Lamellarin α 20-Sulfate, an Inhibitor of HIV-1 Integrase Active against HIV-1 Virus in Cell Culture

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HIV-1 integrase is an attractive target for anti-retroviral chemotherapy, but to date no clinically useful inhibitors have been developed. We have screened diverse marine natural products for compounds active against integrase in vitro and found a series of ascidian alkaloids, the lamellarins, that show selective inhibition. A new member of the family named lamellarin α 20-sulfate (**1**), the structure of which was determined from spectroscopic data, displayed the most favorable therapeutic index. The site of action of lamellarin α 20-sulfate on the integrase protein was mapped by testing activity against deletion mutants of integrase. Inhibition of isolated catalytic domain was detectable though weaker than inhibition of full length integrase; possibly lamellarin α 20-sulfate binds a site composed of multiple integrase domains. Lamellarin α 20-sulfate also inhibited integration in vitro by authentic HIV-1 replication intermediates isolated from infected cells. Lamellarin α 20-sulfate was tested against wild type HIV using the MAGI indicator cell assay and found to inhibit early steps of HIV replication. To clarify the inhibitor target, we tested inhibition against an HIV-based retroviral vector bearing a different viral envelope. Inhibition was observed, indicating that the HIV envelope cannot be the sole target of lamellarin α 20-sulfate in cell culture. In addition, these single round tests rule out action against viral assembly or budding. These findings provide a new class of compounds for potential development of clinically useful integrase inhibitors.

Introduction

HIV encodes three enzymes, reverse transcriptase (RT), protease (PR), and integrase (IN). Inhibitors of the first two have been extremely useful for treating HIV-infected people, particularly when used in combination.[1,2] Integrase is also a promising target, since there are no similar proteins known to be important for normal function of cells. Thus inhibitors of integrase might be relatively nontoxic. Despite extensive efforts, no clinically useful integrase inhibitors have yet been developed.[3−6]

Integrase protein carries out the intial DNA breaking and joining reactions responsible for the attachment of HIV cDNA to host DNA. Prior to integration, two nucleotides are removed from each 3′ end in the linear cDNA precursor.[7−12] This reaction may be important for preparing a defined substrate for subsequent reaction steps, since the reverse transcriptase often adds non-templated bases to the 3′ ends of unintegrated cDNAs.[13,14] The recessed 3′ ends are then joined to 5′ protruding ends of breaks made in the target DNA.[11,15,16] The remaining DNA strands are then attached, probably by the action of host DNA repair enzymes, to complete formation of an integrated provirus. Integration is a required step in retroviral replication, as indicated, for example, by the finding that HIV mutants defective in integration cannot replicate.

In vivo, integrase is assembled with the viral cDNA and other proteins in large complexes called preintegration complexes (PICs). PICs can be obtained by lysing cells freshly infected with a retrovirus, and PICs can carry out integration into DNA targets in vitro.[17−19] HIV-1 PICs contain proteins in addition to integrase, including the viral matrix and reverse transcriptase, a host protein HMG I(Y), and probably other proteins as well.[14,20−22] Stable DNA−protein complexes are present at the ends of the viral cDNA, likely reflecting a productive association with integrase.[14,23]

Many studies indicate that purified integrase protein can be inhibited by diverse compounds, complicating the identification of the most promising leads.[24−44] However, assays with preintegration complexes are more resistant to inhibition and display a response that more closely matches the response of virus in vivo.[36]

Here we describe lamellarin α 20-sulfate (**1**), a new marine natural product with inhibitory activity against integrase, PICs, and HIV virus in cell culture. Previous work has identified a number of sulfated marine natural products that inhibit HIV-1 replication,[5,46] and sulfated molecules have been identified as integrase inhibitors.[29,47] Lamellarin α 20-sulfate (**1**) is of special interest, however, for representing a new chemical class of sulfated compounds that are active against PICs as well

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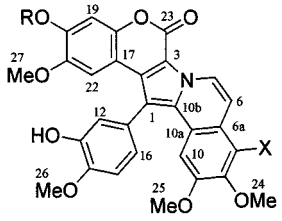
as purified integrase protein and virus. We also introduce assays using HIV-based vectors to help specify the viral protein targeted by the drug action.

Results

Isolation of Lamellarins. Prior chemical studies have shown marine ascidians (tunicates) to be a rich source of a group of DOPA-(2-amino,3-(3′,4′-dihydroxyphenyl)propionic acid)-derived pyrrole alkaloids, called lamellarins. These compounds were first isolated from the prosobranch mollusc *Lamellaria* sp.[48] and subsequently obtained from the didemnid ascidians.[49−51] Some lamellarin alkaloids have demonstrated significant cytotoxicity and immunomodulatory activities.[48,50]



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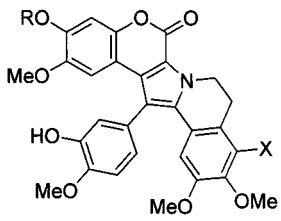

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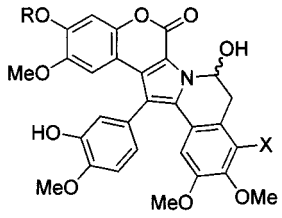

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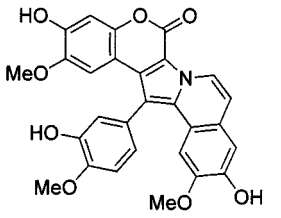

