] Determined by radiolabeled oligonucleotide-based assay as described in the Experimental Section. Values in parentheses, or duplicate values, are the results of multiple runs. [b] Determined through the NCI AIDS Screen using an XTT cytoprotection assay. Replicate values are the results of multiple runs. [c] NR, not reached due to cytotoxicity.

tion, all three compounds displayed significant cellular toxicity and lacked antiviral activity.

Central Linker Chain Length and Mode of Caffeoyl Attachment. Two additional parameters were examined relative to analogue **15**: chain length and mode of caffeoyl attachment. Modification of **15** by the addition of a methylene (**16**) or replacing the caffeoyl ester linkage with an amide linkage (**17**) resulted in significant losses in integration activity (27- and 23-fold decreases, respectively). Although for both **16** and **17** good inhibitory potency was maintained against purified integrase, as with other members of this series, **16** and **17** were toxic and failed to exhibit antiviral activity.

Tetraacetate Derivatives. The catechol moiety has proven critical for potent integrase inhibition over a wide range of inhibitor subclasses. This has been particularly true for those analogues which can be loosely grouped as "CAPE variants". Since the catechol structure could potentially contribute to unwanted collateral cytotoxicity,[17] it would be desirable to eliminate such functionality while maintaining integrase inhibitory potency. Recent efforts in this regard have largely been unsuccessful when applied to CAPE-type inhibitors.[15,16] For example, derivatizing potent catechol-containing inhibitors as their methyl ethers has almost uniformly resulted in a loss of integrase inhibition.[15,16] In contrast to these observations, L-chicoric acid has been reported to show antiviral activity in addition to integrase inhibition.[19−23] Furthermore, recent induction of resistance to antiviral effects of L-chicoric acid in HIV-1-infected cells by a single mutation of integrase has increased support that L-chicoric acid exerts antiviral effects by inhibiting integrase.[24] We therefore examined the catechol functionality in this class of compounds as it relates to integrase inhibitory profile and antiviral activity. Tetraacetates of L- and D-chicoric acid (**6** and **7**, respectively)[25] were examined as chicoric acid analogues having their catechol functionality masked through ester formation. As shown in Table 1, these non-catechol-containing derivatives displayed potent inhibition against purified integrase. Interestingly these non-catechol analogues also had reproducibly low to moderate antiviral activity (**6**, EC_{50} 16.8 μM, IC_{50} 48.8 μM, $n = 4$; **7**, EC_{50} 26.8 μM, IC_{50} 23.2 μM, $n = 5$) with slightly decreased cellular toxicity (**3:** IC_{50} 17.1 μM). Similar observations were made with different viral strains and cell types (see below).

Additional acetylated analogues of chicoric acid were prepared in analogy to the first series of unacetylated chicoric acid derivatives previously discussed. As reported above, while the catechol-containing analogues of the first series (**13**−**17**) displayed high integrase inhibitory potency in the absence of central linker carboxy functionality, this was not the case for acetylated chicoric acid analogues **8**−**12**. In this latter series, removal of this central linker functionality was accompanied by complete loss of inhibitory potency (Table 1). For this class of compounds without central linker carboxyl groups, potent enzyme inhibition can only be obtained with catechol functionality. Stated another way, in the absence of catechol functionality (i.e., as tetraacetates), central linker carboxyl functionality is required. Therefore simultaneous loss of both catechol and carboxylic functionality is not tolerated for integrase inhibition.

Monocarboxylic Analogues. The parent chicoric acids are characterized by the presence of two carboxylic acid groups on their central linkers. As exemplified by the dicaffeoylquinic acids, however, it has been shown that single carboxyls on a central linker between two caffeoyl esters can provide potent integrase inhibition.[19−24] Therefore, it was of interest to examine what effect removal of one carboxylic group from the chicoric acid structures would yield.

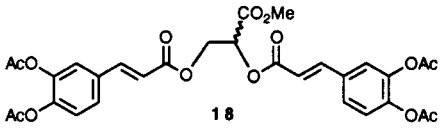
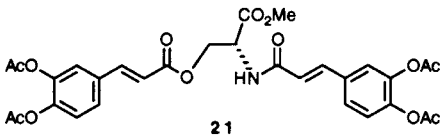
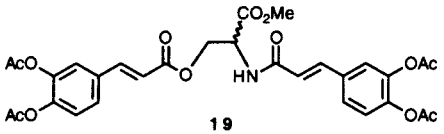
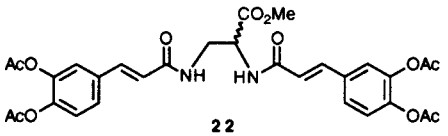
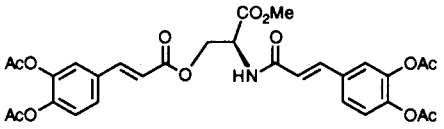
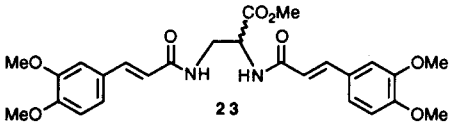
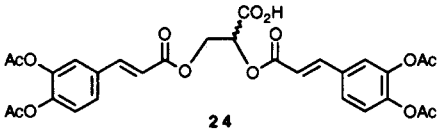
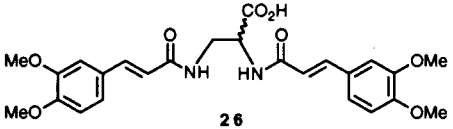
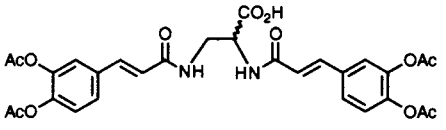
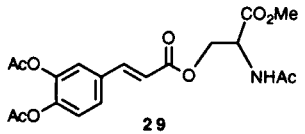
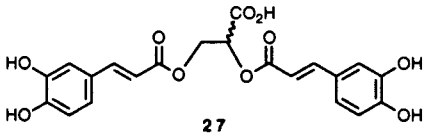
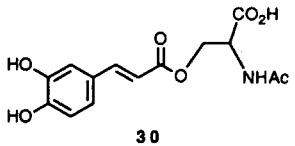
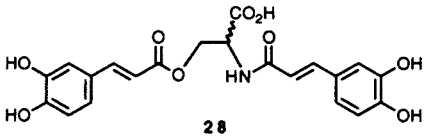
Derivatives of glyceric acid, serine and β-aminoalanine, were therefore prepared as monocarboxylic analogues of the parent chicoric acids. As shown in Table 2, removal of one carboxyl group (compound **27**, $IC_{50} = 2$ μM) resulted in almost no loss of potency. Furthermore, replacement of one caffeoyl ester linkage by an amide linkage (compound **28**, $IC_{50} = 2$ μM) provided equal potency. This latter transformation is significant in that compound **28** is now derived from an amino acid (serine). The tetraacetates were also equally potent regardless of whether the caffeoyl groups were attached to the central linker by means of ester functionality (compound **24**, $IC_{50} = 2$ μM) or amide groups (compound **25**, $IC_{50} = 4$ μM).

Comparison of data in Tables 1 and 2 shows that tetraacetates lacking central linker carboxyl groups (for example, compounds **10** and **12**) are inactive, while similar compounds containing a single central linker carboxyl group (compounds **24** and **25**) exhibit full integrase inhibitory potency. It was of interest to investigate whether anionic character of the carboxyl group was important for this inhibition. Therefore, methyl esters **18**−**22** were prepared. While examining the role of a single carboxyl group, these methyl esters additionally served to investigate: (1) the importance of ester versus amide caffeoyl functionality in attaching the caffeoyl groups to the central linker (compounds **18**, **19**, and **22**) and (2) the role of chirality at the α-carbon (compounds **20** and **21**). As shown in Table 2, methyl esters **18**−**21** inhibited the integrase with nearly equal potency (IC_{50} values of approximately 2−4 μM), with compound **22** being only slightly less potent ($IC_{50} = 10$ μM). These values are essentially unchanged from the parent free carboxylic acids (**27** and **28**).

