Depsides and Depsidones as Inhibitors of HIV-1 Integrase: Discovery of Novel Inhibitors through 3D Database Searching†

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Received November 1, 1996⊗

Seventeen lichen acids comprising depsides, depsidones, and their synthetic derivatives have been examined for their inhibitory activity against HIV-1 integrase, and two pharmacophores associated with inhibition of this enzyme have been identified. A search of the NCI 3D database of approximately 200 000 structures yielded some 800 compounds which contain one or the other pharmacophore. Forty-two of these compounds were assayed for HIV-1 integrase inhibition, and of these, 27 had inhibitory IC_{50} values of less than 100 μM; 15 were below 50 μM. Several of these compounds were also examined for their activity against HIV-2 integrase and mammalian topoisomerase I.

Introduction

Inhibition of HIV-1 replication remains the fulcrum for the treatment of AIDS. To date, successful inhibition of several key enzymes in the HIV life cycle has been achieved. However, due to the increased emergence of drug-resistant strains, limited bioavailability, and high toxicity of known inhibitors, combination therapy with drugs directed toward different targets in the viral life cycle is becoming increasingly important.[1,2] Toward this goal, our laboratory has been engaged in an effort to identify selective inhibitors of HIV-1 integrase.[3−7] This enzyme is responsible for inserting viral DNA into host chromosomal targets. It is known to be essential for effective viral replication[8,9] and thus appears to be an attractive target for selective inhibition. In an attempt to identify lead compounds for activity against integrase, we have embarked on a study to screen compounds that have shown activity in the NCI antiviral drug screen.

Among many natural products that have been tested, several lichen extracts exhibited remarkable activity in the *in vitro* assay against HIV-1 integrase. Two distinctive families of lichen-derived compounds are the depsides and the depsidones.[10,11] These types of compounds are formed by two or three phenylcarboxylic acids, mainly orsellinic or β-orsellinic acids, held together by an ester linkage. Lichens are known to live in symbiotic relationships with algae and fungi, and their extracts have been extensively used for various remedies in folk medicine since ancient Egyptian times.[10,11] Depsides and depsidones isolated from lichens have been shown by several groups to have activity against mycobacteria, Gram-positive bacteria, insects, and nematodes.[10−15] In addition, several well-characterized depsidones and depsides exhibit anti-inflammatory, analgesic, antipyretic, anticancer, and antiviral properties,[10−15] but to our knowledge, the anti-HIV activities of these classes of compounds have not been reported.

Many depsides and depsidones share common 3D structural features, and identification of unique pharmacophores is valuable in understanding the structure−activity relationships of these novel HIV-1 integrase inhibitors and the structural basis for the design and search for other HIV-1 integrase inhibitors. This work is thus concerned with the anti-HIV integrase activity of several naturally occurring and synthetic depsides and depsidones as lead compounds. The effective application of 3D database searches and the identification of several novel inhibitors based on two distinct pharmacophores common in lichen acids are discussed.

Results and Discussion

Pharmacophore Identification. Pharmacophore searching in 3D database is a means of identifying compounds whose 3D structures, in some conformation, contain the pharmacophore used in the search query. This is an approach which has recently gained attention for its ability to find new leads in drug discovery programs.[16,17] We have recently built a 3D database of a total of ca. 407 000 2D structures in the NCI Drug Information System (DIS) database[18] using the program Chem-X.[19] Previous use of this 3D database has led to the discovery of a number of novel protein kinase C activators,[20] HIV-1 protease inhibitors,[21] and HIV-1 integrase inhibitors,[22,23] showing that 3D database searching is a valuable means of drug discovery. Using an *in vitro* assay specific for integrase, we have tested large numbers of compounds from the NCI antiviral drug screening program that were confirmed active or moderately active against HIV-1-infected CEM cells. Through this endeavor, novel inhibitors of HIV-1 integrase were identified. Compound **2** (Figure 1) was initially identified as one such example that exhibited moderate activity in cell-based assays against HIV-1. Subsequently, 16 other depsides and depsidones shown in Figure 1 were examined, and some were shown to possess integrase inhibitory activity (Table 2).

The pharmacophore identification module in Chem-X was used to identify the possible pharmacophore(s) that

† Part 4 in a series of papers describing pharmacophore-based development of HIV-1 integrase inhibitors.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

S0022-2623(96)00759-5

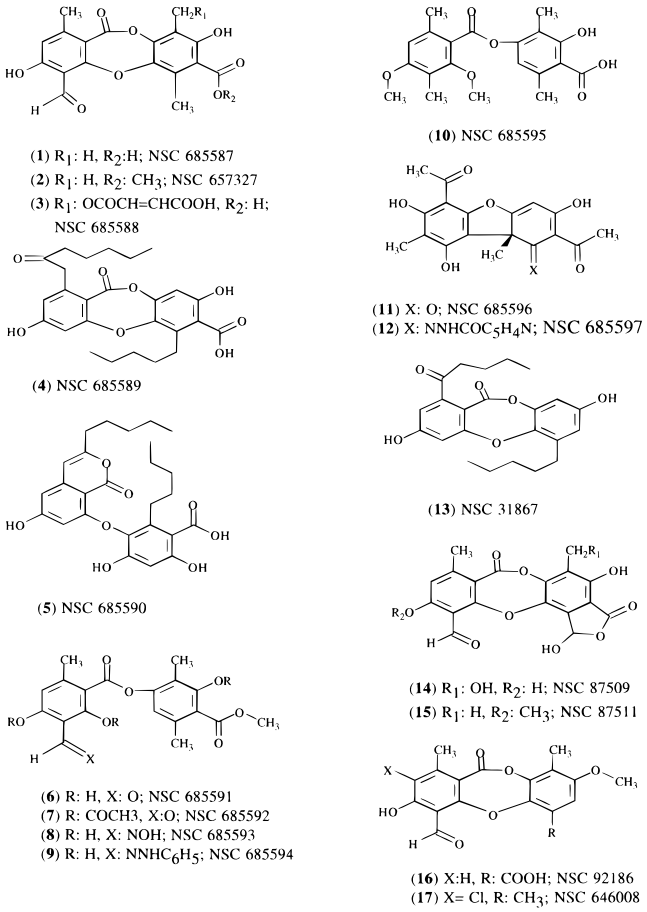

Figure 1. Structures of depsides and depsidones.

