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Synthesis and Binding Mode of Heterocyclic Analogues of Suramin Inhibiting the Human Basic Fibroblast Growth Factor

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Abstract—The design, synthesis, and biological evaluation of a series of pyrrole and pyrazole congeners **2** of suramin, directed toward the development and identification of new ligands that complex the human fibroblast growth factor (bFGF), thereby inhibiting tumor-promoted angiogenesis, is reported. Compounds **2** were evaluated for their ability to inhibit binding of bFGF to its receptor, in vivo bFGF-induced angiogenesis, and neovascularization of the chorio-allantoic membrane in comparison with suramin. These assays showed that ligands **2** exhibit moderate to good activity, comparable to that of suramin, and are less toxic than suramin itself. In this study, affinity data of ligands in combination with the crystal structure of bFGF were used to explain structure–affinity relationships and to gain an insight into the possible mode of ligand–protein interaction. Due to the lack of experimental structural data on the ligand–bFGF complexes, molecular mechanics techniques were used to obtain putative bioactive conformations and to generate docked complexes with the three-dimensional structure of bFGF. These experiments led to suggest that compounds **2** give rise to 1:1 complexes with bFGF through an unprecedented, bidentate attachment of their naphthylsulfonate groups to two main domains, commonly referred to as the heparin binding site and the receptor binding site, on bFGF, thus preventing the interaction of the growth factor with its receptor.

Introduction

The idea of tumor-induced angiogenesis (i.e. tumor-induced growth of new vessels) was first introduced by Virchow in 1863.[1] Although initially this concept was sceptically considered in specialist circles, it was periodically repeated by several scientists, until the Folkman's group reported in 1971 the isolation of a tumor factor responsible for angiogenesis.[2] In 1985 Vallee's group succeded in purifying to homogeneity angiogenin, an angiogenic protein derived from human colon adenocarcinoma.[3] In the last 10 years, a wealth of angiogenic factors produced either by tumor or by normal tissues have been described[4] and it is now widely accepted that angiogenesis is a fundamental process in the development, progression and metastasis of many human tumors, in that it allows the blood supply necessary for tumor growth.[4–8]

Acidic and basic fibroblast growth factors (aFGF and bFGF) are two closely related peptides which, since 1983, have been purified from different tissues.[9] Their amino acid sequence has also been defined: aFGF shares 55% sequence identity with bFGF, whose predominant form is 16–18 kDa. The three-dimensional structure of bFGF has been elucidated through X-ray crystallographic analysis by several authors in the last few years.[10] These studies, in combination with experiments of site-directed mutagenesis,[11] led to the identification on bFGF of a heparin binding domain and a cell attachment domain (receptor binding site) and the

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binding to heparin has been widely exploited for the isolation of this growth factor. bFGF has been detected in a wide variety of normal and malignant tissues and is known to have a diverse physiological profile that includes mitogenic and angiogenic activities as well as a role in cell differentiation and development. Since angiogenesis is a very active process in malignant tissues, bFGF potentially could play a major role in tumor progression by acting as a mitogen for tumor cells, or by stimulating neoangiogenesis. In light of these considerations, compounds capable of inhibiting bFGF show promise in the development of new antitumor agents.

The antitumor activity of the polysulfonated naphthylurea suramin (**1**) is thought to be derived from this mode of action.[12] This compound exhibits antitumor activity in a number of cell lines,[13–15] and it is currently under intense investigation for the treatment of advanced malignancies. Suramin is known to inhibit a large number of important enzymes[16–18] and to block the activity of several growth factors, one of which is bFGF.[19–22] As a drug candidate, suramin is disadvantaged by its low therapeutic index, its extremely long plasma half-life, and its great metabolic stability.