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Structural Elucidation of Lamellarin α 20-Sulfate (1). Lamellarin α 20-sulfate (**1**) was isolated as an optically inactive white solid. The molecular formula, $C_{29}H_{22}NO_{11}SNa$, which requires 19 degrees of unsaturation in addition to the sulfate, was determined by high-resolution FAB mass measurement in conjunction with the ^1H and ^{13}C NMR data. The IR bands at 3425, 1700, 1275, and 1050 cm^{-1} are indicative of phenolic hydroxy, aromatic ester, and sulfate groups, respectively. The UV absorptions at 386, 364, 338 (sh), 324 (sh), 303, 276, and 204 nm indicated a more extensively conjugated chromophore than was observed for lamellarins W and X.[52] The ^1H NMR spectrum in DMSO-d_6 (Table 1) contained signals at δ 9.08 (d, 1 H, $J = 7.5$ Hz) and 7.34 (d, 1 H, $J = 7.5$ Hz) that are typical of an isoquinoline system. The ^1H NMR spectrum also revealed the presence of four methoxyl signals at δ 3.37, 3.38, 3.87, and 3.88 and signals at 7.02 (d, 1 H, $J = 2$ Hz), 7.03 (dd, 1 H, $J = 8$, 2 Hz), and 7.25 (d, 1 H, $J = 8$ Hz), corresponding to a 1,3,4-trisubstituted benzene ring, four singlets at 6.80 (s, 1 H), 7.20 (s, 1 H), 7.43 (s, 1 H), and 7.56 (s, 1 H), and one D_2O exchangeable signal at 9.45 (s, 1 H). The foregoing spectral data indicated that sulfate **1** is a lamellarin alkaloid that differs from lamellarin U 20-sulfate (**3**)[52] by the addition of a 5,6 double bond.

Table 1. NMR Data for Lamellarin α 20-Sulfate (**1**) in DMSO-d_6

				HMBC (C no.)	
C no.	δ_C	δ_H	mult, J (Hz)	$J = 6$ Hz	$J = 8$ Hz
1	111.3				
2	128.1				
3	107.1				
5	122.2	9.08	d, 1 H, 7.5	3, 6a, 6, 10b	
6	113.0	7.34	d, 1 H, 7.5	5, 7, 10a	7, 10a
6a	124.4				
7	108.3	7.43	s, 1 H	6, 8, 9, 10a	6, 8, 9, 10a
8	150.0				
9	149.0				
10	104.8	7.20	s, 1 H	6a, 8, 9, 10a, 10b	6a, 8, 9, 10b
10a	118.3				
10b	133.5				
11	127.0				
12	118.1	7.02	d, 1 H, 2	1, 14, 16	1, 16
13	148.0				
14	147.7				
15	113.7	7.25	d, 1 H, 8	11, 13, 14, 16	11, 14
16	122.0	7.03	dd, 1 H, 8, 2	14	
17	111.6				
18	145.1				
19	108.9	7.56	s, 1 H	17, 18, 20, 21	21
20	143.3				
21	146.7				
22	105.7	6.80	s, 1 H	2, 17, 18, 20, 21	2, 18, 20, 21
23	154.3				
24	55.7	3.88	s, 3 H	8	8
25	54.5	3.38	s, 3 H	9	9
26	56.1	3.87	s, 3 H	14	14
27	55.1	3.37	s, 3 H	21	21
13-OH		9.45	s, 1 H	13	

The positions of the methoxyl, the phenolic hydroxyl, and the sulfate groups were determined by the analysis of HMBC and HMQC experiments (Table 1). The H-19 signal at δ 7.56 (δ_C 108.9), which is shifted downfield from ca. 6.8 in the corresponding phenol by the adjacent sulfate group, showed long-range correlations to C-17, C-18, C-20 and C-21, while the proton signal at 6.80 (δ_C 105.7) was correlated to C-2, C-17, C-18, C-20, and C-21, and the methoxy signal at 3.37 showed a three-bond correlation to C-21. In the trisubstituted aromatic ring, the methoxy signal at δ 3.87 showed a three-bond correlation to C-14 and the exchangeable phenolic signal at 9.45 was correlated to C-13. The proton signal at δ 9.08 (δ_C 122.2) showed long-range correlations to C-3, C-6a, C-6, and C-10b, and the signal at 7.34 (δ_C 113.0) showed long-range correlations to C-5, C-7, and C-10a, indicating that the additional double bond was situated at the 5,6 position. The aromatic proton at δ 7.43 (δ_C 108.3), which was not coupled to any other signal, can only be placed at C-7 and showed long-range correlations to C-6, C-8, C-9, and C-10a. The aromatic proton signal at δ 7.20 (δ_C104.8) showed long-range correlations to C-6a, C-8, C-9, C-10a, and C-10b. The HMBC correlations also established the substitution pattern of the remaining two methoxyl groups. The NMR assignments for lamellarin α 20-sulfate (**1**) were in good agreement with the literature values for similar compounds.[52]

Inhibitor Screening Assaying HIV-1 Integrase in Vitro. In an effort to identify new compounds with antiviral activity, a library of chemicals derived from marine organisms was tested for inhibition of several viral enzymes in vitro. Compounds were titrated into assays containing purified HIV-1 integrase proteins and DNAs mimicing one end of the unintegrated viral DNA and target DNA (Figure 1A). Inhibition was revealed

