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Short communication

Non-peptide inhibitors of HIV-1 protease. Synthesis and structural evaluation of symmetric and non-symmetric naphthalenesulfonic acid analogues

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Summary — In this study, several representative symmetric and non-symmetric naphthalenesulfonic acid derivatives belonging to various structural classes were evaluated for their potential to inhibit HIV-1 protease. The most active compounds were non-symmetrical and possessed hydrophobic pendant groups. In general, the activity of these derivatives was dependent on the number and position of the sulfonic acid moiety and the nature of the appendages. Remarkably, one of the most active compounds also displayed inhibition of DNA polymerase and RNase H activities of HIV-1 reverse transcriptase. This observation provides an insight into designing singular compounds which could inhibit multiple essential enzymes in the HIV-1 life cycle. Since it is unlikely that these agents will reach targeted cellular enzymes due to their polar nature, the discovery of in vitro protease inhibition rationalizes further modification of sulfonic acid derivatives.

sulfonic acid / HIV protease inhibitor / anti-HIV agent

Introduction

The virally encoded HIV-1 protease has been a popular molecular target in the search of novel and effective AIDS chemotherapeutic modalities [1, 2]. HIV-1 protease belongs to the general class of aspartyl proteases [3, 4]. In its active form, the enzyme is homodimeric with a C_2-symmetry and each monomer contributes a catalytic triad of Asp-Thr-Gly [5]. The activity of retroviral protease is essential for viral maturation and replication [6]. The symmetrical nature of the active site of HIV protease offers an opportunity for the design of C_2-symmetric inhibitors [7]. This approach has resulted in the discovery of several tight-binding protease inhibitors, however, the important problem of viral resistance against these nanomolar range inhibitors remains unchallenged [8, 9].

The inhibition of HIV-1 protease by some C_2-symmetrical anionic compounds has previously been reported by some of us [10, 11]. Previous studies from our laboratories have established the usefulness of naphthalenesulfonic acid derivatives as potential anti-AIDS agents displaying anti-HIV activity at non-toxic concentrations. These derivatives have proved to be inhibitory in assays measuring cytopathogenicity, syncytium formation and HIV-1 and HIV-2 reverse transcriptases [12, 13 and references cited therein]. Some of these naphthalenesulfonic acid derivatives also possess C_2-symmetry, which led us to evaluate them for protease inhibition. In this study, we evaluate

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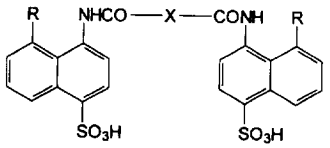
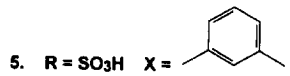
representative compounds from different groups consisting of various spacers and numbers of sulfonic acid moieties on the aromatic ring (figs 1–4). While certain symmetrical derivatives showed moderate HIV protease inhibition, surprisingly two non-symmetrical naphthalenesulfonic acid analogues with hydrophobic pendant groups displayed the best inhibitory activity.

Chemistry and pharmacology

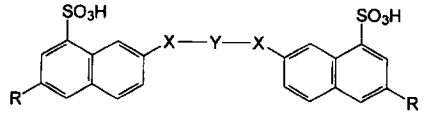
Several new naphthalenesulfonic acid analogues **1**, **5**, **7**, **8**, **12**, **13**, **14**, **16** and **18** were synthesized using single-step reaction schemes from inexpensive, commercially available starting materials. The general synthetic methodology involved reacting an amino or a hydroxyl group on an aromatic nucleus with acyl or sulfonyl halide in anhydrous pyridine or aqueous $NaHCO_3$ reaction medium (table I). Correspondingly, mono- and bisnaphthalenesulfonic acid derivatives were obtained in varying yields.



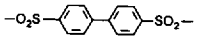
1. R = H, X = $(CH_2)_{10}$
2. R = SO_3H, X = $(CH_2)_5$
3. R = SO_3H, X = $(CH_2)_8$
4. R = SO_3H, X = $(CH_2)_{10}$

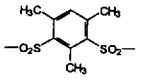


5. R = SO_3H X =

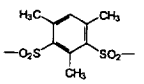


6. R = SO_3H, X = NH, Y =



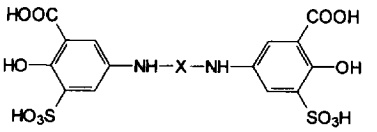


7. R = SO_3H, X = O, Y =

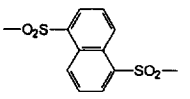


8. R = H, X = O, Y =

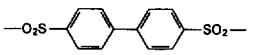
Fig 1. Structures of naphthalenesulfonic acid derivatives (**1–8**).



