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Arylisothiocyanate-Containing Esters of Caffeic Acid Designed as Affinity Ligands for HIV-1 Integrase

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Abstract—Integrase is an enzyme found in human immunodeficiency virus, which is required for the viral life cycle, yet has no human cellular homologue. For this reason, HIV integrase (IN) has become an important target for the development of new AIDS therapeutics. Irreversible affinity ligands have proven to be valuable tools for studying a number of enzyme and protein systems, yet to date there have been no reports of such affinity ligands for the study of IN. As an initial approach toward irreversible ligand design directed against IN, we appended isothiocyanate functionality onto caffeic acid phenethyl ester (CAPE), a known HIV integrase inhibitor. The choice of isothiocyanate as the reactive functionality, was based on its demonstrated utility in the preparation of affinity ligands directed against a number of other protein targets. Several isomeric CAPE isothiocyanates were prepared to explore the enzyme topography for reactive nitrogen and sulfur nucleophiles vicinal to the enzyme-bound CAPE. The preparation of these CAPE isothiocyanates, required development of new synthetic methodology which employed phenyl thiocarbamates as latent isothiocyanates which could be unmasked near the end of the synthetic sequence. When it was observed that β-mercaptoethanol (β-ME), which is required to maintain the catalytic activity of soluble IN (a F185KC280S mutant), reacted with CAPE isothiocyanate functionality to form the corresponding hydroxyethylthiocarbamate, a variety of mutant IN were examined which did not require the presence of β-ME for catalytic activity. Although in these latter enzymes, CAPE isothiocyanate functionality was presumed to be present and available for acylation by IN nucleophiles, they were equally effective against Cys to Ser mutants. One conclusion of these studies, is that upon binding of CAPE to the integrase, nitrogen or sulfur nucleophiles may not be properly situated in the vicinity of the phenethyl aryl ring to allow reaction with and covalent modification of reactive functionality, such as isothiocyanate groups. The fact that introduction of the isothiocyanate group onto various positions of the phenethyl ring or replacement of the phenyl ring with naphthyl rings, failed to significantly affect inhibitory potency, indicates a degree of insensitivity of this region of the molecule toward structural modification. These findings may be useful in future studies concerned with the development and use of HIV-1 integrase affinity ligands. © 2001 Published by Elsevier Science Ltd.

Introduction

The occurrence of tolerance to single target-directed antihuman immunodeficiency virus (HIV) therapies, can potentially be overcome by combination treatments which disrupt multiple points in the viral life cycle.[1] Among the most effective combination regimens, are those employing reverse transcriptase and protease inhibitors;[2] however, development of resistance[3,4] has necessitated continued efforts to derive new agents directed against alternative viral processes. In this regard, HIV integrase (IN) is a particularly attractive target for therapeutic development, because while being essential for viral replication, there is no homologous enzyme in human hosts.[5] Although significant effort devoted to developing IN inhibitors has resulted in large numbers of agents exhibiting potent inhibition of integrase in extracellular enzyme assays, in HIV-infected cells the majority of these agents have either exhibited limiting cytotoxicity or have otherwise failed to elicit potent antiviral effects.[6,7] Investigation of new mod-

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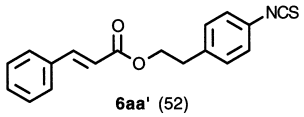
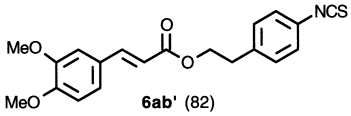
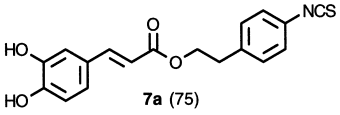
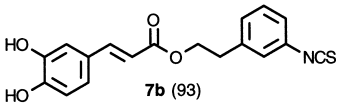
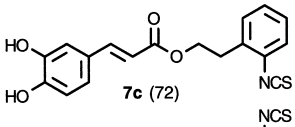
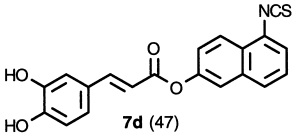
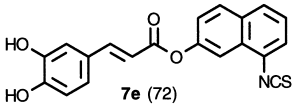
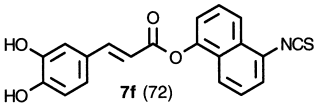
alities of IN inhibition is therefore needed to potentially provide useful leads for the development of more therapeutically relevant agents. Traditionally, one useful approach toward inhibitor development has been the utilization of enzyme/substrate or enzyme/inhibitor crystal structures for computer-assisted design of new highly potent or selective ligands.[8] In the case of IN however, such enzyme/inhibitor structures have been difficult to come by, and to date very little is available to provide the basis for structure-based inhibitor design.

Irreversible affinity ligands have proven to be very useful pharmacological tools in a number of systems.[9] In principle following initial reversible binding, ligands undergo secondary covalent modification which renders them an integral component of the binding protein. Such specific covalent modification can also result in the irreversible inhibition of enzyme function, which may afford physiological advantages over corresponding non-covalent, reversible inhibitors.[10] For purposes of enzyme/ligand structure elucidation, covalent attachment of ligand also potentially affords the opportunity to isolate and re-purify ligated enzyme. Where the size of the appended ligand is insignificant relative to the much larger protein, crystallization and X-ray crystallographic structure elucidation may be possible under conditions similar to those employed for the free, unligated enzyme. Although such irreversible ligands have been important in the study of a number of CNS receptor families, including opiate receptors,[11] benzodiazepine receptors[12] and the dopamine transporter,[13] to date systematic attempts to employ affinity ligands directed at HIV IN have not been reported. Accordingly, herein is presented the preliminary application of potential affinity igands for the study of HIV IN (Table 1).