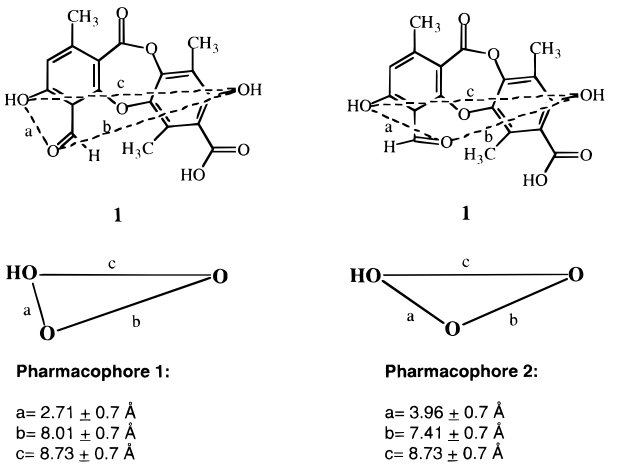

Figure 2. Three-point pharmacophore distance pattern in virensic acid (**1**) and dimensions of the two pharmacophore queries. In searching the NCI 3D database a basic nitrogen atom was allowed in place of oxygen.

Table 1. rms and ΔE Values for the Superposition of Several Lichen Acids onto the X-ray Structure of Compound **2** Using Pharmacophores 1 and 2

compd	rms_1 (Å)[a]	ΔE_1 (kcal/mol)[b]	rms_2 (Å)[a]	ΔE_2 (kcal/mol)[b]
1, NSC 685587	0.041	0.000	0.099	1.152
3, NSC 685588	0.055	3.365	0.131	0.000
4, NSC 685589	0.297	0.000	0.026	0.000
5, NSC 685590	0.410	0.000	0.301	1.612
6, NSC 685591	0.381	0.808	0.298	0.000
8, NSC 685593	0.621	21.859	0.384	21.859
9, NSC 685594	0.762	12.831	0.243	12.831
12, NSC 685597	0.162	4.864	0.385	0.000
14, NSC 87509	0.282	0.000	0.261	0.000
15, NSC 87511	0.230	0.000	0.201	0.000
16, NSC 92186	0.062	0.000	0.129	2.238
17, NSC 646008	0.299	7.121	0.091	0.000

[a] rms_1 and rms_2 refer to the root mean square of superposition of indicated compounds onto compound **2** using pharmacophores 1 and 2, respectively. [b] ΔE_1 and ΔE_2 refer to the energy differences between the lowest energy conformer and superposition conformers of indicated compounds onto compound **2** using pharmacophores 1 and 2, repectively.

may exist among lichen acids. In the first step, a flexible mini-3D database of the 16 compounds shown in Figure 1 was built and keyed using Chem-X. A structural similarity routine within Chem-X broke down this set of compounds into two sets of 6 and 10 compounds. Each of these was submitted to the pharmacophore identification program. This program searches for pharmacophore fragments common to all active compounds but absent from the inactive compounds. One pharmacophore was found in each subset. The two pharmacophores, shown in Figure 2, involve the same set of three oxygens, but with different geometry, and represent two conformations which, in the case of virensic acid, differ by only 0.97 kcal/mol. In our previous report a similar pharmacophore containing a steric exclusion zone was derived based on caffeic acid phenethyl ester.[22]

The results presented in Table 1 show that all of the active compounds were superimposable on one or the other of these two pharmacophores in a low-energy conformation. Several inactive compounds, such as **8** and **9**, did not meet the pharmacophore requirements. Such compounds must pay a substantial energy penalty in order to achieve a conformation that meets the pharmacophore requirements, and therefore, despite their containing the potential pharmacophore(s) in one of their conformers, these compounds are unlikely to achieve good activity.

The superposition of the four most potent compounds, **1**, **2**, **15**, and **17**, in their low-energy conformations, with either pharmacophore 1 or 2, was studied using the program QUANTA. The appropriate three oxygen atoms of compounds **1**, **2**, **15**, and **17** could be superposed onto pharmacophore 1 with a cumulative rms of 0.414 Å. The same three atoms from the same molecules were found to be superposable on pharmacophore 2 with an rms of 0.105 Å. These pharmacophores were used in searches of the NCI 3D structural database of 206 876 compounds in order to identify compounds that possess these structural features and to test if such compounds indeed show activity against HIV-1 integrase.

Selection of Compounds for *in Vitro* Studies against Purified HIV-1 Integrase. A total of 807 compounds were found that contain one or the other of the two pharmacophores shown in Figure 2. Pharmacophore 1 was found in 482 compounds and pharmacophore 2 in 594 compounds; 269 compounds contained both pharmacophores. These sets of structures were reviewed manually, and various structures such as polyanionic compounds, sulfonates, nucleotides, nucleosides, peptides, polymers, obvious DNA binders including intercalators, and minor-groove binders were removed. The remaining structures were divided into chemical classes, and one to three representative compounds from each class were selected for testing. A total of 42 compounds were selected on the basis of chemical

Table 2. Anti-HIV-1 Integrase Activities of Depsides and Depsidones

	% inhibition at 100 μg/mL[a]		IC_{50} (μM)				
			3′-processing		strand transfer		
compd	3′-processing	strand transfer	expt 1	expt 2	expt 1	expt 2	disintegration
1, NSC 685587	95.8	87.5	4.6 ± 1.6		6.5 ± 3.9		33.5
2, NSC 657327	96.0	97.5	5.4 ± 1.9		4.4 ± 0.5		26.0
3, NSC 685588	94.4	89.7	4.9 ± 2.7		4.6 ± 2.6		7.3
4, NSC 685589	94.0	79.5	38.5 ± 25.7		30.9 ± 14.4		32.5
5, NSC 685590	95.0	80.2	52.2 ± 11.4		45.7 ± 3.9		35.0
6, NSC 685591	94.7	76.9	61.0	62.4	36	44.0	128.0
7, NSC 685592	−7.2	7.2					
8, NSC 685593	45.3	51.1					
9, NSC 685594	4.3	5.9					
10, NSC 685595	41.1	34.8					
11, NSC 685596	74.3	70.6	126.4 ± 23.5		123.7 ± 53.4		133.0
12, NSC 685597	90.6	79.2	73.0	60.8	48.5	55.0	39.0
13, NSC 31867	98.8	88.7	59.9	42.2	39.2	28.1	
14, NSC 87509	99.5	76.8	19.0	16.0	12.6	6.2	
15, NSC 87511	98.2	94.9	4.4	2.9	2.6	1.9	11.7
16, NSC 92186	98.9	88.9	17.0	15.1	5.3	12.3	
17, NSC 646008	99.3	93.8	2.2	2.7	1.6	2.7	12.8

[a] Average of two independent experiments. Values with standard deviations are from at least three independent experiments.

diversity for bioassay. As a result, a group of 24 compounds containing pharmacophore 1 or pharmacophore 1 and 2 (Figure 3) and 18 compounds containing pharmacophore 2 (Figure 4) were evaluated in a specific *in vitro* assay for HIV-1 integrase. This initial screening of 42 compounds revealed several novel and structurally unrelated HIV-1 integrase inhibitors. For example, compounds **26**, **32**, and **34** (Table 3) exhibited anti-integrase activities with IC_{50}s around 1 μM. In addition, compounds **43**, **45**, **49**, and **50** (Table 4) were also effective inhibitors at low micromolar concentrations. The superimposition of the appropriate conformation of the three oxygens of compounds **26**, **32**, and **34** on pharmacophore 1 gave an rms value of 0.498 Å, and superimposition of the appropriate oxygens of compounds **45**, **49**, and **50** on pharmacophore 2 gave an rms value of 0.545 Å.