Importance of Acetate Functionality. Catechol functionality has been critical for integrase activity throughout many classes of compounds. For caffeic acid-containing inhibitors, masking the catechol groups has usually resulted in a loss of inhibitory potency. In light of this, the ability of the current series of tetraacetylated chicoric acid analogues to maintain inhibitory potency was unusual. It therefore seemed possible that the inhibitory potency of the tetraacetates in the current series may be due to conversion of the acetates to the free catechols under the conditions of the enzyme assay. To examine this possibility, tetraacetate **25** was incubated under the same conditions as those employed for the integrase assay. Using HPLC analysis, it was observed that **25** was stable during the time course of the assay, indicating that chemical hydrolysis did not occur and that enzyme inhibition was due to the tetraacetate itself and not hydrolysis products.

It was also noted that, in previous studies, masking of catechol functionality was often achieved by converting the catechol hydroxyls to their methyl ethers. In

Table 2. Results of Radionucleotide-Based Integrase Assays

Structure	Integrase Assay[a] (IC_{50} μM) 3'-processing	Integration	Structure	Integrase Assay[a] (IC_{50} μM) 3'-processing	Integration
 **18**	10.1 (10.0)	10.1 (8.0)	 **21**	2.8 (3.0)	3.2 (3.5)
 **19**	2.5 +/- 0.6	3.2 +/- 1.0	 **22**	10.9 (12.3)	10.0 (12.3)
 **20**	2.8 +/- 0.8	3.8 +/- 1.4	 **23**	>1000	>1000
 **24**	2.1 +/- 0.3	2.8 +/- 0.5	 **26**	>1000	>1000
 **25**	4.1 (9.4)	4.1 (4.1)	 **29**	>333	>333
 **27**	4.2+/- 2.5	1.7 +/- 0.9	 **30**	333	111
 **28**	3.3 +/- 2.6	1.7 +/- 0.8			

[a] Determined by radiolabeled oligonucleotide-based assay as described in the Experimental Section. Values in parentheses are the results of multiple runs.

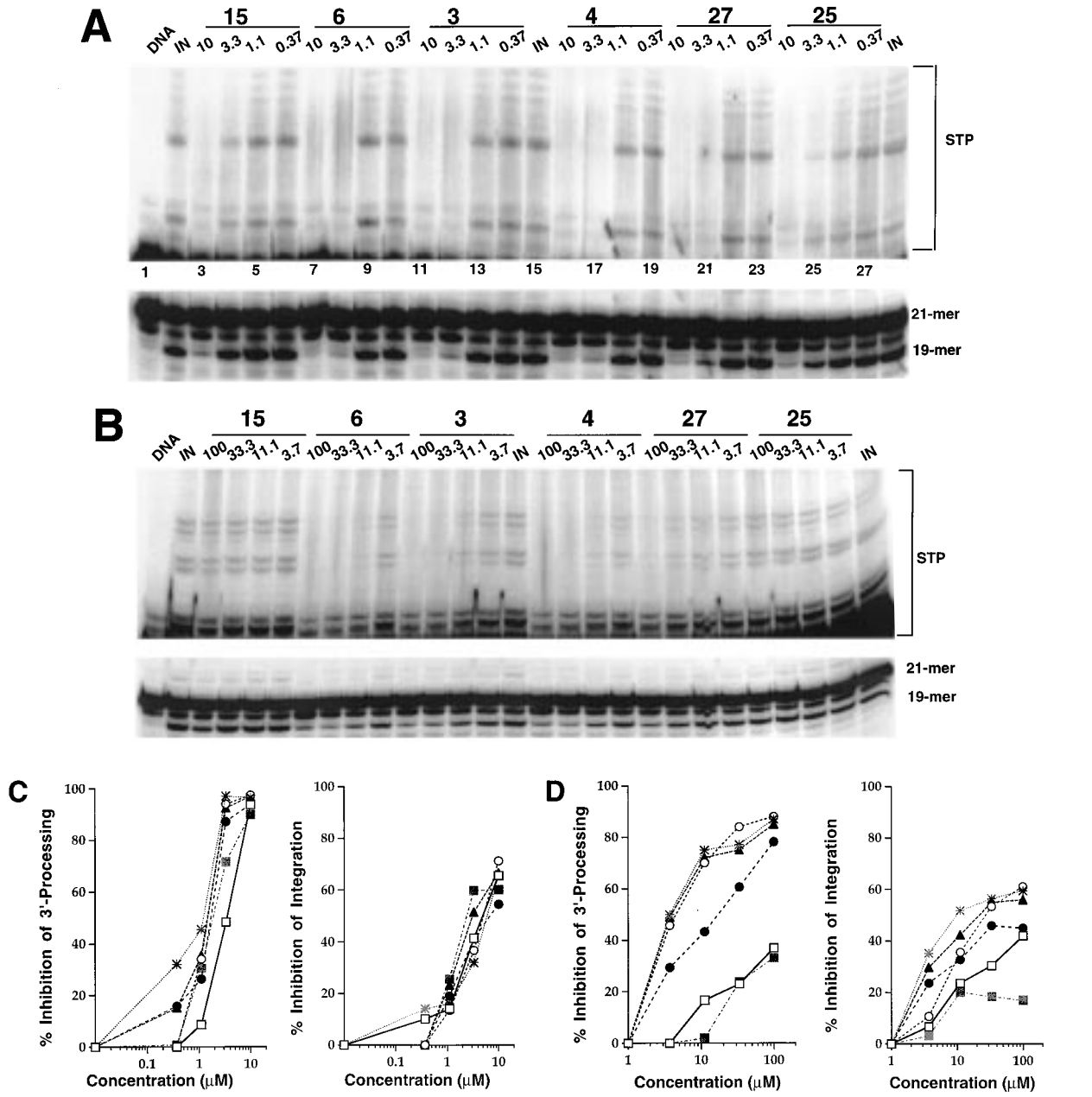


Figure 2. Concentration-dependent inhibition of HIV-1 integrase by chicoric acid analogues **3, 4, 6, 15, 25,** and **27** in the presence of Mn^{2+} (A) or Mg^{2+} (B). Inhibitor concentrations in μM are indicated above each lane. Electrophoresis in a 20% denaturing acrylamide gel showing 19-mer 3′-processing products, the substrate 21-mer oligo, and higher molecular weight strand transfer (integration) products. (C and D) Graphs showing the quantification of results presented in panels A and B, respectively. Inhibition was calculated after Phosphorimager quantitation: (○) **3**, (▲) **4**, (*) **6**, (□) **15**, (■) **25**, and (●) **27**.

most cases the resultant ethers showed significantly reduced integrase inhibitory potency relative to the parent catechols.[15,16] In the present series, the question therefore arose whether tetramethyl ethers would maintain high inhibitory potency similar to the tetraacetate esters. To examine this question, tetramethyl ethers **23** and **26** were prepared. As shown in Table 2, in contrast to the high inhibitory potency of the parent tetraacetates **22** and **25**, respectively, the tetramethyl ethers were inactive. These results indicate that functionality within the acetate esters themselves, not found in the methyl ethers, is critical for high inhibitory potency.

Importance of Two Caffeoyl Groups. Finally, it was of interest to show whether two caffeoyl groups were required for high inhibitory potency. To examine this question, monocaffeoyl analogues **30** and **29** were prepared as free catechol and as acetylated variants, respectively. The inactivity of both analogues against purified integrase (Table 2) indicates that two caffeoyl moieties are important for potency within the series. In summary, however, even though definite structural correlates to integrase inhibitory activity could be identified, the requirements for antiviral activity may be more stringent.