Synthetic

Direct synthesis of isothiocyanates can be accomplished by a number of means, with one of the most common approaches involving reaction of a primary amine with thiophosgene, yielding the isothiocyanate directly.[14] To avoid the use of highly reactive thiophosgene, we recently reported an indirect synthesis of aryl isothiocyanates which utilizes phenylthiocarbamates as

Table 1. Isolated yields of intermediates and products

Amino alcohol **2**	Intermediate **3** (yield %)	Intermediate **5** (yield %)	Isothiocyanate **6** (yield %)	Isothiocyanate **7** (yield %)
2a	**3a** (77)	**5aa′** (92)	 **6aa'** (52)	—
2a	**3a** (77)	**5ab′** (57)	 **6ab'** (82)	—
2a	**3a** (77)	**5ac′** (48)	**6ac′** (98)	 **7a** (75)
2b	**3b** (77)	—	**6bc′** (40)	 **7b** (93)
2c	**3c** (77)	**5cc′** (37)	**6cc′** (73)	 **7c** (72)
2d	**3d** (77)	—	**6dc′** (32)	 **7d** (47)
2e	**3e** (77)	—	**6ec′** (32)	 **7e** (72)
2f	**3f** (77)	—	**6fc′** (32)	 **7f** (72)

latent, 'protected' isothiocyanates, which can be liberated to desired isothiocyanates late in the synthetic sequence.[15] Application of this approach for the preparation of CAPE isothiocanates is shown in Scheme 1. Starting aminophenylethanols (**2a**–**2f**) were transformed to their corresponding phenylthiocarbamates (**3a**–**3f**) by selective aminoacylation using phenylchlorothioformate. Subsequent esterification with appropriately protected caffeoyl chlorides (**4a′**–**4c′**), provided protected CAPE analogues **5**, which upon treatment with trichlorosilane in the presence of NEt_3,[15] gave corresponding isothiocyanates **6** in moderate to high yield. Of note, reactions of phenylthiocarbamates **3b** and **3d**–**3f** with caffeoyl chloride **4c′** yielded CAPE isothiocyanates **6** directly without isolation of the phenylthiocarbamate-containing intermediates. Treatment of silyl-protected **6** with pyridinium hydrogen fluoride, provided the free catechols **7a**–**7f**.

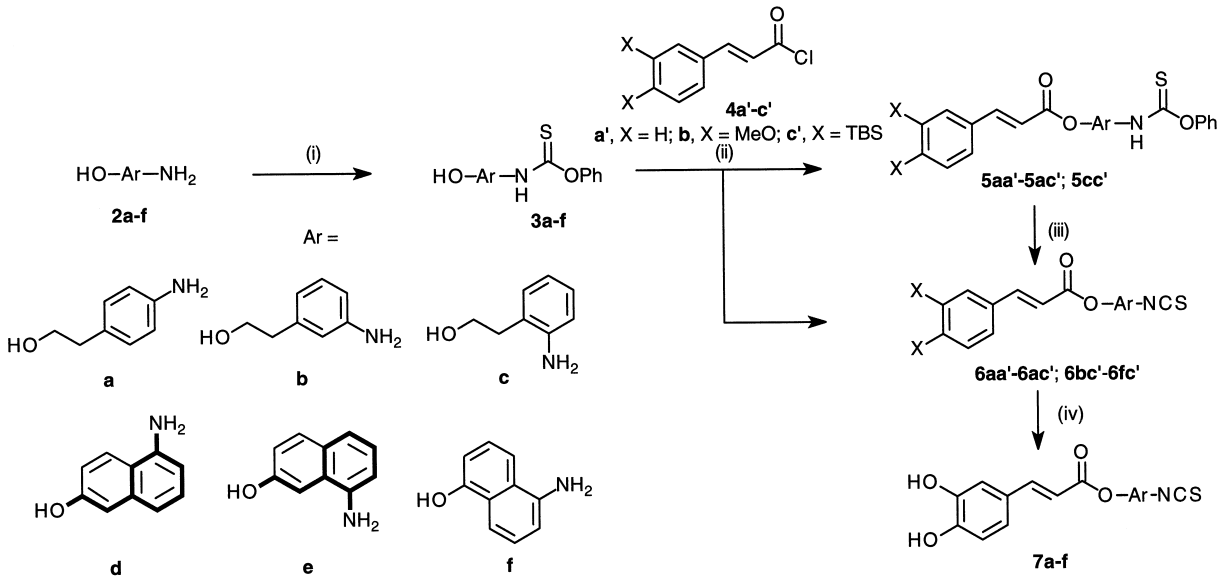
Results and Discussion

Design of CAPE isothiocyanates

In principle, isothiocyanate functionality is capable of undergoing facile reaction with amino and sulfhydryl functionality to form thiourea and thiocarbamate adducts, respectively (Fig. 1).[16] The relative stability of arylisothiocyanate groups toward alcohols including H_2O,[12] coupled with the above mentioned reactivity toward amino and sulfhydryl groups, has made the isothiocyanate moiety a valuable affinity handle for labeling Lys and Cys residues of target proteins. A particularly important application of such affinity labeling, the irreversible fixation of a ligand at its physiological site of binding, requires that once initial reversible binding of ligand has occurred, the isothiocyanate group must be properly situated in the vicinity of protein amino or sulfhydryl-containing amino acid side chains, to allow nucleophilic attack and covalent attachment. This implies not only that Lys or Cys residues must be proximal to the ligand binding site, but also that the isothiocyanate group itself is located on the ligand in suitable spacial orientation for nucleophilic attack. The requirement for proper location of isothiocyanate functionality within the ligand can be facilitated by preparing a series of isomeric inhibitors which vary in the site of isothiocyanate attachment. Therefore, isomeric isothiocyanates **7a**–**7c** were prepared, as well as naphthyl isothiocyanates **7d**–**7f**, which were designed as potential conformationally constrained variants (Scheme 1).

Application of latent isothiocyanate functionality to the synthesis of CAPE isothiocyanates

There are currently several methods of preparing isothiocyanates from amines,[17–23] with the most widely



Scheme 1. Reagents: (i) PhO(C=S)Cl, THF; (ii) pyridine, toluene; (iii) $HSiCl_3$, NEt_3; (iv) HF/pyridine, THF.

