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Synthesis, DNA binding properties and biological evaluation of novel oligo-*meta*-benzamides related to netropsin

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Summary — A series of oligo-*meta*-benzamides structurally related to the natural agent netropsin have been synthesized. Their binding constants to double-stranded polynucleotides as well as their cytostatic activity against tumor cell lines and their *in vitro* activity against a wide variety of DNA and RNA viruses have been determined. Most of these analogues retain the DNA binding capacity of the parent compound but with a notable decrease of selectivity and affinity. Like netropsin, the evaluated analogues did not show significant cytostatic and antiviral activity.

Résumé — Synthèse, propriétés d'association à l'ADN et évaluation biologique de nouveaux oligomères de la *meta*-benzamide apparentés à la nétropsine. *Une série d'oligo-*meta*-benzamides structurellement apparentés à la molécule naturelle nétropsine ont été synthétisés. Leurs constantes d'association à des polynucléotides en double brin ainsi que leurs activités cytostatiques et antivirales ont été déterminées. La plupart de ces analogues conservent l'aptitude à se fixer à l'ADN avec cependant une sélectivité et une affinité réduite. Comme la nétropsine, aucun des analogues évalués n'a montré une activité cytostatique ou antivirale notable.*

***meta*-benzamide analogues / netropsin / DNA binding properties / biological activities**

Introduction

Rational design of sequence-specific DNA binding molecule is of great current importance not only from a fundamental biological point of view but also in a therapeutic approach as antiviral and antitumor agents [2]. In this regard, besides natural and modified synthetic antisense oligonucleotides [3], the structurally related antibiotics netropsin and distamycin (fig 1) have received much attention to date. These polypyrrolocarboxamides exert their biological activities by blocking the template function of DNA by strong non-intercalative binding to $(A \cdot T)_n$ sequences in the minor groove of double-stranded B-DNA [4]. In spite of their reported antiviral and anticancer properties, these 2 molecules are too toxic for clinical use. In order to decrease the toxicity and increase the potency

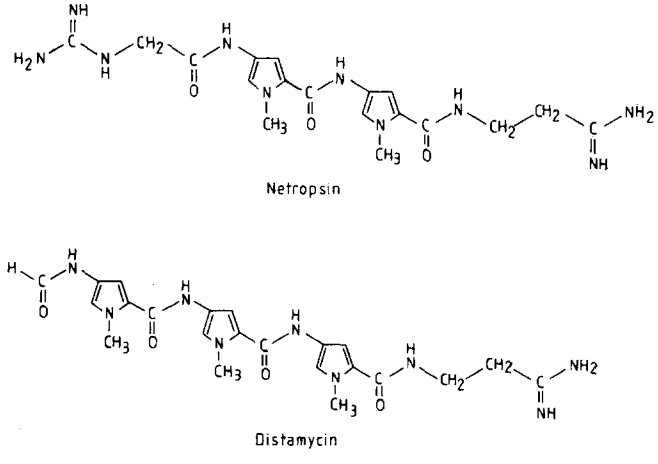

Fig 1. Structure of netropsin and distamycin.

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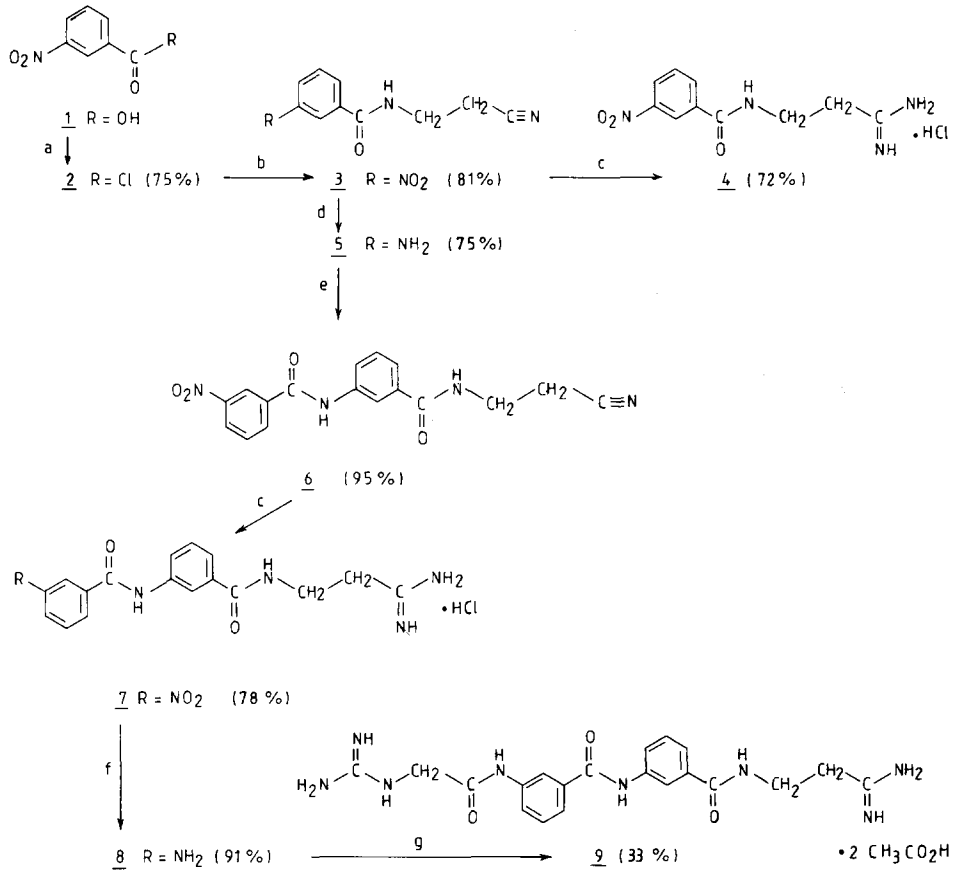
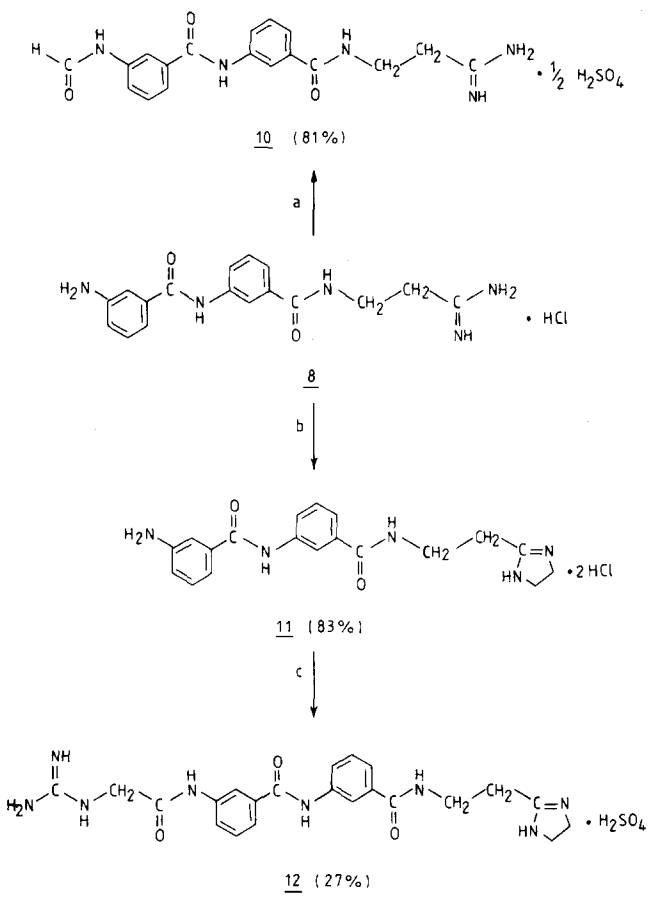
and selectivity of these agents, several attempts of structural modification have been reported. Examples of these modifications include variation of the number of pyrrole units and their replacement by other rings, changes in the mode of pyrrole linkages and substitution of the side chains (surveyed in [5, 6]) [7–15]. While a significant increase of potency compared with the parent natural compounds was achieved for only a few derivatives, it is noteworthy that the replacement of the pyrrole ring(s) by hydrogen bond accepting heterocycles such as imidazole resulted in an alteration of DNA base recognition from A•T to G•C [16–24].