Effects of Divalent Metal Ions. To examine the role of divalent metals in the inhibition of HIV-1 integrase by the chicoric acids, we performed the assays in the presence of Mn^{2+} or Mg^{2+}. Six representative com-

Table 3. Relative Inhibition of Viral Infection in MAGI Cells

compd	concn (μM)	relative no. of infected cells/mL		
		assay 1	assay 2	assay 3
none		100	100	100
6	100	4	2	nd[a]
	10	73	50	42
	1	48	nd	97
	0.1	nd	nd	85
7	100	6	5	nd
	10	88	76	55
	1	83	nd	05
	0.1	nd	nd	91
4	100	11	4	nd
	10	85	78	63
	1	58	nd	114
	0.1	nd	nd	98

[a] nd, not done.

pounds, **3**, **4**, **6**, **15**, **25**, and **27**, containing none, one, or two carboxyl groups were examined. Reaction conditions were optimized for each divalent metal as described. The Mn^{2+} assays were performed in the presence of 7.5 mM manganese chloride. The Mg^{2+} assays were performed in the presence of 5% poly(ethylene glycol) and 7.5 mM magnesium chloride. A representative gel from these experiments is shown in Figure 2A,B. The effect of metals on the inhibition of integrase by these compounds is striking. First, when inhibition assays were run in the presence of Mn^{2+}, all compounds inhibited the enzyme within the same range of concentration (IC_{50} range 1−10 μM). Therefore, effects related to the central linker (number of carboxyl groups) and free or acetylated catechols were minimal in the presence of Mn^{2+}. However, when Mg^{2+} was substituted for Mn^{2+}, compounds **15** and **25** were inactive, while compound **27** exhibited 10-fold reduction in potency, suggesting that for maximal potency in the Mg^{2+} assays at least two carboxyl groups are required. Taken together, our data indicate that the chelation of divalent metals on the active site of integrase may be responsible for the differential potency of these compounds in the presence of different metals. It is also plausible that the integrase enzyme could adopt a different folding pattern in the presence of various metals. Studies are underway to elucidate the role of metals in the inhibition of integrase function.

Inhibition of HIV-1 Integrase in Cell-Based Assays. At present there is no in vivo integrase assay. However, assays could be utilized to examine at what stage inhibitors function. To further test the antiviral activity of compounds, MAGI cells[28] were infected with the NL4-3 isolate of HIV-1. Cells were incubated for 2 days in the presence or absence of tested compounds as detailed in Table 3, and the effects of compounds on viral integration were assessed by scoring the number of blue cells 2 days postinfection. Infections were carried out in duplicate; the average number of infected cells is shown in Table 3. As an additional control, azidothymidine (AZT) was included at 1 μM, which resulted in complete inhibition of viral infection (data not shown). Typically, NL4-3 titers in control infections without compound were approximately 3×10^4 infectious units/mL. The number of infected cells in the control infections without compound was set as an absolute value of 100, and inhibition of viral integration by the compounds was determined relative to the control. The

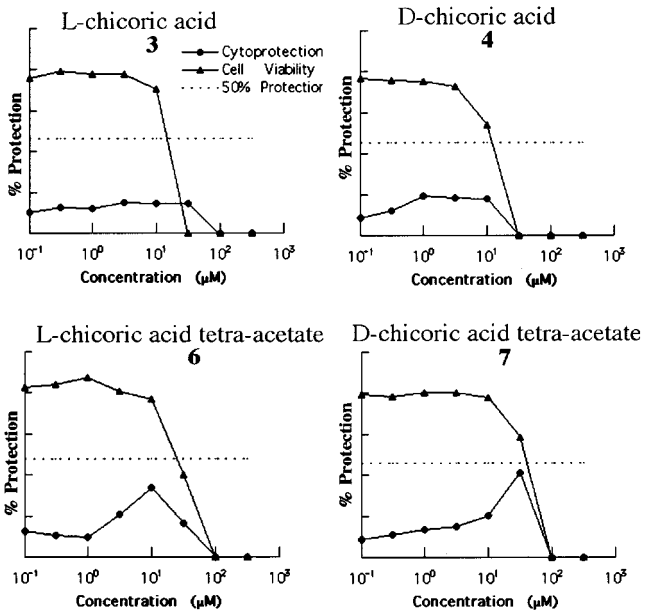


Figure 3. Results of antiviral activity of indicated compounds performed using CEM-SS cells and HIV-1_{RF} using the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]- 2*H*-tetrazolium hydroxide) cytoprotection assay as previously described.[35,36] Levels of cytoprotection are lower than those observed in the standard NCI XTT cytoprotection assay.

results of three independent assays are shown in Table 3. We observed that at 100 μM, **6**, **7**, or **4** inhibited viral infection greater than 90%, whereas when the compounds were added at a concentration of 10 μM, their inhibitory effect was only about 2-fold or less. Questions still remained as to whether these compounds are true integrase inhibitors in vivo. Studies in progress to address this particular issue will be reported elsewhere.

Interpretation of Cell-Based Data. A large number of the catechol-containing compounds were found to be toxic on CEM-SS cells and lacked significant antiviral activity in the XTT cytoprotection assay. Previous reports of L-chicoric acid as a potent antiviral agent in syncytia-based assays[19−23] prompted us to investigate these results more thoroughly. Compounds **3**, **4**, **6**, and **7** were independently assessed on three separate occasions for antiviral activity in the XTT cytoprotection assay using CEM-SS cells, virus stocks, and protocols utilized by the formal NCI screen.[27] The data presented in Figure 3 are representative of these determinations and show that all four compounds failed to achieve 50% protection. Compounds **6** and **7** did elicit slight cytoprotection, but dose-limiting toxicities prevented reaching an EC_{50} value for either compound. Even though consistent antiviral activity could not be identified, these compounds were further examined in target and cell-based assays to determine their mechanism of action. The four compounds were independently found to inhibit integrase function but failed to significantly inhibit reverse transcriptase activity or protease activity. In addition the compounds failed to inhibit viral replication in tumor necrosis factor-α (TNF-α)-induced latently infected U1 cells, indicating that any possible antiviral activity was not associated with a postintegration target.

Compounds **4** and **7** were reassessed for antiviral activity under more rigorous conditions to determine if

Table 4. Effects of Multiplicity of Infection on Cytoprotective Effects of **4** and **7**

	day of infection EC_{50} (μM)[a]		
virus dilution	3	6	9
	Compound **7**		
1:50	>50	>50	>50
1:100	>50	>50	>50
1:200	>50	22.6	>50
1:400	nd[b]	19.6	29.4
	Compound **4**		
1:50	>100	>100	>100
1:100	>100	82.8	>100
1:200	>100	32	39.2
1:400	nd	9.8	27

[a] 50% effective concentration or concentration of compound required to protect 50% of cells against retroviral cytopathic effects. [b] nd, not done.

Table 5. Effects of Order of Addition on Cytoprotection of **4** and **7**

	reciprocal of virus dilution (% of control)		
treatment	100	333	1000
D-chicoric acid (**4**)			
add at infection	141	73	60
pretreat only (100 μM)	108	59	168
pretreatment and add 10 μM	114	82	135
pretreat and add 1 μM	56	92	50
tetraacetylated D-chicoric acid (**7**)			
add at infection	141	81	60
pretreat only (100 μM)	120	163	51
pretreat and add 10 μM	149	39	38
pretreat and add 1 μM	100	57	17
AZT			
pretreat only (100 μM)	114	119	121
add at infection	35	3	5
DIBA-1			
pretreat only (100 μM)	31	4	6

our failure to demonstrate antiviral activity as previously reported[19] was an artifact of our detection system. The role of multiplicity of infection (MOI) in the efficacy of these compounds was determined first (Table 4). CEM-SS cells were pretreated for 30 min with various doses of compounds **4** and **7** after which the cells were infected with serial dilutions of HIV-1_{RF}. Cultures were continued in media supplemented with compound for 3, 6, and 9 days, after which supernatants were collected and p24 antigen content was measured by ELISA. The resulting values were corrected for input p24 antigen (infecting stock) and normalized to percent control (Table 4). Inhibition of HIV-1 replication by **4** and **7** was highly dependent on the MOI, with moderate antiviral activity associated with only the high-test virus dilutions at 6 days of infection. Additionally, extension to 9 days of culture revealed that the antiviral effects measured at 6 days were transient.