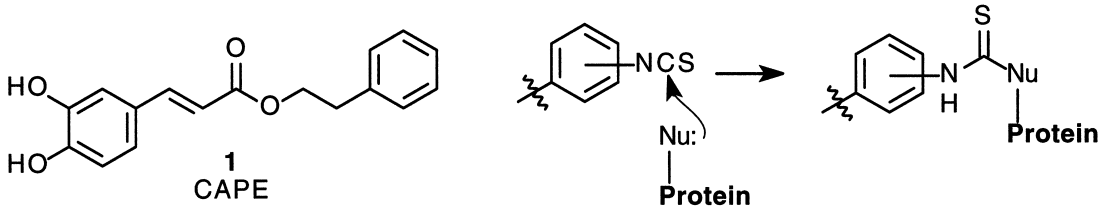


Figure 1. Chemical structure of CAPE (**1**) and hypothetical reaction of protein nucleophile with arylisothiocyanate functionality to yield covalently bound adduct.

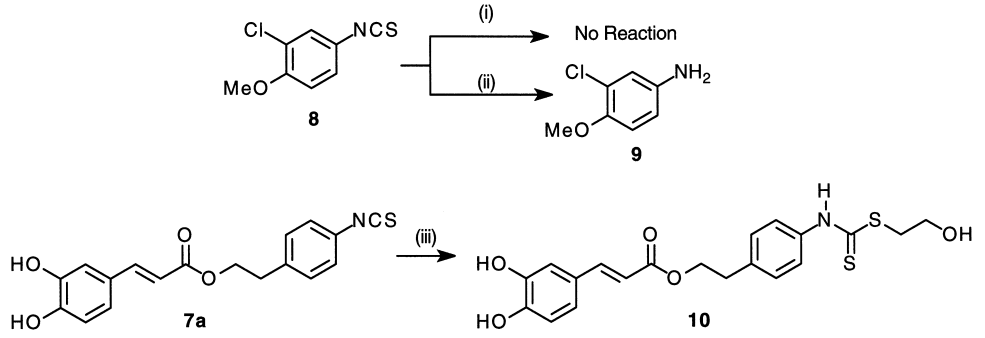
used methods involving isolation of the isothiocyanate directly following reaction of the amine with an activated thiocarbonyl species such as thiophosgene[11,24] thiocarbonyl diimidazole[25] or di-(2-pyridyl) thiocarbonate.[26] However, one potential disadvantage of such approaches, is the use of highly reactive species, which may not be compatible with functionality found elsewhere in the molecule. One means of overcoming these difficulties could rely on use of latent isothiocyanate functionality which could be introduced early in a synthesis, and liberated at a later step. For such purposes, we have recently reported the use of thiocarbamates as stable, 'protected isothiocyanates' which can be liberated to free isothiocyanates when desired.[15] Preparation of the isothiocyanate compounds reported herein, represents the first application of this methodology to a series of potential affinity ligands.

Reaction of CAPE isothiocyanate with β-mecaptoethanol amine

The solubilized HIV-1 integrase normally utilized for in vitro enzyme assays [F185KC280S mutant, here termed 'wild-type' (WT)] contains a number of Cys residues, and enzyme assays must be run under reducing conditions in the presence of β-mercaptoethanol (β-ME). Since arylisothiocyanates are known to react with alkylsulfhydryls,[16] it was of interest to examine whether β-ME would react with CAPE isothiocyanates under conditions approximating those utilized for the enzyme assay. Indeed, it was found that using **7a**, reaction with β-ME occurred, producing the expected product **10** (Scheme 2). When the inhibitory potency of both **7a** and **10** were determined using WT enzyme in the presence of β-ME, essentially identical IC_{50} values were obtained (4 and 3 μM, respectively; Table 2), consistent with the conversion of **7a** to **10** under the assay conditions. Parent CAPE (**1**) as well as isothiocyanate analogues **7e**–**7f**, also provided approximately equivalent IC_{50} values (1–4 μM), indicating that changes to the phenethyl portion of CAPE had little effect on binding potency. Consistent with previous reports,[27] modification of the caffeoyl portion, either by blocking of the catechol hydroxyls as their bis-methyl ethers (**6ab′**; $IC_{50} > 100$ μM), or by deleting them (**6aa′**; $IC_{50} > 100$ μM), rendered the compounds inactive.

Cys to Ser HIV-1 integrase mutants

Since β-ME apparently reacted with CAPE isothiocyanates, rendering them unsuitable for further covalent modification by enzyme nucleophiles, efforts were made to devise conditions under which integrase catalytic activity could be maintained without the use of β-ME in incubation media. For this purpose, assays were run using WT enzyme in the presence of a number of different reducing agents, including ascorbic acid and tris-(carboxyethyl)phosphine hydrochloride (TCEP),[28] both of which are non thiol-containing reducing agents. It was found that although isothiocyanate functionality was stable in the presence of ascorbic acid (Scheme 2), use of this reducing agent was not sufficient to maintain



Scheme 2. Reagents: (i) acscorbic acid, DMSO–H_2O; (ii) TCEP, DMSO–H_2O; (iii) 2-mercaptoethanol, CH_2Cl_2. NEt_3.

Table 2. Inhibition of HIV-1 integrase[a,b]

No.	WT Enzyme[c] IC_{50} (μM)		C56S Mutant IC_{50} (μM)		C65S Mutant IC_{50} (μM)	
	3′-Processing	Integration	3′-Processing	Integration	3′-Processing	Integration
1	5	5	35±5	35±5	11±6	10±4
6aa′	>100	>100	>100	>100	>100	>100
6ab′	>100	>100	>100	>100	>100	>100
7a	4	4	18±3	21±3	7±3	7±3
7b	3	3	23±6	23±6	8±3	8±3
7c	3	3	23±6	23±6	7±3	7±3
7d	3	3	20±5	20±5	7±3	7±3
7e	1	1	11±3	11±3	3±1	3±1
7f	2	2	20±5	20±5	4±2	4±2
10	3	3	23±6	23±6	7±3	7±3

[a]IC_{50}, 50% inhibitory concentration against purified integrase.
[b]Values with standard deviation are from three or more independent experiments.
[c]Soluble mutant F185KC280S.