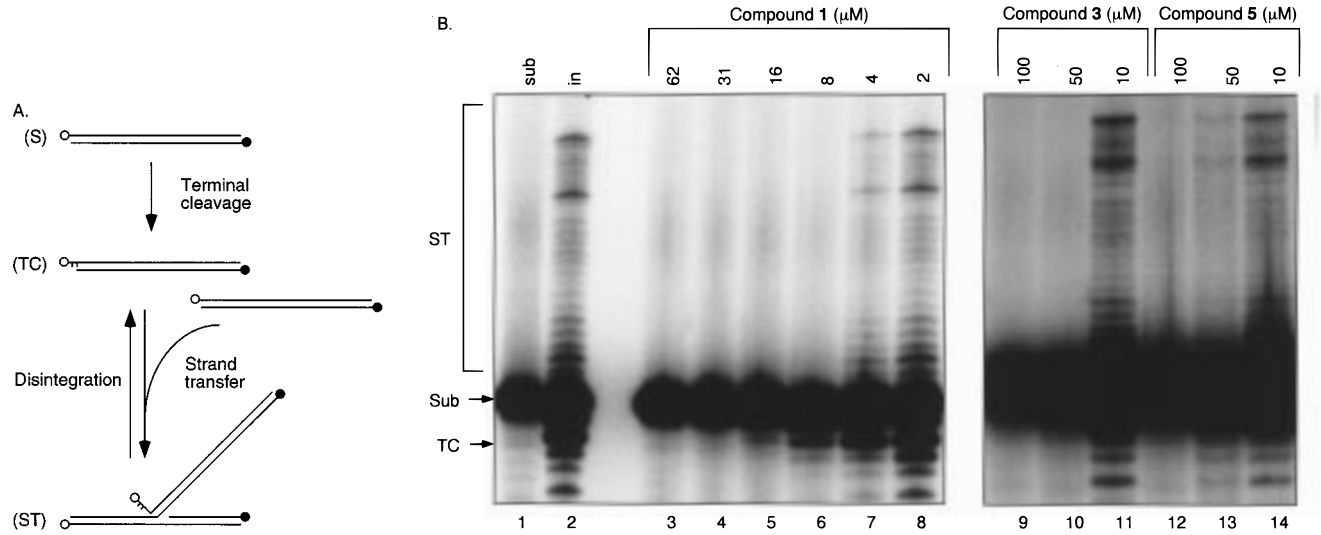

Figure 1. Inhibition of purified HIV-1 integrase by lamellarins. (A) Diagram of the DNA breaking and joining reactions catalyzed by purified HIV-1 integrase in vitro. A double-stranded oligonucleotide matching one end of the unintegrated viral cDNA (indicated as S for substrate) can be cleaved by integrase to yield the terminal cleavage (TC) product. The recessed 3′ end generated thereby can then be joined to another duplex oligonucleotide to yield a strand transfer (ST) product. Note that different strand transfer products will contain different "branch points" in the Y-shaped DNA molecule. DNA 5′ ends are shown as balls. (B) Assays of inhibition by lamellarins. Reactions were carried out in the presence of integrase and an end-labeled substrate oligonucleotide, then separated by electrophoresis, and visualized by autoradiography. Lane 1 (sub): unreacted substrate. Lane 2 (in): positive control with integrase. Lanes 3−8: titration of lamellarin α 20-sulfate (**1**), concentrations as indicted above the gel. Lanes 9−12: titration of lamellarin U 20-sulfate (**3**). Lanes 12−14: titration of lamellarin V 20-sulfate (**5**). Sub indicates unreacted substrate, TC indicates terminal cleavage product, and ST indicates strand transfer product.

Table 2. Inhibitory Activities of Selected Lamellarins

	integration IC50 (μM)					
	integrase					
compound	T. Cl.	Str. Tr.	PIC	live virus	LD_{50} (cytotox) (μM)	counterscreen MCV TOPO (μM)
lamellarin α 20-sulfate (**1**)	16	22	88	8	274	- @ 170
lamellarin U 20-sulfate (**3**)	34	25	ND	12	145	- @ 500
lamellarin V 20-sulfate (**5**)	73	51	ND	20	130	- @ 500
lamellarin T (**6**)	27	24	ND	18	27	- @ 100
lamellarin W (**7**)	19	14	ND	20	28	- @ 170
lamellarin N (**8**)	18	19	ND	1	5	- @ 100

by a reduction in the accumulation of the terminal cleavage and strand transfer products (Figure 1B). Autoradiograms were quantitated by PhosphorImager. IC_{50} values for the lamellarins tested are summarized in Table 2.

Lamellarin α 20-sulfate (**1**) was one of the most active compounds in the series, inhibiting the integrase terminal cleavage activity with an IC_{50} of 16 μM and the strand transfer activity with an IC_{50} of 22 μM. The other lamellarin sulfates tested were also inhibitory.

Inhibitor Screening Assaying MCV Topoisomerase and the Restriction Enzyme *Hin*dIII in Vitro. In our initial screen, candidate compounds and extracts were also tested for inhibition of the type 1B topoisomerase of molluscum contagiosum virus (MCV).[53] In this context the MCV assay serves as a counterscreen for candidate integrase inhibitors, though inhibitors of this enzyme are of interest in their own right. The relaxation activity of MCV topoisomerase was assayed by monitoring reduction in the mobility of supercoiled DNA plasmids upon relaxation using agarose gel electrophoresis. None of the lamellarins inhibited MCV topoisomerase at the concentrations tested (Table 2).

As a second in vitro counterscreen, reactions were carried out in which lamellarin α 20-sulfate (**1**) was titrated in reactions containing the restriction enzyme *Hin*dIII and inhibition quantitated. Lamellarin α 20-sulfate did not inhibit the reaction at the highest concentration tested (50 μM, data not shown).

Lack of Potent Inhibition of the Integrase Catalytic Domain. To begin to specify the mechanism of action of lamellarin α 20-sulfate (**1**), inhibition was tested against the isolated integrase catalytic domain. This domain, comprised of residues 50−212 of the intact protein, is defective for normal integration, but it is capable of catalyzing a reversal of the forward reaction, termed "disintegration".[54,55] To determine whether lamellarin α 20-sulfate was acting against the catalytic domain specifically, the effects on disintegration by 50−212 were tested (Figure 2). Lamellarin α 20-sulfate was found to display inhibitory activity, establishing that it is active against the catalytic domain, but with a high IC_{50} (64 μM). For comparison, the IC_{50} for inhibition of disintegration by the full length protein was determined and found to be only 7 μM. In our experience this is the first example of isolated catalytic domain displaying diminished sensitivity to an inhibitor compared with wild type integrase.[36]

Inhibition of PICs by Lamellarin α 20-Sulfate (1). Since many inhibitors of purified integrase protein do not inhibit integration by PICs isolated from HIV-1 infected cells,[36] lamellarin α 20-sulfate (**1**) was tested