A requirement for preincubation of the virus and compound for optimal antiviral activity has recently been identified.[22] Therefore, a pooled stock HIV-1_{RF} was pretreated for 90 min at 37 °C with 100 μM of either compound **4** or **7**, after which excess compound was removed by centrifugation (18000*g*, 4 °C, 1 h) and cultures were continued with or without re-addition of 10 or 1 μM fresh compound (Table 5). To ensure optimal ratios of virus to target cells, the cells were infected with multiple dilutions of the treated virus. Supernatants were collected at 7 days of culture, and virus replication was assessed by p24 ELISA. Compound **4** at 100 μM had no appreciable effect on viral replication, while at very low MOIs (low input titers of virus) compound **7** at 100 μM reduced but did not completely inhibit virus replication. Pretreatment with 50 μM failed to elicit inhibitory activity with either analogue (data not shown). Pretreatment of virus with AZT failed to inhibit virus replication (cell-mediated metabolism is required to convert AZT into its active form, AZT-TP), while simultaneous addition with virus and cells and maintenance in cell culture resulted in >95% inhibition of virus replication at 1:333 and 1:1000 dilutions of virus. DIBA-1,[29] a directly viricidal NCp7 Zn finger reactive compound, also resulted in >95% inhibition of virus replication at 1:333 and 1:1000. Thus, although target-based assays have identified chicoric acid derivatives as potent inhibitors of purified HIV-1 integrase, demonstration of antiviral activity is highly dependent upon the MOI and order of drug addition.

Table 6. Anti-HIV Activity of Select Analogues

	EC_{50} (μM)[a]		
compound	HIV-1(III_a)	HIV-2(ROD)	IC_{50} (μM)[b]
L-chicoric acid (**3**)	5.3 ± 0.2	21.2	45.0 ± 1.8
tetraacetyl-L-chicoric acid (**6**)	4.1 ± 0.3	10.1	47.1 ± 1.1
D-chicoric acid (**4**)	1.7 ± 0.1	8.6 ± 3.2	39.7 ± 5.2
tetracetyl-D-chicoric acid (**7**)	3.9 ± 1.2	5.1 ± 1.9	60.0 ± 5.2
dextran sulfate[c]	0.4 ± 0.2	0.2 ± 0.1	>125
zidovudine	0.005 ± 0.002	0.005 ± 0.002	100 ± 25

[a] 50% effective concentration or concentration of compound required to protect 50% of cells against retroviral cytopathic effects. [b] 50% inhibitory concentration or concentration of compound required to reduce MT-4 cell viability by 50%. [c] Concentrations of dextran sulfate are expressed in μg/mL.

To address issues concerning antiviral activity, we extended our studies to include other viral strains and cell types. When L-chicoric acid, D-chicoric acid, and their tetraacetyl esters were tested against HIV-1(IIIB) replication, they were found to inhibit by 50% (EC_{50}) at concentrations ranging from 1.7 to 5.3 μM in MT-4 cells (Table 6). On the other hand, the compounds were shown to be toxic to the MT-4 cells with 50% inhibitory concentrations (IC_{50}) ranging from 39.7 to 60.0 μM, resulting in selectivity indices of approximately 10. HIV-2(ROD) was shown to be 2−4 times less sensitive to the inhibitory effects of these compounds in comparison to HIV-1(IIIB). The sulfated polysaccharide dextran sulfate and the reverse transcriptase inhibitor zidovudine (AZT) were tested in parallel (Table 6).

Conclusions

L-Chicoric acid (**3**) and the quinic acids have emerged as interesting classes of caffeoyl-containing catechols exhibiting potent HIV integrase inhibition. These analogues fall within the general category of inhibitors categorized by two aryl groups separated via a central linker, with at least one of the aryl groups being a catechol. While there are a large number of inhibitors within this general class, the multiple reports of anti-HIV activity of L-chicoric acid and quinic acids in cell-based assays have placed them apart from other catechols, which frequently exhibit limiting cytotoxicity. The present study was undertaken to examine features of L-chicoric acid that contribute to both its integrase

enzyme inhibition and its cell-based activity. At the outset of this work, structural features of L-chicoric acid potentially important for biological efficacy included (1) chirality, only the L-enantiomer had been examined for inhibitory potency; (2) carboxylic acids, the presence of two carboxylic acids on a central linker; (3) ester linkages, the attachment of two caffeoyl groups by ester bonds; (4) catechols; the presence of two catechol rings. Through a progressive series of analogues, we have shown against purified integrase that enantiomeric D-chicoric acid (**4**) retains integrase inhibitory potency equal to its L-counterpart and further that removal of either one (compound **27**) or both (compound **15**) carboxylic functionalities results in essentially no loss of integrase inhibitory potency. Additionally, while two caffeoyl moieties are required, (monocaffeoyl analogues **29** and **30** are inactive), attachment of caffeoyl groups to the central linking structure can be achieved via amide or mixed amide/ester linkages. More remarkable is the finding that blockade of the catechol functionality through conversion to tetraacetate esters results in almost no loss of potency, contingent on the presence of at least one carboxyl group on the central linker. This single carboxyl group can be either the free acid or a methyl ester. Taken as a whole, the work has resulted in the identification of new integrase inhibitors typified by **19**−**22**, **25**, and **28**, which may be regarded as biscaffeoyl derivatives of glycidic acid and amino acids such as serine and β-aminoalanine.

While such analysis of chicoric acid-type compounds has identified specific structure−function relationships between these molecules and their ability to inhibit purified integrase, examination of their antiviral activity revealed modest inhibitory activity under highly specific conditions. The XTT cytoprotection assay is a well-established standardized procedure for determining antiviral activity of unknown compounds. The failure of an unknown to mediate antiviral activity may be due to a number of factors. For the purpose of discussion these factors can be divided into intrinsic (assay-determined) and extrinsic (assay-independent) assay factors. In the XTT cytoprotection assay, the most common extrinsic assay factor results from interaction of the XTT dye with the compound under investigation, resulting in quenching or abnormally high optical densities. Chicoric acid derivative concentrations above 200 μM were found to interfere with the XTT cytoprotection assay by depressing the observed optical absorbances. Even though interference occurred at analogue concentrations of approximately 10 times the observed experimental IC_{50} (concentration resulting in 50% cell death), these results led to analysis of subsequent assays by ELISA determination of p24 antigen expression. Measurement of p24 antigen was slightly more sensitive than the XTT assay but resulted in the same general outcome for the antiviral assays. Compounds may fail in the XTT cytoprotection assay due to intrinsic assay factors such as the multiplicity of infection (MOI, ratio of virus particles to target cell) and the timing of compound addition in relation to the infection event. These two parameters were investigated in detail (Tables 4 and 5). Antiviral activity was found to be MOI-dependent requiring preincubation of the virus and chicoric acid analogue followed by subsequent culture in compound-containing media to elicit maximal antiviral activity. Even with optimal MOIs and continuous exposure to the chicoric acid analogues, antiviral activity was far less potent than that mediated by either AZT or DIBA-1. Furthermore under optimized conditions inhibition was transient. Thus, the chicoric acid-mediated inhibition of HIV-1 replication is far less robust and requires extensive in vitro manipulation to elicit antiviral activity that is inferior to known and well-characterized antivirals targeting reverse transcriptase and the NCp7 Zn finger. The fact that better antiviral activities were observed with HIV-1(IIIB) and HIV-2(ROD) in MT-4 cells will be the object of future studies directed at elucidating mechanism(s) by which these compounds exert their antiviral effects.