WT enzyme catalytic activity. Alternatively, although TCEP was capable of substituting for β-ME with maintenance of WT enzyme function, it chemically reduced isothiocyanate functionality (Scheme 2), rendering it unsuitable for use in enzyme assays with CAPE isothiocyanates.

Since compatible conditions could not be found which would allow preservation of isothiocyanate functionality with concomitant maintenance of WT enzyme catalytic activity, investigations were undertaken to examine whether alteration of the WT enzyme could be achieved which would allow preservation of catalytic potency without the need for β-ME co-incubation. Altered HIV-1 enzymes containing Cys to Ser mutations at Cys56 and Cys65 (designated C56S and C65S, respectively)[29] were therefore examined for their ability to maintain function in the absence of β-ME (Fig. 2). As shown in Table 2, these enzymes were catalytically active in the absence of β-ME, with C56S enzyme showing slightly higher inhibition constants relative to the C65S mutant. It was also found that catalytic activity of these mutants in the absence of β-ME was approximately equal to that observed when β-ME was added to the incubation media, indicating that β-ME did not dramatically effect the catalytic activity of the enzymes (data not shown). Since for assays run in the absence of β-ME, it would be expected that inhibitor isothiocyanate functionality would remain intact up to the point of ligand binding, the potential existed for covalent adduct formation with the enzyme through nucleophile attack as shown in Figure 1. However, except for shifting to slightly higher IC_{50} values for C56S and C65S enzymes relative to WT (Table 2), the rank order of potencies of inhibitors against both enzymes was found to be the same as for WT enzyme, where inhibitor isothiocyanate functionality had presumably been inactivated by β-ME. The failure to show significant differences in inhibitory potency of isothiocyanate-containing inhibitors in the presence of β-ME (soluble mutant; Table 2) versus inhibitors in the absence of β-ME (C56S and C65S; Table 2), suggests that in both cases irreversible binding did not occur. This is further supported by near identical potencies of isothiocyanate inhibitors against C56S and C65S in the presence and absence of β-ME.

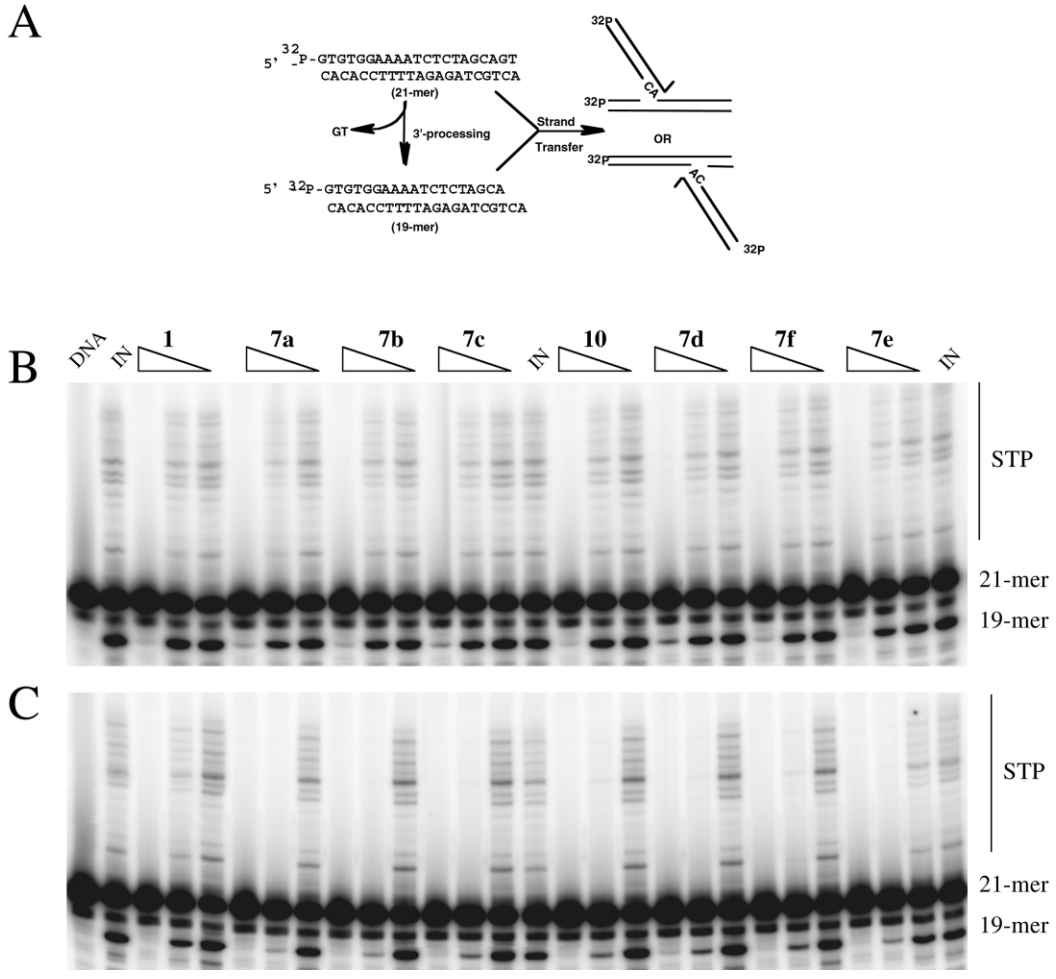


Figure 2. Inhibition of purified HIV-1 integrase by arylisothiocyanates. (A) A 21-mer blunt-end oligonucleotide corresponding to the U5 end of the HIV-1 LTR, 5′ end-labeled with 32P, is reacted with purified IN. The initial step involves nucleolytic cleavage of two bases from the 3′-end, resulting in a 19-mer oligonucleotide. Subsequently, 3′ ends are covalently joined to another identical oligonucleotide that serves as the target DNA (strand transfer reaction). Concentration dependent inhibition of HIV-1 IN by arylisothiocyanates using C56S mutant (B) or C65S mutant (C). Drug concentrations in μM (33, 11, 3.7) are indicated above each lane.