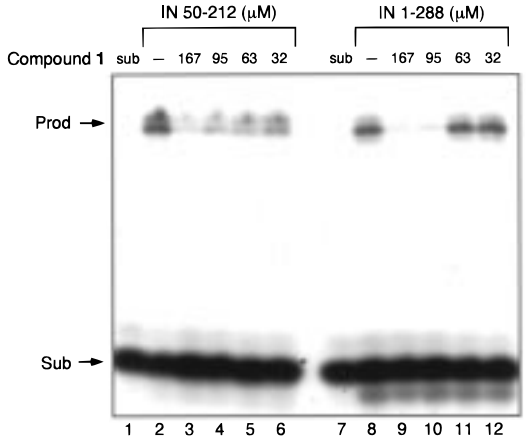

Figure 2. Inhibition of disintegration by wild type integrase and isolated catalytic domain by lamellarin α 20-sulfate (**1**). Reactions were carried out using as substrate the Y-shaped molecule indicated by (ST) in Figure 2A end-labeled on the 5′ end at the lower right. Reactions were separated by electrophoresis and products visualized by autoradiography. Lane 1: unreacted substrate. Lane 2: positive control with purified catalytic domain. Lane 3−6: titration of **1** into reactions with purified catalytic domain. Lane 7: unreacted substrate. Lane 8: reaction with purified integrase protein. Lanes 9−12: titration of **1** into reactions with purified integrase protein. Sub indicates substrate, and Prod indicates disintegration product.

in this more stringent assay. Preintegration complexes were isolated from cells infected with HIV-1 as previously described[19] and used as a source of integration activity in vitro. PICs were mixed with a naked DNA target and incubated at 37 °C for 1 h to allow integration to take place. Reaction products were then deproteinized, separated by electrophoresis on agarose gels, blotted to a solid support, and visualized by hybridization with a labeled DNA probe complementary to the HIV cDNA (Figure 3). Integration activity is visualized by the formation of a new product dependent on addition of target DNA. Addition of lamellarin α 20-sulfate to such reactions revealed inhibition of product formation with an IC_{50} of 88 μM (Figure 3B, lane 5).

Inhibition of HIV Replication by Lamellarin α 20-Sulfate (1). Inhibition of the early steps of replication of HIV-1 in cell culture by lamellarin α 20-sulfate was next assessed. HIV-1 provirus formation was monitored using the "MAGI" assay.[56] The indicator cells used contain DNA encoding the gene for β-galactosidase linked to the HIV-1 transcriptional control signals within the viral long terminal repeat (LTR). Normally the β-galactosidase gene is inactive in these cells, since the HIV LTR requires the viral encoded Tat protein for transcriptional activity. Infection of the MAGI cells with wild type HIV-1 provides a source of Tat, which activates the promoter and allows β-galactosidase protein to accumulate. Staining of cells with the chromogenic substrate X-gal reveals infected centers as clusters of blue cells. For quantitating inhibition of provirus formation, the number of infected centers in standardized infection is compared in the presence of increasing concentrations of inhibitor. Note that this test monitors only a subset of the steps in the viral replication cycle, from initial fusion through integration and gene expression.

Addition of lamellarin α 20-sulfate (**1**) blocked formation of infected centers (Figure 4A). Quantitation of the relative titer yielded an IC_{50} of 8 μM. There was no measurable toxicity of lamellarin α 20-sulfate in this range.

Inhibition of Provirus Formation by an HIV-Based Vector Bearing a Different Viral Envelope. An artifact in tests in vivo could arise if lamellarin α 20-sulfate (**1**) inhibited fusion of the virus with the cell membrane instead of inhibiting integrase. In fact, some inhibitors active against purified integrase have been found previously to inhibit fusion.[29,30] To begin to address this possibility, inhibition was monitored using viral particles containing HIV-1 cores contained within membranes bearing a different viral envelope protein. Such viral "pseudotypes" can be formed by expressing the HIV gag and pol regions, the latter of which encodes integrase, in a cell also expressing a packagable RNA and the envelope protein of a different virus. Particularly useful for this purpose has been the G envelope protein of vesicular stomatitis virus (VSV). Pseudotypes were formed composed of HIV-1 cores within membranes bearing the VSV-G envelope. As a marker for infection, a derivative of the HIV RNA was packaged that transduced the gene for green fluorescent protein (gfp). Thus infected cells can be visualized by fluorescence of gfp produced in the infected cell.

Inhibition of provirus formation was monitored by titrating lamellarin α 20-sulfate (**1**) into test infections and monitoring the amount of total fluorescence after 2 days of growth. Figure 4B shows that the number of fluorescent centers was greatly reduced by the presence of lamellarin α 20-sulfate. Representative fields are shown in Figure 4C. Quantitation of repeated experiments yielded an IC_{50} of 62 μM.

Toxicity of Lamellarins. The toxicity of the lamellarin sulfates was tested using the MTT cytotoxicity assay (Table 2). In this method, HeLa cells in culture are exposed to various concentrations of the cytotoxic agent under investigation. After 3 days, the culture supernatant is removed and the MTT dye added, which is metabolized by living cells to yield a blue color. Thus titration of the inhibitor under investigation can yield a LD_{50} (dose at which the signal is reduced 50% due to cell death).[57] Lamellarin α 20-sulfate (**1**) displayed the least toxicity, with an LD_{50} of 274 μM. Note that the antiviral IC_{50} was 8−62 μM. The other sulfated lamellarins **3** and **5** were toxic in the 100 μM range, while the lamellarins **6**−**8** that lacked the sulfate ester were more toxic.

Discussion

Here we describe the identification of a new HIV-1 integrase inhibitor, lamellarin α 20-sulfate (**1**). This compound is of particular interest since it is active against both preintegration complexes in vitro and HIV-1 virus in cell culture.

Structurally, lamellarin α 20-sulfate (**1**) resembles several classes of previously described inhibitors of integrase. Embedded within the lamellarins is a coumarin moiety. Coumarins have been previously found to be active as integrase inhibitors, though the 1,2-dihydroxy substituents previously found to be important[37] are not present in the lamellarins. Polyhydroxylated phenols have been widely reported to inhibit

