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INHIBITION OF HIV INTEGRASE BY 4-HYDROXYCOUMARIN DIMER BEARING ANILINE MUSTARD MOIETY

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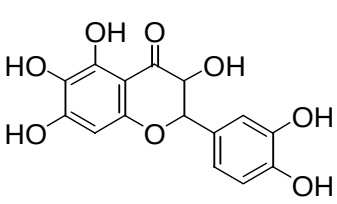
Abstract-The design, synthesis of novel HIV-integrase (HIV-IN) inhibitors possessing the 4-hydroxycoumarin dimer bearing aniline mustard moiety are described. These compounds showed good inhibitory activity against HIV-IN.

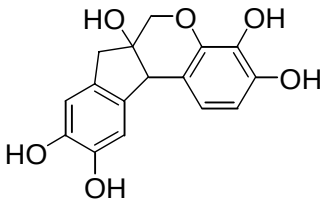
Current therapy for AIDS (acquired immunodeficiency syndrome) is inadequate; hence efforts are directed to develop new treatment strategies.[1] Human immunodeficiency virus integrase (HIV-IN) is responsible for incorporation of reverse-transcribed proviral DNA into the host genome. The incorporation occurs by a sequence involving DNA splicing (3'-processing) and coupling (integration) reaction.[2-4] Because the integration process is essential for the replication of HIV and the enzyme appears to be absent in the mammalian host, HIV-IN represents a potential target for the development of non-toxic antiviral therapeutic agents.

A number of heterocyclic compounds including quercetagetin (**1**)[5], hematoxylin (**2**)[6], and quercetin (**3**)[7] are known to inhibit HIV-IN activity. The tetrameric 4-hydroxycoumarin (NSC 158393) was first reported against HIV-IN by Mazumder *et al.*[8] with potent inhibition of both 3'-processing (IC_{50} = 1.5 μM) and strand transfer (IC_{50} = 0.8 μM). In order to delineate structural features of NSC 158393 necessary for high inhibitory activity, Zhao *et al.* disclosed the minimum active pharmacophore (**4**)[8,9], *i.e.* a coumarin dimer containing an aryl substituent on the central linker methylene.

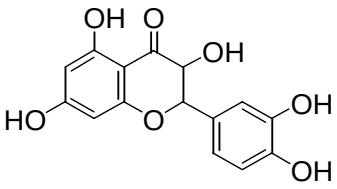
It has been well known that nitrogen mustards are reactive compounds that act on DNA, RNA, and enzymes by forming a covalent bond with one or more types of functional groups near the binding site of



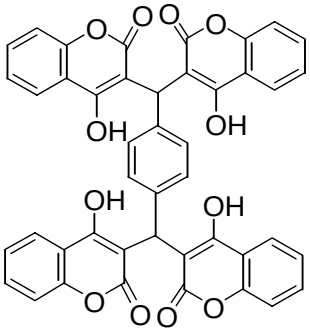
quercetagetin (**1**)



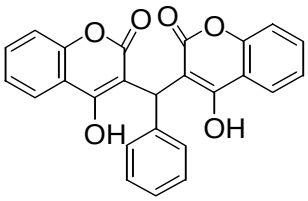
hematoxylin (**2**)



quercetin (**3**)