9. X =



10. X =



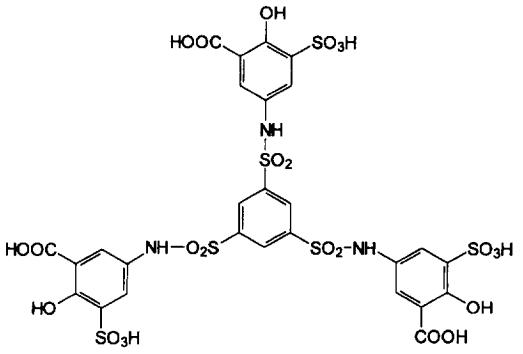


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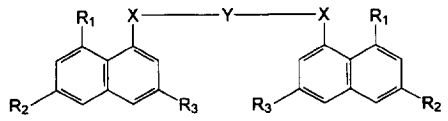
Fig 2. Structures of naphthalenesulfonic acid derivatives (**9–11**).

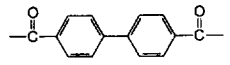
These new analogues, along with other representative sulfonic acid derivatives, were tested for their inhibitory potential against HIV-1-induced cytopathogenicity and HIV protease. The protease enzyme used in this study was chemically synthesized and a literature protocol was used for measuring enzyme inhibition.

Results and discussion

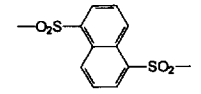
Symmetrical derivatives of 4-amino-1,5-naphthalenedisulfonic acid, tethered by flexible aliphatic spacers, displayed inhibitory activity against HIV-1 protease. This activity was found to be dependent on the separation of aromatic rings, because upon increasing spacer length an increase in inhibition was observed. An octamethylene spacer analogue **3** (IC_{50} = 30 μM) and a decamethylene spacer analogue **4** (IC_{50} = 18 μM) (table II) were considerably more effective than the pentamethylene spacer analogue **2** (IC_{50} = 110 μM). A similar trend was observed in the whole virus assay



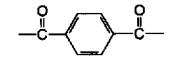
12. $R_1 = H$, $R_2 = SO_3H$, $R_3 = SO_3H$, X = O, Y =



13. $R_1 = H$, $R_2 = SO_3H$, $R_3 = SO_3H$, X = O, Y =



14. $R_1 = H$, $R_2 = SO_3H$, $R_3 = SO_3H$, X = O, Y =



15. $R_1 = OH$, $R_2 = SO_3H$, $R_3 = SO_3H$, X = NH, Y = $-CO(CH_2)_8CO-$

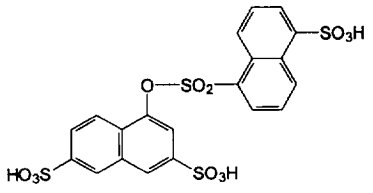


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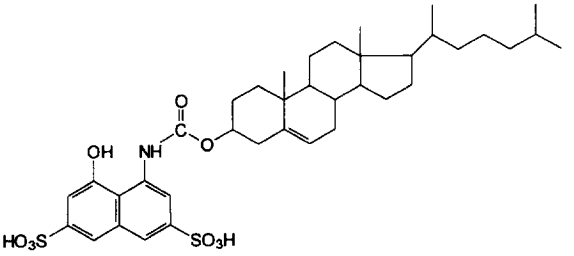
Fig 3. Structures of naphthalenesulfonic acid derivatives (**12–16**).



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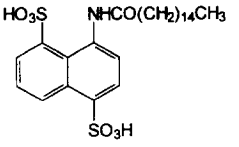

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Fig 4. Naphthalenesulfonic acid derivatives with lipophilic side chain (**17** and **18**).

(table III) where derivative **4** demonstrated better antiviral activity than analogues **2** and **3**. Interestingly, by eliminating one sulfonic acid group as in decamethylene spacer analogue **1**, the enzyme inhibitory activity was attenuated.