In an effort to find or design novel compounds able to complex bFGF and consequently block the angiogenic process more effectively than suramin, some analogues (**2**, Figure 1) were synthesized. This effort led us to develop a new class of compounds endowed with better antitumor activity and lower toxicity than suramin.[23]

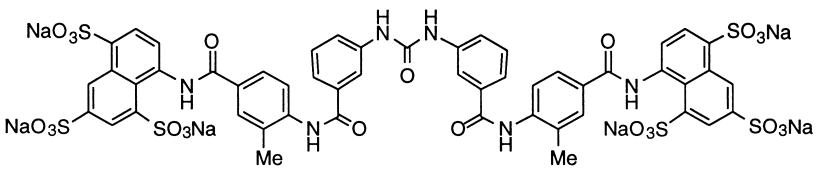
In the present paper we wish to give full account on the synthesis of a group of heterocyclic analogues **2a–s** (Figure 1 and Table 1) of suramin, which have been selected as the most representative of the whole family of synthesized compounds. Compared to **1**, they generally have a lower number of sulfonic groups either on the α- or β-aminonaphthalene moieties, in order to modulate the binding to plasma proteins, while the benzene rings have been substituted by heterocycles (namely pyrrole and/or pyrazole) which might confer less metabolic stability. Moreover, structure–activity relationship (SAR) is discussed and a possible mode of action for compounds **2** is derived through a computational approach based on docking experiments between derivatives **2** and bFGF.

Results

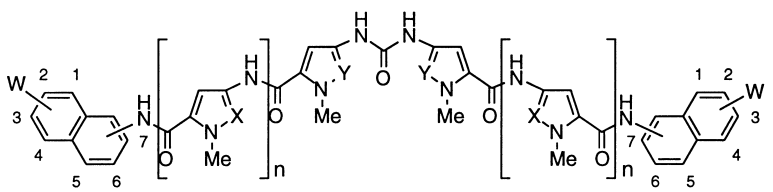
Chemistry

The preparation of the new compounds (Scheme 1) is straightforward and entails the acylation of sulfonated or phosphonated naphthylamines **3a–m** with 1-methyl-4-nitropyrrole-2-carbonyl chloride **4a** or 3-benzyloxycarbonylamino-1-methylpyrazole-5-carbonyl chloride **4b** to give amides **5a–m** in a yield ranging between 50 and 90% (Method A), followed by reduction to the corresponding aminoamides **6a–m** in quantitative yield by catalytic hydrogenation (Method B).

When these reactions were repeated once or twice starting from **6a–m**, amines **8a–o** and **10**, respectively, were



Suramin **1**



2a-s W = SO_3Na, PO_3HNa, $P(O)(OEt)_2$

X, Y = CH, N

n = 0-2

Figure 1. Structure of suramin (**1**) and congeners **2a–s**.