Probing the Integrase Binding Sites for Lead Compounds. During viral infection, integrase catalyzes two consecutive reactions. Initially, it processes linear viral DNA by removing two nucleotides from each 3′-end, leaving the recessed 3′-OH termini. This is followed by transesterification of phosphodiester bonds in which a host DNA strand is cut and the 5′-end of the cut is joined to a processed viral 3′-terminus. These two steps, known as 3′-processing and DNA strand transfer, can be readily measured in an *in vitro* assay employing purified recombinant integrase and a 21-mer duplex oligonucleotide corresponding to the U5-end of the HIV LTR sequence (Figure 5A). A representative gel illustrating inhibition of both 3′-processing and strand transfer reactions by compounds **34**, **26**, **49**, and **32** is shown in Figure 5B. Since inhibition of the strand transfer step could arise due to inhibition of the 3′-processing, we used an assay specific for strand transfer in which a "precleaved" substrate containing a 19-mer oligonucleotide lacking the terminal GT dinucleotide annealed to the complementary 21-mer was employed as described in the Experimental Section. The results (Figure 5C) demonstrated that the same four compounds inhibited the strand transfer reaction with IC_{50} values of 2.8, 3.1, 4.3, and 0.97 μM, respectively. Thus, these compounds inhibited both steps of the integration reaction, the 3′-processing and strand transfer.

The 3′-processing reaction involves hydrolysis of a single phosphodiester bond 3′ of the conserved CA-3′ dinucleotide. However, in addition to this hydrolysis reaction, retroviral integrases can use glycerol or the hydroxyl group of the viral DNA terminus as the nucleophile in the 3′-processing reaction, yielding a glycerol esterified to the 5′-phosphate on a circular di- or trinucleotide, respectively[24−26] (Figure 6A). In order to examine the effect of several inhibitors on the choice of nucleophiles in the 3′-processing reaction, a substrate DNA labeled at the 3′-end was employed.[30] Compounds **34**, **26**, **49**, and **32** inhibited glycerolysis, hydrolysis, and circular nucleotide formation to essentially the same extent, as can be seen in Figure 6B. This indiscriminate block of nucleophilic attack in the 3′-processing reaction is consistent with the binding of these drugs at the HIV-1 integrase active site.

In addition to the 3′-processing and strand transfer reactions described above, integrase can also catalyze reversal of strand transfer, leading to the "disintegration" step shown in Figure 7A. For the disintegration reaction, an integrase deletion mutant (IN^{50-212}) can be used to probe the site of drug−enzyme binding. As shown in Figure 7B compounds **34**, **26**, **49**, and **32** inhibited disintegration by IN^{50-212} with IC_{50} values of 7.1, 12.0, 13.0, and 16.7 μM, respectively, implying that the binding of these inhibitors to the integrase core region is responsible for integrase inhibition. Similar inhibiton of the core integrase (IN^{50-212}) was observed with the lichen acids (Table 2).

The catalytic domain of HIV-1 integrase contains a conserved region, Asp-X_{39-58}-Asp-X_{35}-Glu (the D,D(35)E motif), in which the invariable Asp residues are located at positions 64 and 116 and the Glu residue is at position 152. It has also been well established that integrases require a divalent cation (manganese or magnesium) as a cofactor for catalysis and that the divalent metal ion probably binds to the D,D(35)E motif.[8,9,27] A recent report indicates that *in vitro* integrase assays can be performed when magnesium is substituted for manganese.[28] We compared the activity of several inhibitors in the presence of either manganese or magnesium and found that compounds **1**, **2**, **23**, **26**, **32**, and **34** exhibited comparable potencies with the presence of magnesium or manganese. Lichen acids are