NSC158393



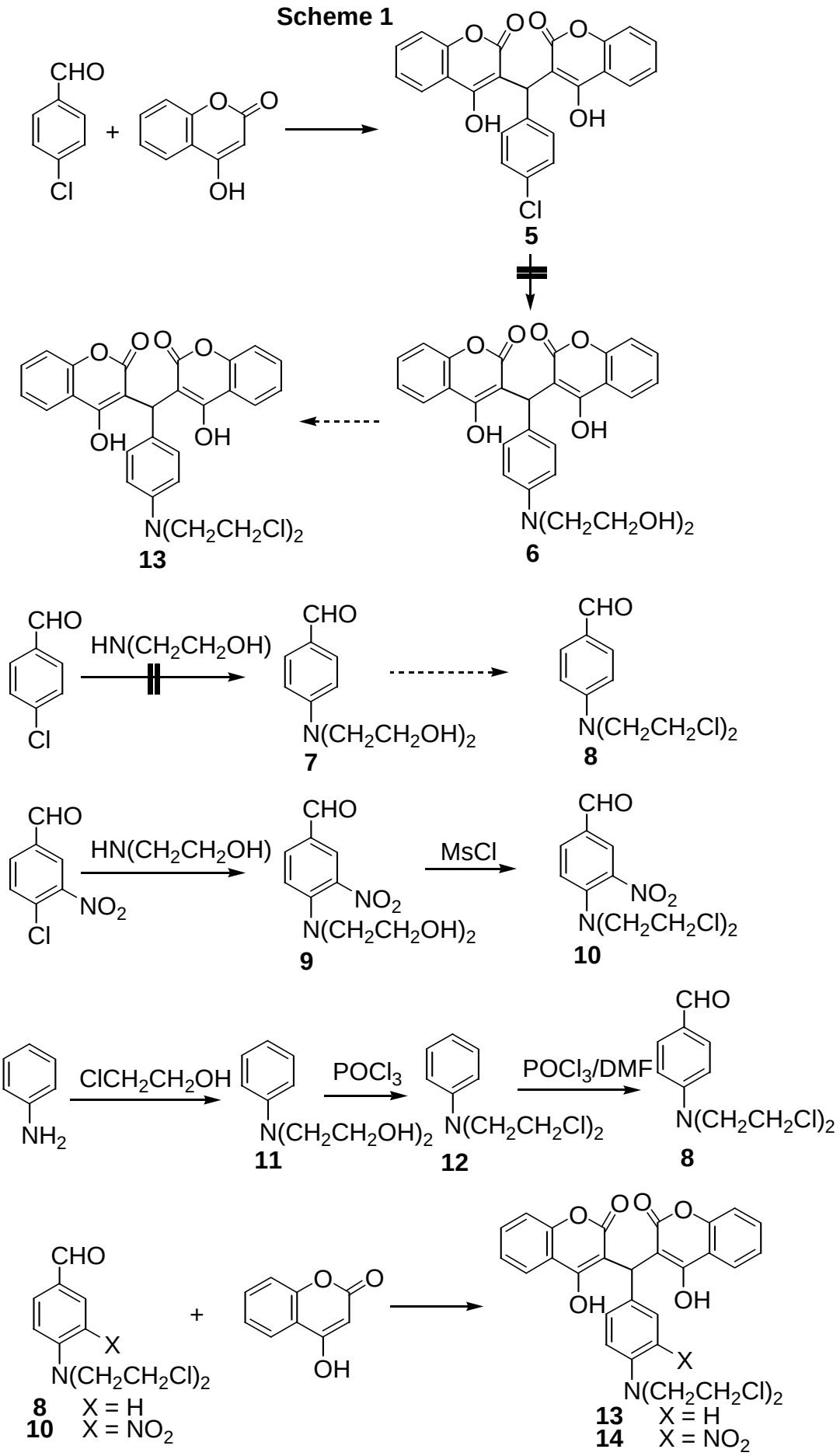
minimun active pharmacophore (**4**)

the enzyme. We thought the compound containing a reactive species such as nitrogen mustard attaching on the aromatic ring of the minimum active pharmacophore (**4**) would confer potent inhibitory activity. Thus, we initiated the preparation and evaluation of several 4-hydroxycoumarin dimer analogues containing aniline mustard moiety to explore the inhibitory effect of nitrogen mustard moiety on HIV-integrase,.

RESULTS AND DISCUSSION

The synthetic routes to the target compounds (**13**) and (**14**) are outlined in Scheme 1. Compound (**5**) was prepared by a known procedure[10], *i.e.* the condensation of two equivalent of 4-hydroxycoumarin with *p*-chlorobenzaldehyde. The approach to the preparation of **6,** which could serve as the intermediate for the synthesis of **13** was unsuccessful, presumably because it is absence of an electron-withdrawing group on the chlorobenzene ring of **5**. A possible route to the synthesis of **13** through the condensation of two equivalent of 4-hydroxycoumarin and 4-[*N*,*N*-bis(2-chloroethyl)amino]benzaldehyde (**8**) instead of 4-chlorobenzaldehyde could be applied. However, synthesis of 4-[bis(2-hydroxyethyl)amino]benz-aldehyde (**7**), a precursor of **8**, by treatment of 4-chlorobenzaldehyde with diethanolamine was fruitless, presumably because the electron-withdrawing effect of *para*-substituted formyl group to the chlorine on benzene was not strong enough to facilitate a successful nucleophilic aromatic substitution. Thus, with the strong electron-withdrawing group (*i.e.* nitro group) assistance, nucleophilic substitution reaction of 4-chloro-3-nitro-benzaldehyde with diethanolamine led to **9**. Compound (**9**) was treated with methanesulfonyl chloride in dry pyridine under reflux furnished **10**. In order to obtain **8**, we chose an alternative route utilizing aniline as starting material. Treatment of aniline with readily available