As a part of our ongoing work in attempting to understand the structural requirements of oligopeptides for biological activities and DNA-binding properties we have previously described some analogues of netropsin showing substitution of the aminopropionamidine side chain by either a polyamine or by mono-, di- or tri-L-alanyl residues [6]. In the present study we report the synthesis, characterization and biological evaluation of new netropsin analogues which bear both 1,3-disubstituted benzene moieties in place of *N*-methylpyrrole and various side chains including aminopropionamidine, aminoethylimidazoline and spermidine functions. The rationale for these structural changes results from an attempt to assess the influence of both the backbone curvature of these molecules and the nature of the side chains on their DNA-binding selectivity and biological activities. Thus model building studies have shown that benzene rings in place of *N*-methylpyrroles increase the curvature of the molecule as compared to netropsin and distamycin. Such a modification, as well as the change in the natural side chains, might lead to different van der Waals and hydrogen-bond interactions, and consequently, altered DNA affinity and selectivity. There have been previous reports in the literature of synthesis of bis- and tris(*meta*-benzamido)netropsin or distamycin analogues [7, 8, 10, 25, 26]. However,



Scheme 1. Reaction conditions: (a) $SOCl_2$, Δ; (b) H_2N-$(CH_2)_2$-CN/pyridine; (c) HCl in $CHCl_3$-C_2H_5OH, then dry NH_3 in C_2H_5OH; (d) H_2, Pd/C in CH_3OH-DMF; (e) **2**/pyridine; (f) H_2, Pd/C in C_2H_5OH; (g) HCl•H_2NC (= NH) $NHCH_2CO_2H$, DCC in DMF.



Scheme 2. Reaction conditions: (a) $CH_3C(=O)OC(=O)H$; (b) $H_2N(CH_2)_2NH_2/C_2H_5OH$; (c) $HCl{\cdot}H_2NC(=NH)NHCH_2CO_2H$, DCC in DMF.

so far no modification has been introduced in their side chains and only a few data on their physicochemical and biological properties have been reported.

Results and Discussion

Chemistry

The preparation of the 1,3-disubstituted benzene netropsin homologue **9** was carried out from the commercial 3-nitrobenzene-1-carboxylic acid **1** essentially according to the strategy originally reported [25]; the main features are outlined in scheme 1.

The synthesis of derivatives **10** and **12** carrying a formyl or an ethylimidazoline function in place of the natural guanidinoacetyl of propionamidine side chains respectively was accomplished from the intermediate **8**, previously obtained during the preparation of **9**. On the one hand, the exocyclic amine of **8** was condensed with formic acetic anhydride [27] to afford the already known **10** [7] in good yield. On the other hand, the amidine function of **8** was first transformed with ethylenediamine [28] to an imidazoline function, after wich the amine of the resulting **11** was reacted with guanidinoacetic acid hydrochloride [29] in the presence of dicyclohexylcarbodiimide (DCC) to yield the hitherto unknown analogue **12** (scheme 2).

Concerning the introduction of spermidine as a side chain, 2 procedures were applied for amide bond formation, namely either a direct reaction of an amine with an acid chloride in the presence of base or a one-step active ester procedure implementing benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent [30, 31].

Thus condensation of N^4,N^8-bis(*tert*-butoxycarbonyl)spermidine [32] with the acid chloride **2** in a pyridine-acetonitrile mixture gave **13**. The latter was reduced by catalytic hydrogenation to give the amine **14** which was acylated with **2** to afford **15**. This nitro compound **15** was reduced catalytically to give the amine **16** which was allowed to react with guanidinoacetic acid hydrochloride in the presence of DCC to give **17**. From the latter compound, removal of the *tert*-butoxycarbonyl protecting groups on treatment with trifluoroacetic acid proceeded satisfactorily to yield the target compound **18** (scheme 3).