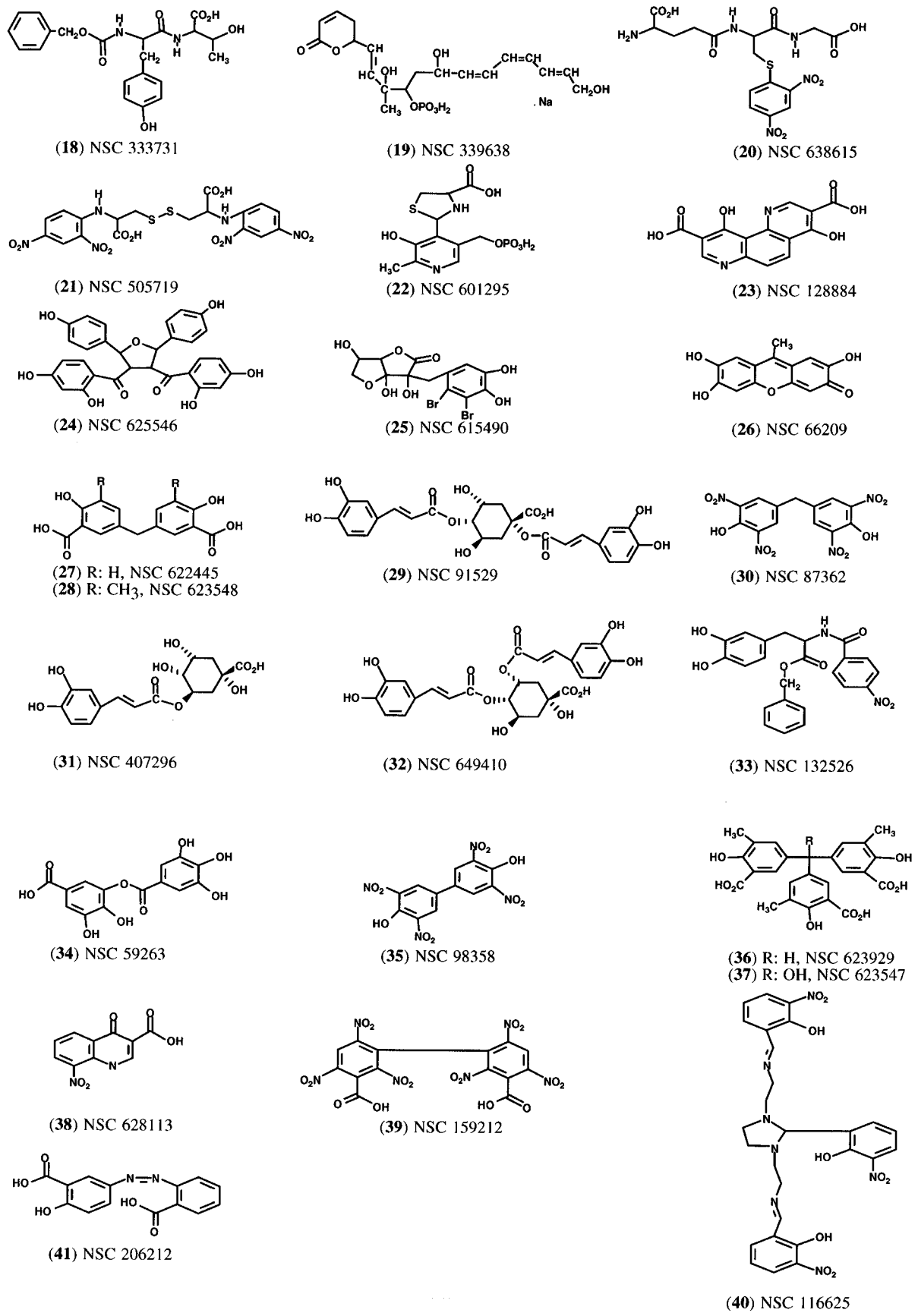

Figure 3. Structures containing pharmacophore 1 or pharmacophores 1 and 2.

found crystalline on the outer surface of the medullary hyphae on the mycobiont in the lichen thallus.[10,29] These acids can chelate various metals as nutrient, and in this position they are readily available for abstraction of minerals in their microenvironment. Since several of the potent inhibitors derived from the pharmacophore 1 and 2 search contain either a catechol and/or a carboxylic acid group, they can chelate metals.[30] It is

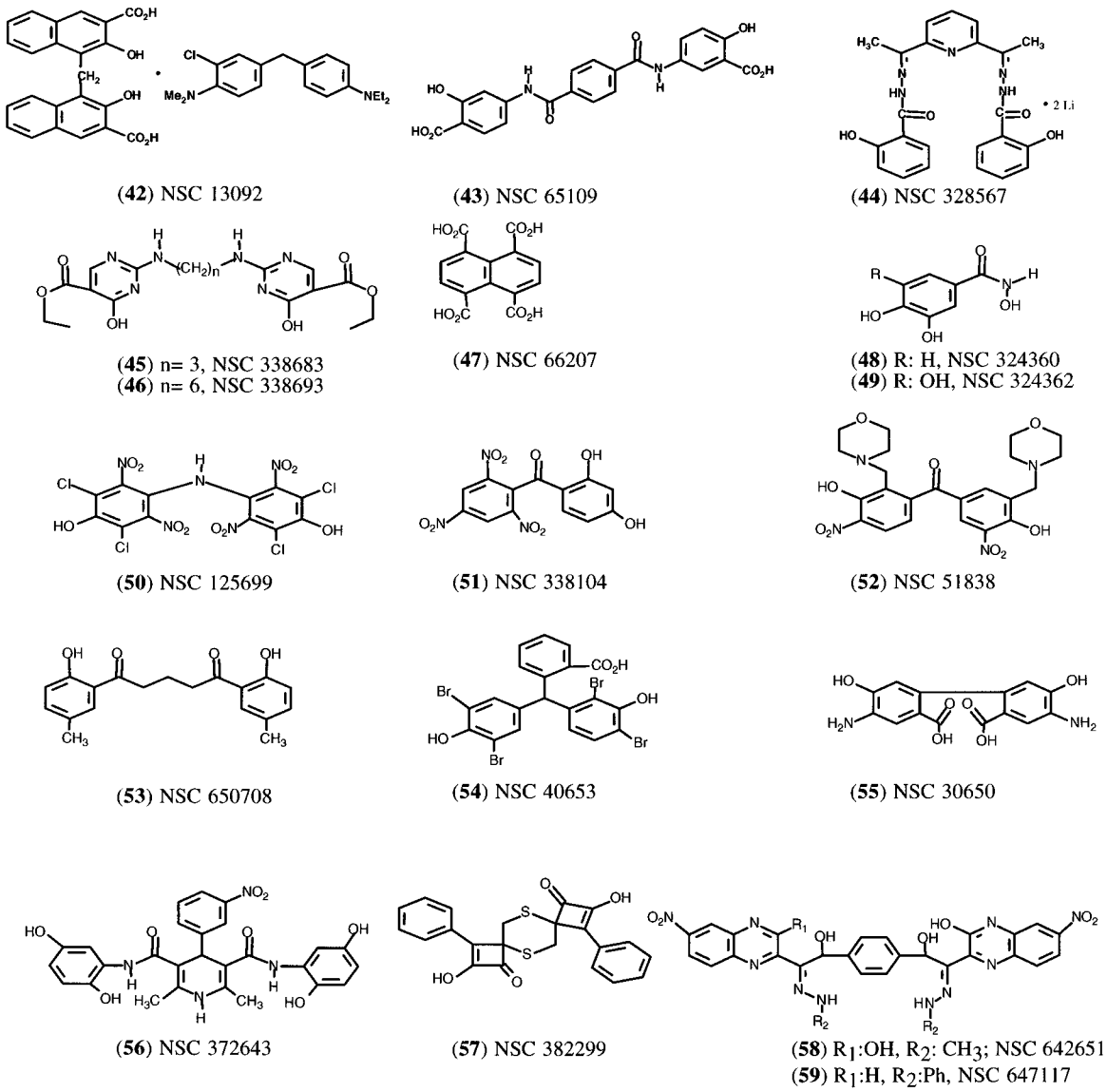

Figure 4. Structures containing pharmacophore 2.

tempting to speculate that some of the activity of the free acids or the catechol-containing compounds reported in the current study might be due to selective chelation of the divalent cations at the integrase catalytic site. Further studies will be required to elucidate this point.