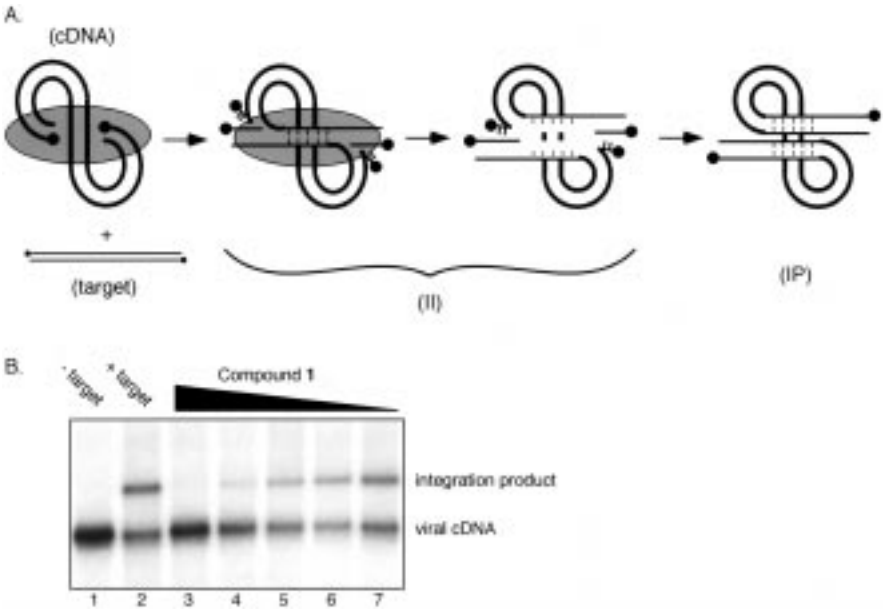

Figure 3. Inhibition of integration by purified PICs by lamellarin α 20-sulfate (**1**). (A) Diagram of the integration reaction with purified preintegration complexes. HIV cDNA bound with integrase and other viral proteins integrates cDNA 3′ ends into a target DNA in vitro to yield an integration intermediate (II). Subsequent repair of this intermediate, probably by host DNA repair proteins, yields the integration product (IP). Reactions with preintegration complexes in vitro yield the integration intermediate. (B) Assay of inhibition of integration by PICs by **1**. Presented is an autoradiogram of a Southern blot monitoring integration by HIV-1 PICs. Bands corresponding to the unreacted viral cDNA and integration product are as marked. Control reactions were carried out in the absence (lane 1) or presence (lane 2) of target DNA. Compound **1** was tested at 250 μM (lane 3), 125 μM (lane 4), 62.5 μM (lane 5), 31 μM (lane 6), or 15 μM (lane 7). Analysis of band intensities by PhosphorImager yielded an IC_{50} of 88 μM.

integrase, though the utility of this series has been questioned due to its potential toxicity.[32,36,38,58,59] Lamellarin α 20-sulfate contains several aromatic structures though no polyhydroxylated phenyl moieties. Sulfated compounds have also been found to be inhibitory in some cases (e.g., suramin[29]). Possibly the sulfate group resembles a DNA phosphate normally contacted by integrase. In many cases negative charges can be detrimental to inhibitor function, since polyanions do not enter cells efficiently. However, the lamellarins contain only the single sulfate, which judging by the efficacy in cell culture does not block bioavailability.

We tested inhibition of viral replication using the single-step MAGI assay. This method monitors the viral replication steps from initial infection through to the expression of a marker gene from the integrated provirus. Thus we can be confident that lamellarin α 20-sulfate (**1**) is not acting in cell culture by inhibiting viral assembly or budding. The use of a single-step assay is more stringent than the popular MTT or XTT assays, which monitor viral replication over several replication cycles and have the potential to magnify small effects.

One source of confusion in studing integrase inhibitors has been the finding that some compounds can act against integrase protein in vitro but against other targets in vivo. Particularly common has been cross-inhibition of initial cell binding and membrane fusion in vivo. To argue against this possibility, we have tested, in parallel, inhibition of HIV and of an HIV-based vector bearing the vesicular stomatitis envelope protein (VSV-G) in place of the HIV envelope.[60] We found that lamellarin α 20-sulfate (**1**) inhibits both viruses, with IC_{50}s of 8 μM for HIV and 62 μM for HIV bearing the VSV-G envelope (an "HIV VSV-G pseudo-type"). This supports the model that lamellarin α 20-sulfate is not acting exclusively on the HIV envelope. The difference in IC_{50} values could be explained by partial activity against HIV envelope or differences in the two assays. For example, the efficiency of expression of the marker gene transduced by either of the two viruses could differ and quantitatively affect the apparent degree of inhibition. Regardless, lamellarin α 20-sulfate is clearly acting against the virus in cell culture in a way that cannot be fully explained by action against the HIV envelope protein. Use of HIV-based vectors should prove generally useful in characterizing inhibitors in vivo.

The relatively weak inhibition of purified catalytic domain by lamellarin α 20-sulfate (**1**) was surprising, since most compounds studied previously have acted equivalently against the catalytic domain and complete protein. This observation could be explained if lamellarin α 20-sulfate binds to both the catalytic domain and also other parts of integrase. According to this model, weak inhibition of the catalytic domain only would be a consequence of the presence of only a partial binding site for the drug. It will be of interest to map the binding site in more detail by assaying additional HIV integrase derivatives bearing deletions and point mutations.

In conclusion, our data indicate that lamellarin α 20-sulfate can inhibit integrase protein in vitro and viral replication in cultured cells. Lamellarin α 20-sulfate acts in a part of the viral life cycle consistent with inhibition of integration. Firm data arguing for inhibition of integrase in cultured cells awaits the isolation of viral mutants with reduced sensitivity to lamellarin α 20-sulfate. Mapping of such mutations to the integrase coding region could argue strongly for inhibition of integrase during viral replication.