Experimental Section

Preparation of Oligonucleotide Substrates. High-performance liquid chromatography-purified oligonucleotides AE117 (5′-ACTGCTAGAGATTTTCCACAC-3′) and AE118 (5′-GTGTGGAAAATCTCTAGCAGT-3′) were purchased from Midland Certified Reagent Co. (Midland, TX). The expression vector for the wild-type integrase was a generous gift of T. Jenkins and R. Craigie, Laboratory of Molecular Biology, NIDDKD, NIH, Bethesda, MD. To analyze the extent of 3′-processing and strand transfer using 5′-end-labeled substrates, AE118 was 5′-end-labeled with T_4 polynucleotide kinase (Gibco BRL, Gaithersberg, MD) and [γ-^{32}P]ATP (DuPont-NEN). The kinase was heat-inactivated, and AE117 was added to the same final concentration. The mixture was heated at 95 °C, allowed to cool slowly to room temperature, and run on a G-25 Sephadex quick spin column (Boehringer Mannheim, Indianapolis, IN) to separate annealed double-stranded oligonucleotide from unincorporated label.

Integrase Assays. The detailed in vitro assays used to study integrase inhibitors were recently described elsewhere.[30] In brief, integrase was preincubated at a final concentration of 200 nM with the inhibitor in reaction buffer (50 mM NaCl, 1 mM HEPES (pH 7.5), 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (w/v), 7.5 mM $MnCl_2$, 0.1 mg of bovine serum albumin/mL, 10 mM 2-mercaptoethanol, 10% dimethyl sulfoxide (DMSO), and 25 mM MOPS (morpholinepropanesulfonic acid) (pH 7.2)) at 30 °C for 30 min. The 5′-end ^{32}P-labeled** linear oligonucleotide substrate (20 nM) was then added, and incubation was continued for an additional 1 h. Reactions were quenched by the addition of an equal volume (16 μL) of loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue). An aliquot (5 μL) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M Tris-borate (pH 8.3), 2 mM EDTA, 20% acrylamide, 8 M urea). Gels were dried, exposed in a PhosphorImager cassette, and analyzed with a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Percent inhibition was calculated using the following equation: % inhibition = $100 \times [1 - (D - C)/(N - C)]$, where C, N, and D are the fractions of 21-mer substrate converted to 19-mer (3′-processing product) or strand transfer products for DNA alone, DNA plus integrase, and integrase plus drug, respectively. The 50% inhibitory concentrations (IC_{50}) were determined by plotting the log of drug concentration versus percent inhibition and identifying the concentration which produced an inhibition of 50%.

XTT Cytoprotection Assay. Testing performed at the NCI AIDS Drug Screening Laboratory (results shown in Table 1) is based on a protocol described by Weislow et al.[27] In brief, all compounds were dissolved in DMSO and diluted 1:100 in cell culture medium. Exponentially growing T4 lymphocytes (CEM-SS cell line) were added at 5000 cells/well. Frozen virus stock solutions were thawed immediately before use, suspended in complete medium to yield the desired multiplicity of infection, and added to the microtiter wells, resulting in a 1:200 final dilution of the compound. Uninfected cells with the

compound served as a toxicity control, and infected and uninfected cells without the compound served as basic controls. Cultures were incubated at 37 °C in a 5% CO_2 atmosphere for 6 days. The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT) was added to all wells, and cultures were incubated to allow formazan color development by viable cells. Individual wells were analyzed spectrophotometrically to quantitate formazan production and in addition were directly viewed microscopically for detection of cytotoxic effects and confirmation of protective activity.

Mechanistic and Target-Based Assays. All positive control compounds for individual assays except AZTTP were obtained from the NCI chemical repository. The reference reagents for the individual assays are as follows: attachment, Farmatalia (NSC 65016)[31] and dextran sulfate (NSC 620255); reverse transcriptase inhibition, rAdT template/primer-AZTTP (Sierra BioResearch, Tuscon, AZ) and rCdG template/primer-UC38[32] (NSC 629243); protease inhibition, KNI-272[33] (NSC 651714); integrase inhibition, ISIS 5320[34] (NSC 665353) and DIBA-1[29] (NSC 654077), a NCp7 Zn finger inhibitor.

Antiviral Assay. Antiviral Assays Performed Independently of the AIDS Drug Screen. Antiviral activity was performed independently of the NCI AIDS Drug Screen with CEM-SS cells and HIV-1_{RF} (MOI = 0.1) (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, Bethesda, MD) using the XTT cytoprotection assay as previously described.[34,35] Effective antiviral concentrations providing 50% cytoprotection (EC_{50}) and cellular growth inhibitory concentrations causing 50% cytotoxicity (IC_{50}) were calculated. 3′-Azido-2′,3′-dideoxythymidine (AZT) and dextran sulfate were utilized as reference compounds for antiviral activity.

Experiments designed to determined MOI sensitivity were carried out in the following manner. CEM-SS cells were preincubated for 15−30 min with various concentration of test compounds. Serial dilutions of HIV-1_{RF} were added, and virus adsorption was carried out for 2 h. After 2 h media containing unabsorbed virus was removed, and cultures were continued with the appropriate concentrations of compounds in media consisting of RPMI 1640, 10% FCS, and 20 mg/mL gentamicin with 200 mM L-glutamate. At days 3, 6, and 9 of incubation sister plates were scored for syncytia formation and 100 μL of cell-free supernatant was collected for measurement of HIV-1 p24 by ELISA (AIDS Vaccine Program, NCI−FCRDC, Frederick, MD). Cell viability was determined by XTT dye reduction. All calculations were corrected for residual p24 from the infection protocol. Results are shown in Table 4.

Experiments designed to determine whether pretreatment of HIV-1 with selected chicoric acid analogues enhanced antiviral activity were carried out as follows. Briefly, 1 mL of HIV-1_{RF} stock was preincubated with 100 μM chicoric acid analogue, DIBA-1 (a virucidal nucleocapsid zinc finger inhibitor), or 10 μM AZT (reverse transcriptase inhibitor) for 90 min at 37 °C. Following incubation, compound was removed by centrifugation (18000*g*, 1 h at 4 °C) and the virus pellet resuspended in RPMI 1640, 10% FCS, and 20 μg/mL gentamicin−200 mM L-glutamate with or without supplemental compound. Virus was serially diluted onto CEM-SS cells (5000 cells/well) and adsorbed for 2 h, and cultures were continued for 6 days with or without supplemental compound. Cell-free supernatants were collected for measurement of HIV-1 p24 by ELISA, and cell viability was determined by the XTT dye reduction method. All calculations were corrected for residual p24 from the infection protocol. Results are shown in Table 5.

Antiviral Assay. Cell viability of MT-4 cells[36] infected at 200 times the $CCID_{50}$ (50% cell culture infective dose) of HIV-1(IIIB)[37] and HIV-2(ROD)[38] was determined by a tetrazolium colorimetric method (MTT), as described previously.[39] This method was used to determine the 50% effective concentrations and 50% inhibitory concentrations (EC_{50} and IC_{50}, respectively) of the test compounds.

HIV-1 Cell and Target-Based Assays. Latently infected tumor necrosis factor-α (TNF-α)-inducible U1 cells were used to determine the effects of the compounds on late-phase virus replication as previously described.[40] Briefly, U1 cells (5×10^4 cells/0.2 cm well) were simultaneously treated with 5 ng/mL TNF-α and test compound. Cultures were continued for 48 h after which cell viability was determined by XTT dye reduction and cell-free supernatants were collected for determination of p24 by the ELISA method.