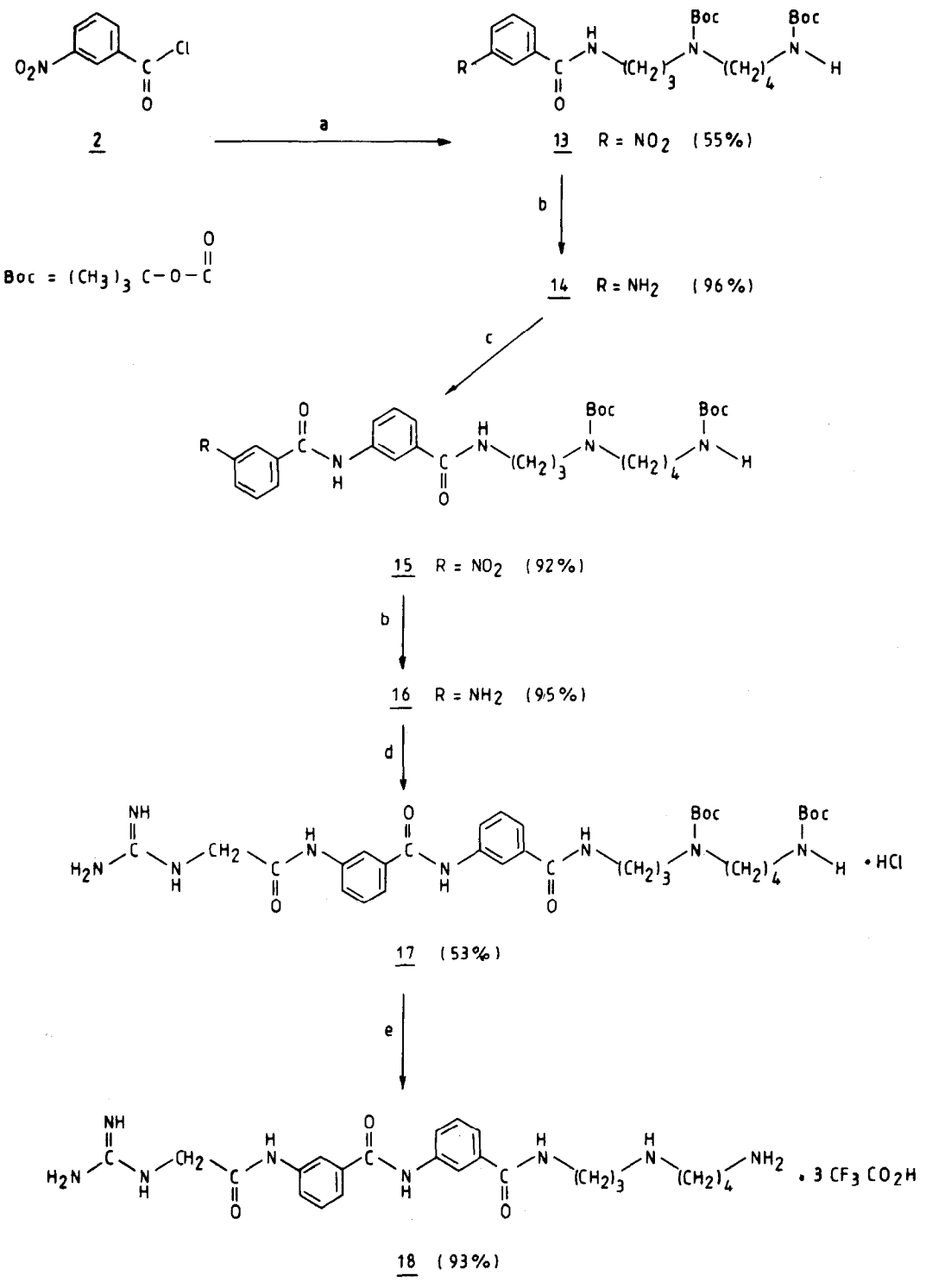
Furthermore, the amine **16** was also transformed into its succinamido derivative **19**, which was subsequently condensed with N^4,N^8-bis(*tert*-butoxycarbonyl)spermidine using BOP as coupling reagent to yield the desired fully protected compound **20**. Finally, removal of the *tert*-butoxycarbonyl groups from **20** on treatment with trifluoroacetic acid yielded the last target analogue **21** (scheme 4).

It is noteworthy that the low yields observed during the synthesis of **9**, **12** and **17** were caused by a known side reaction [33] between the carbodiimide and aminobenzene moieties as exemplified by the isolation of N^1-([3-[3-(*N*,*N*'-dicyclohexylguanilyl)-benzene-1-carboxamido]-benzen-1-yl]carbonyl)-N^4,N^8-bis(*tert*-butoxycarbonyl)spermidine during the purification of the crude reaction mixture resulting from the coupling of the amine **16** with guanidinoacetic acid in the presence of DCC.

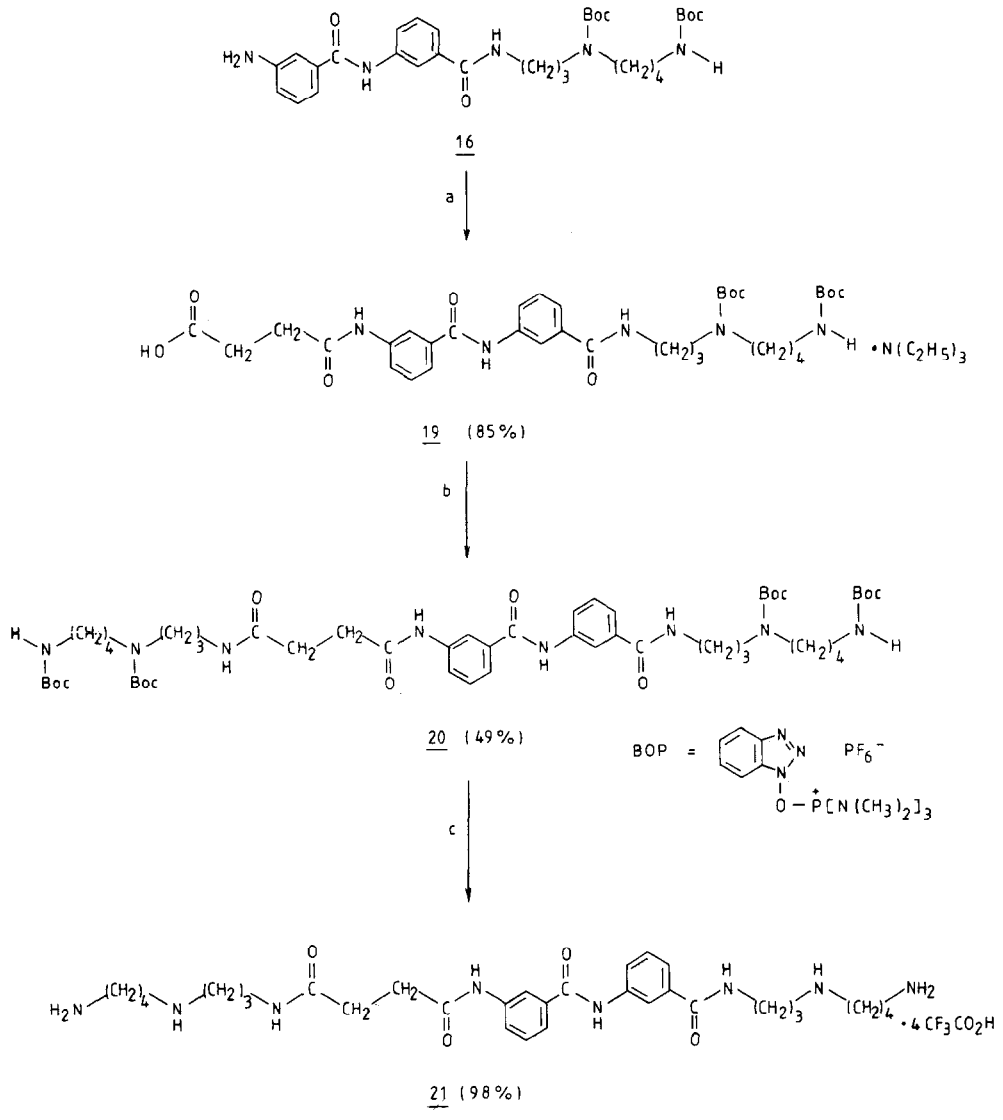
Binding of compounds to polynucleotides

The binding constants of netropsin analogues **4**, **7–10**, **12**, **18** and **21** to poly(dA-dT)•poly(dA-dT), and poly(dG-dC)•poly(dG-dC) are presented in table I.

Compound **9** which differs from netropsin only by the substitution of the original *N*-methylpyrrole rings



Scheme 3. Reaction conditions: (a) $H_2N\ (CH_2)_3\ N(Boc)(CH_2)_4NHBoc$/pyridine–$CH_3CN$; (b) H_2, Pd/C in CH_3OH; (c) **2**/pyridine–CH_3CN; (d) $HCl{\cdot}H_2N\ C\ (=NH)\ NHCH_2CO_2H$, DCC in DMF; (e) CF_3CO_2H.