In another mono- and naphthalenedisulfonic acid series, derivative **6** coupled to a biphenyl spacer displayed an IC_{50} value of 14 μM. Shortening of spacer distance with a mesitylene group and employing a sulfonyloxy linker as in analogue **7** drastically reduced inhibitory potency (35% inhibition at 275 μM). However, removing one sulfonic acid from the naphthalene ring while keeping the mesitylene spacer linked with a sulfonyloxy group as in derivative **8** (IC_{50} = 45 μM) could partially restore inhibitory activity.

Derivatives **9–11** (fig 2) were synthesized from 5-amino-2-hydroxy-3-sulfobenzoic acid employing different aromatic spacers. The symmetrical tris-derivative **11** (IC_{50} = 45 μM) emerged as a moderately active compound in this series (table II). Considering the 2,7-naphthalenedisulfonic acid series, the symmetrical analogue **12** (IC_{50} = 7 μM) containing a biphenyl spacer was much more active than derivatives **13** and **14**, both of which have shorter aromatic spacers. These observations suggest that spacer length could alter potency in this aromatic framework. If an amino functionality was added to the naphthalene ring, the activity was partially retained as observed for amide-linked octamethylene spacer analogue **15** (IC_{50} = 15 μM). The most promising results were obtained from non-symmetric naphthalenesulfonic acid derivatives, as exemplified by analogues **16–18** (figs 3 and 4). The analogue **17**, bearing a cholesteryl appendage, and derivative **18**, bearing a palmitoyl side chain, were equipotent in protease inhibition assay and displayed IC_{50} values of 4 μM, emerging as the most active compounds in the present study.

It is tempting to attribute HIV protease inhibitory activity to the increased hydrophobic character of these derivatives. However, the enzyme inhibition was not generally observed for other compounds bearing cholesteryl and palmitoyl groups (unpublished results). Structural studies through molecular modeling are being planned and chemical manipulation of derivatives **17** and **18** is being pursued to completely understand the mechanism of HIV protease inhibition by this class of compounds.

Table I. Physical properties of naphthalenesulfonic acid derivatives.

Compound	*Yield (%)*	*Mp (°C), dec*	*Analysis*	*Method of synthesis*[a]
1	31	307–308	C, H, N	Pyridine
2[b]	42	288–290	C, H, N	Pyridine
3[b]	34	310–311	C, H, N	Pyridine
4[b]	43	325–326	C, H, N	Pyridine
5	17	326–327	C, H, N	Pyridine
6[c]	14	332–334	C, H, N	$NaHCO_3$ (aq)
7	51	315 dec	C, H	$NaHCO_3$ (aq)
8	15	278 dec	C, H	$NaHCO_3$ (aq)
9[b]	10	283–284	C, H, N	$NaHCO_3$ (aq)
10[c]	10	248–250	C, H, N	$NaHCO_3$ (aq)
11[b]	8	250–252	C, H, N	$NaHCO_3$ (aq)
12	88	> 360	C, H	Pyridine
13	50	> 360	C, H	$NaHCO_3$ (aq)
14	72	> 360	C, H	Pyridine
15[c]	38	330–332	C, H, N	Pyridine
16	25	> 360	C, H	$NaHCO_3$ (aq)
17[d]	22	227–229	C, H, N	Pyridine
18	11	266–268	C, H, N	Pyridine

[a]Reaction of naphthalenesulfonic acid and acyl/sulfonyl halide in the stated medium; [b]data obtained from reference [12]; [c]data obtained from reference [13]; [d]data obtained from reference [14].

Table II. Inhibition of HIV-1 protease activity by naphthalenesulfonic acid derivatives.

Compound	IC_{50} *(μM)*[a]
1	275
2	110
3	30
4	18
5	70
6	14
7	35% at 275
8	45
9	80
10	30% at 275
11	45
12	7
13	40
14	38% at 137
15	15
16	37% at 137
17	4
18	4
Pepstatin	2.5

[a]IC_{50} = 50% inhibitory concentration.