Assays for activity against HIV-1 reverse transcriptase rAdT (template/primer) and rCdG (template/primer) using recombinant HIV-1 reverse transcriptase (a kind gift from S. Hughes, ABL Basic Research, NCI−FCRDC, Frederick, MD) have been previously described.[41] The substrate cleavage of recombinant HIV-1 protease in the presence of test compounds was quantified using an HPLC-based methodology with the artificial substrate Ala-Ser-Glu-Asn-Try-Pro-Ile-Val-amide (Multiple Peptide Systems, San Diego, CA) as previously described.[29,42]

Inhibition of Viral Integration Measured by MAGI Assay. Infection of the HeLa CD4 LTR-β-gal cell line (MAGI cells)[28] with HIV-1 molecular clone pNL4-3[43] was performed as previously described.[28] Virus stocks were obtained by transfecting HeLa cells with HIV-1 molecular clone pNL4-3. Two days posttransection, virus supernatants were harvested, filtered, and assayed for reverse transcriptase activity.[44] MAGI cells were infected with 8×10^4 RT units of NL4-3, 2 days postinfection the cells were fixed and stained for expression of β-galactosidase with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and the number of blue cells was scored under the light microscope as described.[28] Briefly, each MAGI cell carries an integrated β-galactosidase gene under the control of a truncated HIV-LTR. Therefore expression of the enzyme is dependent on the synthesis of HIV-1 Tat protein by integrated proviruses, after infection. Thus, changes in the number of infected cells, as visualized by virtue of their blue color after staining with X-Gal, can be used as a readout to assess the potential effects of drugs on viral integration. Compounds were added to the cells 4 h before infection and remained present during infection and the 2-day incubation period. Each compound was tested at 100, 10, 1, or 0.1 μM, and all infections were performed in duplicate. The number of blue cells in the control infections without compound was set as 100, and inhibition of viral integration by the compounds was determined.

Synthetic. Melting points were determined on either a Gallenkamp or a Mel Temp II melting point apparatus and are uncorrected. Elemental analyses were obtained from Atlantic Microlab Inc., Norcross, GA, and are within 0.4% of the theoretical values unless otherwise indicated. Fast atom bombardment mass spectra (FABMS) were acquired with a VG Analytical 7070E mass spectrometer under the control of a VG 2035 data system. ^{1}H NMR data were obtained on a Bruker AC250 spectrometer (250 MHz) and are reported in ppm relative to TMS and referenced to the solvent in which they were run. Anhydrous solvents were obtained commercially and used without further drying. Flash column chromatography was performed using E. Merck silica gel 60 (particle size, 230−400 mesh). HPLC was done on a Waters PrepLC 4000 system using Vydac C18 peptide/protein column. Optical rotation was taken on a Perkin-Elmer 241 digital polarimeter at ambient temperature.

General Method for the Preparation of Compounds 8−12. To a solution of commercially available diol or ethanolamine (1 mmol) in anhydrous pyridine (3 mL) was added dropwise under argon a solution of freshly prepared 3,4-diacetylcaffeoyl acid chloride[25] (**5**) (2.5 mmol) in toluene (5 mL). The mixture was stirred at room temperature (overnight); then pyridine was removed under reduced pressure. The residue was taken to dryness from toluene (three times), then purified by silica gel chromatography, and crystallized to provide the desired product.

(+)-(2S,3S)-*O*,*O*-Bis(3,4-diacetoxycinnamoyl)-2,3-butanediol (8). Reaction of (2*S*,3*S*)-butanediol as described above provided **8** as a foam (92% yield): $[\alpha]^{20}_D$ +54° (MeOH, $c = 0.14$); ^{1}H NMR ($CDCl_3$) δ 7.6 (d, $J = 16.0$ Hz, 2H), 7.39−7.35 (m, 4H), 7.19 (d, $J = 8.2$ Hz, 2H), 6.37 (d, $J = 16.0$ Hz,

2H), 5.14 (dq, J = 6.0, 3.8 Hz, 2H), 2.28 (s, 3H), 2.27 (s, 3H), 1.3 (d, J = 6.0 Hz, 6H). Anal. ($C_{30}H_{30}O_{12}\cdot H_2O$) C, H.

(+)-(1*S*,2*S*)-*O*,*O*-Bis(3,4-diacetoxycinnamoyl)-1,2-cyclohexanediol (9). Reaction of (1*S*,2*S*)-cyclohexanediol as described above provided **9** as a solid (91% yield): mp 62−64 °C (EtOAc−hexane); $[\alpha]^{20}_D$ + 220° (MeOH, c = 0.13); ^{1}H NMR ($CDCl_3$) δ 7.55 (d, J = 16.0 Hz, 2H), 7.36−7.3 (m, 4H), 7.17 (d, J = 8.3 Hz, 2H), 6.3 (d, J = 16.0 Hz, 2H), 5.0−4.97 (m, 2H), 2.27 (s, 3H), 2.26 (s, 3H), 2.16−2.11 (m, 2H), 1.78 (m, 2H), 1.47−1.4 (m, 4H). Anal. ($C_{32}H_{32}O_{12}\cdot H_2O$) C, H.

***O*,*O*-Bis(3,4-diacetoxycinnamoyl)-1,2-ethanediol (10).** Reaction of 1,2-ethanediol as described above provided **10** as a solid (89% yield): mp 153−156 °C (EtOAc−hexane); ^{1}H NMR (DMSO-d_6) δ 7.72−7.64 (m, 6H), 7.31 (d, J = 8.4 Hz, 2H), 6.7 (d, J = 16.1 Hz, 1H), 4.43 (s, 4H). Anal. ($C_{28}H_{26}O_{12}$) C, H.

***O*,*O*-Bis(3,4-diacetoxycinnamoyl)-1,3-propanediol (11).** Reaction of 1,3-propanediol as described above provided **11** as a solid (91% yield): mp 194.5−196 °C (EtOAc−hexane); ^{1}H NMR (DMSO-d_6) δ 7.7−7.62 (m, 6H), 6.09 (d, J = 8.4 Hz, 2H), 6.66 (d, J = 16.1 Hz, 1H), 4.47 (t, J = 6.2 Hz, 4H), 2.283 (s, 6H), 2.278 (s, 6H), 2.04 (quintuplet, J = 6.3 Hz, 2H). Anal. ($C_{29}H_{28}O_{12}\cdot H_2O$) C, H.

***N*,*O*-Bis(3,4-diacetoxycinnamoyl)-2-hydroxyethylamine (12)**. Reaction of 2-hydroxyethylamine as described above provided **12** as a solid (96% yield): mp 177−179.5 °C (EtOAc−hexane); ^{1}H NMR ($CDCl_3$) δ 7.66 (d, J = 16.1 Hz, 1H), 7.57 (d, J = 15.6 Hz, 1H), 7.42−7.32 (m, 4H), 7.21 (d, J = 8.5 Hz, 1H), 7.18 (d, J = 8.4 Hz, 1H), 6.38 (d, J = 16.0 Hz, 1H), 6.34 (d, J = 15.4 Hz, 1H), 6.00 (t, J = 5.3 Hz, 1H), 4.36 (t, J = 5.1 Hz, 2H), 3.71 (dt, J = 5.1, 5.3 Hz, 2H), 2.29 (s, 3H), 2.28 (s, 3H), 2.280 (s, 3H), 2.276 (s, 3H). Anal. ($C_{28}H_{21}NO_{11}$) C, H, N.

General Method for Preparation of Compounds 13−17. Diesters **8**−**11** or amide ester **12** (0.5 mmol) in acetone (15 mL) were refluxed (3 h) with 3 N HCl (5 mL), then cooled to room temperature, diluted with EtOAc (100 mL), and washed with brine. After drying (Na_2SO_4) samples were concentrated and purified by either crystallization or HPLC to give the desired products.

(+)-(2*S*,3*S*)-*O*,*O*-Bis(3,4-dihydroxycinnamoyl)-2,3-butanediol (13). Treatment of diester **8** as described above and purification by HPLC (linear gradient, MeOH in H_2O from 0% to 100% MeOH over 30 min) provided **13** as a white solid (73% yield): $[\alpha]^{20}_D$ +98° (MeOH, c = 0.13); ^{1}H NMR (DMSO-d_6) δ 7.47 (d, J = 16.0 Hz, 2H), 7.03−6.96 (m, 4H), 6.73 (d, J = 8.1 Hz, 2H), 6.24 (d, J = 15.9 Hz, 2H), 5.05 (dq, J = 6.0, 3.8 Hz, 2H), 1.21 (d, J = 6.0 Hz, 6H); FABMS m/z 413 (M − H). Anal. ($C_{22}H_{22}O_8\cdot H_2O$) C, H.