Conclusions

Structure-based design of enzyme inhibitors predicated on X-ray structures of ligated proteins, has proven to be highly effective in a number of enzyme systems. However, the difficulty in obtaining crystals of enzyme/ligand complexes suitable for X-ray crystallography, has limited this approach for development of HIV integrase inhibitors. As one approach toward overcoming this difficulty, we sought to prepare irreversible HIV integrase inhibitors which could potentially form covalent adducts with the enzyme. Theoretically, subsequent repurification of the enzyme following adduct formation and potential crystal formation under conditions similar to those utilized for the unligated enzyme could provide one means of overcoming this problem. Our approach toward irreversible ligand design, was to append isothiocyanate functionality onto CAPE, a known HIV integrase inhibitor. The choice of isothiocyanate as the reactive functionality, was based on its demonstrated utility in the preparation of affinity ligands directed against a number of other protein targets. Several isomeric CAPE isothiocyanates were prepared to explore the enzyme topography for reactive nitrogen and sulfur nucleophiles vicinal to the enzyme-bound CAPE. This represents the first reported study of HIV integrase inhibitors designed as irreversible ligands. The preparation of these CAPE isothiocyanates, required development of new synthetic methodology employing phenyl thiocarbamates as latent isothiocyanates which could be unmasked near the end of the synthetic sequence. Although it proved to be the case that isothiocyanate functionality was incompatible with β-ME needed to maintain the activity of WT enzyme, this provided the impetus to investigate integrase mutants which did not require β-ME for their catalytic activity. One conclusion which can be reached from these studies, is that upon binding of CAPE to the integrase, nitrogen or sulfur nucleophiles may not be properly situated in the vicinity of the phenethyl aryl ring to allow reaction with and covalent modification of reactive functionality, such as isothiocyanate groups. The fact that introduction of the isothiocyanate group onto various positions of the phenethyl ring or replacement of the phenethyl ring with naphthyl rings, failed to significantly affect inhibitory potency, indicates a degree of insensitivity of this region of the molecule toward structural modification. These findings may be useful in future studies concerned with the development and use of HIV-1 integrase affinity ligands.

Experimental

Biological materials, chemicals, and enzymes

All compounds were dissolved in DMSO with stock solutions being stored at −20°C. [γ-^{32}P]-ATP was purchased from DuPont NEN (Boston, MA, USA). The expression system for the soluble mutant F185KC280S, was a generous gift of Drs. T. Jenkins and R. Craigie, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD, USA. The expression systems for the cysteine mutant enzymes were a generous gift of Drs. Anna Marie Skalka and Jizu Yi, Fox Chase Cancer Center, Philadelphia, PA, USA and the proteins were prepared as described.[29,30]

Preparation of oligonucleotide substrates

HPLC purified oligonucleotides AE117, 5′-ACTGC-TAGAGATTTTCCACAC-3′ and AE118, 5′-GTGT-GGAAAATCTCTAGCAGT-3′, were purchased from Midland Certified Reagent Company (Midland, TX, USA). To analyze the extent of 3′-processing and strand transfer using 5′-end labeled substrates, AE118 was 5′-end labeled using T_4 polynucleotide kinase (Gibco BRL) 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 through a G-25 Sephadex quick spin column (Boehringer Mannheim, Indianapolis, IN, USA) to separate annealed double-stranded oligonucleotide from unincorporated label.

Integrase assays

To determine the extent of 3′-processing and strand transfer, IN was preincubated at a final concentration of 200 nM with 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/mL bovine serum albumin, 10 mM β-ME, 10% DMSO, and 25 mM MOPS, pH 7.2) at 30 °C for 30 min. Then, 20 nM of the 5′-end ^{32}P-labeled linear oligonucleotide substrate was 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 and 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 using a Molecular Dynamics PhosphorImager (Sunnyvale, CA, USA). Percent inhibition was calculated using the following equation: $\%I = 100\times[1-(D-C)/(N-C)]$, 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 IN, and IN plus drug, respectively. All IC_{50} values were determined by plotting drug concentration versus percent inhibition and determining the concentration, which produced 50% inhibition.

General synthetic methods

Commercially available reagents were used as supplied. Column flash chromatography was performed using silica gel 60 (E. Merck 40–60 μm Darmstadt, Germany) and product solutions were dried over Na_2SO_4 prior to rotary evaporation under reduced pressure. Melting points were determined on a Mel Temp II melting point apparatus and are uncorrected. Elemental analyses were

obtained from Atlantic Microlab Inc., Norcross, GA, USA and fast atom bombardment mass spectra (FAB-MS) were acquired either with a VG Analytical 7070E mass spectrometer under the control of a VG 2035 data system or with a JEOL SX-102 mass spectrometer using 'magic-bullet' (1:5 dithiothrietol/dierythritol), glycerol or nitrobenzyl alcohol matrix as appropriate. Chemical ionization mass spectra were obtained on a Finnigan 4500 quadrupole mass spectrometer using ammonia gas in both positive and negative ion mode. ^{1}H NMR data were obtained on Bruker AC250 (250 MHz and are reported in ppm relative to TMS and referenced to the solvent in which they were run.

General procedure for synthesis of phenylthiocarbamates (3) from aminophenylethanols or aminonaphthols

Synthesis of 2-(4-((phenoxythioxomethyl)amino)phenyl)-ethan-1-ol (3a). A mixture of 2-(4-aminophenyl)ethan-1-ol (**2a**) (274 mg, 2.0 mmol) and phenyl chlorothionoformate (173 mg, 1.0 mmol) in THF (5 mL) was stirred at room temperature (30 min) then insoluble material was removed by filtration through Celite™ 521.The resulting solution was taken to dryness under reduced pressure and residue was chromatographed (EtOAc/hexane, 1:5) to afford product **3a** as a solid[15] (205 mg, 75%).

