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Carbonyl J Derivatives: A New Class of HIV-1 Integrase Inhibitors

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Integration of a DNA copy of the HIV-1 genome is required for viral replication and pathogenicity, and this highly specific molecular process is mediated by the virus-encoded integrase protein. The requirement for integration, combined with the lack of a known analogous process in mammalian cells, makes integrase an attractive target for therapeutic inhibitors of HIV-1 replication. While many reports of HIV-1 IN inhibitors exist, no such compounds have yet emerged to treat HIV-1 infection. As such, new classes of integrase inhibitors are needed. We have combined molecular modeling and combinatorial chemistry to identify and develop a new class of HIV-1 integrase inhibitors, the Carbonyl J [*N*,*N*′-bis(2-(5-hydroxy-7-naphthalenesulfonic acid)urea] derivatives. This new class includes a number of compounds with sub-micromolar IC_{50} values for inhibiting purified HIV-1 integrase *in vitro*. Herein we describe the chemical characteristics that are important for integrase inhibition and cell toxicity within the Carbonyl J derivatives.

INTRODUCTION

Retroviral integration, the covalent ligation of a complete copy of the viral genome into the host cell DNA, is an essential step in the retroviral life cycle and is mediated by the virus-encoded integrase (IN) protein (1). Following receptor-mediated cell entry, virus-encoded reverse transcriptase (RT) uses the viral RNA as a template for synthesis of a DNA copy of the viral genome. IN then removes two nucleotides from the 3′-end of each strand of the viral DNA, referred to as 3′-processing. While still in a nucleoprotein complex, also called the preintegration complex, the viral genome migrates into the nucleus where IN acts to link covalently the DNA copy of the viral genome to the host cell DNA. The latter step, called strand transfer, generates the provirus that serves as a template for future virus production and ensures that all daughter cells contain a complete copy of the virus genome.

IN is one of three enzymes common to all retroviruses, the other two being RT and protease (PR). RT and PR are well characterized, their crystallographic structures have been determined, and each is a target of clinically available anti-retroviral agents (*2*,*3*). While anti-RT and anti-PR therapeutics have made significant advances in controlling HIV-1 infection and improving the lives of people with AIDS, problems with toxicity and resistance continue to plague the current anti-retroviral armamentarium (*2*,*3*). As such, new anti-retroviral agents, directed against new targets, remain an important goal of AIDS research.

The essential role of IN in viral replication, and the lack of a known functional analog in human cells, make IN an attractive anti-retroviral target (*1*). Multiple classes of

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IN inhibitors have been reported to date, including nucleotides/nucleosides (*4,5*), oligonucleotides (*5–10*), DNA binding molecules (*11,12*), proteins (*13,14*), and peptides (*15*). The majority of reports, however, have involved hydroxylated and polyhydroxylated aromatic compounds including aurintricarboxylic acid and its derivatives (*16,17*), cosalines (*18,19*), and topoisomerase inhibitors (*12*). While the initial searches for IN inhibitors were rather broad in scope, many subsequent ones have been driven by the search for better hydroxylated aromatic compounds (*20–23*) and by searching the NCI drug database for compounds that share a pharmacophore with published IN inhibitors (*24–28*). While there are numerous reported inhibitors, only one has suggested a direct inhibition of IN activity in culture (*29,30*). As such, new anti-IN lead compounds are needed, and this will most likely happen by taking new approaches to identifying such compounds.

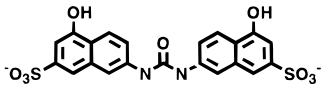
We have used a combination of structure-based computer modeling and combinatorial chemistry to identify new inhibitors of HIV-1 RT and HIV-1 IN. During one of our structure-based searches for new IN inhibitors, we noticed a structural motif similarity between a new set of potential IN inhibitor compounds and a class of compounds that were already under evaluation for their anti-RT properties, the Carbonyl J class of compounds (*31*). The lead RT inhibitor compound at that time, Carbonyl J (Fig. 1; compound **1**), was therefore tested for its anti-IN activity. It had an IC_{50} of 4 μM for *in vivo* IN-mediated 3′-processing and strand transfer. Subsequent structural similarity searches identified Calcomine orange (Fig. 1; compound **2**) with much improved anti-IN activity. Herein we describe our structure–function analysis of this new class of HIV-1 IN inhibitors, the Carbonyl J derivatives, which have the benefit of also possessing significant anti-RT activity (*31*). The combined anti-IN and anti-RT effect in a single compound makes the Carbonyl J derivatives an intriguing class of compounds for further study and development.

EXPERIMENTAL

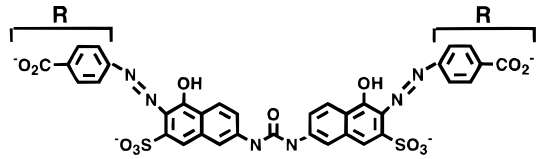
Materials. Carbonyl J (*N,N′*-bis(2-(5-hydroxy-7-naphthalenesulfonic acid)) urea) was purchased from Pfaltz and Bauer, Inc. (Waterbury, CT). Calcomine orange was purchased from Sigma (St. Louis, MO). All other chemicals were purchased from Aldrich Chemical (Milwaukee, WI).

Synthesis. A general procedure was used for production of the di(diazo) inhibitors reported. This procedure may be scaled from approximately 25 to 500 mg of aniline or an aniline derivative. A sample of the aniline to be coupled (1.9 mM) was slurried in 2 ml of water and 1.25 ml of 20% H_2SO_4 was added followed by the addition of 1.8 mM $NaNO_2$ in 1 ml water. The reaction was mixed until >95% of the precipitate dissolved (generally <5 min) and was then added to 15 ml of water. A saturated Na_2CO_3 solution was added (typically close to 6 ml) to achieve a pH of 9. A solution of compound **1** or analog (0.95 mM fully dissolved in 10 ml warm water) was added and the reaction mixture immediately turned dark orange-red. Saturated Na_2CO_3 solution (3 ml) was added to maintain basicity. The resulting solution was stirred at room temperature for 2.5–4 h, acidified with 20% H_2SO_4 to a pH of approximately 0 (approximately 5 ml), and then diluted with water to 140 ml. The precipitate was isolated by centrifugation, washed with water (2×80 ml) to remove salts, methanol, or ethanol (2×40 ml) to remove residual organics, and then vacuum dried overnight. Electron spray and liquid secondary ion mass spectroscopy analyses were performed at the UCSF Mass Spectrometry Facility (A.L. Burlingame, Director).