Table 1. Chemical and physical data of compounds **2**, **5–10**

Compd	NH pos.	X	Y	W	n	Formula	FAB-MS (m/z)	Yield (%)
2a	7	CH	CH	1,3-SO_3Na	1	$C_{45}H_{36}N_{10}Na_4O_{17}S_4$	1231 $(M+Na)^+$ 1209 $(M+H)^+$	55
2b	7	CH	CH	1,3,5-SO_3Na	1	$C_{45}H_{34}N_{10}Na_6O_{23}S_6$	1411 $(M-H)^-$ 1389 $(M-Na)^-$	24[a]
2c	7	CH	CH	1,3-SO_3Na	2	$C_{57}H_{48}N_{14}Na_4O_{19}S_4$	1451 $(M-H)^-$ 1429 $(M-Na)^-$	19
2d	8	CH	CH	1,3-SO_3Na	1	$C_{45}H_{36}N_{10}Na_4O_{17}S_4$	1231 $(M+Na)^+$ 1209 $(M+H)^+$ 1187 $(M-Na+H)^+$	22
2e	8	CH	CH	1,3,5-SO_3Na	1	$C_{45}H_{34}N_{10}Na_6O_{23}S_6$	1411 $(M-H)^-$ 1389 $(M-Na)^-$	20[b]
2f[c]	7	CH	CH	1,3-SO_3Na	1	$C_{45}H_{36}N_{10}Na_4O_{16}S_5$	1223 $(M-H)^-$ 1201 $(M-Na)^-$	22
2g	7	CH	CH	1,3-SO_3Na	0	$C_{33}H_{24}N_6Na_4O_{15}S_4$	987 $(M+Na)^+$ 965 $(M+H)^+$	20
2h	7	CH	CH	3,5-SO_3Na	1	$C_{45}H_{36}N_{10}Na_4O_{17}S_4$	1209 $(M+H)^+$ 1187 $(M-Na+H)^+$	17
2i	8	CH	CH	3,5-SO_3Na	1	$C_{45}H_{36}N_{10}Na_4O_{17}S_4$	1209 $(M+H)^+$	35[a]
2j	7	CH	CH	4,8-SO_3Na	1	$C_{45}H_{36}N_{10}Na_4O_{17}S_4$	1207 $(M-H)^-$ 1185 $(M-Na)^-$ 1105 $(M-SO_3Na)^-$	98
2k	7	CH	CH	3,6-SO_3Na	1	$C_{45}H_{36}N_{10}Na_4O_{17}S_4$	1209 $(M+H)^+$ 1187 $(M-Na+H)^+$	14
2l	7	N	N	1,3-SO_3Na	1	$C_{41}H_{32}N_{14}Na_4O_{17}S_4$	1213 $(M+H)^+$ 1190 $(M-Na+H)^+$	81
2m	7	CH	CH	2,4-SO_3Na	1	$C_{45}H_{36}N_{10}Na_4O_{17}S_4$	1231 $(M+Na)^+$ 1209 $(M+H)^+$	6[d]
2n	7	CH	CH	1,5-PO_3HNa	1	$C_{45}H_{40}N_{10}Na_4O_{17}P_4$	1209 $(M+H)^+$	99
2o	8	CH	CH	1,5-SO_3Na	1	$C_{45}H_{36}N_{10}Na_4O_{17}S_4$	1231 $(M+Na)^+$ 1209 $(M+H)^+$	26
2p	7	CH	N	1,3-SO_3Na	1	$C_{43}H_{34}N_{12}Na_4O_{17}S_4$	1209 $(M-H)^-$ 1187 $(M-Na)^+$	65