Scheme 4. Reaction conditions: (a) Succinic anhydride, DMAP, $(C_2H_5)_3N/CH_2Cl_2$; (b) $H_2N(CH_2)_3$ N(Boc) $(CH_2)_4$ NH Boc, BOP/CH_3CN; (c) CF_3CO_2H.

by benzene moieties, displays a much lesser affinity for poly(dA-dT)•poly(dA-dT) than the parent molecule (decrease of K_{app}: more than 2 orders of magnitude). Since this compound is able to better bind to poly(dG-dC)•poly(dG-dC) than netropsin (increase of K_{app}: ≈ 1 order of magnitude), the result is a loss of sequence selectivity recognition for **9**. From this analogue **9**, change of the left guanidinoacetamido side chain by a nitro function to give **7** lowers the affinity constant for double-stranded polynucleotides, while its change by an amino or a formylamino function (compounds **8** and **10**) leads to the disappearance of significant binding capacity. On the other hand, cyclising the propionamidino right side chain of **9** into an ethylimidazoline function to give **12** does not appreciably modify the affinity for polynucleotides. Finally, it can be noted that the spermidine derivatives **18** and **21** keep the ability of the parent compound, netropsin, to bind to poly(dA-dT)•poly(dA-dT) albeit with reduced affinity. In addition, these spermidine derivatives also bind better to poly(dG-dC)•poly(dG-dC) than netropsin.

Table I. Association constants (K_{app}) of compounds with polynucleotides.

Compd	K_{app}, M^{-1}		*Ratio* K_{app} *poly(dA-dT)/ poly(dG-dC)*
	poly(dA-dT)	*poly(dG-dC)*	
4	6.3×10^3	1.2×10^3	5.25
7	1.8×10^4	3.4×10^3	5.29
8	$< 10^2$	$< 10^2$	
9	3.5×10^5	2.3×10^5	1.52
10	$< 10^2$	$< 10^2$	
12	4.7×10^5	2.1×10^5	2.24
18	4.1×10^6	6.7×10^4	61.19
21	8.2×10^6	5.4×10^5	15.19
Netropsin	7.3×10^8	3.0×10^4	23548
Distamycin	8.5×10^7	2.5×10^4	3400

Table II. Inhibitory effects of compounds on the proliferation of murine leukemia (L1210), human B-lymphoblast (Raji) and human T-lymphoblast (Molt/4F) cells.

Compd	CD_{50}[a] *(μg/ml)*		
	L1210	*Raji*	*Molt/4F*
4	> 200	171 ± 13	> 200
7	> 200	> 200	> 200
8	> 200	≥ 200	> 200
9	> 200	145 ± 30	> 200
10	> 200	> 200	> 200
12	> 200	> 200	> 200
18	> 200	153 ± 42	≥ 200
21	> 200	> 200	> 200
Netropsin	≥ 100	≥ 100	> 200

[a]50% cytotoxic dose or dose required to inhibit cell proliferation by 50%.

Biological evaluation

Cytostatic activity

Among the 8 netropsin analogues tested for their inhibitory effects on the proliferation of exponentially growing murine leukemia L1210 and human B-lymphoblast Raji and T-lymphoblast Molt/4F cells, compounds **4**, **9** and **18** proved to be slightly cytostatic to Raji tumor cells (table II). None of the other compounds showed any cytostatic activity at the highest concentration tested (200 μg/ml).

Antiviral activity

The netropsin analogues were evaluated for their *in vitro* inhibitory effects on the replication of a number of DNA viruses (*ie* herpes simplex virus type 1 (KOS) and type 2 (G), vaccinia virus) and RNA viruses (vesicular stomatitis virus, Coxsackie virus B4, polio virus-1, parainfluenza virus-3, reovirus-1, Sindbis virus and Semliki forest virus) in primary rabbit kidney, HeLa and Vero B cell cultures (table III; for comparative purposes, the antiviral data of the well-known antiviral compounds tubercidin, (S)-DHPA, ribavirin, and carbocyclic 3-deazaadenosine are included). With a few exceptions, none of the analogues showed a marked antiviral effect at doses up to 200 or 400 μg/ml. Compounds **7** and **18** were active at 100 and 300 μg/ml, respectively, against vesicular stomatitis virus in HeLa cells; they were also effective at 150 μg/ml against reovirus-1 in Vero B cells. None of the compounds showed any detectable alteration of host cell morphology at the highest concentration tested (400 μg/ml).

When evaluated in an anti-human immunodeficiency virus assay, none of the analogues proved effective against HIV-induced cytopathogenicity in human MT-4 cells at nontoxic concentrations (table IV).

Conclusion

According to the present results, it is obvious that the change of the *N*-methylpyrrole rings by benzene moieties results in a loss of DNA-binding specificity, perhaps owing to the increase in the curvature of the molecules. However, the A•T specificity can be in part recovered when the original side chains are substituted by a spermidine function. While the evaluated analogues showed no significant biological activity, it is noteworthy that, in contrast to previously published data [34–40], netropsin itself was also inactive in our assays. These findings provide relevant information in the design of future sequence-specific agents structurally related to netropsin.

Experimental protocols

Chemical synthesis

General procedures and instrumentation used have been described in [6]. High-performance liquid chromatographic (HPLC) analyses were also carried out on a third Waters Associate Unit (apparatus C) equipped with 2 Model 510 EF

Table III. Antiviral activity of compounds against different viruses in different cell systems.

Compd	*Minimum cytotoxic conc*[b] *μg/ml*			*Minimum inhibitory concentration,* [a] *μg/ml*										
				Primary rabbit kidney (PRK) cells				*HeLa cells*		*African green monkey kidney (Vero B) cells*				
	PRK cells	*HeLa cells*	*Vero B cells*	*Herpes simplex virus-1 (KOS)*	*Herpes simplex virus-2 (G)*	*Vaccinia virus*	*Vesicular stomatitis virus*	*Polio virus-1*	*Coxsackie virus B4*	*Sindbis virus*	*Parain-fluenza virus-3*	*Reovirus*	*Semliki forest virus*	
4	400	400	≥ 200	> 200	> 200	> 100	> 200	> 200	> 200	> 200	> 200	> 100	> 100	> 200
7	400	400	400	> 200	> 200	> 200	100	> 200	> 200	> 200	> 200	> 200	150	> 200
8	≥ 400	≥ 400	400	> 400	> 400	> 400	> 200	> 400	> 400	> 400	> 200	> 200	> 200	> 200
9	> 400	≥ 400	400	> 400	> 400	> 400	> 400	> 400	> 400	> 400	> 200	> 200	> 200	> 200
10	> 400	≥ 400	400	> 400	> 400	> 400	> 400	> 400	> 400	> 400	> 200	> 200	> 200	> 200
12	> 400	≥ 400	400	> 400	> 400	> 400	> 400	> 400	> 400	> 400	> 200	> 200	> 200	> 200
18	> 400	≥ 400	400	> 400	> 400	> 200	300	> 400	> 200	> 400	> 200	> 200	> 200	150	> 200
21	> 400	> 400	≥ 400	> 400	> 400	> 400	> 400	> 400	> 400	> 400	> 200	> 200	> 200	> 200	> 200
Netropsin	≥ 40	≥ 100	200	> 40	> 40	> 10	> 40	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
Tubercidin	≥ 0.4	≥ 1	0.4	> 0.1	> 0.1	> 0.1	0.2	0.04	0.2	0.07	> 0.1	> 0.1	> 0.1	> 0.1	> 0.1
(S)-DHPA	> 400	> 400	≥ 400	> 400	> 400	70	20	150	> 400	> 400	> 400	> 400	100	40	> 400
Ribavirin	> 400	≥ 200	> 400	> 400	> 400	20	300	20	150	70	> 400	70	150	200	70
Carbocyclic 3-deazaadenosine	> 400	> 400	≥ 400	> 400	> 400	2	2	2	> 400	> 400	70	400	2	0.7	> 400