A



CARBONYL J (1)



CALCOMINE ORANGE (2)

B

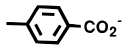
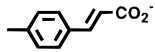
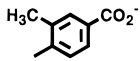
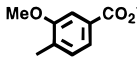
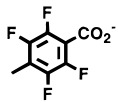
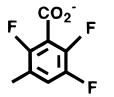
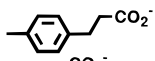
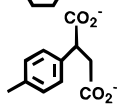
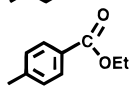
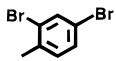
COMPOUND #	R	IC_{50} (μM) 3'-P	IC_{50} (μM) ST	TC_{50} (μM)	T.I. (TC_{50}/IC_{50})
1	N/A	4.0	4.0	ND	—
2		0.35	0.50	85	170
3		2.7	3.2	150	47
4		0.4	0.70	90	129
5		0.5	0.70	70	100
6		0.20	0.33	165	507
7		1.0	1.0	335	335
8		0.50	0.50	140	280
9		0.50	1.0	300	300
10		0.30	0.70	ND	—
11		2.0	4.0	ND	—
12		2.7	1.3	ND	—
13		4.0	0.60	ND	—

FIG. 1. Structure–function analysis and toxicity data for Carbonyl J derivatives. (A) The two drawings at the top show the chemical structure of Carbonyl J (**1**) and Calcomine Orange (**2**). The "R" group of Calcomine Orange is relevant to compounds **2–13** in B. (B) Carbonyl J (**1**) has no "R" group as shown in A. The IC_{50} values are from *in vitro* assays and represent the concentration of inhibitor that yields a 50% reduction in IN activity compared to control reactions performed in the absence of inhibitor. The TC_{50} represents the concentration of inhibitor that yields a 50% reduction in live cells after 5 days of continuous exposure to the compound as compared to control cells grown for 5 days in the absence of inhibitor. Compounds **10–13** were not tested (ND) for TC_{50} due to poor solubility. A T.I. (therapeutic index: TC_{50}/IC_{50}) value is therefore provided for compounds **2–9.**

Matrix-assisted laser desorption ionization mass spectroscopy (MALDIMS) was performed on a PerSeptive Biosystems Voyager-DE instrument and were internally calibrated by close proximity spotting. NMR spectra (^{1}H and ^{13}C) were taken on a GE 300 MHz instrument, and shifts were measured against tetramethylsilane. Data presented come from compounds of >95% purity as judged by NMR spectra, without any unidentified peaks or peaks attributable to synthetic by-products.

*N,N′-bis-(2-(5-hydroxy-7-naphthalenesulfonic acid))oxalamide (**18**).* A mixture of oxallyl di-imidazole (1.98 g, 10.4 mM), J-acid (6-amino-1-hydroxy-3-naphthalenesulfonic acid; 5.00 g, 2(1 mM, dried on pump overnight) and imidazole (1.60 g, 23.5 mM, recrystallized from benzene) was diluted with DMF (20 ml, dry). After stirring for a few hours, the reaction became cloudy and remained so for the duration of the reaction. After 4 days the reaction mixture was centrifuged, and the pelleted material was washed with ethanol (4 × 10–15 ml) until the liquid layer was only lightly colored. The material was dried in vacuum to yield 2.67 g of an off-white powder containing trace DMF. Negative ion liquid secondary ion mass spectroscopy found (MH-) 531^{1}H NMR (300 MHz, DMSO) 11.10 (s, 2H) 9.03 (s, 2H) 8.45 (s, 2H) 8.10 (d, *J* = 9 Hz, 2H) 7.93 (d, *J* = 9 Hz, 2H) 7.67 (s, 4H) 7.54 (s, 2H) 7.14 (s, 2H) ^{13}C NMR (75 MHz, DMSO) 158.86, 152.75, 146.58, 135.96, 134.44, 133.61, 122.68, 121.74, 119.68, 119.46, 117.55, 114.79, 105.52

*N,N′-bis-(2-(5-hydroxy-6-(azo-(4-benzoic acid))-7-naphthalenesulfonic acid))urea (**2**).* Negative ion electron spray mass spectroscopy (ESMS) found (MH-) 799, (MNa-) 821 ^{1}H NMR (300 MHz, DMSO) 15.81 (s, 2H) 12.5–13.0 (broad s, 2 H) 9.53 (s, 2H) 8.21 (d, *J* = 8.7 Hz, 2H) 7.99 (d, *J* = 8.1 Hz 4H) 7.82 (d, *J* = 8.1 Hz, 4H) 7.76 (s, 2H) 7.50 (s, 2H) ^{13}C NMR (75 MHz, DMSO) 177.81, 166.88, 151.85, 146.14, 144.82, 143.50, 137.00, 130.84, 129.40, 128.65, 126.90, 125.04, 122.29, 117.91, 116.62, 116.35

*N,N′-bis-(2-(5-hydroxy-6-(azo-(4-cinnaminic acid))-7-naphthalenesulfonic acid)) urea (**3**).* Negative ion ESMS found (MH-) 851, (MNa-) 873 ^{1}H NMR (300 MHz, DMSO) 15.94 (s, 2H) 12.08 (broad s, 2H) 9.58 (s, 2H) 8.20 (d, *J* = 8.4 Hz, 2H) 7.77 (m, 12H) 7.62 (d, *J* = 15.9 Hz, 2H) 7.51 (s, 2H) 6.56 (d, *J* = 15.9 Hz, 2H) ^{13}C NMR (75 MHz, DMSO) 177.00, 167.34, 151.55, 144.25, 143.87, 143.71, 143.12, 136.72, 131.08, 129.37, 128.76, 128.16, 124.70, 121.05, 117.89, 117.37, 117.11, 115.89