Table III. Inhibition of HIV-1 (HTLV-III_B) by naphthalenesulfonic acid derivatives in MT-4 cells.*

Compound	EC_{50} *(μM)*[e]	CC_{50} *(μM)*[f]
1[a]	260	> 500
2[c]	143	> 500
3[a]	66	> 500
4[c]	46	> 500
5[c]	55	> 500
6[c]	29	> 500
7[c]	42	228
8[a]	40	> 500
9[c]	52	> 500
10[c]	52	247
11[d]	11	> 500
12[c]	81	> 500
13[c]	39	322
14[c]	89	324
15[c]	10	289
16[b]	288	> 500
17[a]	> 500	> 500
18[a]	112	224

*All data are the mean result of at least two separate experiments; [a]disodium salt; [b]trisodium salt; [c]tetrasodium salt; [d]hexasodium salt; [e]50% effective antiviral concentration; [f]50% cytotoxic concentration.

Conclusion

At the present time, the mechanism of protease inhibition by these derivatives is unclear. However, the protease inhibition data in its own right represents a useful starting point for further rational structural manipulation in this series of compounds to improve antiviral activity. We recognize the fact that these derivatives are considerably less inhibitory than several reported HIV-1 protease inhibitors. *However, the fact that several of these potent analogues have stimulated emergence of resistant viral strains and have failed to arrest HIV infection provides an incentive to search for other novel protease inhibitors.*

It is also noteworthy that cholesteryl derivative **17** potently inhibited both the DNA polymerase and ribonuclease H (RNase H) activities associated with HIV-1 reverse transcriptase (IC_{50} values for RNA-dependent DNA-polymerase, DNA-dependent DNA-polymerase and ribonuclease H are 0.06, 7.9 and 21.8 μM, respectively) [14]. This observation raises the possibility of designing compounds which are capable of inhibiting multiple targets in viral life cycle, an approach which might offer a route to counter HIV-1 drug resistance.

Experimental protocols

Chemistry

Melting points were determined on a Mel-Temp II apparatus and are uncorrected. NMR spectra were recorded on a Varian XL-300 (300 MHz) instrument in DMSO-d_6. Chemical shifts are reported in parts per million relative to tetramethylsilane as internal standard. Analytical thin-layer chromatography was performed on Baker-flex silica-gel IB2-F sheets. Elemental analyses were carried out at the Midwest Microlab (Indianapolis, IN, USA) and C, H, N were within ±0.4% of theoretical values. Pyridine was freshly distilled from potassium hydroxide and stored over potassium hydroxide. Preparative gel permeation chromatography was performed using Spectra/Gel 05 (Fisher; Itasca, IL; filtration range 300–2500 MW) and using water as eluent under N_2 pressure.

Sodium 4,4'-[1,10-decanediylbis(carbonylamino)]bis(1-naphthalenesulfonate) ***1***

4-Amino-1-naphthalenesulfonic acid (0.32 g, 1.3 mmol) was heated to a temperature of 120 °C for 15 min. Dodecanedioyl dichloride (0.24 g, 0.9 mmol) was added, and the mixture was stirred for another 15 min. Dry pyridine (3 mL) was then added and the reaction was refluxed for 4.5 h at 120–125 °C. Pyridine was evaporated under vacuum and the resulting solid was dissolved in methanol (20 mL) and bubbled with ammonia gas for 10 s. The methanolic solution was evaporated to dryness, and the solid was washed with heptane (20 mL) and chloroform (20 mL). The residue was redissolved in methanol (30 mL) and filtered. The desired product was obtained as a white residue (0.28 g, 31%). An analytically pure sample was prepared by recrystallizing from water: mp 307–308 °C (dec). ^{1}H NMR δ 9.89 (s, 2H, exchangeable with D_2O), 8.87 (m, 2H), 8.04 (m, 2H), 7.90 (d, J = 7.9 Hz, 2H), 7.56 (d, J = 7.7 Hz, 2H), 7.52 (m, 4H), 2.47 (m, 4H), 1.66 (m, 4H), 1.35 (m, 12H).