Scheme 1


CHO
Cl
OH
5
6
13
7
8
9
10
11
12
HN(CH2CH2OH)
ClCH2CH2OH
POCl3
POCl3/DMF
MsCl
NO2
NH2
N(CH2CH2Cl)2
N(CH2CH2OH)2
X = H
X = NO2

ethylenechlorohydrin in the presence of sodium carbonate gave *N*,*N*-bis(2-hydroxyethyl)aniline (**11**). Chlorination of **11** with phosphorus oxychloride produced *N*,*N*-bis(2-chloroethyl)aniline (**12**). Vilsmeier reaction[11] was applied by using phosphorus oxychloride / *N*,*N*-dimethylformamide for introducing an formyl group into the *para* position of aromatic ring of **12**, and yielded **8** (70%). Condensation of **8** and **10** with two equivalent of 4-hydroxycoumarin provided **13** (88%) and **14** (36%), respectively.

Table 1. HIV-integrase inhibitory activity of **5**, **13**, **14** and NSC158393.

Entry	Compounds	3'-Processing (μM)	Strand transfer (μM)
1	**4**	43.0[a]	nd
2	**5**	27.4	15.0
3	**13**	6.6	12.0
4	**14**	8.5	4.0
5	NSC158393[a]	1.1 (1.5)[a]	0.4 (0.8)[a]

a: Data from ref 9.
nd: no determination

BIOLOGICAL STUDIES

HIV integrase inhibition assays were conducted with low amount of purified recombinant integrase (50 nM) in the presence of magnesium as the cationic cofactor, using 21-mer double-stranded oligonucleotide substrate. 3'-Processing assays were performed as described previously.[12] Strand transfer inhibition assays were carried out in a 96-wells plate assay which uses a radiolabeled 21-mer double-stranded oligonucleotide as viral substrate and a 3'-biotinylated 20-mer oligonucleotide as target. Insertion of substrate DNA into target DNA results in 5'-radiolabeled, 3'-biotinylated oligonucleotides that are captured on magnetic beads coated with streptavidin (Dynabeads^{TM}). The amount of radioactivity associated with the magnetic beads yields the integrase activity. The inhibitory effects are summarized in Table 1. All three compounds (**5**), (**13**), and (**14**) were found to have good inhibitory activity against recombinant wild type HIV integrase. NSC158393 served as a control and had an IC_{50} value of 1.1 μM for 3'-processing and 0.4 μM for strand transfer in this assay. The reported inhibitory activity[9] on NSC158393 are 1.5 μM (3'-processing) and 0.8 μM (strand transfer). Compound (**5**) with chloro substituent on the benzene ring was almost twice as more potent than **4**. Compound (**13**) and (**14**) both with nitrogen mustard moiety on the benzene ring have exhibited an IC_{50} value of 6.6 μM and 8.5 μM respectively, about a 6-fold increase over **4**. It is clear that the introduction of a reactive nitrogen mustard into the aromatic ring of **4** do improve the inhibitory activity against HIN-IN. Introduction of an electron withdrawing group (*i.e.* nitro

group) into **13** (Entry 4) had no improvement on potency against 3'-processing but led to a better effect against strand transfer activity, thus confirming that the molecular determinants of activity against the both steps of integration reaction can be dissociated.[13]

In conclusion, the design and synthesis of 'aniline mustard' coumarin dimer is described. Introducing nitrogen mustard into the minimum active pharmacophore (**4**) increases the inhibitory effect on HIV-integrase. Compounds (**13)** and (**14**) represent the first example of coumarin dimer containing aniline mustard that have been found to have potent anti HIV-IN activity. Furthermore, these compounds could serve as candidates for cocrystallization study with HIV-IN to elucidate the binding mechanism of the enzyme.