*N,N′-bis-(2-(5-hydroxy-6-(azo-(2-methyl-4-benzoic acid))-7-naphthalenesulfonic acid)) urea (**4**).* Negative ion ESMS found (MH-) 827, (MNa-) 849 ^{1}H NMR (300 MHz, DMSO) 16.17 (s, 2H) 9.64 (s, 2H) 8.19 (d, *J* = 7.8 Hz, 2H) 8.09 (d, *J* = 7.5 Hz, 2H) 7.85 (s, 6H) 7.62 (d, *J* = 7.8 Hz, 2H) 7.57 (s, 2H) 2.45 (s, 6H) ^{13}C NMR (75 MHz, DMSO) 177.77, 167.06, 151.77, 144.78, 144.07, 143.33, 136.92, 132.09, 130.14, 128.71, 128.55, 126.64, 125.17, 124.93, 122.39, 117.91, 116.20, 115.33, 56.09 (EtOH) 18.60 (EtOH) 16.72

*N,N′-bis-(2-(5-hydroxy-6-(azo-(2-methoxy-4-benzoic acid))-7-naphthalenesulfonic acid))urea (**5**).* Negative ion ESMS found (MH-) 799, (MNa-) 821 ^{1}H NMR (300 MHz, DMSO) 15.94 (s, 2H) 9.69 (s, 2H) 8.21 (d, *J* = 8.4 Hz, 2H) 8.04 (d, *J* = 7.8 Hz, 2H) 7.85 (s, 2H) 7.65 (m, 6H) 7.56 (s, 2H) 4.06 (s, 6H) ^{13}C NMR (75 MHz, DMSO) 178.00, 166.93, 151.85, 147.44, 144.78, 143.45, 136.91, 134.94, 130.15, 128.80, 127.36, 125.11, 123.86, 122.28, 117.86, 116.28, 115.38, 112.13, 56.23

*N,N′-bis-(2-(5-hydroxy-6-(azo-(3-benzoic acid))-7-naphthalenesulfonic acid)) urea (**8**).* Negative ion ESMS found (MH-) 799, (MNa-) 821 ^{1}H NMR (300 MHz, DMSO) 16.03 (s, 2H) 9.55 (s, 4H) 8.23 (d + s, 2H) 8. 08 (d, *J* = 8.1 Hz, 2H) 7.77 (s, 6H) 7.60 (t, *J* = 7.8 Hz 2H) 7.52 (s, 2H) ^{13}C NMR (75 MHz, DMSO) 176.74, 167.02, 151.99, 151.91, 144.80,

143.35, 143.17, 136.88, 132.25, 129.94, 128.69, 126. 39, 125.09, 121.58, 121.29, 118.16, 117.88, 116.19, 56.16 (EtOH), 18.64 (EtOH)

*N,N′-bis-(2-(5-hydroxy-6-(azo-(4-(3-propanoic acid)phenyl)-7-naphthalenesulfonic acid)) urea (**9**)*. Negative ion MALDIMS found (MH-) 855, (MNa-) 877 ^{1}H NMR (300 MHz, DMSO) 16.22, (s, 2H) 12.07 (broad s, 2H) 9.53 (s, 2H) 9.21 (d, 8.4 Hz, 2H) 7.78–7.68 (m, 8H) 7.50 (s, 2H) 7.33 (d, *J* = 7.8 Hz, 4H)2.87 (t, poorly resolved, 4H) 2.57 (t, *J* = 7.2 Hz, 4H) ^{13}C NMR (75 MHz, DMSO) 175.22, 173.80, 170.39, 151.96, 144.30, 143.48, 141.10, 139.16, 136.64, 129.41, 128.09, 124.99, 120.48, 117.75, 117.52, 115.92, 59.81(EtOAc), 56.09(EtOH), 35. 29, 30.06, 20.81 (EtOAc), 18.59 (EtOH), 14.14 (EtOAc).

*N,N′-bis-(2-(5-hydroxy-6-(azo-(4-(2-succinic acid)phenyl)-7-naphthalenesulfonic acid)) urea (**10**)*. Negative ion MALDIMS found (MH-) 944, (MNa-) 965 ^{1}H NMR (300 MHz, DMSO) 16.05 (broas S, 2H) 9.49 (s, 2H) 8.21 (d, *J* = 8.4 Hz, 2H) 7.77–7.01 (m, 8H) 7.48 (s, 2H) 7.38 (d, *J* = 7.8 Hz, 4H) 3.94 (dd, *J* = 4.5, 10.2 Hz, 2H) 2.99 (dd, *J* = 10.2, 16.5 Hz, 2H) 2.58 (dd, *J* = 4.5, 16.8 Hz, 2H) ^{13}C NMR (75 MHz, DMSO) 175.96, 174.00, 172.74, 152.01, 144.46, 143.40, 141.95, 136.81, 136.40, 128.97, 128.32, 125.11, 120.96, 117.96, 117.60, 116.16 46.55, 37.49

*N,N′-bis-(2-(5-hydroxy-6-(azo-(3-(carboxyethoxy)phenyl))-7-naphthalenesulfonic acid))urea (**11**)*. Negative ion MALDIMS found (MH-) 855, (MNa-) 877 ^{1}H NMR (300 MHz, DMSO) 15.91 (s, 2H) 9.59 (s, 2H) 8.19 (d + s, 4H) 8.10 (d, *J* = 7.8 Hz, 2H) 7.77 (m, 6H) 7.59 (t, *J* = 7.5 Hz, 2H) 7.52 (s, 2H) 4.34 (q, *J* = 6.9, 4H) 1.35 (t, *J* = 7.5 Hz, 6H) ^{13}C NMR (75 MHz, DMSO) 176.68, 165.33, 151.80, 144.66, 143.19, 136.77, 131.19, 129.91, 128.76, 128.37, 125.92, 124.95, 121.75, 121.26, 118.03, 117.82, 116.09, 61.04, 56.12 (EtOH), 18.58 (EtOH), 14.18

*N,N′-bis-(2-(5-hydroxy-6-(azo-(4-(carboxyethoxy)phenyl))-7-naphthalenesulfonic acid))urea (**12**)*. Negative ion MALDIMS found (MH-) 855, (MNa-) 877 ^{1}H NMR (300 MHz, DMSO) 15.77 (s, 2H) 9.55 (s, 2H) 8.18 (d, *J* = 8.4 Hz, 2H) 7.98 (d, *J* = 8.4 Hz, 4H) 7.82 (d, *J* = 8.4 Hz, 4H) 7.76 (s, 2H) 7.71 (d, *J* = 9.0 Hz, 2H) 7.51 (s, 2H) 4.31 (q, *J* = 6.9 Hz, 4H) 1.34 (t, *J* = 6.9 Hz, 6H) ^{13}C NMR (75 MHz, DMSO) 177.84, 165.25, 151.65, 146.27, 144.81, 143.08, 136.82, 130.59, 129.48, 128.57, 125.79, 124.92, 122.71, 117.83, 116.58, 116.28, 60.61, 14.21