Sodium 4,4'-[1,3-benzenediylbis(carbonylamino)]bis(1,5-naphthalenedisulfonate) ***5***

4-Amino-1,5-naphthalenedisulfonic acid, monosodium salt (0.30 g, 0.92 mmol) was heated at 110–120 °C for 30 min. After this time, isophthaloyl chloride (0.10 g, 0.49 mmol) was added and stirring was continued for 15 min. Dry pyridine (3 mL) was added and the reaction mixture was refluxed for 2 h at 110–120 °C. The reaction mixture was evaporated under vacuum to remove excess pyridine, dissolved in 40 mL methanol and filtered. The filtrate was concentrated to 20 mL and bubbled with ammonia gas for 30 s. The resulting ammoniacal methanolic solution was evaporated to dryness to yield a yellow solid (0.32 g) as the crude product. An analytically pure sample was obtained by passing the crude solid twice through a long gel permeation column and thrice through a short gel permeation column, as a tetrasodium salt (0.06 g, 17%): mp 326–327 °C. ^{1}H NMR δ 12.72 (s, 2H), 9.12 (d, J = 8.6 Hz, 2H), 8.65 (s, 1H), 8.43 (d, J = 7.9 Hz, 2H), 8.32 (d, J = 6.1 Hz, 2H), 8.1 (s, 4H), 7.64 (t, J = 7.7 Hz, 1H), 7.49 (t, J = 8 Hz, 2H).

Sodium 7,7'-[2,4-mesitylenediylbis(sulfonyloxy)]bis(1,3-naphthalenedisulfonate) ***7***

A mixture of 7-hydroxy-1,3-naphthalenedisulfonic acid (0.50 g, 1.64 mmol), 2,4-mesitylenedisulfonyl chloride (0.15 g, 0.47 mmol) and $NaHCO_3$ (0.80 g, 9.52 mmol) in water (25 mL) was stirred at 45–50 °C for 22 h. An additional quantity of 2,4-mesitylenedisulfonyl chloride (0.15 g, 0.47 mmol) and $NaHCO_3$ (0.80 g, 9.52 mmol) was added after 10 h. The reaction mixture was filtered and the filtrate (25 mL) was triturated with acetone (200 mL). The resulting suspension was filtered and to the filtrate (225 mL) was added another 200 mL acetone to afford a white precipitate. The precipitate was filtered to yield 0.44 g of crude product. It was dissolved in 3 mL water and triturated with acetone (200 mL) to afford the pure product as a tetrasodium salt (0.36 g, 51%): mp 315 °C (dec). ^{1}H NMR δ 8.71 (s, 2H), 8.28 (s, 2H), 8.15 (s, 2H), 8.00 (d, J = 8.9 Hz, 2H), 7.40 (s, 1H), 7.10 (d, J = 9.0, 2H), 3.05 (s, 3H), 2.43 (s, 6H).

Sodium 7,7'-[2,4-mesitylenediylbis(sulfonyloxy)]bis(1-naphthalenesulfonate) ***8***

A mixture of 7-hydroxy-1-naphthalenesulfonic acid, monosodium salt (0.80 g, 3.33 mmol), 2,4-mesitylenedisulfonyl chloride (0.30 g, 0.95 mmol), and $NaHCO_3$ (1.00 g, 11.90 mmol) in water (20 mL) was stirred at 45–50 °C for 46 h. A further quantity of 2,4-mesitylenedisulfonyl chloride (0.30 g, 0.95 mmol) and $NaHCO_3$ (1.00 g, 11.90 mmol) was added after 24 h. The reaction mixture was filtered to yield a white residue. This residue was dissolved in methanol (30 mL), filtered and the filtrate was evaporated to dryness to yield 0.42 g of crude product. The crude product was recrystallized once from water/ethanol (9.5:0.5) to afford pure product as a disodium salt (0.18 g, 15%): mp 278 °C (dec). ^{1}H NMR δ 8.72 (s, 2H), 8.01 (d, J = 6.9 Hz, 2H), 7.88 (d, J = 8.1 Hz, 2H), 7.77 (d, J = 9.2 Hz, 2H), 7.51 (t, J = 7.6 Hz, 2H), 7.45 (s, 1H), 7.04 (d, J = 9.0 Hz, 2H), 3.37 (s, 3H), 2.98 (s, 6H).