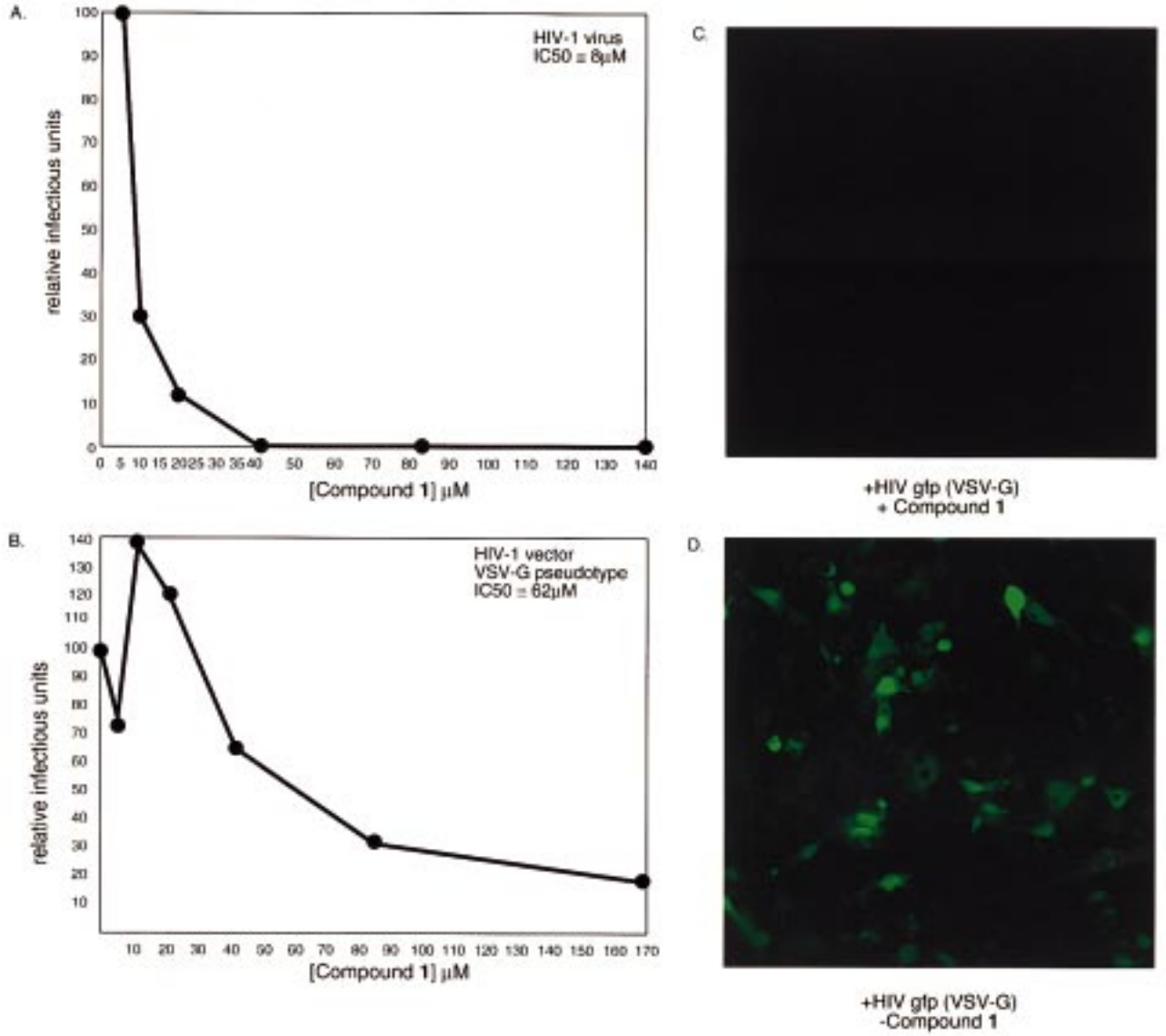

Figure 4. Inhibition of the early steps of HIV replication in cell culture by lamellarin α 20-sulfate (**1**). (A) Inhibition of formation of infected centers by wild type HIV measured in a MAGI assay. The 100% value represented 98 infected centers from 280 pg p24 capsid antigen. (B) Inhibition of formation of infected centers by an HIV-based VSV-G pseudo-type transducing gfp. The 100% value represented 56 infected centers in a typical field. Representative fields from cell cultures infected the HIV VSV-G pseudotype in the absence (C) or presence (D) of lamellarin α 20-sulfate.

Experimental Section

General Experimental Procedures. ^{13}C NMR spectra were recorded on a Varian Gemini 400 MHz spectrometer, and ^{1}H NMR, HMQC, and HMBC experiments were recorded on a Varian Inova 300 MHz spectrometer. Chemical shifts are reported in ppm based on $\delta_{TMS} = 0$, and coupling constants (J) are reported in hertz. High-resolution FAB mass spectra were run on a ZAB-E4F mass spectrometer at the regional mass spectrometry facility at UC Riverside. IR and UV spectra were recorded on Perkin-Elmer 1600 and Lambda 3B instruments, respectively.

Animal Material. The unidentified ascidian yielding lamellarin α 20-sulfate (IIC-197) was collected from the Arabian Sea near Trivandrum, India, by skin diving (−10 m) in January 1995. A voucher specimen (IIC-197) has been deposited at the National Institute of Oceanography, Goa, India.

Extraction and Isolation. The freshly collected specimens were soaked in MeOH at the collection site and stored until workup. The MeOH was decanted and concentrated in vacuo to obtain an aqueous suspension that was lyophilized to obtain a solid residue. The specimens (43 g dry wt) were then lyophilized, extracted with MeOH:CH_2Cl_2 (1:1, 2 × 700 mL), and concentrated to obtain a solid residue. The combined residues were extracted with MeOH (2 × 300 mL), and the concentrated extract (226 mg) was chromatographed on Sephadex LH-20 using 1:1 MeOH/CH_2Cl_2 as eluant to afford two major and one minor UV-active fractions. We had earlier reported that the two major UV-active fractions afforded lamellarin T, U (**3**), V (**5**), and Y 20-sulfates and lamellarins N (**8**), T (**6**), U, V, W (**7**), and X.[52] The minor UV-active fraction was subjected to flash chromatography on a reversed phase C-18 column using MeOH and H_2O as eluents followed by reversed phase HPLC on a preparative 60A C-18 column using CH_3CN/H_2O (1:4) as eluent to afford lamellarin α 20-sulfate (**1**, 10 mg, 0.025% dry wt) and lamellarin E 20-sulfate (**2**, 1 mg, 0.0025% dry wt), which is the 20-sulfate ester of the known metabolite lamellarin E (**4**).[49]

Lamellarin α 20-Sulfate (1): white solid, mp 145−148 °C; IR (AgCl) 3425, 1700, 1415, 1275, 1170, 1050, 835 cm^{-1}; UV (MeOH) 386 (ϵ 7000), 364 (ϵ 5500), 338 (sh), 324 (sh), 303 (ϵ 16 000), 276 (ϵ 19 500), 204 (ϵ 31 500) nm; ^{1}H NMR (300 MHz, DMSO-d_6) see Table 1; ^{13}C NMR (100 MHz, DMSO-d_6) see Table 1; HRFABMS m/z 592.0944 $(M - Na)^-$ (calcd for $C_{29}H_{22}NO_{11}S$, 592.0914).