Selectivity of Inhibition. In order to determine the selectivity of these compounds toward other integrases and other types of DNA binding enzymes, four representative compounds (**34**, **26**, **49**, **32**) were tested for inhibition of HIV-2 integrase and eukaryotic topoisomerase I. As illustrated in Figure 8 the IC_{50}s for 3′-processing and strand transfer for HIV-2 integrase were similar to those for HIV-1 (IC_{50} values for 3′-processing and strand transfer respectively were (**34**) 2.5 and 2.2, (**26**) 2.3 and 1.5, (**49**) 1.6 and 1.6, and (**32**) 0.5 and 0.5 μM). In order to determine the extent of topoisomerase I cleavable complex formation and inhibition of cleavable complex induced by camptothecin, a 33-mer oligonucleotide bearing a unique topoisomerase I cleavage site in its center was used. None of the four compounds (**34**, **26**, **49**, **32**) tested induced any detectable cleavable complex or inhibited the ability of topoisomerase I to generate camptothecin-mediated cleavable complex at concentrations below 10 μg/mL (data not shown).

Structure−Activity Relationships. Virensic acid (**1**) belongs to the depside family of compounds isolated from *Alectoria tortuosa* Merr.[14,31] It and its methyl ester derivative granulatine (**2**), as well as stictic acid (**15**) and chloroparellic acid (**17**), all exhibited similar potency against integrase with IC_{50} values of 3 μM for both 3′-processing and strand transfer reactions. Other depsidones such as physodic acid (**4**), norlobaric acid (**13**), salazinic acid (**14**), and the parellic acid (**16**) were also active at low micromolar concentrations. Open chain derivatives were generally inactive, implying the importance of a rigid polycyclic system for activity. All the acetylated or methylated derivatives were inactive; atranorin (**6**), for example, exhibited moderate activity, while its acetylated derivative (**7**) was inactive, suggesting the importance of free acids or hydroxyls for potency. Moreover, the oxime (**8**) of atranorin **(6)** was dramatically less potent and the corresponding phenylhydrazone (**9**) was completely inactive. Interestingly, however, physodic acid (**4**) showed very similar potency to that of its rearranged metabolite (**5**). Usnic acid (**11**),

Table 3. Anti-HIV-1 Integrase Activities of Pharmacophore 1- and Pharmacophore 1 and 2-Containing Compounds

			IC_{50} (μM)			
	% inhibition at 100 μg/mL[a]		3′-processing		strand transfer	
compd	3′-processing	strand transfer	expt 1	expt 2	expt 1	expt 2
18, NSC 333731	55.7	60.5				
19, NSC 339638	79.3	85.4	41.3	117.7	12.8	52.7
20, NSC 638615	31.5	30.0				
21, NSC 505719[b]	86.7	88.1	45.1	82.0	43.0	51.0
22, NSC 601295	61.8	74.1	91.7	75.1	93.4	31.7
23, NSC 128884	100	92.1	7.6	6.1	9.8	6.1
24, NSC 625546[b]	65.6	71.1	164.6	168.8	143	128.7
25, NSC 615490[b]	89.9	93	64.7	28.7	50.2	24.3
26, NSC 66209	93.3	93.1	1.1	1.5	1.1	0.9
27, NSC 622445[b]	93.1	95.8	91.3	51.8	60.4	57.8
28, NSC 623548[b]	89.9	93.5	61.8 ± 8.6		68.5 ± 13.5	
29, NSC 91529[b]	75.1	98.1	9.5 ± 2.0		7.8 ± 1.4	
30, NSC 87362[b]	92.7	94.3	49.7	61.8	43.9	40.5
31, NSC 407296	76.9	87.7	87.8	58.2	45.8	31.3
32, NSC 649410[b]	85.6	97.2	1.3	0.7	0.6	0.7
33, NSC 132526	64.9	91.8	163.4	253.0	55.4	76.6
34, NSC 59263[b]	90.2	89.5	0.9	1.5	0.6	0.6
35, NSC 98358[b]	92.6	90.0	61.7	65.0	57.3	35.8
36, NSC 623929[b]	93.8	97.6	20.4	<23.4	16.7	<23.4
37, NSC 623547[b]	93.7	96.7	35.8	32.8	21.8	23.0
38, NSC 62811 [b]	46.9	54.9				
39, NSC 159212[b]	97.9	94.1	18.2	46.1	15.0	43.3
40, NSC 116625[b]	83.5	74.8	52.7	60.8	36.7	49.5
41, NSC 206212	97.6	91.4	116	211.2	116	224

[a] Average of two independent experiments. Values with standard deviations are from at least three independent experiments. [b] Pharmacophore 1 and 2-containing compounds.

Table 4. Anti-HIV-1 Integrase Activities of Pharmacophore 2-Containing Compounds

			IC_{50} (μM)			
	% inhibition at 100 μg/mL[a]		3′-processing		strand transfer	
compd	3′-processing	strand transfer	expt 1	expt 2	expt 1	expt 2
42, NSC 13092	72.1	79.5	61.8	78.6	45.8	38.6
43, NSC 65109	92.4	82.6	23.6	25.2	16.3	20.0
44, NSC 328567	57.5	65.6				
45, NSC 338683	94.1	90.2	12.8	11.6	16.7	7.9
46, NSC 338693	28.4	34.9				
47, NSC 66207	84.1	73.8	121.7	174.0	176	182.2
48, NSC 324360	58.6	65				
49, NSC 324362	88.5	86.3	1.1	3.2	0.5	1.6
50, NSC 125699	94.7	71.2	11.0	8.3	4.4	4.0
51, NSC 338104	93.5	84.2	66.2	67.0	88.2	54.4
52, NSC 51838	41.1	49.3				
53, NSC 650708	32.8	43.3				
54, NSC 40653	93.3	83.9	43.7	25.6	38.8	27.2
55, NSC 30650	73.6	64.9	212.2		233.6	
56, NSC 372643	48.2	34.8				
57, NSC 382299	81.9	62.6	131.5	60.0	172.1	54.8
58, NSC 642651	34.0	32.5				
59, NSC 647117	24.7	37.0				

[a] Average of two independent experiments. Values with standard deviations are from at least three independent experiments.

with its abundant occurrence in many species of lichens and its use in several formulations, was also included in our assay against integrase. (+)-Usnic acid exhibited only moderate potency but its *N*-isonicotinoylhydrazone derivative (**12**) was more potent.