2q	7	N	CH	1,3-SO_3Na	1	$C_{43}H_{34}N_{12}Na_4O_{17}S_4$	1209 $(M-H)^-$ 1187 $(M-Na)^+$	27
2r	8	CH	CH	3-SO_3Na	1	$C_{45}H_{38}N_{10}Na_2O_{11}S_2$	1027 $(M+Na)^+$ 1005 $(M+H)^+$	46
2s	7	CH	CH	1,5-$PO(OEt)_2$	1	$C_{61}H_{76}N_{10}O_{17}P_4$	1344 $(M-H)^-$	77
5a	7	CH	—	1,3-SO_3Na	—	$C_{16}H_{11}N_3Na_2O_9S_2$	498 $(M-H)^-$	87
5b	7	CH	—	1,3,5-SO_3Na	—	$C_{16}H_{10}N_3Na_3O_{12}S_3$	600 $(M-H)^-$	ND[e]
5c	8	CH	—	1,3-SO_3Na	—	$C_{16}H_{11}N_3Na_2O_9S_2$	498 $(M-H)^-$	ND
5d	8	CH	—	1,3,5-SO_3Na	—	$C_{16}H_{10}N_3Na_3O_{12}S_3$	600 $(M-H)^-$	94
5e	7	CH	—	3,5-SO_3Na	—	$C_{16}H_{11}N_3Na_2O_9S_2$	498 $(M-H)^-$	85
5f	8	CH	—	3,5-SO_3Na	—	$C_{16}H_{11}N_3Na_2O_9S_2$	498 $(M-H)^-$	ND
5g	7	CH	—	4,8-SO_3Na	—	$C_{16}H_{11}N_3Na_2O_9S_2$	498 $(M-H)^-$	85
5h	7	CH	—	3,6-SO_3Na	—	$C_{16}H_{11}N_3Na_2O_9S_2$	498 $(M-H)^-$	90
5i	7	N	—	1,3-SO_3Na	—	$C_{23}H_{18}N_4Na_2O_9S_2$	603 $(M-H)^-$	89
5j	7	CH	—	2,4-SO_3Na	—	$C_{16}H_{11}N_3Na_2O_9S_2$	498 $(M-H)^-$	50
5k	7	CH	—	1,5-$PO(OEt)_2$	—	$C_{24}H_{31}N_3O_9P_2$	567 $(M)^+$[f]	90
5l	8	CH	—	1,5-SO_3Na	—	$C_{16}H_{11}N_3Na_2O_9S_2$	498 $(M-H)^-$	95
5m	8	CH	—	3-SO_3Na	—	$C_{16}H_{12}N_3NaO_6S$	396 $(M-H)^-$	97
6a	7	CH	—	1,3-SO_3Na	—	$C_{16}H_{14}ClN_3Na_2O_7S_2$	446 $(M-HCl-Na)^-$	93
6b	7	CH	—	1,3,5-SO_3Na	—	$C_{16}H_{13}ClN_3Na_3O_{10}S_3$	548 $(M-HCl-Na)^-$	ND
6c	8	CH	—	1,3-SO_3Na	—	$C_{16}H_{14}ClN_3Na_2O_7S_2$	446 $(M-HCl-Na)^-$	ND
6d	8	CH	—	1,3,5-SO_3Na	—	$C_{16}H_{13}ClN_3Na_3O_{10}S_3$	548 $(M-HCl-Na)^-$	99
6e	7	CH	—	3,5-SO_3Na	—	$C_{16}H_{14}ClN_3Na_2O_7S_2$	446 $(M-HCl-Na)^-$	ND
6f	8	CH	—	3,5-SO_3Na	—	$C_{16}H_{14}ClN_3Na_2O_7S_2$	446 $(M-HCl-Na)^-$	ND