Integration Assays. Integrase protein and isolated catalytic domain were purified and assayed essentially as described.[54,61] Quantiation of inhibition of reactions with purified integrase was performed as described.[6,12,36] Preparation and assay of PICs was carried out as described.[19,36] Assays of DNA relaxation by MCV topoisomerase and inhibition of relaxation were carried out as described.[53] Assays of inhibition of cleavage by *Hin*dIII were carried out as described.[59]

Assays of Inhibition of the Early Steps of HIV Infection in Cell Culture. Assays of infection by wild type HIV were carried out using p4-2 cells[62] according to the method described.[56] Assays of infection by HIV (VSV-G) pseudo-types were carried out essentially as described.[60] HIV (VSV-G) pseudo-types were produced from a viral producer cell line kindly given to us by Kafri and Verma (Salk Institute). Transduction of green fluorescent protein was scored using a Nikon Diaphot 300 epi-fluorescent microscope, and images were analyzed using OS-Slidebook software. Assays of cytotoxicity were carried out using MTT dye as described.[57]

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References

(1) Bushman, F. D.; Landau, N. R.; Emini, E. A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *95*, 11041−11042.
(2) Coffin, J. M.; Hughes, S. H.; Varmus, H. E. *Retroviruses*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1997.
(3) Plasterk, R. H. A. *Struct. Biol.* **1995**, *2*, 87−90.
(4) Coffin, J. M. In *Virology*; Fields, B. N., Knipe, D. M., Howley, R. M., Eds.; Lippincott-Raven Publishers: Philadelphia, 1996; Vol. 2, pp 1767−1848.
(5) Farnet, C. M.; Bushman, F. D. *AIDS* **1996**, *10* (Supplement A), 3−11.
(6) Chow, S. A. *Methods* **1997**, *12*, 306−317.
(7) Brown, P. O.; Bowerman, B.; Varmus, H. E.; Bishop, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2525−2529.
(8) Fujiwara, T.; Mizuuchi, K. *Cell* **1988**, *54*, 497−504.
(9) Katzman, M.; Katz, R. A.; Skalka, A. M.; Leis, J. *J. Virol.* **1989**, *63*, 5319−5327.
(10) Sherman, P. A.; Fyfe, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5119−5123.
(11) Craigie, R.; Fujiwara, T.; Bushman, F. *Cell* **1990**, *62*, 829−837.
(12) Bushman, F. D.; Craigie, R. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1339−1343.
(13) Patel, P. H.; Preston, B. D. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 549−553.
(14) Miller, M. D.; Farnet, C. M.; Bushman, F. D. *J. Virol.* **1997**, *71*, 5382−5390.
(15) Bushman, F. D.; Fujiwara, T.; Craigie, R. *Science* **1990**, *249*, 1555−1558.
(16) Katz, R. A.; Merkel, G.; Kulkosky, J.; Leis, J.; Skalka, A. M. *Cell* **1990**, *63*, 87−95.
(17) Brown, P. O.; Bowerman, B.; Varmus, H. E.; Bishop, J. M. *Cell* **1987**, *49*, 347−356.
(18) Ellison, V. H.; Abrams, H.; Roe, T.; Lifson, J.; Brown, P. O. *J. Virol.* **1990**, *64*, 2711−2715.
(19) Farnet, C. M.; Haseltine, W. A. *Proc. Natl. Acad. Sci U.S.A.* **1990**, *87*, 4164−4168.
(20) Bukrinsky, M. I.; Sharova, N.; McDonald, T. L.; Pushkarskaya, T.; Tarpley, G. W.; Stevenson, M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6125−6129.
(21) Gallay, P.; Swingler, S.; Song, J.; Bushman, F.; Trono, D. *Cell* **1995**, *17*, 569−576.
(22) Farnet, C.; Bushman, F. D. *Cell* **1997**, *88*, 1−20.
(23) Wei, S.-Q.; Mizuuchi, K.; Craigie, R. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10535−10540.
(24) Billich, A.; Schauer, M.; Frank, S.; Rosenwirth, B.; Billich, S. *Antiviral Chem. Chemother.* **1992**, *3*, 113−119.
(25) Fesen, M. R.; Kohn, K. W.; Leteurtre, F.; Pommier, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 2399−2403.
(26) Carteau, S.; Mouscadet, J. F.; Goulaouic, H.; Subra, F.; Auclair, C. *Biochem. Biophys. Res. Commun.* **1993**, *192*, 1409−1414.
(27) Mouscadet, J. F.; Carteau, S.; Goulaouic, H.; Subra, F.; Auclair, C. *J. Biol. Chem.* **1994**, *269*, 21635−21638.
(28) Mouscadet, J. F.; Ketterle, C.; Goulaouic, H.; Carteau, S.; Subra, F.; Le Bret, M.; Auclair, C. *Biochemistry* **1994**, *33*, 4187−4196.
(29) Carteau, S.; Mouscadet, J. F.; Goulaouic, H.; Subra, F.; Auclair, C. *Arch. Biochem. Biophys.* **1993**, *305*, 606−610.
(30) Cushman, M.; Sherman, P. *Biochem. Biophys. Res. Commun.* **1992**, *185*, 85−90.