*N,N′-bis-(2-(5-hydroxy-6-(azo-(2,4-dibromophenyl))-7-naphthalenesulfonic acid))urea (**13**)*. Negative ion MALDIMS found (MH-) 1025, 1027 (MNa-) 1048 ^{1}H NMR (300 MHz, DMSO) 16.00 (s, 2H) 9.56 (s, 2H) 8.16 (d, *J* = 8.4 Hz, 2H) 8.00 (d, *J* = 8.7 Hz, 2H) 7.93 (s, 2H) 7.75 (s, 2H) 7.67 (m, 2H) 7.55 (s, 2H) ^{13}C NMR (75 MHz, DMSO) 177.33, 151.62, 144.78, 143.34, 140.09, 136.91, 134.45, 131.94, 130.03, 128.71, 124.59, 122.69, 119.10, 117.81, 117.23, 116.29, 111.39

*N,N′-bis-(2-(5-hydroxy-6-(azo-(2,3,5,6-tetrafluoro-4-benzoic acid)-7-naphthalenesulfonic acid))urea (**6**)*. Negative ion MALDIMS found (M(−3H)-) 939, (M(−4H)Na-) 961 ^{1}H NMR (300 MHz, DMSO) 15.82(s, 2H) 10.88 (s, 2H) 9.58 (s, 2H) 8.24, (d, *J* = 8.7, 2H) 7.84 (s, 2H) 7.79 (d, *J* = 9. 0 Hz, 2H) 7.57 (s, 2H) ^{13}C NMR (75 MHz, DMSO, 50°C) 172.67, 160.82, 151.35, 146.42 (m), 144.34, 143.14 (m), 141.73, 141.10 (m), 139.44 (m), 137.71 (m), 136.57, 134.67, 134.50, 128.64, 127.95, 123.24, 121.96, 118.26, 115.94, 180.92 (m) (multiplets due to ^{19}F splitting).

*N,N′-bis-(2-(5-hydroxy-6-(azo-(2,4,5-trifluoro-3-benzoic acid)-7-naphthalenesulfonic acid))urea (**7**)*. Negative ion MALDIMS found (M(−3H)-) 903, (M(−4H)Na-) 925 ^{1}H

NMR (300 MHz, DMSO) 16.04 (broad s, 2H) 9.50 (s, 2H) 8.24, (d, *J* = 8.7 Hz, 2H) 7.93 (dd, *J* = 6.9, 10.8 Hz, 2H) 7.84 (s, 2H) 7.75 (d, *J* = 8.7 Hz, 2H) 7.57 (s, 2H) ^{13}C NMR (75 MHz, DMSO) 171.22, 166.10, 151.90, 150.10, 148.76, 146.91, 145.41, 145.02 (m), 144.02, 143.16, 136.59, 129.09, 127.85, 124.45 (m), 123.84, 121.11, 118.26, 115.86, 110.41 (m), 106.90 (m) (multiplets due to ^{19}F splitting).

N,N′-bis-(2-(5-hydroxy-6-(azo-(4-benzoic acid))-7-naphthalenesulfonic acid))oxalamide ***(14)***. Negative ion MALDIMS found (MH-) 827, (MNa-) 849 ^{1}H NMR (300 MHz, DMSO) 15.81 (broad S, 2H) 11.36 (s, 2H) 8.23 (m, 4H) 8.05 (d, *J* = 9.0 Hz, 2H) 7.99 (d, *J* = 8.1 Hz, 4H) 7.83 (d, *J* = 8.4 Hz, 4H) 7.47 (s, 2H) ^{13}C NMR (75 MHz, DMSO) 177.19, 166.57, 158.71, 146.04, 144.10, 142.28, 136.55, 130. 61, 129.39, 127.97, 127.11, 126.78, 121.45, 119.65, 119.02, 116.67

N,N′-bis-(2-(5-hydroxy-6-(azo-(4-(2-cinnaminic acid)phenyl))-7-naphthalenesulfonic acid))oxalamide ***(15)***. Negative ion ESMS found (MH-) 879, (MNa-) ^{1}H NMR (300 MHz, DMSO, note spectra is poorly resolved) 15.94 (s, 2H), 11.28 (s, 2H) 8.23 (s, 4H) 8.01 (d, *J* = 6 Hz, 2H) 7.78 (s, 4H) 7.58 (d, *J* = 15.9 Hz, 2H) 7.48 (s, 2H) 6.53 (d, *J* = 15.3 Hz, 2H) ^{13}C NMR (75 MHz, DMSO) 176.50, 167.69, 158.79, 144.14, 143.38, 142.22, 136.56, 131.69, 129.68, 129.02, 128.02, 126.83, 121.01, 119.71, 119.10, 118.96, 118.34, 117.68

N,N′-bis-(2-(5-hydroxy-6-(azo-(3-benzoic acid))-7-naphthalenesulfonic acid))oxalamide ***(19)***. Negative ion MALDIMS found (MH-) 827, (MNa-) 849 ^{1}H NMR (300 MHz, DMSO) 16.03 (s, 2H) 11.36 (s, 2H) 8.26 (m, 6H) 8.07 (m, 4H) 7.79 (d, *J* = 7.2 Hz, 2H) 7.60 (t, *J* = 7.8 Hz, 2H) 7.47 (s, 2H)) ^{13}C NMR (75 MHz, DMSO) 175.90, 166.83, 158.86, 144.26, 143.19, 142.15, 136.55, 132.16, 129.79, 128.80, 127.91, 126.75, 126.50, 121.36, 120.68, 119.70, 118.94, 118.36