EXPERIMENTAL

General. Melting points were taken on a BUCHI 530 apparatus and are uncorrected. The silica gel used for chromatography was silica gel 60 70-230 mesh (E. Merck, Darmstadt, Germany), TLC was performed on prescored DC-Alufolien Kieselgel 60F_{254} (E. Merck, Darmstadt, Germany). Compounds were visualized by illuminating under UV light (254 nm). Evaporations were carried out at < 50 ^{o}C using a rotary evaporator at reduced pressure (water aspirator). Solvent ratios are reported as v/v. ^{1}H and ^{13}C NMR spectra were obtained at Varian 300 NMR spectrometer. Where necessary, deuterium exchange experiments were used to obtained proton shift assignments. Mass spectra were recorded on a JEOL J.M.S-300 spectrophotometer. Analytical samples were dried under reduced pressure at 78 ^{o}C in the presence of P_2O_5 for at least 12 h unless otherwise specified. Elemental analyses were obtained from Perkin-Elmer 2400 Elemental Analyzer.

3,3'-(4-Chlorobenzylidene)bis(4-hydroxycoumarin) (5) 4-Chlorobenzaldehyde (7.08 g, 50.4 mmol) was added to a solution of 4-hydroxycoumarin (20.4 g, 126 mmol) in EtOH (200 mL) and the mixture was refluxed for 6 h. The mixture was evaporated under reduced pressure and crystallized with ethanol to give pure **5** (15.2 g, 67%). R_f 0.63 ($CHCl_3$/EtOH 9:1). mp 256-257 ^{o}C (EtOH). ^{1}H-NMR (DMSO-d_6) δ: 6.25 (s, 1H, CH), 7.09-7.83 (m, 12H, ArH). MS (*m/z*): 446 (M^+). Anal. Calcd for $C_{25}H_{15}O_6Cl$: C, 67.20; H, 3.38. Found: C, 67.11; H, 3.18.

4-[Bis(2-hydroxyethyl)amino]-3-nitrobenzaldehyde (9) A solution of 4-chloro-3-nitrobenaldehyde (4.03 g, 21 mmol), diethanolamine (3.43 g, 33 mmol) in toluene (10 mL) was heated under reflux for 6 h. The mixture was concentrated under *vacuum* and then, the residue was chromatographed (silica gel, hexane/EtOAc 1:1) to give pure **9** (0.78 g, 14%). R_f 0.30 (hexane/EtOAc 1:1). mp 169-170 ^{o}C (EtOH). ^{1}H-NMR ($CDCl_3$) δ: 3.58 (m, 4H, NCH_2), 3.77 (m, 4H, CH_2O), 7.42, 7.92 (d, J = 8 Hz, 1H each, H_5, H_6), 8.18 (s, 1H, H_2), 9.84 (s, 1H, CHO). MS (*m/z*): 254 (M^+). Anal. Calcd for $C_{11}H_{14}N_2O_5$: C, 51.97; H, 5.55; N, 11.02. Found: C, 52.07; H, 5.65; N, 11.32.

4-[Bis(2-chloroethyl)amino]-3-nitrobenzaldehyde (10) MsCl (1.79 g, 16 mmol) was added dropwise at 0 ^{o}C to a solution of **9** (0.99 g, 4 mmol) in dry pyridine (5 mL). The solution was allowed stirred at 0 ^{o}C for 15 min and then at room temperature for 4 h. The solution was poured into cold dilute HCl (10% w/v). The mixture was extracted with EtOAc. The organic layer was separated and washed thoroughly with saturated aqueous $NaHCO_3$, brine, dried with $MgSO_4$, evaporated under vacuum, and the residue was subjected to column chromatography (silica gel, hexane/EtOAc 1:1) to give **10** (1.04 g, 79%) as an oil. R_f 0.45 (hexane/EtOAc 1:1). ^{1}H-NMR ($CDCl_3$) δ: 3.60-3.67 (m, 8H, NCH_2CH_2O), 7.39, 8.01 (d, *J* = 8 Hz, 1H each, H_5, H_6), 8.25 (s, 1H, H_2), 9.91 (s, 1H, CHO). MS (*m/z*): 291 (M^+). Anal. Calcd for $C_{11}H_{12}N_2O_3Cl_2$: C, 45.38; H, 4.15; N, 9.62. Found: C, 45.18; H, 4.05; N, 9.42.

***N,N*-Bis(2-hydroxyethyl)aniline (11)** A mixture of aniline (3.68 g, 31 mmol), 2-chloroethanol (14.07 g, 179 mmol), and anhydrous $NaHCO_3$ (4.41 g, 52 mmol) was heated under reflux for 48 h. The mixture was poured into water and extracted with ether. The organic layer was separated, dried with $MgSO_4$, evaporated under vacuum, and the residue was subjected to column chromatography (silica gel, hexane / EtOAc 4:1) to give pure **11** (4.92 g, 69%). R_f 0.34 (hexane/EtOAc 1:1). mp 56-57 ^{o}C (lit.,[14] 55 ^{o}C).