Previously it has been demonstrated that hydroxylated aromatics are effective inhibitors of integrase. From a representative list of structures containing pharmacophore 1, several carry a carboxyl or catechol group. Other compounds, however, such as **21**, **30**, **35**, **39**, and **40**, containing a nitro group on an aromatic ring, exhibited moderate activity against HIV-1 integrase, an observation consistent with our recent studies of anti-integrase activities of a series of diaryl sulfones.[32] Structures **42**−**59** comprise a diverse group of compounds containing pharmacophore 2. In this list, several compounds exhibited inhibitory activity at low micromolar concentrations, but 50% of the compounds were inactive; only compounds **43**, **45**, **49**, and **50** displayed significant activity. The moderate activity exhibited by nonacidic compounds **51** and **57** was intriguing. Several of the aurintricarboxylic acid monomers such as **27**, **28**, **36**, and **37**, which contain pharmacophore 1, were previously reported as inhibitors of HIV-1 integrase, and our results are largely in agreement with this previous report.[33] In addition, other aurintricarboxylic acid monomer analogs such as **30** and **54** also exhibited similar potency, suggesting that the electron-withdrawing nitro and bromo groups could replace a carboxylic acid group without loss in potency.

Relevance to Antiviral Activity. Among the lichen acids (compounds **1**−**17**) submitted for testing for anti-HIV-1 activity against CEM cells, only the first two compounds showed moderate activity. Compound **1**

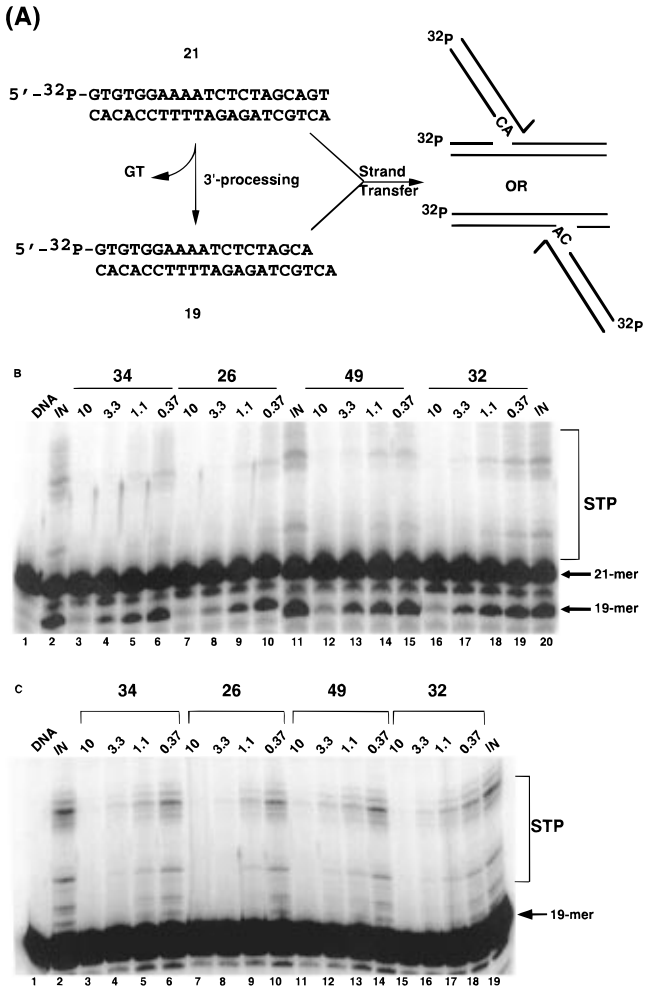

Figure 5. HIV-1 integrase catalytic assays. (A) A 21-mer blunt-end oligonucleotide corresponding to the U5-end of the HIV-1 proviral DNA, 5′-end-labeled with ^{32}P, is reacted with purified HIV-1 integrase. The initial step involves nucleolytic cleavage of two bases from the 3′-end, resulting in a 19-mer oligonucleotide. The second, or strand transfer step, involves joining this recessed 3′-end to the 5′-end of an integrase-induced break in another identical oligonucleotide, which serves as the target DNA. (B) Concentration-dependent inhibition of HIV-1 integrase by compounds **34**, **26**, **49**, and **32**. (C) Concentration-dependent inhibition of the HIV-1 integrase strand transfer step by compounds **34**, **26**, **49**, and **32**. A precleaved 19-mer oligonucleotide substrate was used instead of the original 21-mer blunt-end oligonucleotide as described in the Experimental Section. Drug concentrations (μg/mL) are indicated above each lane.

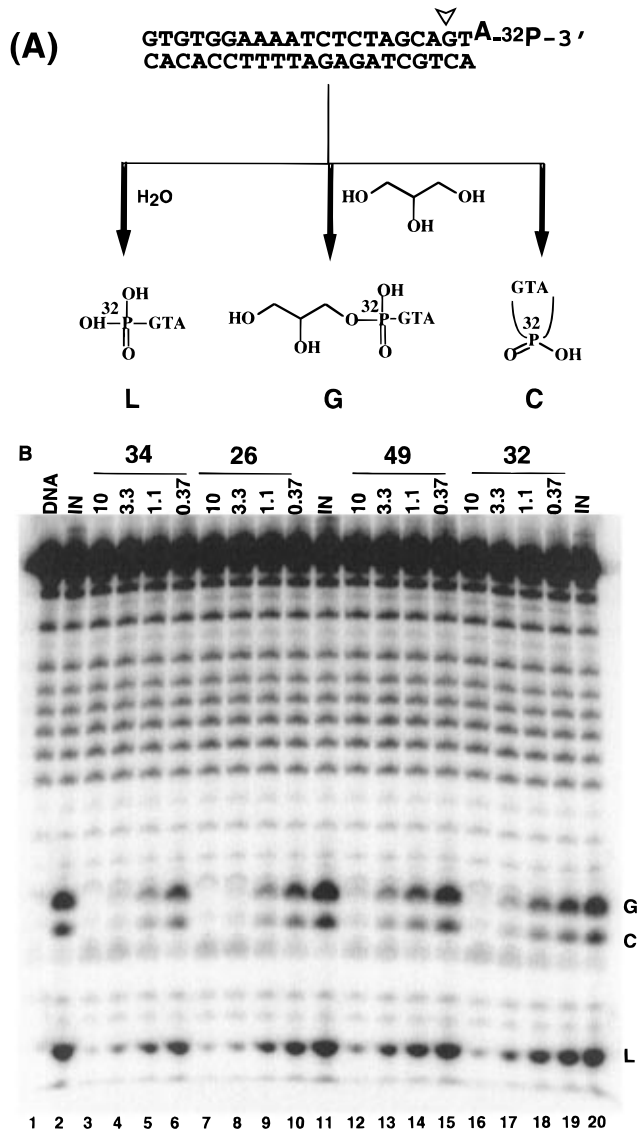

Figure 6. Site-specific cleavage of 3′-end-labeled substrate by HIV-1 integrase. (A) Nucleophilic substitution of water, glycerol, or the 3′-hydroxyl group of the viral DNA terminus yielding a linear trinucleotide with a 5′-phosphate (L), a linear trinucleotide with a glycerol esterified to the 5′-phosphate (G), and a cyclic trinucleotide (C). (B) Global inhibition of nucleophilic attack in the 3′-processing reaction by compounds **34**, **26**, **49**, and **32**. Drug concentrations (μg/mL) are indicated above each lane.