N,N′-bis-(2-(5-hydroxy-6-(azo-(4-succinic acid))-7-naphthalenesulfonic acid))oxalamide (racemic mixture) ***(20)***. Negative ion MALDIMS found (MH-) 972, (MNa-) 993 ^{1}H NMR (300 MHz, DMSO) 16.2 (very broas S, 2H) 11.35 (s, 2H) 8.28 (d + s, 4H) 8.05 (d, *J* = 8.7 Hz, 2H) 7.75 (d, *J* = 8.1 Hz, 4H) 7.48 (s, 2H) 7.40 (d, *J* = 8.4 Hz, 4Hz) 3.95 (dd, *J* = 4.8, 9.6 Hz, 2H) 3.00 (dd, *J* = 10.2, 16.5, 2H) 2.59 (dd, *J* = 4.8, 16.8 Hz, 2H)) ^{13}C NMR (75 MHz, DMSO) 175.44, 174.31, 173.05, 159.18, 143.62, 142.28, 137.17, 136.59, 129.37, 128.62, 128.19, 127.30, 121.03, 120.38, 119.51, 118.26, 46.90, 37.74

Integrase activity and inhibition assays. Oligonucleotide-based assays for *in vitro* HIV-1 IN 3′-processing and strand transfer were performed essentially as described previously (32). The IN protein assayed was expressed in yeast and purified as described (*32*). Briefly, 15-μl reaction volumes included 20 mM morpholinepropanesulfonic acid (MOPS) pH 7.0, 5 mM DTT, 10 mM $MnCl_2$, 5 pmol of purified, full-length HIV-1 IN, and 0.5 pmol of radiolabeled, duplex *att* site oligonucleotide (U5-29+: 5′-TTTAGTCAGTGTGGAAAATCTCTAG**CA**GT-3′, and U5-29-: 5′-ACTGCTAGAGATTTTCCACACTGACTAAA-3′; the bold nucleotides indicate the invariant CA dinucleotide near the viral end). The U5-29+ was radiolabeled at its 5′-end to follow the fate of the duplex during a reaction (*32*). Approximately 20 mM NaCl is present as a "carry over" from the protein storage buffer. Inhibitors were preincubated for 10 min at 30°C in complete reaction mix lacking the *att* site oligonucleotide. The reaction was initiated by adding the oligonucleotide substrate, and the reaction was stopped after 20 min at 37°C by the adding gel loading dye (95% formamide) and heating to 100°C for 2 min. Reaction mixtures were electrophoresed through 7 M urea 15% acrylamide denaturing gels, the gels were dried on 3 mm paper, and the signal was quantified using a PhosphorImager (Molecular Dynamics).

Inhibitor concentrations were always performed in duplicate and positive controls (no inhibitor) were always performed in triplicate. Results presented are an average of two or more independent experiments.

Cell toxicity assays. Toxicity assays were performed using a slight modification to an assay frequently used to test for the toxicity of possible HIV-1 inhibitors (*33*). We changed from the standard assay substrate tetrazolium XTT (Polysciences) to the fluorescent substrate Calcein AM (Molecular Probes, Inc.) because many of our compounds are colored and therefore interfered with the absorbence characteristics of the standard substrate. CEM-SS cells, a human T-cell line, were grown in RPMI media supplemented with 10% fetal bovine serum, 100 units of penicillin G sodium and 0.1 mg of streptomycin sulfate per ml. Assays were performed using 100 μl of cells at 50,000/ml and inhibitor at final concentrations of 10, 100, 200, 500, 1000 μM. Cells were incubated in the presence of the compounds for 5 days. One hundred microliters of Calcein AM (Molecular Probes, Inc.) at 4 μM in phosphate-buffered saline was then added to each well. Calcein AM enters cells with intact membranes and is converted by intracellular esterase from a virtually nonfluorescent compound to an intensely fluorescent one (*34*). The plate was incubated at room temperature for 30–45 min and read with PerSeptive Biosystems' Fluorescence Multi-well Plate reader at 485 nm for excitation and 530 nm for emission, using the Cyto-Flour II software. Toxicity assays are performed in quadruplicate, and all data presented represent the average of two or more experiments.

Restriction enzyme inhibition assays. One unit of ApaL I (New England Biolabs), the manufacturer's recommended buffer, and inhibitor at either one- or fivefold the 3′-processing IC_{50} concentration were pre-incubated in 15 μl for 10 min at 30°C. Five microliters of plasmid DNA at 80 ng/μl was added to the reaction followed by a 90-min incubation at 37°C. The reaction was stopped by addition of 6X loading dye and the restriction digests were electrophoresed in a 0.8% agarose gel in TAE buffer. The DNA bands indicative of enzyme activity were quantified with the IS1000 Digital Imaging System (Alpha Innotech Corporation). The amount of enzyme and the time of the reaction were both within the linear range of enzyme activity for the reaction conditions.

RESULTS

Carbonyl J inhibits HIV-1 IN in vitro. Carbonyl J (**1**), the initial compound screened, demonstrated an IC_{50} value of 4 μM for 3′-processing and for strand transfer when tested for anti-IN activity using in vitro assays that employ purified HIV-1 IN and synthetic mimics of the HIV-1 *att* site (*32*). A series of Carbonyl J derivatives, largely compounds in which the hydroxyl or sulfonic acid groups were repositioned or removed, either lessened, or failed to improve, the anti-IN activity seen with Carbonyl J (data not shown).

Similarity search yields more potent Carbonyl J derivative. The failure to improve upon the anti-IN activity of Carbonyl J by derivatives described above led us to search the available chemical data base for compounds structurally similar to Carbonyl J. One such compound, Calcomine Orange (**2**), resembles Carbonyl J but contains two symmetrically attached phenyl-azo groups (Fig 1). Calcomine Orange demonstrated an IC_{50} of 350 nM for 3′-processing and 500 nM for strand transfer (Figs. 1 and 2).

Synthesis of Carbonyl J derivatives. Having identified Calcomine Orange as a potent lead compound for the development of IN inhibitors, we made a number of symmetrical

analogues by varying the appended aryl groups. Calcamine Orange can be seen as arising from the addition of an aryl diazonium salt to Carbonyl J, with the azo addition being directed by the phenol group in the adjacent (ortho) position (*35, 36*). While aryl diazonium salts are not generally commercially available, they are readily produced from a wide variety of commercially available anilines via reaction with nitrous acid (37,38).