***N,N*-Bis(2-chloroethyl)aniline (12)** $POCl_3$ (1.65 g, 11 mmol) was added dropwise at 0 ^{o}C to **11** (0.5 g, 3 mmol). The mixture was heated under reflux for 1 h. To the mixture toluene (100 mL) was added. The mixture was poured into ice water. The aqueous layer was extracted with toluene three times. The combined extract was dried with $MgSO_4$, evaporated under vacuum, and the residue was subjected to column chromatography (silica gel, hexane/EtOAc 4:1) to give pure **12** (0.34 g, 55%). R_f 0.49 (hexane / EtOAc 4:1). mp 41-42 ^{o}C (lit.,[14] 41-45 ^{o}C).

4-[*N,N*-Bis(2-chloroethyl)amino]benzaldehyde (8) $POCl_3$ (14 g, 92 mmol) was added at 0 ^{o}C to DMF (10 mL). Then, the mixture was added dropwise to a solution of **12** (2 g, 9 mmol) in DMF (10 mL). The mixture was stirred at room temperature for 2 h and then was poured into ice water. The mixture was filtered at once. The filtrate was stood overnight to precipitate **8** (1.52 g, 70%). R_f 0.40 (hexane/EtOAc 4:1). mp 86-88 ^{o}C (lit.,[14] 85-88 ^{o}C).

3,3'-[4-[Bis(2-chloroethyl)amino]benzylidene]bis(4-hydroxycoumarin) (13) The aldehyde (**8**) (0.52 g, 2.1 mmol) was added to a solution of 4-hydroxycoumarin (6.81 g, 4.2 mmol) in DMF/MeOH (1/5, 30 mL) and the mixture was refluxed for 2 h. The mixture was evaporated under reduced pressure and the residue was crystallized with ethanol to give pure **13** (1.01 g, 87%). R_f 0.52 (hexane/EtOAc 1:1). mp 192-194 ^{o}C. ^{1}H-NMR (DMSO-d_6) δ: 3.60-3.70 (m, 8H, NCH_2CH_2), 6.01 (s, 1H, CH), 6.60-7.62 (m, 12H, ArH). MS (*m/z*): 552 (M^+). Anal. Calcd for $C_{29}H_{23}NO_6Cl_2$: C, 63.05; H, 4.20; N, 2.54. Found: C, 63.02; H, 416; N, 2.24.

3,3'-[4-[Bis(2-chloroethyl)amino]-3-nitrobenzylidene]bis(4-hydroxycoumarin) (14) The aldehyde (**10**) (1.11 g, 3.8 mmol) was added to a solution of 4-hydroxycoumarin (1.35 g, 8.3 mmol) in acetone (30 mL) and the mixture was reflux for 6 h. The mixture was evaporated under reduced pressure and the residue was crystallized with ethanol to give pure**14** (0.81 g, 36%). R_f 0.58 (hexane/EtOAc 4:1). mp 193-195 ^oC. ^1H-NMR (DMSO-d_6) δ 3.50-3.57 (m, 8H, NCH_2CH_2), 6.03 (s, 1H, CH), 7.30-8.07 (m, 11H, ArH). MS (*m/z*): 597 (M^+). Anal. Calcd for $C_{29}H_{22}N_2O_8\ Cl_2$: C, 58.30; H, 3.71; N, 4.69. Found: C, 58.42; H, 3.49; N, 4.42.

3,3',3'',3'''-(1,4-Dimethylenophenyl)tetrakis-4-hydroxycoumarin (NSC 158393) 4-Terephthaldehyde (0.3 g, 2.2 mmol) was added to a solution of 4-hydroxycoumarin (1.43 g, 8.8 mmol) in acetone (30 mL) and the mixture was reflux for 6 h. The mixture was evaporated under reduced pressure and the residue was crystallized with ethanol to give NSC 158393 (0.66 g, 39%). R_f 0.13 (hexane/EtOAc 1:4). mp 281-283 ^oC. ^1H-NMR (DMSO-d_6) δ 6.33 (s, 2H, CH), 7.03-7.91 (m, 20H, ArH). MS (*m/z*): 762 (M^+). Anal. Calcd for $C_{44}H_{26}O_{12}$: C, 70.78; H, 3.51. Found: C, 70.56; H, 3.72.