(+)-(1*S*,2*S*)-*O*,*O*-Bis(3,4-dihydroxycinnamoyl)-1,2-cyclohexanediol (14). Treatment of diester **9** as described above provided **14** as a solid (88% yield): mp 216−218 °C (EtOAc−$CHCl_3$); $[\alpha]^{20}_D$ +373° (MeOH, c = 0.18); ^{1}H NMR (DMSO-d_6) δ 7.42 (d, J = 15.9 Hz, 2H), 6.98−6.92 (m, 4H), 6.7 (d, J = 8.1 Hz, 2H), 6.16 (d, J = 15.9 Hz, 2H), 4.9 (m, 2H), 1.98 (m, 2H), 1.68 (m, 2H), 1.4 (m, 4H); FABMS m/z 439 (M − H). Anal. ($C_{24}H_{24}O_8\cdot 1^1/_4\ H_2O$) C, H.

***O*,*O*-Bis(3,4-dihydroxycinnamoyl)-1,2-ethanediol (15).** Treatment of diester **10** as described above provided **15** as a solid (89% yield): mp > 330 °C (MeOH−$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 9.61 (s, 2H), 9.13 (s, 2H), 7.5 (d, J = 15.9 Hz, 2H), 7.04−6.99 (m, 4H), 6.75 (d, J = 8.1 Hz, 2H), 6.29 (d, J = 15.9 Hz, 1H), 4.37 (s, 4H); FABMS m/z 385 (M − H). Anal. ($C_{20}H_{18}O_8\cdot H_2O$) C, H.

***O*,*O*-Bis(3,4-dihydroxycinnamoyl)-1,3-propanediol (16).** Treatment of diester **11** as described above provided **16** as a solid (83% yield): mp 205−207 °C (MeOH−$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 7.48 (d, J = 5.9 Hz, 2H), 7.03 (d, J = 1.8 Hz, 2H), 6.97 (dd, J = 8.2, 1.8 Hz, 2H), 6.68 (d, J = 8.1 Hz, 2H), 6.24 (d, J = 15.9 Hz, 2H), 4.21 (t, J = 6.2 Hz, 4H), 2.0 (t, J = 6.1 Hz, 2H); FABMS m/z 399 (M − H). Anal. ($C_{21}H_{20}O_8\cdot H_2O$) C, H.

***N*,*O*-Bis(3,4-dihydroxycinnamoyl)-2-hydroxyethylamine (17).** Treatment of diester **12** as described above provided **17** as a solid (88% yield): mp 214−215.5 °C (MeOH−$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 9.59 (s, 1H), 9.35 (s, 1H), 9.14 (s, 1H), 9.11 (s, 1H), 8.17 (t, J = 5.4 Hz, 1H), 7.5 (d, J = 15.9 Hz, 1H), 7.25 (d, J = 15.7 Hz, 1H), 7.04−6.93 (m, 2H), 6.85−6.71 (m, 2H), 6.34 (d, J = 15.7 Hz, 1H), 6.25 (d, J = 15.5 Hz, 1H), 4.16 (t, J = 5.4 Hz, 2H), 3.45 (m, 2H); FABMS m/z 384 (M − H). Anal. ($C_{20}H_{19}NO_7\cdot 0.5\ H_2O$) C, H, N.

Methyl *O*,*O*-Bis(3,4-diacetoxycinnamoyl)-2,3-dihydroxypropanoate (18). To a solution of 2,3-dihydroxypropanoic acid methyl ester (5.0 mmol) in anhydrous pyridine (20 mL) was added a solution of 3,4-diacetylcaffeoyl acid chloride (**5**) (12.5 mmol) in toluene (30 mL), and the resulting cloudy solution was stirred at room temperature overnight. Solvent was removed by rotary evaporator and residue purified by flash chromatography (ether:$CHCl_3$), to afford **18** as a pale-white solid (2.70 g, 88% yield): mp 159−160 °C; ^{1}H NMR (DMSO-d_6) δ 7.80 (d, J = 16.1 Hz, 1H), 7.73 (d, J = 16.2 Hz, 1H), 7.30−7.48 (m, 6H), 6.57 (d, J = 16.1 Hz, 1H), 6.48 (d, J = 16.1 Hz, 1H), 5.60 (t, J = 4.9 Hz, 1H), 4.74 (m, 2H), 3.91 (s, 3H), 2.39 (s, 12H); FABMS m/z 613 (M + H). Anal. ($C_{30}H_{28}O_{14}$) C, H.

Methyl *N*,*O*-Bis(3,4-diacetoxycinnamoyl)serinate (19). Using racemic methyl serinate hydrochloride in a procedure similar to that described above for the synthesis of **18**, product **19** was obtained as a white solid (84% yield): mp 105.5−107 °C; ^{1}H NMR ($CDCl_3$) δ 7.70 (d, J = 16.0 Hz, 1H), 7.67 (d, J = 15.6 Hz, 1H), 7.29−7.40 (m, 6H), 6.55 (d, J = 8.0 Hz, 1H), 6.50 (d, J = 15.6 Hz, 1H), 6.44 (d, J = 16.1 Hz, 1H), 5.11 (m, 1H), 4.69 (q, J = 3.4 Hz, 2H), 3.90 (s, 3H), 2.38 (s, 12H); FABMS m/z 612 (M + H). Anal. ($C_{30}H_{29}NO_{13}$) C, H, N.

Methyl *N*,*O*-Bis(3,4-diacetoxycinnamoyl)-L-serinate (20). Using methyl L-serinate hydrochloride in a procedure similar to that described above for the synthesis of **18**, product **20** was obtained as a white powder (76% yield). NMR spectral data was the same as that reported above for **19**. **20**: $[\alpha]^{20}_D$ +105.5° ($CHCl_3$, c = 0.56). Anal. ($C_{30}H_{29}NO_{13}\cdot 0.5H_2O$) C, H, N.

Methyl *N*,*O*-Bis(3,4-diacetoxycinnamoyl)-D-serinate (21). Using methyl D-serinate hydrochloride in a procedure similar to that described above for the synthesis of **18**, product **21** was obtained as a white powder (81% yield). NMR spectral data was the same as that reported above for **19**. **21**: $[\alpha]^{20}_D$ −108.6° ($CHCl_3$, c = 0.63). Anal. ($C_{30}H_{29}NO_{13}\cdot 0.5H_2O$) C, H, N.

Methyl *N*,*N*-Bis(3,4-diacetoxycinnamoyl)-2,3-diaminopropanoate (22). To a mixture of *N*-methylmorpholine (52.1 mmol), methyl 2,3-diaminopropanoate dihydrochloride (5.23 mmol), 3,4-diacetoxycinnamic acid (10.5 mmol), and 1-hydroxybenzotriazole hydrate (10.5 mmol) in CH_2Cl_2 (30 mL) was added diisopropylcarbodiimide (13.60 mmol). The resulting solution was stirred at room temperature (overnight), then partitioned between CH_2Cl_2 (~200 mL) and H_2O (~70 mL), washed with brine, and dried (Na_2SO_4). Solvent was removed by rotary evaporator and residue purified by flash column chromatography ($CHCl_3$ and $CHCl_3$:EtOAc) to afford **22** as a white crystalline solid (1.40 g, 44% yield): mp 151−152 °C; ^{1}H NMR (DMSO-d_6) δ 7.65 (d, J = 15.8 Hz, 1H), 7.64 (d, J = 15.6 Hz, 1H), 7.26−7.34 (m, 6H), 6.54 (br s, 2H), 6.51 (d, J = 15.8 Hz, 1H), 6.41 (d, J = 15.6 Hz, 1H), 4.85 (q, J = 4.6 Hz, 1H), 3.92 (d, J = 4.6 Hz, 2H), 3.89 (s, 3H), 2.39 (s, 6H), 2.38 (s, 6H); FABMS m/z 611 (M + H). Anal. ($C_{30}H_{30}N_2O_{12}$) C, H, N.