2-(3-((Phenoxythioxomethyl)amino)phenyl)ethan-1-ol (3b). ^{1}H NMR ($CDCl_3$) δ 7.47–7.11 (m, 9H), 3.89 (t, 2H, J = 6.4 Hz), 2.90 (t, 2H, J = 6.4 Hz). FAB-MS ($^+$VE) m/z 274 (MH^+). Anal. calcd for $C_{15}H_{15}NO_2S$: C, 65.97; H, 5.53; N, 5.13. Found: C, 65.93; H, 5.64; N, 5.11.

2-(2-((Phenoxythioxomethyl)amino)phenyl)ethan-1-ol (3c). ^{1}H NMR ($CDCl_3$) δ 7.50–7.20 (m, 9H), 4.02 (t, J = 5.3 Hz, 2H), 3.02 (t, J = 5.3 Hz, 2H). FAB-MS ($^+$VE) m/z 274 (MH^+). Anal. calcd for $C_{15}H_{15}NO_2S$: C, 65.97; H, 5.53; N, 5.13. Found: C, 65.84; H, 5.51; N, 5.12.

5-((Phenoxythioxomethyl)amino)napthalen-2-ol (3d). ^{1}H NMR (DMSO-d_6) δ 11.66 (s, 1H), 9.88 (s, 1H), 7.85 (d, 1H, J = 8 Hz), 7.68 (d, 1H, J = 8 Hz), 7.45–7.15 (m, 8H), 7.02 (d, 1H, J = 8 Hz). FAB-MS ($^+$VE) m/z 296 (MH^+). Anal. calcd for $C_{17}H_{13}NO_2S$: C, 69.20; H, 4.78; N,4.74. Found: C, 68.75; H, 4.74; N, 4.50.

8-((Phenoxythioxomethyl)amino)napthalen-2-ol (3e). ^{1}H NMR ($CDCl_3$) δ 8.71 (s, 1H), 7.83 (s, 1H), 7.78 (d, 1H, J = 9 Hz), 7.57–7.24 (m, 8H), 7.17 (d, 1H, J = 9 Hz), 7.05 (d, 1H, J = 8 Hz). FAB-MS ($^+$VE) m/z 296 (MH^+). Anal. calcd for $C_{17}H_{13}NO_2S$: C, 69.20; H, 4.78; N,4.74. Found: C, 68.88; H, 4.40; N, 4.91.

5-((Phenoxythioxomethyl)amino)napthalen-1-ol (3f). ^{1}H NMR (acetone-d_6) δ 10.44 (br, 1H), 9.10 (s, 1H), 8.18 (d, J = 8 Hz, 1H), 7.62–6.88 (m, 10H). FAB-MS ($^+$VE) m/z 296 (MH^+). Anal. calcd for $C_{17}H_{13}NO_2S$: C, 69.20; H, 4.78; N,4.74. Found: C, 68.83; H, 4.45; N, 4.98.

General procedure for synthesis of caffeoyl esters (5) by reaction of phenylthiocarbamates (3) with caffeoyl chlorides 4

Synthesis of 2-(4-((phenoxythioxomethyl)amino)phenyl)-ethyl *O,O*-(3,4-bis(1,1,2,2-tetramethyl-1-silapropyl))caffeic acid ester (5ac′). To a solution of freshly prepared *tert*-butyldimethylsilyl-protected caffeoyl chloride[31] **4c′** (0.81 mmol) in toluene (8 mL) was added **3a** (221 mg, 0.81 mmol) and pyridine (1.2 mL) and the mixture was stirred at room temperature (overnight) then filtered through Celite™ 521. The resulting solution was taken to dryness under reduced pressure and residue was chromatographed (EtOAc/hexane, 1:4) to afford product **5ac′** (256 mg, 48%).[15]

2-(4-((Phenoxythioxomethyl)amino)phenyl)ethyl cinnamic acid ester (5aa′). ^{1}H NMR ($CDCl_3$) δ 11.76 (s, 1H), 7.71–7.60 (m, 4H), 7.42–7.14 (m, 11H), 6.53 (d, J = 16 Hz, 1H), 4.36 (m, 2H), 2.97 (m, 2H) FAB-MS ($^+$VE) m/z 404 (MH^+). HR-FAB-MS calcd for $C_{24}H_{22}NO_3S$: 404.1320, Found. 404.1325.

2-(4-((Phenoxythioxomethyl)amino)phenyl)ethyl *O,O*-(3,4-bis(methyl)) caffeic acid ester (5ab′). ^{1}H NMR ($CDCl_3$) δ 7.69 (d, 1H, J = 16 Hz), 7.51–7.12 (m, 11H), 6.94 (d, 1H, J = 8 Hz), 6.36 (d, 1H, J = 16 Hz), 4.50 (b, 2H), 3.99 (s, 6H), 3.10 (br, 2H). FABMS ($^+$VE) m/z 464 (MH^+). Anal. calcd for $C_{26}H_{25}NO_5S$: C, 67.42; H, 5.44; N, 3.02. Found: C, 67.22; H, 5.51; N, 2.96.

2-(2-((Phenoxythioxomethyl)amino)phenyl)ethyl *O,O*-(3,4-bis(1,1,2,2-tetramethyl-1- silapropyl))caffeic acid ester (5cc′). ^{1}H NMR ($CDCl_3$) δ 7.65–6.77 (m, 14H), 4.46 (t, 2H, J = 6 Hz), 3.15 (br, 2H), 0.98 (s, 9H), 0.97 (s, 9H), 0.22 (s, 6H), 0.20 (s, 6H). FAB-MS ($^+$VE) m/z 664.6 (MH^+). Anal. calcd for $C_{36}H_{49}NO_5SSi_2$: C, 65.11; H, 7.43; N, 2.10. Found: C, 64.91; H, 7.51; N, 2.09.