(31) Mazumder, A.; Cooney, D.; Agbaria, R.; Gupta, M.; Pommier, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5771−5775.
(32) Fesen, M. R.; Pommier, Y.; Leteurtre, F.; Hiroguchi, S.; Yung, J.; Kohn, K. W. *Biochem. Pharmacol.* **1994**, *48*, 595−608.
(33) Mazumder, A.; Raghavan, K.; Weinstein, J.; Kohn, K. W.; Pommier, Y. *Biochem. Pharmacol.* **1995**, *49*, 1165−1170.
(34) Mazumder, A.; Gupta, M.; Perrin, D. M.; Sigman, D. S.; Rabinovitz, M.; Pommier, Y. *AIDS Res. Hum. Retroviruses* **1995**, *11*, 115−125.
(35) LaFemina, R. L.; Graham, P. L.; LeGrow, K.; Hastings, J. C.; Wolfe, A.; Young, S. D.; Emini, E. A.; Hazuda, D. J. *Antimicrob. Agents Chemother.* **1995**, *39*, 320−324.
(36) Farnet, C.; Lipford, R.; Wang, B.; Bushman, F. D. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 9742−9747.
(37) Zhao, H.; Neamati, N.; Hong, H.; Mazumder, A.; Wang, S.; Sunder, S.; Milne, G. W. A.; Pommier, Y.; Burke, T. R. *J. Med. Chem.* **1997**, *40*, 242−249.
(38) Robinson, W. E.; Reinecke, M. G.; Abdel-Malek, S.; Jia, Q.; Chow, S. A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6326−6331.
(39) Robinson, W. E.; Cordeiro, M.; Abdel-Malek, S.; Jia, Q.; Chow, S. A.; Reinecke, M. G.; Mitchell, W. M. *Mol. Pharmacol.* **1996**, *50*, 846−855.
(40) Cushman, M.; Golegiewski, W. M.; Pommier, Y.; Mazumder, A.; Reymen, D.; De Clercq, E.; Graham, L.; Rice, W. G. *J. Med. Chem.* **1995**, *38*, 443−452.
(41) Mazumder, A.; Gazit, A.; Levitzki, A.; Nicklaus, M.; Yung, J.; Kohlhagen, G.; Pommier, Y. *Biochemistry* **1995**, *34*, 15111−15122.
(42) Neamati, N.; Hong, H.; Mazumder, A.; Wang, S.; Sunder, S.; Nicklaus, M. C.; Milne, G. W. A.; Proksa, B.; Pommier, Y. *J. Med. Chem.* **1997**, *40*, 942−951.
(43) Nicklaus, M. C.; Neamati, N.; Hong, H.; Mazumder, A.; Sunder, S.; Chen, J.; Milne, G. W. A.; Pommier, Y. *J. Med. Chem.* **1997**, *40*, 920−929.
(44) Eich, E.; Pertz, H.; Kaloga, M.; Schulz, J.; Fesen, M. R.; Mazumder, A.; Pommier, Y. *J. Med. Chem.* **1996**, *39*, 89−95.
(45) McKee, T. C.; Cardellina, J. H.; Riccio, R.; D′Auria, M. V.; Iorizzi, M.; Minale, L.; Moran, R. A.; Gulawkowski, R. J.; McMahon, J. B.; Buckheit, R. W.; Snader, K. M.; Boyd, M. R. *J. Med. Chem.* **1994**, *37*, 793−797.
(46) Beutler, J. A.; McKee, T. C.; Fuller, R. W.; Tischler, M.; Cardellina, J. H.; Snader, K. M.; McCloud, T. G.; Boyd, M. R. *Antiviral Chem. Chemother.* **1993**, *4*, 167−172.
(47) Pommier, Y.; Pilon, A. A.; Bajaj, K.; Mazumder, A.; Neamati, N. *Antiviral Chem. Chemother.* **1997**, *8*, 463−483.
(48) Anderson, R. J.; Faulkner, D. J.; Cun-heng, H.; Van Duyne, G. D.; Clardy, J. *J. Am. Chem. Soc.* **1985**, *107*, 5492−5495.
(49) Lindquist, N.; Fenical, W.; Van Duyne, G. D.; Clardy, J. *J. Org. Chem.* **1988**, *53*, 4570−4574.
(50) Carroll, R. A.; Bowden, B. F.; Coll, J. C. *Aust. J. Chem.* **1993**, *46*, 489−501.
(51) Urban, S.; Capon, R. J. *Aust. J. Chem.* **1996**, *49*, 711−713.
(52) Reddy, M. V. R.; Faulkner, D. J.; Venkateswarlu, Y.; Rao, M. R. *Tetrahedron* **1997**, *53*, 3457−3466.
(53) Hwang, Y.; Wang, B.; Bushman, F. D. *J. Virol.* **1998**, *72*, 3401−3406.
(54) Bushman, F. D.; Engelman, A.; Palmer, I.; Wingfield, P.; Craigie, R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3428−3432.
(55) Vink, C.; Oude Groeneger, A. M.; Plasterk, R. H. A. *Nucleic Acids Res.* **1993**, *21*, 1419−1425.
(56) Kimpton, J.; Emerman, M. *J. Virol.* **1992**, *66*, 2232−2239.
(57) Schwartz, O.; Henin, Y.; Marechal, V.; Montagnier, L. *AIDS Res. Hum. Retroviruses* **1988**, *4*, 441−448.
(58) Burke, T. R.; Fesen, M. R.; Mazumder, A.; Wang, J.; Carothers, A. M.; Grunberger, D.; Driscoll, J.; Kohn, K.; Pommier, Y. *J. Med. Chem.* **1995**, *38*, 4171−4178.
(59) Farnet, C. M.; Wang, B.; Hansen, M.; Lipford, J. R.; Zalkow, L.; Robinson, W. R. J.; Siegel, J.; Bushman, F. D. *Antimicrob. Agents Chemother.* **1998**, *42*, 2245−2253.
(60) Naldini, L.; Blomer, U.; Gallay, P.; Ory, D.; Mulligan, R.; Gage, F. H.; Verma, I. M.; Trono, D. *Science* **1996**, *272*, 263−267.
(61) Jenkins, T. M.; Engelman, A.; Ghirlando, R.; Craigie, R. *J. Biol. Chem.* **1996**, *271*, 7712−7718.
(62) Charneau, P.; Mirambeau, G.; Roux, P.; Paulous, S.; Buc, H.; Clavel, F. *J. Mol. Biol.* **1994**, *241*, 651−662.

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