[a]Required to reduce virus-induced cytopathogenicity by 50%. Viral cytopathogenicity was recorded as soon as it reached completion in the control virus-infected cell cultures. The multiplicity of infection (MOI) was invariability 100 x $CCID_{50}$, ie 100 times the virus dose needed to infect 50% of the cells. [b]Required to cause a microscopically detectable alteration of normal cell morphology, when incubated with cells for the same duration as required to measure antivirial activity.

solvent delivery systems, a Model 680 automated gradient controller, a Model U6K sample injector, a Waters 990 photodiode array detector, and a NEC-APC IV microprocessor-controlled data system (standard program Waters 990+).

Compounds **2–9** were synthesized with only minor modifications following similar conditions as those used by Julia and Gombert during the original synthesis of **9** [25]. The strategy of their preparation is shown in scheme 1 and their physical properties are presented below.

3-(3-Nitrobenzene-1-carboxamido)propionitrile ***3***

Mp = 110–111°C (lit mp = 104–105°C [25]); ^1H NMR (Me_2SO–d_6) δ 2.85 (t, 2H, CH_2-CN), 3.6 (m, 2H, NH-CH_2), 7.6–8.8 (m, 4H, C_6H_4), 9.25 (t, 1H, NH); MS (matrix, 3-nitrobenzyl alcohol) FAB > O: 220 $(M+H)^+$.

3-(3-Nitrobenzene-1-carboxamido)-propionamidine hydrochloride ***4***

Mp = 210–213°C (lit mp = 210–212°C [25]); ^1H NMR (Me_2SO–d_6) δ 2.72 (t, 2H, NH-CH_2-CN_2), 3.6 (m, 2H, NH-

Table IV. Anti-HIV-1 and cytostatic activities of compounds in human T-lymphocyte (MT-4) cells.

Compd	CD_{50}[a] *(μg/ml)*	ED_{50}[b] *(μg/ml)*
4	97.6 ± 13.3	> 40
7	> 200	> 200
8	> 200	> 200
9	> 200	> 200
10	> 200	> 200
12	> 200	> 200
18	> 200	> 200
21	> 200	> 200
Netropsin	63.9 ± 17.9	> 40

[a]50% cytotoxic dose; [b]50% effective dose

CH_2-CH_2), 7.8–8.7 (m, 4H, C_6H_4), 8.5–9.1 [br s, 4H, (C=NH_2^+)-NH_2], 9.24 (t, 1H, NH); MS (matrix, glycerol) FAB > O: 237 $[M+H-HCl]^+$.

3-(3-Aminobenzene-1-carboxamido)propionitrile ***5***

Mp = 118–119°C (lit mp = 123–124°C [25]); ^1H NMR (Me_2SO–d_6) δ 2.78 (t, 2H, CH_2CN), 3.5 (m, 2H, NH-CH_2), 5.3 (br s, 2H, NH_2), 6.6–7.3 (m, 4H, C_6H_4), 8.6 (t, 1H, NH); MS (matrix, 3-nitrobenzyl alcohol) FAB > O: 190 $[M+H]^+$.

3-[3-(3-Nitrobenzene-1-carboxamido)-benzene-1-carboxamido]propionitrile ***6***

Mp = 187–188°C (lit mp = 180°C [25]); ^1H NMR (Me_2SO–d_6) δ 2.85 (t, 2H, CH_2-CN), 3.6 (m, 2H, NH-CH_2), 7.5–8.8 (m, 8H, 2 C_6H_4), 8.9 (t, 1H, *NH*-CH_2), 10.8 (br s, 1H, *NH*-C_6H_4); MS (matrix, 3-nitrobenzyl alcohol) FAB > O: 339 $[M+H]^+$.

3-[3-(3-Nitrobenzene-1-carboxamido)-benzene-1-carboxamido]propionamidine hydrochloride ***7***

Mp = 148–150°C (lit mp = 142–145°C [25]); ^1H NMR (Me_2SO–d_6) δ 2.71 (t, 2H, CH_2-CH_2-), 3.6 (m, 2H, NH-CH_2), 7.4–8.5 and 8.83 (m and s, 7 and 1H respectively, 2 C_6H_4), 8.78 (t, 1H, *NH*-CH_2), 8.7–9.2 [br s, 4H, (C=NH_2^+)NH_2], 10.8 (br s, 1H, *NH*, C_6H_4); MS (matrix, glycerol) FAB > O: 356 [M+H-HCl]$^+$; HPLC retention time 14.9 min, spectrophotometric purity at 254 nm: > 97% [UV, λ_{max} 224 and 256 (sh) nm] (Apparatus B; column C_{18} 'Radial Pak' (100 x 8 mm id, 10 μm particle size) in a Waters Associates Radial compression module RCM 100, protected by a prefilter and a precolumn C_{18} 'Guard Pak'; solvent A, 100% 0.1 M ammonium acetate buffer, pH 5.9; solvent B, 50% of acetonitrile in the same buffer; linear gradient 0–50% B in 10 min, then 50–100% B in 5 min; flow rate 3.0 ml/min).

3-[3-(3-Aminobenzene-1-carboxamido)-benzene-1-carboxamido]propionamidine hydrochloride ***8***

^1H NMR (Me_2SO–d_6) δ 2.71 (t, 2H, NH-CH_2-CH_2-), 3.6 (m, 2H, NH-CH_2), 5.3 (br s, 2H, NH_2), 6.7–8.3 (m, 8H, 2 C_6H_4), 8.7 [m, 3H, *NH*-CH_2 and (C=NH_2^+)NH_2], 9.1 [br s, 2H, (C=NH_2^+)NH_2], 10.2 (s, 1H, *NH*-C_6H_4); MS (matrix, 3-nitrobenzyl alcohol) FAB > O: 326 $[M+H-HCl]^+$; HPLC retention time 11.4 min, spectrophotometric purity at 254 nm: > 96% (UV, λ_{max} 227 nm) (same apparatus, column, solvents and conditions as described for the characterization of **7**).