Sodium 4,4'-[4,4'-biphenyldiylbis(carbonyloxy)]bis(2,7-naphthalenedisulfonate) ***12***

4-Hydroxy-2,7-naphthalenedisulfonic acid, disodium salt (0.35 g, 1 mmol) and 4,4'-biphenyldicarbonyl chloride (0.17 g, 0.6 mmol) were heated at 170–180 °C for 10 min. Dry pyridine (5 mL) was added and the reaction mixture was refluxed for 20 h at 120–125 °C. Pyridine was evaporated under vacuum and the residue was dissolved in methanol, and filtered. The filtrate was

evaporated to yield crude product (0.40 g, 88%). A portion of this solid (0.20 g) was dissolved in water (3 mL) and purified through a long gel permeation column. An analytically pure sample was prepared by evaporating fractions containing pure product and redissolved in methanol (2 mL); the methanolic solution was triturated with ether to produce a light brown powder: mp > 360 °C. ^{1}H NMR δ 8.44 (d, J = 7.4 Hz, 4H), 8.25 (s, 2H), 8.14 (d, J = 6.6 Hz, 6H), 7.87 (d, J = 8.4 Hz, 2H), 7.81 (d, J = 8.9 Hz, 2H), 7.67 (s, 2H).

Sodium 4,4'-[1,5-naphthalenediylbis(sulfonyloxy)]bis(2,7-naphthalenedisulfonate) ***13*** *and sodium 4-(1-sulfonyloxy-5-naphthalenesulfonato)-2,7-naphthalenedisulfonate* ***16***

A mixture of 1-hydroxy-3,6-naphthalenedisulfonic acid (0.35 g, 1 mmol), 1,5-naphthalenedisulfonyl chloride (0.25 g, 0.75 mmol) and $NaHCO_3$ (0.17 g, 2 mmol) in water (20 mL) was stirred at 40–50 °C for 40 h. After evaporating water, the residue was dissolved in methanol and filtered. The filtrate was concentrated and triturated with ether to produce a white solid (0.42 g). A portion of this solid (0.25 g) was dissolved in water (2 mL) and purified through a long gel filtration column. Elution of the column with water first yielded pure fractions of **13** (0.12 g, 50%): mp > 360 °C. ^{1}H NMR δ 9.30 (d, J = 8.9 Hz, 2H), 8.57 (d, J = 7.2 Hz, 2H), 8.27 (s, 2H), 8.20 (s, 2H), 8.15 (d, J = 8.0 Hz, 2H), 7.94 (d, J = 8.8 Hz, 2H), 7.82 (d, J = 8.8 Hz, 2H), 7.40 (s, 2H). Following the elution of **13**, pure fractions of **16** were obtained (0.08 g, 25%): mp > 360 °C. ^{1}H NMR δ 9.38 (d, J = 8.9 Hz, 1H), 8.80 (d, J = 8.7 Hz, 1H), 8.26 (d, J = 7.3 Hz, 1H), 8.18 (d, J = 8.0 Hz, 2H), 8.09 (s, 1H), 7.69–7.88 (m, 4H), 7.38 (s, 1H).

Sodium 4,4'-[1,4-benzenediylbis(carbonylamino)]bis(2,7-naphthalenedisulfonate) ***14***

1-Hydroxy-2,7-naphthalenedisulfonic acid (0.35 g, 1.00 mmol) and terephthaloyl chloride (0.12 g, 0.60 mmol) were heated at 120–130 °C for 5 min. Pyridine (8 mL) was added and the reaction mixture was heated under reflux for 8 h at 120–125 °C. Excess pyridine was evaporated and the residue was washed with chloroform (5 x 20 mL), filtered and dried to yield the product (0.32 g, 72%). A portion of this solid (0.20 g) was dissolved in water (2 mL) and purified through a long gel filtration column. An analytically pure sample was prepared by evaporating fractions containing product, redissolved in methanol (3 mL) and triturated with ether to yield a white product **14**: mp > 360 °C. ^{1}H NMR δ 8.53 (m, 4H), 8.32 (s, 2H), 8.22 (s, 2H), 7.88 (s, 4H), 7.77 (s, 2H).