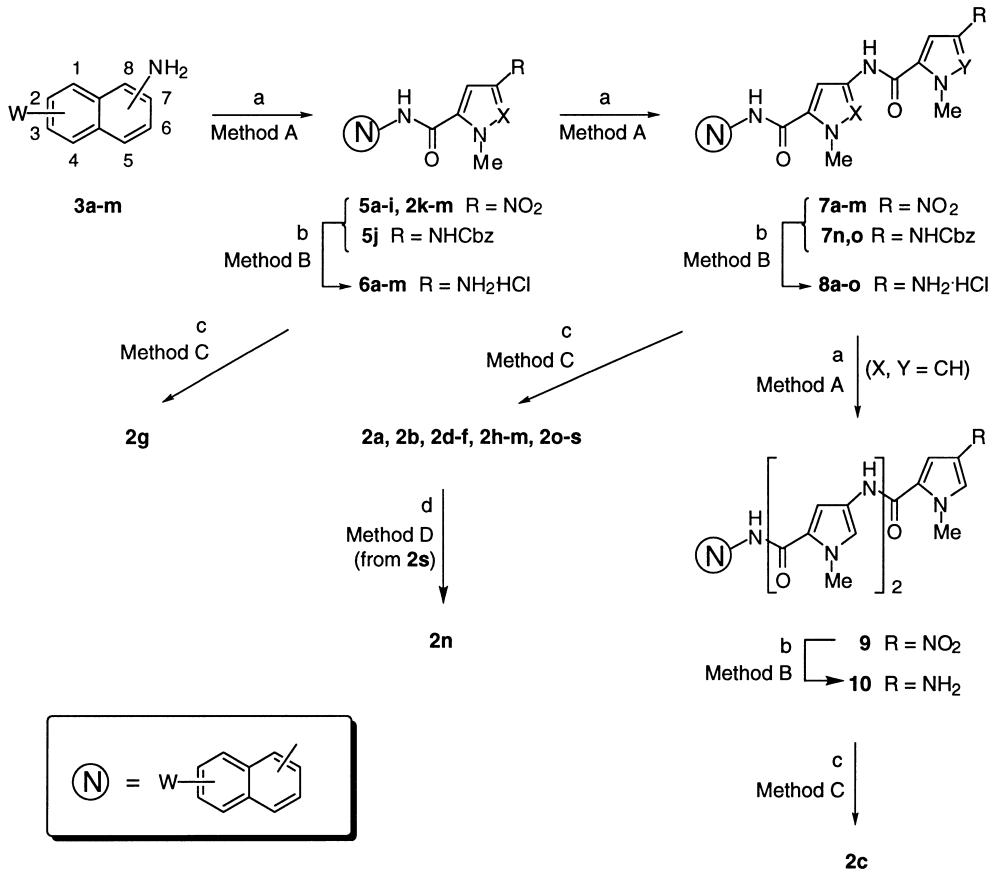
(continued)