General procedure for conversion of phenylthiocarbamates (5) to isothiocyanates

Synthesis of 2-(4-isothiocyanatophenyl)ethyl *O,O*-(3,4-bis(1,1,2,2-tetramethyl-1-silapropyl))caffeic acid ester (6ac′). To a solution of **5ac′** (80 mg, 0.12 mmol) in benzene (5 mL) was added triethylamine (19 mg, 0.19 mmol) followed by trichlorosilane (26 mg, 0.19 mmol) and the mixture was stirred at room temperature (1 h) then filtered through Celite™ 521. The resulting solution was taken to dryness under reduced pressure and residue was chromatographed (EtOAc/hexane, 1:10) to afford product **6ac′** as a solid (69 mg, 98%).[15]

2-(4-Isothiocyanatophenyl)ethyl cinnamic acid ester (6aa′). ^{1}H NMR ($CDCl_3$) δ 7.67 (d, J = 16 Hz, 1H), 7.55–7.51 (m, 2H), 7.41–7.38 (m, 2H), 7.27–7.17 (m, 5H), 6.41 (d, J = 16 Hz, 1H), 4.41 (t, J = 7 Hz, 2H), 3.02 (t, J = 7 Hz, 2H). FAB-MS ($^+$VE) m/z 310 (MH^+). Anal. calcd for $C_{18}H_{15}NO_2S$: C, 69.88; H, 4.89; N, 4.53. Found: C, 70.02; H, 4.92; N, 4.33.

2-(4-Isothiocyanatophenyl)ethyl *O,O*-(3,4-bis(methyl))-caffeic acid ester (6ab′). ^{1}H NMR ($CDCl_3$) δ 7.68 (d,

1H, $J=16$ Hz), 7.34–7.11 (m, 6H), 6.94 (d, 1H, $J=8$ Hz), 6.35 (d, 1H, $J=16$ Hz,), 4.48 (t, 2H, $J=7$ Hz), 3.99 (s, 6H), 3.08 (t, 2H, $J=7$ Hz). FAB-MS ($^+$VE) m/z 370 (MH^+). Anal. calcd for $C_{20}H_{19}NO_4S$: C, 65.02; H, 5.18; N, 3.79. Found: C, 64.87; H, 5.17; N, 3.72.

2-(3-Isothiocyanatophenyl)ethyl *O,O*-(3,4-bis(1,1,2,2-tetramethyl-1-silapropyl))caffeic acid ester (6bc′). ^{1}H NMR ($CDCl_3$) δ 7.55 (d, 1H, $J=16$H), 7.28 (d, 1H, $J=8$ Hz), 7.18–7.00 (m, 5H), 6.82 (d, 1H, $J=8$ Hz), 6.20 (d, 1H, $J=16$ Hz), 0.99 (s, 9H), 0.98 (s, 9H), 0.22 (s, 6H), 0.21 (s, 6H). FAB-MS ($^+$VE) m/z 570(MH^+). Anal. calcd for $C_{30}H_{43}NO_4SSi_2$: C, 63.22; H, 7.60; N, 2.45. Found: C, 63.14; H, 7.67; N, 2.40.

2-(2-Isothiocyanatophenyl)ethyl *O,O*-(3,4-bis(1,1,2,2-tetramethyl-1-silapropyl))caffeic acid ester (6cc′)

^{1}H NMR ($CDCl_3$) δ 7.57 (d, 1H, $J=16$ Hz), 7.28–7.24 (m, 4H), 7.05–7.02 (m, 2H), 6.84 (d, 1H, $J=8.8$ Hz), 6.25 (d, 1H, $J=1$ 6 Hz), 4.44 (t, 2H, $J=6.6$ Hz), 3.13 (t, 2H, $J=6.6$ Hz), 1.02 (s, 9H), 1.00 (s, 9H), 0.24 (s, 6H), 0.23 (s, 6H). FAB-MS ($^+$VE) m/z 570 (MH^+) Anal. calcd for $C_{30}H_{43}NO_4SSi_2$: C, 63.22; H, 7.60; N, 2.45. Found: C, 63.22; H, 7.62; N, 2.41.

5-Isothiocyanato-2-naphthyl *O,O*-(3,4-bis(1,1,2,2-tetramethyl-1-silapropyl))caffeic acid ester (6dc′). ^{1}H NMR ($CDCl_3$) δ 8.17 (d, 1H, $J=9$ Hz), 7.83 (d, 1H, $J=16$ Hz,), 7.77–7.70 (m, 3H), 7.47–7.43 (m, 2H), 7.13–7.09 (m, 2H), 6.88 (d, 1H, $J=9$ Hz), 6.48 (d, 1H, $J=16$ Hz), 1.03 (s, 9H), 1.01 (s, 9H), 0.26 (s, 6H), 0.25 (s, 6H). FAB-MS ($^+$VE) m/z 592.5 (MH^+). Anal. calcd for $C_{32}H_{41}NO_4SSi_2$: C, 65.00; H, 6.98; N, 2.37. Found: C, 64.70; H, 7.11; N, 2.19.

8-Isothiocyanato-2-naphthyl *O,O*-(3,4-bis(1,1,2,2-tetramethyl-1-silapropyl))caffeic acid ester (6ec′). ^{1}H NMR ($CDCl_3$) δ 7.94–7.79 (m, 4H), 7.50–7.39 (m, 3H), 7.14–7.10 (m, 2H), 6.88 (d, 1H, $J=8.8$ Hz), 6.48 (d, 1H, $J=16$ Hz), 1.03 (s, 9H), 1.01 (s, 9H), 0.26 (s, 6H), 0.25 (s, 6H). FAB-MS ($^+$VE) m/z 592.6 (MH^+). HR-FAB-MS calcd for $C_{32}H_{41}NO_4SSi_2$: 592.2373. Found: 592.2369 (MH^+).