3-[3-[3-(Guanidinoacetamido)benzene-1-carboxamido]-benzene-1-carboxamido]propionamidine bis(acetate) ***9***

This compound, first obtained as a sulfate salt [25] with a 42% yield, was then transformed in its acetate salt to improve its aqueous solubility. ^1H NMR (Me_2SO–d_6) δ 1.65 (s, 6H, 2 CH_3CO_2-), 2.62 (t, 2H, NH-CH_2-CH_2), 3.6 (m, 2H, NH-CH_2-CH_2), 4.08 (s, 2H, NH-CH_2-CO), 7.4–8.3 (m, 8H, 2 C_6H_4), 8.4–10.5 [br s, 12H, 2(C=NH_2^+)NH_2 and 4NH]; MS (matrix, glycerol) FAB > O: 425 $[M+H-2CH_3CO_2H]^+$; HPLC retention time 11.3 min, spectrophotometric purity at 254 nm: > 95% (UV, λ_{max} 248 nm) (Apparatus B; column, Ultrasphere XLODS cartridge Beckman, 70 x 4.6 mm id, 3 μm particle size, protected by prefilters and a XLODS precolumn (5 x 4.6 mm id, 3 μm); solvent A, 100% ammonium acetate buffered to pH 2.5 by formic acid; solvent B, 50% acetonitrile in the same buffer; linear gradient 0–50% B in 10 min, then 50–100% B in 5 min; flow rate 1 ml/min).

3-[3-[3-(Formylamino)benzene-1-carboxamido]-benzene-1-carboxamido]propionamidine hemi(sulfate) ***10***

Acetic formic anhydride (0.203 ml) [27] was added to compound **8** (0.253 g, 0.7 mmol). The resulting solution was first stirred at room temperature for 1 h, then diluted with methanol (4 ml) and finally poured into diethyl ether. The obtained precipitate was collected, dissolved in water (1.5 ml) at 50°C and to the solution was added sodium sulfate (0.1 g, 0.7 mmol). By cooling to room temperature, the sulfate salt of **10** crystallized (0.228 g, 81% yield); mp: 187–189°C; ^1H NMR (Me_2SO–d_6) δ 2.64 (t, 2H, NH-CH_2CH_2-), 3.6 (m, 2H, NH-CH_2), 7.3–8.3 (m, 8H, 2 C_6H_4), 8.27 [s, 1H, H-C(=O)], 8.5–9.3 [m, 7H, (C=NH_2^+)NH_2 and 3 NH]; MS (matrix, glycerol) FAB > O: 354 $[M+H-1/2H_2SO_4]^+$; HPLC retention time 17.9 min, spectrophotometric purity at 254 nm: 100% (UV, λ_{max} 229 nm) (same apparatus, column and solvents as described for the characterization of **7**; linear gradient 0–50% B in 20 min, flow rate 3.0 ml/min).

2-[2-[3-(3-Aminobenzene-1-carboxamido)-benzene-1-carboxamido]ethyl]imidazoline bis(hydrochloride) ***11***

A solution of compound **8** (0.253 g, 0.7 mmol) and anhydrous ethylenediamine (0.051 ml, 0.77 mmol) in absolute ethanol (1 ml) was refluxed for 1.5 h [28] and then cooled to room temperature. The solution was evaporated to dryness, the residue was dissolved in ethanol, and 12 N HCl (0.05 ml) was added. The resulting crystalline solid was collected by filtration to give **11** as its bis-hydrochloride salt (0.248 g, 83% yield); mp: 178–180°C; ^1H NMR (Me_2SO–d_6) δ 2.78 (t, 2H, NH-CH_2CH_2), 3.6 (m, 2H, NH-CH_2CH_2), 3.8 (s, 4H, N-CH_2-CH_2-N), 7.4–8.3 (m, 8H, 2 C_6H_4), 8.5 [br s, 2H, C(=N)NH_2^+-CH_2] 8.9 (t, 1H, *NH*-CH_2), 10.3 (br s, 3H, NH_3^+), 10.6 (s, 1H, *NH*-C_6H_4); MS (matrix, glycerol) FAB > O: 352 $[M+H-2HCl]^+$. This compound was sufficiently pure to be used without further purification for the preparation of **12**.

2-[2-[3-(3-(Guanidinoacetamido)benzene-1-carboxamido]-benzene-1-carboxamido]ethyl]imidazoline sulfate ***12***

To a solution of compound **11** (0.12 g, 0.28 mmol) and guanidinoacetic acid hydrochloride [29] (0.043 g, 0.28 mmol) in anhydrous DMF (1.5 ml) was added dicyclohexylcarbodiimide (DCC; 0.116 g, 0.56 mmol). After the mixture was stirred for 7 h at room temperature, the formed precipitate of dicyclohexylurea was removed by filtration, and the filtrate was evaporated. The resulting residue was dissolved in water (3 ml) at 60°C, and to the solution was added sodium sulfate (0.06 g, 0.42 mmol). By cooling to room temperature, the sulfate salt of **12** crystallized (0.041 g, 27 % yield); mp: 204–205°C; ^1H NMR (Me_2SO–d_6) δ 2.8 (m, 2H, NH-CH_2-CH_2), 3.6 (m, 2H, NH-CH_2-CH_2), 3.7 (s, 4H, N-CH_2-CH_2-N), 4.1 (br s, 2H, NH-CH_2-CO), 7.4–8.6 [m, 12H, 2 C_6H_4 and (C=NH_2^+)NH_2], 8.9 [m, 1H, *NH*-CH_2), 9.0–10.6 [very br s, 3H, NH_2-C-*NH* and C(=N)NH_2^+-CH_2), 10.8 (br s, 2H, 2*NH*-C_6H_4); MS (matrix, glycerol) FAB > O: 451 $[M+H-H_2SO_4]^+$; HPLC retention time 9.7 min, spectrophotometric purity at 254 nm: > 97% (UV, λ_{max} 224 nm) (same apparatus, column and solvents as described for the characterization of **7**; linear gradient 0–100% B in 10 min; flow rate 3.0 ml/min).

N^1-*[(3-Nitrobenzen-1-yl)carbonyl]*-N^4,N^8-*bis*(tert-*butoxycarbonyl)spermidine* ***13***

To a solution of N^4,N^8-bis(*tert*)butoxycarbonyl)spermidine [32] (2.1 g, 6.15 mmol) in anhydrous pyridine (3 ml) was dropwise

added at 0°C a solution of the acyl chloride **2** (1.14 g, 6.15 mmol) in anhydrous acetonitrile (3 ml), and the mixture was stirred at ambient temperature for 3 h. After dilution with CH_2Cl_2, the mixture was poured into a saturated $NaHCO_3$ aqueous solution. The organic phase was separated, twice washed with water, dried over sodium sulfate, and evaporated. Chromatography of the residue on a silica gel column using as eluent a stepwise gradient of acetone (0–40%) in CH_2Cl_2 led, after evaporation of the appropriate fractions, to the isolation of pure **13** (1.67 g, 55% yield); ^1H NMR ($CDCl_3$) δ 1.3–2.3 (m, 6H, $3CH_2$), 1.45 and 1.5 [2s, 2 x 9H, $2(CH_3)_3$], 3.0–3.7 (m, 8H, 4 CH_2), 4.75 (m, 1H, *NH*BOC), 7.7–8.7 (m, 4H, C_6H_4), 9.0 (m, 1H, CO-*NH*-CH_2); MS (matrix, glycerol) FAB > O: 495 $[M+H]^+$, 395 $[M+2H\text{-}BOC]^+$, 295 $[M+3H\text{-}2BOC]^+$.