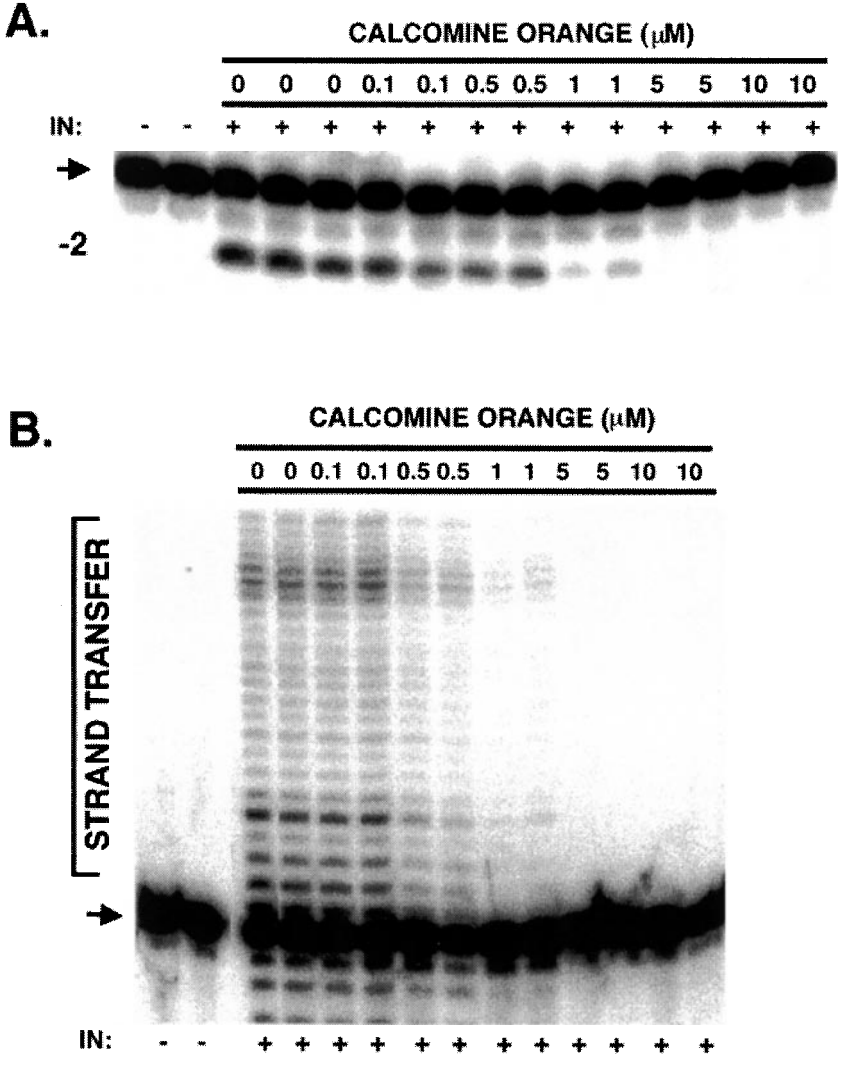
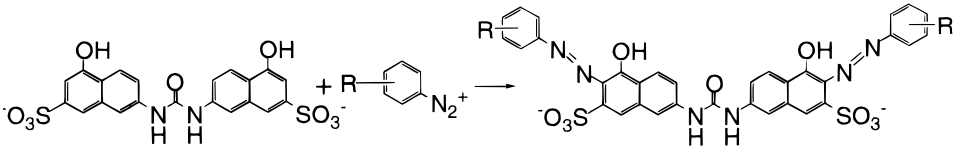


FIG. 2. Dose–response curve for Calcomine Orange inhibition of HIV-1 IN activity *in vitro.* The Calcomine Orange dose–response curve for 3′-processing (top) and strand transfer (bottom) represent the type of data obtained for all compounds described. Arrows indicate the starting material for each experiment, –2 indicates the products of 3′-processing that migrate faster than the starting material in A. The products of strand transfer migrate slower than the starting material and are indicated in B. Inhibitor concentration (μM) is indicated at the top of each panel.



The formation of the azo species was performed initially in an array style, running 12 simultaneous single-compound reactions to produce compounds for initial screening. After initial screening, promising compounds were individually resynthesized, purified, and retested for their anti-IN activity. Data presented here are from the individually synthesized and purified compounds.

Calcomine Orange structure–function relationships. Calcomine Orange has a central hinge region or linker, the urea moiety, two sulfonated phenol groups, and two symmetrically placed phenyl-azo groups (Fig. 1). Since Calcomine Orange differs from Carbonyl J by the simple addition of the two phenyl-azo groups, we sought to determine which structural aspects of phenyl-azo groups account for the improved anti-IN activity (Fig. 1).

The di-cinnamate derivative, compound **3,** was synthesized to determine if the "reach" of the carboxylic acid residues influences the anti-IN activity. Moving the carboxylic acids further apart in compound **3** reduced the anti-IN activity by nearly eightfold for 3′-processing and sixfold for strand transfer. With compound **8** having IC_{50} values of 500 nM for both 3′-processing and strand transfer, a meta or para positioning of the carboxylic acid group appears to have no significant effect on anti-IN activity (**2** vs. **8**). To explore the effects of steric hindrance on the aryl ring, and a possible tilting of the ring relative to the azo bond, methylated (**4**) and O-methylated (**5**) derivatives were made. These changes also had very little effect on anti-IN activity relative to Calcomine Orange (**2**), with IC_{50} values for 3′-processing and strand transfer of 400 and 700 nM, respectively, for compound **4** and 500 and 700 nM, respectively, for compound **5.** Taken together, these data suggest that it is favorable to have the carboxylic acid groups directly attached to the benzene portion of the aryl ring, but its position and the presence of small groups attached to the aryl ring have little effect on anti-IN activity (Fig. 1).

With the di-cinnamate derivative (**3**) demonstrating six to eightfold less anti-IN activity relative to Calcomine Orange (**2**), we wished to test the effect of increased flexibility of the distal carboxylic acid by removing the double bond in the linkage connecting the carboxylic acid to the ring structure in compound **3.** We postulated that more flexibility would increase the chance for the carboxylic acid residues to "find" their optimal orientation, while the increased entropy could work against this approach. The resulting derivative, compound **9,** demonstrated a fivefold improvement in 3′-processing activity and a threefold improvement in strand transfer when compared to the di-cinnamate (**3**). While improving the flexibility improves the anti-IN activity, it still failed to achieve a level of anti-IN activity seen when the carboxylic acid group is directly attached to the benzene portion of the aryl ring as in **2** and **8**.