5-Isothiocyanato-1-naphthyl *O,O*-(3,4-bis(1,1,2,2-tetramethyl-1-silapropyl))caffeic acid ester (6fc′). ^{1}H NMR ($CDCl_3$) δ 8.05 (d, 1H, $J=8.5$ Hz), 7.91–7.84 (m, 2H), 7.63 (t, 1H, $J=8$ Hz), 7.47–7.39 (m, 3H), 7.15–7.12 (m, 2H), 6.88 (d, 1H, $J=8.5$ Hz), 6.57 (d, 1H $J=16$ Hz), 1.02 (s, 9H), 1.01 (s, 9H), 0.01 (s, 12H). FAB-MS ($^+$VE) m/z 592.2 (MH^+). HR-FAB-MS calcd for $C_{32}H_{42}NO_4SSi_2$: 591.2295. Found: 592.2369 (MH^+).

General procedure for removal of silyl-protecting groups

Synthesis of 2-(4-isothiocyanatophenyl)ethyl caffeic acid ester (7a). To a stirred solution of silyl-protected **6ac′** (72 mg, 0.13 mmol) in THF (3 mL) was added pyridinium hydrogen fluoride (0.1 mL) and the mixture was stirred at room temperature (4 h) then filtered through Celite™ 521. The resulting solution was taken to dryness under reduced pressure and residue was chromatographed (EtOAc/hexane, 1:2) to afford product **7a** as a solid (34 mg, 75%).[15]

2-(3-Isothiocyanatophenyl)ethyl caffeic acid ester (7b). ^{1}H NMR (methanol-d_4) δ 7.54 (d, 1H, $J=16$ Hz), 7.37 (d, 1H, $J=8$ Hz), 7.30–6.94 (m, 5H), 6.79 (d, 1H, $J=8$ Hz), 6.25 (d, 1H, $J=16$ Hz), 4.41 (t, 2H, $J=6$ Hz), 3.04 (t, 2H, $J=6$ Hz). FAB-MS ($^+$VE) m/z 342(MH^+). Anal. calcd for $C_{18}H_{15}NO_4S$: C, 63.33; H, 4.42; N, 4.10; S, 9.4. Found: C, 63.63; H, 4.60; N, 4.01; S, 9.20.

2-(2-isothiocyanatophenyl)ethyl caffeic acid ester (7c). ^{1}H NMR (methanol-d_4) δ 7.48 (d, 1H, $J=16$ Hz), 7.32–7.27 (m, 4H), 6.99 (s, 1H), 6.90 (d, 1H, $J=7$ Hz), 6.75 (d, 1H, $J=8$ Hz), 6.19 (d, 1H, $J=16$ Hz), 4.37 (t, 2H, $J=6$ Hz), 3.07 (t, 2H, $J=6$ Hz). FAB-MS ($^+$VE) m/z 342 (MH^+). Anal. calcd for $C_{18}H_{15}NO_4S$: C, 63.33; H, 4.42; N, 4.10. Found: C, 63.05; H, 4.60; N, 3.94.

5-Isothiocyanato-2-naphthyl caffeic acid ester (7d). ^{1}H NMR (methanol-d_4) δ 8.06 (d, 1H, $J=9$ Hz), 7.82–7.70(m, 3H), 7.46–7.41 (m, 3H), 7.12–6.98 (m, 2H), 6.78 (d, 1H, $J=8$ Hz), 6.48 (d, 1H, $J=16$ Hz). FAB-MS ($^+$VE) m/z 364(MH^+). HR-FAB-MS calcd for $C_{20}H_{12}NO_4S$: 362.0487. Found: 362.0496 ($^-$VE).

8-Isothiocyanato-2-naphthyl caffeic acid ester (7e). ^{1}H NMR (methanol-d_4) δ 8.01 (d, 1H, $J=9$ Hz), 7.91–7.77 (m, 3H), 7.55–7.41 (m, 3H), 7.14–7.03 (m, 2H), 6.81 (d,1H, $J=8$ Hz), 6.53 (d,1H, $J=16$ Hz). FABMS ($^+$VE) m/z 364 (MH^+). HR-FAB-MS ($^-$VE) calcd for $C_{20}H_{12}NO_4S$ (M−H): 362.0487. Found: 362.0485.

5-Isothiocyanato-1-naphthyl caffeic acid ester (7f). ^{1}H NMR (methanol-d_4) δ 7.91 (d, 1H, $J=8$ Hz), 7.81–7.72 (m, 2H), 7.58 (t, 1H, $J=8$ Hz), 7.44–7.41 (m, 3H), 7.08–6.96(m, 2H), 6.74 (d, 1H, $J=8$ Hz), 6.54 (d, 1H, $J=16$ Hz). FAB-MS ($^+$VE) m/z 364(MH^+). HR-FABMS ($^-$VE) calcd for $C_{20}H_{12}NO_4S$ (M−H): 362.0487. Found: 362.0493.

Reaction of isothiocyanate analogue 7a with 2-mer captoethanol

Synthesis of 2-(4-(((2-hydroxyethylthio)thioxyomethyl)-amino)phenyl)ethyl caffeic acid ester (10). To a stirred mixture of **7a** (16 mg, 0.045 mmol) in CH_2Cl_2, was added β-ME (16 μL, 0.22 mmol) along with NEt_3 and the mixture was stirred at room temperature (1 h). It was then diluted with CH_2Cl_2, washed with H_2O, dried (Na_2SO_4) and taken to dryness under reduced pressure and residue was chromatographed (EtOAc/hexanes/MeOH, 1:2:0.5) to afford adduct **10** (9 mg, 46%).[15] ^{1}H NMR ($CDCl_3$) δ 7.80–7.48 (m, 3H), 7.29 (d, 2H, $J=8$ Hz), 7.02 (d,1H, $J=2$ Hz), 6.93 (dd, 1H, $J=8$ Hz, 2 Hz), 6.77 (d, 1H, $J=8$ Hz), 6.23 (d, 1H, $J=16$ Hz), 4.37 (t, 2H, $J=7$ Hz), 3.76 (t, 2H, $J=6.6$ Hz), 3.44 (t, 2H, $J=6.6$ Hz), 3.00 (t, 2H, $J=7$ Hz). FAB-MS ($^+$VE) m/z 420 (MH^+). HR-FAB-MS calcd for $C_{20}H_{22}NO_5S_2$ (MH^+): 420.0939. Found: 420.958.

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