Sodium 4-(palmitoylamino)-1,5-naphthalenedisulfonate ***18***

4-Amino-1,5-naphthalenedisulfonic acid (1.00 g, 3.08 mmol) in 0.1 M NaOH (10 mL) was heated to 90 °C for 1 h. The solution was evaporated to dryness to yield the disodium salt (1.45 g, 4.18 mmol). The disodium salt was heated to 110 °C for 30 min, then palmitoyl chloride (0.5 mL, 1.25 mmol) was added and the reaction mixture was heated for another 15 min. To this mixture, pyridine (4 mL) was added and the reaction mixture was stirred for 24 h at 100 °C. Excess pyridine was evaporated to dryness and the residue was dissolved in methanol (20 mL) and filtered. The filtrate was evaporated to 1 mL and to it ether was added. This resulted in the formation of a light-orange crude product which was filtered. The crude product was dissolved in methanol (25 mL) and bubbled with ammonia gas and then filtered. The methanolic solution was evaporated to dryness and dissolved in minimum amount of methanol (20 mL) and triturated with ether. A pale-yellow powder was obtained (0.08 g, 11%): mp 266–268 °C. ^{1}H NMR δ 12.16 (s, 1H, exchangeable with D_2O), 9.04 (d, J = 7.4 Hz, 1H), 8.42 (d, J = 7.3 Hz, 1H), 8.03 (d, J = 8.1 Hz, 1H), 7.96 (d, J = 8.1Hz, 1H), 7.44 (t, J = 8.1 Hz, 1H), 2.2 (t, 2H), 1.62 (br, s, 2H), 1.25 (br, s, 24H), 0.86 (t, 3H).

Biological evaluation

In vitro HIV-1 protease assay

HIV-1 protease (modified SF2 sequence) was chemically synthesized according to a published method [15] involving the replacement of cysteines 67, 95, 67' and 95' with 2-aminobutyric acid to improve handling. Otherwise the protein was identical to the unmodified enzyme.

A fluorometric assay for HIV-1 protease activity [16] was used in this work. It utilizes a substrate [2-aminobenzoyl-Thr-Ile-Nle-(*p*-nitroPhe)-Gln-Arg-amide] which shows weak fluorescence due to intra-molecular quenching by the *p*-nitrophenyl moiety. Enzymatic cleavage of the substrate, confirmed by HPLC analysis, releases the fluorescent product (2-aminobenzoyl-Thr-Ile-Nle) with excitation and emission peaks at 325 and 420 nm. Assays of the compounds as HIV protease inhibitors were performed using a Perkin Elmer LS 50B spectrofluorimeter, a Jasco IFP770 spectrofluorimeter or a Flow Labs Fluoroskan II microtitre plate reader. Assays were carried out in 100 mM MES buffer, 10% glycerol, pH 6.5 at 37 °C using an excitation wavelength of 325 nm and an emission wavelength of 420 nm. Continuous assays were performed on three to five inhibitor concentrations carried out in duplicate using either of the spectrofluorimeters.

The inhibition of each compound was measured in single time point assays in quadruplicate of five inhibitor concentrations, using positive (with enzyme) and negative (without enzyme) controls. Stock solutions (10 mM) of inhibitors in DMSO were diluted with 100 mM MES buffer, 10% glycerol, pH 6.5, and then preincubated for 15 min at 37 °C with 10 μL of ~ 300 nM enzyme, in a total volume of 180 μL made up with buffer. Substrate was then added (20 μL), giving a final concentration of 50 μM, and the enzymatic reaction was terminated after 5 min with 100 mL of 6 M guanidine•HCl in buffer. Fluorescence was monitored on the Titertek Fluoroskan II microtitre plate reader set with 355 nm excitation and 460 nm emission filters. By this method the K_m for the substrate was 37.5 ± 7.5 μM. An aspartyl protease inhibitor, pepstatin (IC_{50} = 2.5 μM; K_i = 1.8 μM) was used as a reference. Results were corrected where necessary for quenching of the 'internal filter effect', at inhibitor concentrations > 50 μM.

Viral cytopathogenesis assay

The activity of the compounds against the replication of HIV-1 was determined from the inhibition of virus-induced cytopathogenicity in MT4 cells, as previously described [17]. Briefly, MT-4 cells were infected with HTLV-III_B (a laboratory strain of HIV-1) at a multiplicity of infection of 0.02 and incubated in the presence of various concentrations of test compounds. After a 4 day incubation, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) method, as previously described [17]. Cytotoxicity of the compounds for mock-infected MT4 cells was also assessed by the MTT method.

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