*N^1-[(3-Aminobenzen-1-yl)carbonyl–N^4,N^8-bis(*tert*-butoxycarbonyl)spermidine* ***14***

Methanol (10 ml) cooled to –20°C was added to 10% palladium on charcoal (243 mg) and **13** (1.67 g, 3.38 mmol) was added. The solution was hydrogenated at atmospheric pressure and room temperature. After the calculated amount of hydrogen (≈ 250 ml) was taken up (≈ 4 h), the catalyst was removed by filtration, the methanol was removed *in vacuo*, and the evaporation was repeated with some methanol. The intermediate amine **14** (1.5 g, 96% yield) was not further purified but used directly for the synthesis of **15**; MS (matrix, glycerol) FAB > O: 465 $[M+H]^+$, 365 $[M+2H\text{-}BOC]^+$, 265 $[M+3H\text{-}2BOC]^+$.

*N^1-[[3-(3-Nitrobenzene-1-carboxamido)-benzen-1-yl]-carbonyl]-*N^4,N^8*-bis(*tert*-butoxycarbonyl)spermidine* ***15***

To a solution of amine **14** (0.646 g, 1.39 mmol) in anhydrous pyridine (2 ml) was dropwise added at 0°C a solution of the acyl chloride **2** (0.258 g, 1.39 mmol) in anhydrous acetonitrile (1 ml), and the mixture was stirred at ambient temperature for 1 h. After dilution with CH_2Cl_2, the mixture was poured into a saturated $NaHCO_3$ aqueous solution. The organic phase was separated, twice washed with water, dried over sodium sulfate, and evaporated. Chromatography of the residue on a silica gel column using as eluent a stepwise gradient of acetone (0–50%) in CH_2Cl_2 led to the isolation of pure **15** (0.79 g, 92% yield); ^1H NMR (Me_2SO–d_6) δ 1.4–1.6 (m, 6H, $3CH_2$), 1.42 and 1.44 [2s, 2 x 9H, $2(CH_3)_3$], 3.1–3.3 (m, 8H, $4CH_2$), 4.7 (br s, 1H, *NH*BOC), 7.4–9.7 (m, 8H, 2 C_6H_4), 7.9 (br s, 1H, *NH*-CH_2), 8.1 (br s, 1H, *NH*-C_6H_4); MS (matrix, glycerol) FAB > O: 614 $[M+H]^+$, 514 $[M+2H\text{-}BOC]^+$.

*N^1-[[3-(3-Aminobenzene-1-carboxamido)-benzen-1-yl]-carbonyl]-*N^4,N^8*-bis(*tert*-butoxycarbonyl)spermidine* ***16***

This compound was synthesized (following a similar procedure as described for the preparation of **14**) from the nitro-derivative **15** (1.75 g, 2.85 mmol) by catalytic hydrogenation with 10% palladium on charcoal (243 mg) in methanol (10 ml). After a similar workup, the residue obtained by evaporation of methanol was chromatographed on a silica gel column using as eluent a stepwise gradient of methanol (0–20%) in CH_2Cl_2 to afford pure **16** (1.58 g, 95% yield); ^1H NMR (Me_2SO–d_6) δ 1.1–1.4 [m, 22H, $2(CH_3)_3C$ and $2CH_2$], 1.7 (m, 2H, CH_2), 2.9–3.3 (m, 8H, $4CH_2$), 5.28 (s, 2H, NH_2), 6.7 (m, 1H, CH_2-*NH*BOC), 6.7–8.2 (m, 8H, $2C_6H_4$), 8.37 (t, 1H, CO-*NH*-CH_2), 10.16 (s, 1H, *NH*-C_6H_4); MS (matrix, glycerol) FAB > O: 584 $[M+H]^+$, 484 $[M+2H\text{-}BOC]^+$, 384$[M+3H\text{-}2BOC]^+$.

*N^1-[[3-[3(Guanidinoacetamido)benzene-1-carboxamido]-benzen-1-yl]carbonyl-*N^4,N^8*-bis(*tert*-butoxycarbonyl)spermidine hydrochloride* ***17***

To a solution of compound **16** (0.583 g, 1.0 mmol) and guanidinoacetic acid hydrochloride [29] (0.154 g, 1.0 mmol) in anhydrous DMF (1 ml) was added a solution of DCC (0.309 g, 1.5 mmol) in DMF (1 ml). After the mixture was stirred for a night at room temperature, the formed precipitate of dicyclohexylurea was removed by filtration, and the filtrate was evaporated. The residue was chromatographed on a silica gel column using as eluent a stepwise gradient of methanol (0–30%) with 1% of water in CH_2Cl_2. After evaporation of the appropriate fractions, lyophilization from dioxane gave pure **17** (0.38 g, 53%); ^1H NMR (Me_2SO-d_6) δ 1.3–1.4 [m, 22H, $2(CH_3)_3C$ and $2CH_2$], 1.7 (m, 2H, CH_2), 2.9 (m, 2H, CH_2), 3.1–3.3 (m, 6H, $3CH_2$), 4.10 (d, 2H, NH-CH_2-CO), 6.76 (t, 1H, CH_2-*NH*-BOC), 7.3 [br s, 4H, $C(=NH_2^+)NH_2$], 7.64 [t, 1H, CH_2-*NH*-$(C=NH_2^+)NH_2$], 7.4–8.2 (m, 8H, $2C_6H_4$), 8.43 [t, 1H, CO-*NH*-CH_2], 10.39 and 10.50 (2s, 1H each, 2 CO-*NH*-C_6H_4); MS (matrix glycerol) FAB > O: 683 $[M+H\text{-}HCl]^+$, 483 $[M+2H\text{-}HCl\text{-}BOC]^+$; HPLC retention time 15.5 min, spectrophotometric purity at 254 nm: 100% (UV, λ_{max} 227 nm) (same apparatus, column and solvents as described for the characterization of **7**; linear gradient 0–100% B in 10 min, then 100% B for 10 min; flow rate 3.0 ml/min).

N^1-[[3-[3-(Guanidinoacetamido)benzen-1-carboxamido]-benzen-1-yl–carbonyl]-spermidine tris(trifluoroacetate) ***18***

Compound **17** (0.05 g, 0.07 mmol) was dissolved in trifluoroacetic acid (TFA, 1 ml). After 20 min, the solution was evaporated to dryness, the resulting residue was dissolved in water and the aqueous layer was twice washed with ethyl ether. After evaporation of water, lyophilization from water gave pure **18** (0.054 g, 93% yield); ^1H NMR (Me_2SO–d_6) d 1.6 and 1.9 (2 m, 4 and 2H, $3CH_2$), 2.8, 3.0 and 3.3 (3m, 2, 4 and 2H, 4 CH_2-N), 4.08 (d, 2H, NH-CH_2-CO), 7.5–8.6 (m, 8H, 4 C_6H_4), 7.4, 7.7, 7.9 and 8.6 [4 br s, 4, 1, 3 and 2H respectively, $C(=NH_2^+)NH_2$, NH, NH_3^+ and NH_2^+], 8.63 (t, 1H, CO-*NH*-CH_2), 10.39 and 10.41 (2s, 2H, 2 CO-*NH*-C_6H_4); MS (matrix, glycerol) FAB > O: 597 $[M+H\text{-}2CF_3CO_2H]^+$, 483 $[M+H\text{-}3\ CF_3CO_2H]^+$; HPLC retention, time 9.2 min, spectrophotometric purity at 254 nm: 100% (UV, λ_{max} 227 nm) [same apparatus, column, solvents and conditions as described for the characterization of **17**].