exhibited lower potency than its methyl ester analog (**2**), perhaps due to a decreased cellular uptake. On the other hand, derivatives **3**−**12** exhibited cytotoxicity in the range of 10−60 μM with no obvious protection against HIV-1 in infected CEM cells. From a set of integrase inhibitors containing pharmacophores 1 and 2, we tested compounds **19**, **23**, **26**, **29**, **31**, **32**, **36**, **43**, and **54** for antiviral activity in the NCI antiviral drug screen. Among this series, compound **36** exhibited moderate activity against infected CEM cells; however, its analog **54** was inactive. Compounds **23** and **31** showed no toxicity and no protection at 200 μM; on the other hand, **19** and **26** with IC_{50} values of 18 and 3.8 μM were toxic in uninfected cells. The caffeoylquinic acids **29** and **32** exhibited IC_{50} values of 30 and 158 μM, respectively, and no protection against HIV-1 in infected CEM cells. Compound **43** exhibited an IC_{50} value of 140.1 ± 55.0 μM and no protection.

It is interesting to note that many of the inhibitors discovered in this study contain ionizable carboxyl groups which in many instances would impede their cellular uptake. Although a variety of polyhydroxycarboxylates have been reported to inhibit HIV-1 replication, perhaps by interaction with gp120 and prevention of virus binding to the CD_4 receptor,[34,35] none has been shown to inhibit integrase function in the cell. For example, Mahmood et al.[35] reported that the 4,5-di-*O*-caffeoylquinic acid interacts irreversibly with gp120. More recently, however, Robinson et al.[36] have shown that 3,5-di-*O*-caffeoylquinic acid is an effective inhibitor of HIV-1 integrase *in vitro*, which is consistent with our results. Several caffeoylquinic acids including compound **32** reported in this study were shown to protect MT-2 cells in tissue culture.[36] The fact that the galloyl

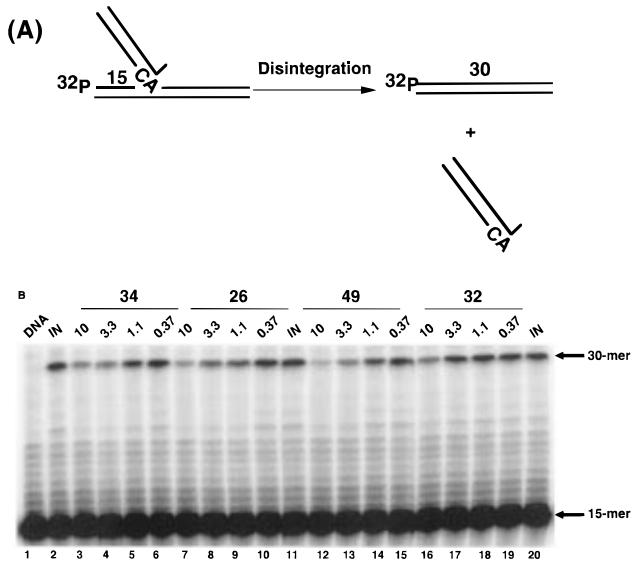

Figure 7. HIV-1 integrase disintegration assay using the core truncated mutant IN^{50-212}. (A) The substrate oligonucleotide mimics a strand transfer product, i.e., a Y oligonucleotide containing 15-mer oligonucleotide 5′-end-labeled with ^{32}P. HIV-1 integrase mediates the disintegration generating a 30-mer oligonucleotide. (B) Concentration-dependent inhibition of HIV-1 integrase-catalyzed disintegration by compounds **34**, **26**, **49**, and **32.** The electrophoresis in a 20% denaturing acrylamide gel shows the original 15-mer and the 30-mer disintegration products. Drug concentrations (μg/mL) are indicated above each lane.

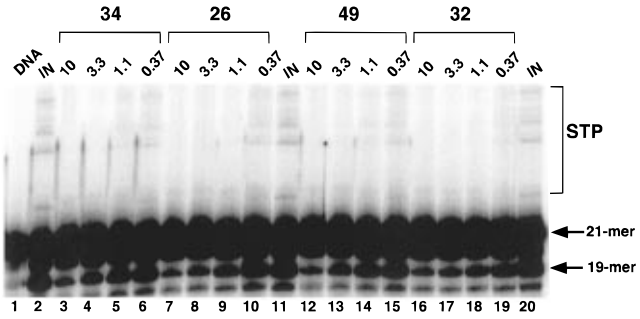

Figure 8. Concentration-dependent inhibition of HIV-2 integrase by compounds **34**, **26**, **49**, and **32** as described for Figure 5. Drug concentrations (μg/mL) are indicated above each lane.

derivative of **32**[37] was previously reported to possess several biological activities,[38] including inhibition of HIV-1 reverse transcriptase[39,40] as well as human DNA polymerases α, β, and γ,[37] calls into question the effectiveness of these compounds as selective inhibitors of cellular HIV-1 integrase. In addition, Mahmood et al.[35] have shown that 4,5-di- and 3,4,5-tri-*O*-caffeoylquinic acids were more effective inhibitors of HIV infection when they were present during virus adsorption than when added 1 or more hours after infection, suggesting that they influence binding of the virus to cells. They concluded that these compounds inhibit HIV replication by inhibiting the binding of gp120 to CD_4, reducing fusion of chronically infected and uninfected cells, and reducing syncytium formation.

Conclusions

Our efforts of combining rational drug selection of compounds with known activity against HIV-1 with molecular modeling and three-point pharmacophore searching of the NCI 3D database have enabled us to select a small set of structurally diverse HIV-1 integrase inhibitors from a large collection. Several of the potent inhibitors reported in this study are water-soluble and could serve as candidates for cocrystallization studies with wild-type HIV-1 integrase.

Experimental Section

Chemicals. All compounds were dissolved in DMSO, and all aliquots were also made in DMSO prior to each experiment. The stock solutions were kept at −20 °C.