Fluorine atoms were attached to the benzene rings to test for the effect of modifying the acidity of the distal carboxylic acid groups in the phenyl-azo groups of compound **2**. Four fluorine atoms (**6**) resulted in a nearly 50% improvement in anti-IN activity when compared with compound **2**. However, when only three positions on the

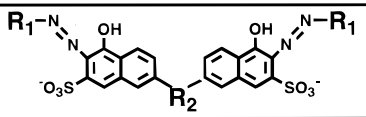
benzene ring are fluorinated (**7**), the anti-IN activity is approximately two- to threefold less than that seen with compound **2.** While compounds **6** and **7** differ in the position of their carboxylic acid moiety in addition to the number of fluorine atoms, we feel that the activity difference is more likely due to the number of fluorine atoms because the difference in carboxylic acid position alone had no significant difference in activity (compare **2** and **8**).

To assess the role of overall charge on the anti-IN activity, we wished to synthesize a derivative of compound **2** with an additional carboxylic acid group directly attached to the phenyl groups. The required diazonium salt for the production of a phthalate Carbonyl J derivative could not be synthesized in sufficient purity, so we instead synthesized a succinate derivative (**10**) to compare with the activity of compound **9.** The additional carboxylic acid group and its associated charge provide a modest improvement in anti-IN activity (**10** vs. **9**). The anti-IN activity profile of compound **2** suggests that the additional negative charge of compound **10** is less important for anti-IN activity than is having fewer negative charges with the carboxylic acid group directly attached to the benzene ring of the aryl group. The role of the single terminal carboxylic acid on each wing was assessed by developing ethyl ester (**11** and **12**) and dibromide derivatives (**13**). With the exception of strand transfer inhibition seen with compound **13**, the loss of the terminal carboxylic acid residues resulted in 5- to 10-fold less activity against 3′-processing and 2.5- to 6-fold less activity against strand transfer. Interestingly, each change had a greater effect on the inhibition of 3′-processing than it had against strand transfer. Taken together, these findings suggest that the carboxylic acid group is important for anti-IN activity but its exact positioning may not be critical for the underlying mechanism.

Functionality of the central linker that joins the two symmetrical halves of the Carbonyl J derivatives was assessed by replacing the urea linker with an oxallyl linker in compounds **2**, **3**, **8**, and **10** yielding compounds **14**, **15**, **19**, and **20**, respectively (Fig. 3). In general, the oxallyl linker lessened or left unchanged the anti-IN activity, with the exception of strand transfer for **15**. Our data do not, however, reveal the mechanism that underlies the superiority of the urea linker. Knowing that the carboxylic acid group directly attached to the two symmetrically placed phenyl-azo groups (**2**) yields greater IN inhibition than when the carboxylic acid groups are more widely separated (**3**), it is possible that the activity differences associated with the linkers simply reflect their effect on the overall distance separating the two distal carboxylic acids.

The compounds in Fig. 1 have molecular weights in range of 800–950, with the fluorinated compounds being more than 1000. Recognizing that these sizes may be detrimental to the development of the Carbonyl J derivatives as therapeutic agents, we synthesized compounds **16** and **17**, derivatives of compounds **2** and **3** but with only a single naphthalene sulfonic acid group (Fig. 4). The smaller compounds (**16** and **17**) demonstrate improved cell toxicity with variable effects on in vitro IN inhibition (Fig. 4).

Cell toxicity profiles for Carbonyl J derivatives provide a good therapeutic index. The anti-IN activity of the Carbonyl J derivatives positions them as a relatively potent new class of lead compounds for the development of therapeutic HIV-1 integrase inhibitors. However, since therapeutic value is a balance between efficacy and toxicity, we also



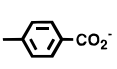
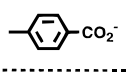
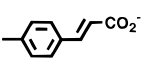
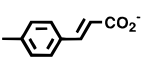
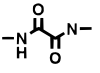
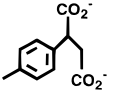
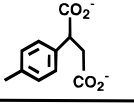
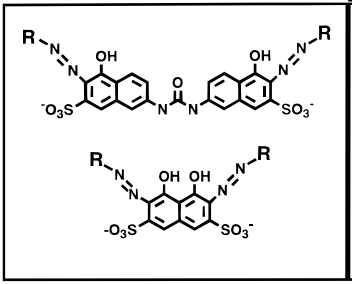
COMPOUND #	R_1	R_2	IC_{50} (μM) 3'-P	IC_{50} (μM) ST	TC_{50} (μM)	T.I. (TC_{50}/IC_{50})
2			0.35	0.50	85	170
14			3.0	1.0	ND	—
3			2.7	3.2	150	47
15			5.0	2.3	60	12
8			0.50	0.50	140	280
19			1.0	2.5	85	34
10			0.30	0.70	ND	—
20			0.80	1.0	320	320

FIG. 3. Assessing the significance of the central urea linker. The role of the urea linker in anti-IN activity of compounds **2, 3, 8,** and **10** was assessed by making the oxallyl linker derivatives, compounds **14, 15, 19,** and **20,** respectively. With the exception of strand transfer for compound **15,** the urea linker consistently provided superior anti-IN activity.

assessed their effect on CEM-SS cells using a toxicity assay commonly used in the screening phase of new HIV-1 inhibitors (*33*). The compounds have a remarkably good therapeutic index, ranging from 47 to over 500 (Fig. 1). Interestingly, changing from the urea linker to an oxallyl linker significantly increased the toxicity and thereby worsened the therapeutic index (Fig. 3). For the two cases tested, removal of the linker and one of the two naphthalene groups significantly improved the toxicity profiles (Fig 4).