*N^1-[[3-[3-(Succinamido)benzene-1-carboxamido]-benzen-1-yl]carbonyl]-*N^4,N^8*-bis(*tert*-butoxycarbonyl)spermidine triethylammonium* ***19***

Compound **16** (0.498 mg, 0.853 mmol) was first coevaporated 3 times with anhydrous pyridine and then dissolved in pyridine, and 4-dimethylaminopyridine (DMAP, 0.183 g, 1.5 mmol) was added to the solution.

The solution was evaporated to dryness, and after 3 coevaporations with anhydrous acetonitrile, the mixture of **16** and DMAP was dissolved in anhydrous CH_2Cl_2 (4.5 ml). To the stirred solution succinic anhydride (0.150 g, 1.5 mmol) and triethylamine (0.21 ml, 1.5 mmol) were added. The reaction was stirred for 2 h at ambient temperature. Saturated aqueous $NaHCO_3$ (30 ml) was added, and the resulting mixture was extracted with CH_2Cl_2. The combined organic layers were washed once with water, dried over sodium sulfate and evaporated to dryness. The residue was chromatographed on a silica gel column using as eluent a strepwise gradient of methanol (0–20%) with 1% of triethylamine in CH_2Cl_2 to

afford **19** (0.57 g, 85% yield). This compound was sufficiently pure (TLC and mass spectrum analyses [matrix, glycerol; FAB < O: 682 [M-H-$(C_2H_5)_3N$]$^-$, 582 [M-H-$(C_2H_5)_3N$-BOC]$^-$] to be used directly for the preparation of **20**.

N^1-*[[3-{3-[*N^4,N^8-*bis(*tert-*butoxycarbonyl)spermidinosuccinamido]benzene-1-carboxamido}-benzen-1-yl]carbonyl]-*N^4,N^8-*bis(*tert-*butoxycarbonyl)spermidine* ***20***

To a solution of compound **19** (0.57 g, 0.73 mmol) and of N^4, N^8-bis(*tert*-butoxycarbonyl)spermidine [32] (0.376 g, 1.09 mmol) in anhydrous acetonitrile (11 ml) was added benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate [30, 31] (BOP, 0.321 g, 0.73 mmol). The mixture was stirred at room temperature for 2 h and then saturated NaCl solution (35 ml) was added. Compound **20** was extracted with ethyl acetate (3 x 10 ml). The combined organic layers were washed successively with 2 N HCl (50 ml), water (50 ml), 5% $NaHCO_3$ (50 ml) and water and then dried over sodium sulfate and filtered. The filtrate was evaporated to dryness. The resulting residue was chromatographed on a silica gel column using as eluent a stepwise gradient of methanol (0–10%) in CH_2Cl_2. After evaporation of the appropriate fractions, lyophilization from dioxane gave pure **20** (0.36 g, 49% yield); ^{1}H NMR (Me_2SO–d_6) δ 1.3–1.4 [m, 44H, 4$(CH_3)_3C$ and 4CH_2], 1.6 and 1.7 (2m, 2H each, CH_2 each), 2.41 and 2.58 (2t, 2H each, CO-CH_2-CH_2-CO), 2.9 and 3.2 (2m, 4H and 12H respectively, 8CH_2), 6.8 (br s, 2H, 2 CH_2-*NH*-BOC), 7.8 [br s, 1H, CO-*NH*-$(CH_2)_3$]. 7.4–8.2 (m, 8H, 4 C_6H_4), 8.4 (m, 1H, CO-*NH*-CH_2), 10.12 and 10.35 (2s, 1H each, 2CO-*NH*-C_6H_4); MS (matrix, glycerol) FAB > 0: 1011 [M+H]$^+$, 911 (M+2H-BOC]$^+$, 811 [M+3H-2BOC]$^+$, 711 [m+4H-3BOC]$^+$, 611 [M+5H-4BOC]$^+$.

N^1-*[[3-{3-[*N^1-*(spermidino)succinamido]benzene-1-carboxamido}-benzen-1-yl]carbonyl]-spermidine tetra(trifluoroacetate)* ***21***

Compound **20** (0.05 g, 0.049 mmol) was dissolved in TFA (2 ml). After 15 min, the solution was evaporated to dryness, the residue was dissolved in water and the aqueous layer was twice washed with ethyl ether and lyophilized from water to give pure **21** (0.051 g, 98% yield); ^{1}H NMR (Me_2SO–d_6) δ 1.6, 1.7 and 1.9 (3m, 8, 2 and 2H respectively, 6CH_2), 2.44 and 2.61 (2t, 2H each, CO-CH_2-CH_2-CO), 2.8–3.0, 3.1 and 3.4 (3m, 12, 2 and 2H respectively, 8 CH_2), 7.9 (br s, 6H, 2NH_3^+), 8.09 (t, 1H, *NH*-CO), 7.4–8.3 (m, 8H, 2C_6H_4), 8.6 (m, 5H, 2NH_2^+ and CO-*NH*-CH_2), 10.18 and 10.39 (2s, 1H each, 2CO-*NH*-C_6H_4); MS (matrix, glycerol) FAB > 0: 611 [M+H-4CF_3CO_2H]$^+$; HPLC retention time 9.0 min, spectrophotometric purity at 254 nm: ≥ 99% (apparatus C; same column as described for the characterization of **9**; solvent A, 100% 0.1 M ammonium acetate buffer, pH 5.9; solvent B, 50% of acetonitrile in the same buffer; linear gradient 0–100% B in 10 min; flow rate 1.0 ml/min).

Determination of association constant values to polynucleotides

The binding constants (K_{app}) of the test compounds to poly(dA-dT)•poly(dA-dT) and poly(dG-dC)•poly(dG-dC) were determined based on their ability to compete with the binding of ethidium bromide, according to Baguley *et al* [41] and as described previously for other netropsin analogues [6].

Biological methods

Cytostatic activity assays
The cytostatic activity assays were performed according to previously established procedures [42, 43].

Anti-human immunodeficiency virus (HIV) assay
The method for determining the cytopathic effect of HIV in MT-4 cells has been previously described [46]. Briefly, MT-4 cells were seeded at 10^6 cells/ml and infected with HIV at 1000 $CCID_{50}$/ml. Then, 10^5 cells were brought into wells of a microtiter tray containing 100 µl of various dilutions of the test compounds. After 5 d incubation at 37°C, the number of viable cells was determined microscopically in a hematocytometer by trypan blue exclusion.

Antiviral activity assays
The antiviral assays were performed as previously reported [44, 45].

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