Table 1—*contd*

6g	7	CH	—	4,8-SO_3Na	—	$C_{16}H_{14}ClN_3Na_2O_7S_2$	446 $(M-HCl-Na)^-$	51
6h	7	CH	—	3,6-SO_3Na	—	$C_{16}H_{14}ClN_3Na_2O_7S_2$	446 $(M-HCl-Na)^-$	68
6i	7	N	—	1,3-SO_3Na	—	$C_{15}H_{13}ClN_4Na_2O_7S_2$	447 $(M-HCl-Na)^-$	77
6j	7	CH	—	2,4-SO_3Na	—	$C_{16}H_{14}ClN_3Na_2O_7S_2$	446 $(M-HCl-Na)^-$	98
6k	7	CH	—	1,5-$PO(OEt)_2$	—	$C_{24}H_{34}ClN_3O_7P_2$	536 $(M-HCl-H)^-$	99
6l	8	CH	—	1,5-SO_3Na	—	$C_{16}H_{14}ClN_3Na_2O_7S_2$	446 $(M-HCl-Na)^-$	99
6m	8	CH	—	3-SO_3Na	—	$C_{16}H_{15}ClN_3NaO_4S$	344 $(M-HCl-Na)^-$	97
7a	7	CH	CH	1,3-SO_3Na	—	$C_{22}H_{17}N_5Na_2O_{10}S_2$	620 $(M-H)^-$	64
7b	7	CH	CH	1,3,5-SO_3Na	—	$C_{22}H_{16}N_5Na_3O_{13}S_3$	722 $(M-H)^-$	ND
7c	8	CH	CH	1,3-SO_3Na	—	$C_{22}H_{17}N_5Na_2O_{10}S_2$	620 $(M-H)^-$	ND
7d	8	CH	CH	1,3,5-SO_3Na	—	$C_{22}H_{16}N_5Na_3O_{13}S_3$	722 $(M-H)^-$	ND
7e	7	CH	CH	3,5-SO_3Na	—	$C_{22}H_{17}N_5Na_2O_{10}S_2$	620 $(M-H)^-$	ND
7f	8	CH	CH	3,5-SO_3Na	—	$C_{22}H_{17}N_5Na_2O_{10}S_2$	620 $(M-H)^-$	ND
7g	7	CH	CH	4,8-SO_3Na	—	$C_{22}H_{17}N_5Na_2O_{10}S_2$	620 $(M-H)^-$	99
7h	7	CH	CH	3,6-SO_3Na	—	$C_{22}H_{17}N_5Na_2O_{10}S_2$	620 $(M-H)^-$	ND
7i	7	N	CH	1,3-SO_3Na	—	$C_{21}H_{16}N_6Na_2O_{10}S_2$	621 $(M-H)^-$	78
7j	7	CH	CH	2,4-SO_3Na	—	$C_{22}H_{17}N_5Na_2O_{10}S_2$	620 $(M-H)^-$	ND
7k	7	CH	CH	1,5-$PO(OEt)_2$	—	$C_{30}H_{37}N_5O_{10}P_2$	688 $(M-H)^-$	89
7l	8	CH	CH	1,5-SO_3Na	—	$C_{22}H_{17}N_5Na_2O_{10}S_2$	620 $(M-H)^-$	29
7m	8	CH	CH	3-SO_3Na	—	$C_{22}H_{18}N_5NaO_7S$	518 $(M-H)^-$	98
7n	7	CH	N	1,3-SO_3Na	—	$C_{29}H_{24}N_6Na_2O_{10}S_2$	725 $(M-H)^-$	84
7o	7	N	N	1,3-SO_3Na	—	$C_{28}H_{23}N_7Na_2O_{10}S_2$	726 $(M-H)^-$	99
8a	7	CH	CH	1,3-SO_3Na	—	$C_{22}H_{20}ClN_5Na_2O_8S_2$	568 $(M-HCl-Na)^-$	90
8b	7	CH	CH	1,3,5-SO_3Na	—	$C_{22}H_{19}ClN_5Na_3O_{11}S_3$	670 $(M-HCl-Na)^-$	ND
8c	8	CH	CH	1,3-SO_3Na	—	$C_{22}H_{20}ClN_5Na_2O_8S_2$	568 $(M-HCl-Na)^-$	90[a]
8d	8	CH	CH	1,3,5-SO_3Na	—	$C_{22}H_{19}ClN_5Na_3O_{11}S_3$	670 $(M-HCl-Na)^-$	ND
8e	7	CH	CH	3,5-SO_3Na	—	$C_{22}H_{20}ClN_5Na_2O_8S_2$	568 $(M-HCl-Na)^-$	91[b]
8f	8	CH	CH	3,5-SO_3Na	—	$C_{22}H_{20}ClN_5Na_2O_8S_2$	568 $(M-HCl-Na)^-$	ND
8g	7	CH	CH	4,8-SO_3Na	—	$C_{22}H_{20}ClN_5Na_2O_8S_2$	568 $(M-HCl-Na)^-$	41
8h	7	CH	CH	3,6-SO_3Na	—	$C_{22}H_{20}ClN_5Na_2O_8S_2$	568 $(M-HCl-Na)^-$	80[b]
8i	7	N	CH	1,3-SO_3Na	—	$C_{21}H_{19}ClN_6Na_2O_8S_2$	569 $(M-HCl-Na)^-$	89
8j	7	CH	CH	2,4-SO_3Na	—	$C_{22}H_{20}ClN_5Na_2O_8S_2$	568 $(M-HCl-Na)^-$	ND
8k	7	CH	CH	1,5-$PO(OEt)_2$	—	$C_{30}H_{40}ClN_5O_8P_2$	658 $(M-HCl-H)^-$	96
8l	8	CH	CH	1,5-SO_3Na	—	$C_{22}H_{20}ClN_5Na_2O_8S_2$	568 $(M-HCl-Na)^-$	94
8m	8	CH	CH	3-SO_3Na	—	$C_{22}H_{21}ClN_5NaO_5S$	466 $(M-HCl-Na)^-$	94
8n	7	CH	N	1,3-SO_3Na	—	$C_{21}H_{19}ClN_6Na_2O_8S_2$	569 $(M-HCl-Na)^-$	95
8o	7	N	N	1,3-SO_3Na	—	$C_{20}H_{18}ClN_7Na_2O_8S_2$	570 $(M-HCl-Na)^-$	60
9	7	CH	CH	1,3-SO_3Na	—	$C_{28}H_{23}N_7Na_2O_{11}S_2$	742 $(M-HCl-Na)^-$	95
10	7	CH	CH	1,3-SO_3Na	—	$C_{28}H_{26}ClN_7Na_2O_9S_2$	690 $(M-HCl-Na)^-$	90