HIV-1 Integrase Inhibitory Assay

Oligonucleotides. Oligonucleotides were purchased from Eurogentec and further purified on 18% acrylamide/urea denaturing gel. U5B: GTGTGGAAAATCTCTAGCA; U5B-2: GTGTGGAAAATCT-CTAG; U5A: 5'-ACTGCTAGAGATTTTCCACAC; ST1: AGTGAATTAGCCCTTGGTCA-biotine; ST2: 5' TGACCAAGGGCTAATTCACT-biotine; U5B and U5B-2 were radiolabeled using T4 polynucleotide kinase for respectively 3'-processing and strand transfer reactions.

HIV-1 Integrase assays. Wild-type HIV-1 integrase was purified as described previously.[15] 3'-Processing assay was performed in a reaction volume of 20 µL containing 0.025 pmoles of labled U5A/U5B double-stranded DNA substrate and 1 pmole of integrase in buffer A [20 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT]. Products were separated on a 18% acrylamide/urea denaturing gel and quantified on a phosphoimager using ImageQuant software (AmershamPharmaciaBiotech). Strand transfer reactions were performed in triplicate in 96-well plates using 0.25 pmoles of labeled U5A/U5B-2 double–stranded DNA substrate, 12 pmoles of ST1/ST2 3'-biotinylated target DNA and 2 pmoles of integrase in buffer A in a final volume of 40 µL. Radiolabeled reaction products were bound to Streptavidin-coated magnetic beads (DynaL), washed twice in buffer B (PBS buffer supplemented with 0.025% tween 20 and 10 µg/mL BSA) and quantified on a beta radiation counter. Inhibition in the presence of drugs is expressed as the fractional product in percent of the control without drug.

ACKNOWLEDGEMENTS

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REFERENCES:

1. M. I. Johnston and D. F. Hoth, *Science* 1993, **260**, 1286.
2. R. A. Katz and A. M. Skalka, *Ann.Rev. Biochem.*, 1994, **63**, 133.
3. P. Rice, R. Craigie, and D. R. Davies, *Curr. Opin. Struct. Biol*., 1996, **6**, 76.
4. C. M. Farnet, B. Wang, J. R. Lipford, and F. D.Bushman, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 9742.
5. M. R. Fesen, Y. Pommier, F. Leteurtre, S. Hiroguchi, J. Yung, and K.W. Kohn, *Biochem. Pharmacol*., 1994, **48**, 595.
6. R. L. LaFemina, P. L. Graham, K. LeGrow, J. C. Hastings, A. Wolfe, S. D. Young, E. A. Emini, and D. J. Hazuda, *Antimicrob. Agents Chemother*., 1995, **39**, 320.
7. M. R. Fesen, K. W. Kohn, F. Leteurtre, and Y. Pommier, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 2399.
8. A. Mazumder, S. Wang, N. Neamati, M. Nicklaus, S. Sunder, J. Chen, G. W. Milne, W. G. Rice, T. R. Jr. Burke, and Y. Pommier, *J. Med. Chem.*, 1996, **39**, 2472.
9. H. Zhao, N. Neamati, H. Hong, A. Mazumder, S. Wang, S. Sunder, G. W. Milne, Y. Pommier, and T. R. Jr. Burke, *J. Med. Chem.*, 1997, **40**, 242.
10. W. R. Sullivan, C. F. Huebner, M. A. Stahman, and K. P. Link, *J. Am. Chem. Soc*., 1943, **65**, 2288.
11. A. Vilsmeier and A. Haack, *Ber*., 1927, **60**, 119.
12. K. Mekouar, J. F. Mouscadet, D. Desmaele, F. Subra, H. Leh, D. Savoure, C. Auclair, and, J. d'Angelo, *J. Med. Chem.*, 1998, **41**, 2846.
13. D. J. Hazuda, P. Felock, M. Witmer, A. Wolfe, K. Stillmock, J. A. Grobler, A. Espeseth, L. Gabryelski, W. Schleif, C. Blau, and M. D. Miller, *Science*, 2000, **287**, 646.
14. W. C. J. Ross, *J. Chem. Soc*., 1949, 183.
15. H. Leh, P. Brodin, J. Bischerour, E. Deprez, P. Tauc, J. C. Brochon, E. LeCam, D. Coulaud, C. Auclair, and J. F. Mouscadet, *Biochemistry*, 2000, **39**, 9285.