Carbonyl J derivatives differentially inhibit DNA-processing enzymes. While it would be useful to have agents that inhibit multiple essential steps in the retroviral life cycle, broad inhibition of DNA-processing enzyme function would likely be detrimental to the



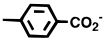
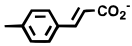
	R:					
						
	CPD #	IC_{50} (μM) 3′-P S.T.	TC_{50} (μM)	CPD #	IC_{50} (μM) 3′-P S.T.	TC_{50} (μM)
	2	0.35 0.50	85	3	2.7 3.2	150
	16	2.5 3.5	800	17	1.8 0.75	540

FIG. 4. The effect of removing one naphthalenesulfonic acid group. To reduce the size of the compounds, compounds **16** and **17** were synthesized with the two aryl-azo groups directly attached to a single naphthalenesulfonic acid structure. This resulted in a reduction in cell toxicity as reflected by the increase in TC_{50}, but with variable effects on anti-IN activity.

infected host. We therefore assayed compounds **2**, **5**, **6**, and **8** for their effect on inhibiting the ApaL I restriction enzyme. We found that each inhibitor at its IC_{50} concentration for HIV-1 IN 3′-processing had no inhibition on Apa I activity. Compounds **2**, **6**, and **8** also had no inhibition of ApaL I activity at 5X the IC_{50} concentration for 3′-processing, while 5X the IC_{50} concentration for compound **6** resulted in approximately 50% inhibition of ApaI activity. Combined with the favorable cell toxicity profiles and the variable effects these compounds have on a number of mammalian polymerases (*31*), the data suggest that the Carbonyl J derivatives are not simply universal inhibitors of DNA-processing enzymes.

DISCUSSION

The development of viral resistance to current anti-retroviral agents (*39*), the clear benefit of combination therapy (*40*), and compliance problems with current multi-agent drug regimens highlight the serious need for new anti-HIV drugs directed against new targets. While numerous compounds with anti-IN activity have been described in the literature, none has yet yielded a clinically approved drug to treat HIV infection. New lead compounds with the potential for development into therapeutic agents are therefore critical. In this paper we describe our initial characterization of a new class of compounds with inhibitory activity against HIV-I IN, the Carbonyl J derivatives.

We initially identified Carbonyl J as part of an effort to develop new HIV-1 RT inhibitors (*31*), but its structural similarity to anti-IN compounds under investigation led us to test it for anti-IN activity. Not only did we find Carbonyl J to possess significant anti-IN activity (IC_{50} = 4 μM), similarity searches of the available chemical databases identified a derivative, Calcomine Orange (**2**), with IC_{50} values of 350 nM and 500 nM for inhibiting HIV-1 IN 3′-processing and strand transfer, respectively. Through the synthesis and testing of numerous synthetic derivatives of **2**, we have found that the distal carboxylic acid moieties are important for the anti-IN activity, that maximal anti-IN activity occurs with the carboxylic acid groups directly attached

to the benzene portion of the aryl ring, and that increasing the acidity of the molecule using fluorine substitution improves the anti-IN activity. Six Carbonyl J derivatives, compounds **2**, **4**, **5**, **6**, **8**, and **10**, have IC_{50} values of 200–500 nM for 3′-processing and 500–700 nM for strand transfer, making them among the more potent anti-IN compounds described to date. The favorable anti-IN activity and cell toxicity profiles (Figs. 1, 3, and 4), and inhibitory activity on HIV-1 RT (*31*) justify further development of the Carbonyl J derivatives as new lead compounds in the search for therapeutic inhibitors of HIV-1 replication.

While sulfonated aromatic compounds have been shown to inhibit HIV replication in culture (*41–44*), the compounds we describe differ significantly from compounds in the literature. Our Carbonyl J derivatives have one sulfonic acid group located on each of two naphthalene sulfonic acids connected by a urea or oxallyl linker. Suramin, an agent with 6 sulfonic acid groups used in the treatment of African trypanosomiasis, has been reported to have an inhibitory effect on nearly every step of the virus life cycle but failed to demonstrate therapeutic benefit in clinical trials (*41,45–48*). Sulfonated organic polymers have demonstrated anti-HIV activity mediated through disruption of viral entry (*49*). Four more closely related, but still dissimilar, naphthalenesulfonic acids tested for HIV-1 IN inhibition resemble one half of our symmetric compounds (*50*). The most active one, with IC_{50} values of 16 mM for 3′-processing and 11 mM for strand transfer, respectively, has two sulfonic acid groups on a single naphthalene structure and is more than an order of magnitude less active than many of our compounds (Fig. 1). Interestingly, a single sulfonic acid per naphthalene group yielded no significant IN inhibition, a distinct contrast to our Carbonyl J derivatives. In studies with the catalytic core domain of avian sarcoma virus (ASV) IN, the small, bisulfonated naphthalene sulfonic acid cocrystallized in close proximity to the IN active site, altering the conformation of the active site residues (*50*). We have not yet identified the IN binding sites for any of our Carbonyl J derivatives, but co-crystal growth trials are underway.

An attractive characteristic of our Carbonyl J derivatives is that many of them inhibit both HIV-1 reverse transcriptase and integrase. The exact mechanism of action for inhibiting these two viral enzymes remains speculative, but recent data suggest an interference of RT binding to nucleic acids (*31*). While we do not yet have data to support the mechanism of action against IN, it is interesting that the small naphthalenesulfonic acid compound co-crystallized with ASV IN bound in the active site (*50*), a location that is consistent with perturbing an IN-nucleic acid interaction. It is important to note that while the Carbonyl J derivatives are active against both HIV-1 RT and HIV-1 IN, their structure-activity relationships are divergent in some critical areas. Most notably, whereas compound **2** is a much more potent inhibitor of IN than is compound **3,** the reverse activity profile is seen for RT (*31*).

Some of the polyhydroxylated IN inhibitors may work through binding of divalent cations critical for IN function (*21,51*). Given that the IC_{50} values for our Carbonyl J derivatives are in the low- to submicromolar range, and that Mn^{2+} is present at 10 mM in our reactions, it seems unlikely that simply chelating divalent cations is the primary mechanism of action for our compounds. This is supported by their varying inhibitory activity against a number of polymerase enzymes, each of which requires a divalent cation for activity (*31*).

The Carbonyl J derivatives represent a new class of IN inhibitors that have the benefit of blocking more than one step in the virus life cycle. This feature, combined with favorable cell toxicity data, provide ample reason to better understand the underlying mechanism of action against IN and against RT. It will be necessary to determine the effect of these compounds on viral replication in culture. For those that show a block in viral infectivity it will be necessary to determine where in the virus life-cycle the block(s) occurs. Our *in vitro* data suggest that it might occur during both reverse transcription and integration.

ACKNOWLEDGMENTS

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