Methyl *N*,*N*-Bis(3,4-methoxycinnamoyl)-2,3-diaminopropanoate (23). Using 3,4-dimethoxycinnamic acid in a procedure similar to that described above for the synthesis of **22**, product **23** was obtained as a pale solid (67% yield): mp 125−126 °C; ^{1}H NMR (DMSO-d_6) δ 7.65 (d, J = 15.6 Hz, 1H), 7.64 (d, J = 15.5 Hz, 1H), 7.23 (d, J = 6.8 Hz, 1H), 6.90−7.13 (m, 6H), 6.62 (t, J = 6.7 Hz, 1H), 6.45 (d, J = 15.6 Hz, 1H), 6.37 (d, J = 15.5 Hz, 1H), 4.90 (q, J = 6.6 Hz, 1H), 3.98 (s, 12H), 3.92 (m, 2H), 3.88 (s, 3H); FABMS m/z 499 (M + H). Anal. ($C_{26}H_{30}N_2O_8$) C, H, N.

General Procedure for Demethylation of Compounds 18, 22, and 23 To Provide Products 24−26, Respectively. A suspension of methyl ester (0.4 mmol) and lithium iodide (3.2 mmol) in pyridine (3 mL) was stirred at reflux (1 h). Pyridine was removed, and the residue was taken up in H_2O (30 mL), acidified with 6 N HCl (to pH 3), extracted with

EtOAc (2 × 50 mL), washed with brine, and dried (Na_2SO_4). Removal of solvent provided products as solids.

***O,O*-Bis(3,4-diacetoxycinnamoyl)-2,3-dihydroxypropanoic Acid (24).** Treatment of methyl ester **18** as described above in the general procedure provided **24** as a white solid (63% yield): mp 130 °C dec; ^{1}H NMR (DMSO-d_6) δ 13.50 (br s, 1H), 7.88 (m, 2H), 7.38−7.80 (m, 6H), 6.81 (d, J = 16.4 Hz, 1H), 6.74 (d, J = 16.1 Hz, 1H), 5.48 (t, J = 3.5 Hz, 1H), 4.65 (d, J = 3.5 Hz, 2H), 2.34 (br s, 12H); FABMS m/z 597 (M − H). Anal. ($C_{29}H_{26}O_{14}$·2.5H_2O) C, H.

***N,N*-Bis(3,4-diacetoxycinnamoyl)-2,3-diaminopropanoic Acid (25).** Treatment of methyl ester **22** as described above in the general procedure provided **25** as a white solid (67% yield): mp 120 °C dec; ^{1}H NMR (DMSO-d_6) δ 12.90 (br s, 1 H), 10.15 (br d, 1H), 9.94 (br d, 1H), 8.47 (m, 2H), 7.06−7.54 (m, 6H), 6.69 (m, 2H), 4.55 (m, 1H), 3.70 (br s, 2H), 2.34 (s, 12H); FABMS m/z 595 (M − H). Anal. ($C_{29}H_{28}N_2O_{12}$·0.4C_5H_5N) C, H, N.

***N,N*-Bis(3,4-dimethoxycinnamoyl)-2,3-diaminopropanoic Acid (26).** Treatment of methyl ester **23** as described above in the general procedure provided **26** (84% yield): mp 108 °C dec; ^{1}H NMR (DMSO-d_6) δ 12.90 (br s, 1H), 8.30 (d, J = 7.8 Hz, 1H), 8.16 (t, J = 5.9 Hz, 1H), 7.42 (d, J = 15.9 Hz, 1H), 7.41 (d, J = 15.6 Hz, 1H), 7.02−7.19 (m, 6H), 6.66 (d, J = 15.9 Hz, 1H), 6.58 (d, J = 15.6 Hz, 1H), 4.52 (m, 1H), 3.84 (s, 3H), 3.83 (s, 6H), 3.82 (s, 3H), 3.52−3.68 (m, 2H); FABMS m/z 483 (M − H). Anal. ($C_{25}H_{28}N_2O_8$) C, H, N.

***O,O*-Bis(3,4-dihydroxycinnamoyl)-2,3-dihydroxypropanoic Acid (27).** A suspension of **18** in a mixture of 3 N HCl: acetone (v/v 1:3) was refluxed (3 h), then cooled to room temperature, diluted with an equal volume of H_2O, and extracted with EtOAc. Combined organic extracts were dried (Na_2SO_4) and taken to dryness, and residue was purified by silica gel flash chromatography (EtOAc/AcOH) to provide **27** as a white solid (30% yield): mp 85−90 °C; ^{1}H NMR (DMSO-d_6) δ 11.15 (br s, 1H), 8.45 (br s, 4H), 7.69 (d, J = 15.9 Hz, 1H), 7.64 (d, J = 15.9 Hz, 1H), 6.92−7.24 (m, 6H), 6.40 (m, 2H), 5.56 (dd, J = 3.2, 8.6 Hz, 1H), 4.72 (m, 2H); FABMS m/z 429 (M − H). Anal. ($C_{21}H_{18}O_{10}$·0.6H_2O) C, H.

***N,O*-Bis(3,4-dihydroxycinnamoyl)serine (28).** Treatment of **19** as described above for the conversion of **18** to **27** provided **28** as a white solid (71% yield): mp 112 °C dec; ^{1}H NMR (DMSO-d_6) δ 13.05 (br s, 1H), 9.67 (s, 1H), 9.44 (s, 1H), 9.21 (s, 1H), 9.19 (s, 1H), 8.49 (d, J = 8.0 Hz, 1H), 7.55 (d, J = 15.9 Hz, 1H), 7.32 (d, J = 15.6 Hz, 1H), 6.80−7.04 (m, 6H), 6.55 (d, J = 15.6 Hz, 1H), 6.27 (d, J = 15.9 Hz, 1H), 4.78 (m, 1H), 4.44 (d, J = 4.6 Hz, 2H); FABMS m/z 428 (M − H). Anal. ($C_{21}H_{19}NO_9$·0.7H_2O) C, H, N.

Methyl *N*-Acetyl-*O*-(3,4-diacetoxycinnamoyl)serinate (29). To a suspension of methyl serinate hydrochloride (6.43 mmol), 3,4-diacetoxycinnamic acid (12.86 mmol), and (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP-Cl) (13.1 mmol) in CH_2Cl_2 (120 mL) was added *N*-methylmorpholine (52.1 mmol), followed by a subsequent addition of 1-hydroxybenzotriazole hydrate (12.9 mmol). The resulting brownish solution was stirred at room temperature (24 h) and then partitioned between CH_2Cl_2 (150 mL) and brine (2 × 50 mL). The organic extracts were dried (Na_2SO_4), taken to dryness, and purified by silica gel flash column chromatography ($CHCl_3$ and $CHCl_3$:EtOAc) to afford **29** as a gum (1.56 g, 40% yield): ^{1}H NMR ($CDCl_3$) δ 7.67 (d, J = 15.6 Hz, 1H), 7.27−7.48 (m, 3H), 6.55 (s, 1H), 6.48 (d, J = 15.6 Hz, 1H), 5.06 (m, 1H), 4.55 (m, 2H), 3.90 (s, 3H), 2.39 (s, 3H), 2.38 (s, 3H), 2.15 (s, 3H); HRMS m/z calcd for $C_{19}H_{21}NO_9$ 408.129 (M + H), found 408.123. (Note: assignment of relative sites of acylation is not unambiguous.)

***O*-(3,4-Dihydroxycinnamoyl)serine (30).** Treatment of **29** as previously described for the conversion of **18** to **27** provided **30** as a white solid (43% yield): mp 202 °C dec; ^{1}H NMR (DMSO-d_6) δ 12.65 (br s, 1H), 8.15 (br s, 1H), 8.10 (br s, 1H), 7.97 (d, J = 8.1 Hz, 1H), 7.41 (d, J = 15.7 Hz, 1H), 6.85−7.11 (m, 3H), 6.71 (d, J = 15.6 Hz, 1H), 5.11(br s, 1H), 4.59 (m, 1H), 3.85 (q, J = 11.0 Hz, 2H); FABMS m/z 266 (M − H). Anal. ($C_{12}H_{13}NO_6$·0.5H_2O) C, H, N.

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