Preparation of Oligonucleotide Substrates. The HPLC-purified oligonucleotides AE117, 5′-ACTGCTAGAGATTTTC-CACAC-3′, AE118, 5′-GTGTGGAAAATCTCTAGCAGT-3′, AE157, 5′-GAAAGCGACCGCGCC-3′, AE146, 5′-GGACGC-CATAGCCCCGGCGCGGTCGCTTTC-3′, AE156, 5′-GTGTG-GAAAATCTCTAGCAGGGGCTATGGCGTCC-3′, and RM22M, 5′-TACTGCTAGAGATTTTCCACAC-3′, were purchased from Midland Certified Reagent Co. (Midland, TX). Purified recombinant HIV-1 integrase deletion mutant, IN^{50-212}, was a generous gift of Drs. T. Jenkins and R. Craigie, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD. Dr. Craigie also provided the expression system for the wild-type HIV-1 integrase. To analyze the extents 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). To determine the extent of 30-mer target strand generation during disintegration AE157 was 5′-end-labeled and annealed to AE156, AE146, and AE117. 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.

To analyze the extents of site-specific cleavage of 3′-end-labeled substrate by HIV-1 integrase, AE118 was 3′-end-labeled using [γ-^{32}P]cordycepin triphosphate (DuPont-NEN) and terminal transferase (Boehringer Mannheim). The transferase was heat-inactivated, and RM22M was added to the same final concentration. The mixture was heated at 95 °C, allowed to cool slowly to room temperature, and analyzed on a G-25 spin column as before.

Integrase Assay. 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/mL bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethyl sulfoxide, 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, 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).

Disintegration reactions were performed as above with a Y oligonucleotide (i.e., the branched substrate in which the U5-end was "integrated" into target DNA). Gels were dried, exposed in a Molecular Dynamics phosphorimager cassette, and analyzed using a Molecular Dynamics phosphorimager (Sunnyvale, CA). 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 integrase, and integrase plus drug, respectively. IC_{50} values were determined by plotting the drug concentration versus percent inhibition and determining the concentration which produced 50% inhibition.

Topoisomerase Reactions. Reactions were performed in 10 μL of reaction buffer (0.01 M Tris-HCl, pH 7.5, 150 mM

KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 mg/mL bovine serum albumin) with the following duplex oligonucleotide substrate labeled with [α-^{32}P]cordycepin at the 3′-end of the upper strand (asterisk):[41]

5′-GATCTAAAAGACTTˇGGAAAAATTTTTAAAAAA*

ATTTTCTGAA-CCTTTTTAAAAATTTTTTCTAG-5′

This oligonucleotide contains a single topoisomerase I cleavage (caret on the upper strand). Approximately 50 fmol of oligonucleotide/reaction was incubated with 10 units of calf thymus DNA topoisomerase I (Gibco BRL, Gaithersburg, MD). Reactions were stopped by adding sodium dodecyl sulfate (0.5% as final concentrations). Proteolysis was halted by the addition of 36 μL of 2.5× loading buffer (98% formamide, 0.01 M EDTA, 1 mg/mL xylene cyanol, 1 mg/mL bromophenol blue).

Anti-HIV Assays in Cultured Cell Lines. The anti-HIV drug testing performed at NCI is based on a protocol described by Weislow et al.[42] In brief, all compounds were dissolved in dimethyl sulfoxide and diluted in 1:100 in cell culture medium. Exponentially growing T4 lymphocytes (CEM 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 (MOI ≃ 0.1), 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 tetrazolium 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 viewed microscopically for detection of viable cells and confirmation of protective activity.

Pharmacophore Identification. The 2D structure of each of these 16 HIV-1 integrase inhibitors was generated using the Edit/2D Draw program in Chem-X.[19] A 3D structure was created automatically by the program from the 2D structure and entered into a database. Initial use of the pharmacophore searching program upon this database gave ambiguous results, and so the data set was subjected to cluster analysis based upon 3D structural similarity as has been described elsewhere.[23] Three clusters were obtained, and each of these subsets was submitted to the pharmacophore search, find, and validate programs in Chem-X. A total of 11 distinct pharmacophores were obtained from this process, and each of them was used to search the original data set. The two pharmacophores shown in Figure 2 emerged as the best of the 11 pharmacophores, and these were then used in searches of the large database.

The location of one or the other of these two three-center pharmacophores in compounds **1**−**17** was determined as follows. Starting with the X-ray crystal structure data of compound **2**, molecular models of compounds **1**−**4** and **14**−**16**, which have similar ring skeletons, were built. All of the structures were minimized, and the global energy minima were identified by conformational searching in QUANTA. Pharmacophore mapping techniques were used to superimpose these low-energy conformations onto the two pharmacophores identified by Chem-X. All of the compounds, in some arbitrary conformation, could be mapped onto one or the other of the pharmacophores. The conformation involved was minimized, and its energy was then compared to that of the global minimum for the structure. The difference constitutes the energy penalty facing that compound as it attempts to assume a conformation containing the pharmacophore.

NCI 3D Database and Search Software. The details of the NCI 3D database and the Chem-X program used in both the 3D database build and search processes have been described elsewhere.[43] The current version of the NCI 3D database consists of 206 876 open and 201 036 discrete (proprietary) structures, for a total of 407 912 structures. All searches reported in this paper were conducted on only the open part of the database. The conformationally flexible search algorithm implemented in Chem-X (July 1994 version, running on a SGI workstation) was employed. For flexible compounds, multiple conformations were generated and analyzed during both building and searching of the database. Searches were conducted with the tolerances shown in Figure 2.

Molecular Modeling. All molecular modeling studies were performed with the QUANTA[44] 4.0/CHARMm 2.2[45] molecular modeling package, running on a Silicon Graphics IRIS Indigo workstation. Energy minimization was typically computed with 5000 iterations or until convergence (defined as an energy gradient of 0.001 kcal/mol or less), using an adjusted basis Newton−Raphson algorithm as implemented in CHARMm. The structures of compounds were built using the ChemNote module with QUANTA and energy-minimized using CHARMm. Conformational searches were conducted using either the systematic or the Monte Carlo random search algorithms implemented in QUANTA.

Acknowledgment. The assistance of the staff of the Drug Synthesis and Chemistry Branch, National Cancer Institute, is gratefully acknowledged. We also thank Drs. T. Jenkins and R. Craigie (Laboratory of Molecular biology, NIDDK, NIH) for the mutant HIV-1 integrase proteins and the expression system for wild-type HIV-1 integrase and Dr. K. Kohn for stimulating discussion and the preparation of this manuscript.

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JM960759E