[a]Overall yield from starting material.
[b]Overall yield for two steps.
[c]Thiourea derivative.
[d]Overall yield for three steps.
[e]ND = Not determined.
[f]EI-MS.

obtained in 20–90% overall yield, through the intermediacy of the corresponding nitro derivatives **7a–o** and **9**. Treatment of amines **6a**, **8a–o**, and **10** with phosgene in the presence of sodium acetate (Method C) afforded the final ureido derivatives **2a–m** and **2o–s** (6–98% yield). Compound **2n** was obtained by reaction of **2s** with trimethylsilyl bromide followed by hydrolysis with acetone/water (Method D). The overall yield of the whole preparation is quite good, ranging between 17 and 60%, depending on the number of heterocyclic spacers introduced. Chemical and physical data of the new compounds are reported in Table 1. All the compounds gave analytical data in accordance with the reported structures. Compounds **2a–s** analyzed within ±0.4% of theoretical values for C, H, N and S; however, no definite melting points could be taken for these compounds, which decompose in the range 200–250 °C. It is worth noting that 200 MHz proton NMR spectra showed highly symmetrical signals for the two portions of the molecules with respect to the ureido carbonyl group.



Scheme 1. Reagents: (a) 1-methyl-4-nitropyrrole-2-carbonyl chloride (**4a**) or 3-benzyloxycarbonylamino-1-methyl-pyrazole-5-carbonyl chloride (**4b**) AcONa/dioxane/H_2O or Et_3N/CH_2Cl_2; (b) H_2, 10% Pd/C, HCl, H_2O or MeOH; (c) $COCl_2$ or $CSCl_2$, AcONa/dioxane/H_2O or Et_3N/CH_2Cl_2; (d) ME_3SiBr, CH_2Cl_2, then H_2O/acetone and $NaHCO_3$. For W, X, Y, see Table 1.

Biology

Compounds **2a–r** were evaluated[23,24] for their ability to inhibit bFGF binding,[25] in vivo bFGF-induced angiogenesis,[12] and neovascularization of the chorioallantoic membrane (CAM)[26] in comparison with suramin (Table 2).[12,27] Most of the tested compounds showed moderate to good activity in the above assays. Both the number of the sulfonate groups and their position relative to the amino moiety on the naphthalene rings of **2** have a little influence on the biological properties, whereas the length of the molecule seems to be a crucial factor: while good activity is still exhibited by the longer molecule **2c**, compound **2g**, having only two pyrrole spacers, shows reduced activity in all of the tests. Also the substitution of pyrazole for pyrrole ring(s) (compounds **2l**, **2p**, and **2q**) does not positively affect the biological profile, in terms of either activity or toxicity.

Compound **2a**, a molecule selected for clinical development, was tested on different solid murine tumor models in comparison with suramin.[28] Both molecules were effective in inhibiting tumor growth in the M5076 murine reticulosarcoma, MXT, and S180 murine fibrosarcoma models and were inactive against B16F10 melanoma. Moreover, **2a** was found to be active against M5076/CTX and UV2237 fibrosarcoma, although with borderline values of activity, and to be equally effective on the subline made resistant to doxorubicin, UV2237/ADM. Suramin appeared to be more toxic than **2a**, mainly due to the higher mortality observed on an equimolar basis. Moreover, as regards hematology findings, **2a** induced a transient decrease in platelets, whereas suramin had a long-lasting effect on thrombocytes, which was associated with severe anemia. The liver necrosis tended to regress after treatment and did not appear to threaten the life of affected animals. **2a** did not induce any nephrotoxicity. The renal lesions observed with suramin persisted over a long observation period, showing a minimal trend toward recovery. Compound **2a** is presently in phase II clinical trials as a drug candidate.

Table 2. Biological data of compounds **2a-r** in comparison to suramin (1)

Compd	Inhibition of bFGF binding (ID_{50}, μM)[a]	log(ID_{50})	Inhibition of bFGF-induced angiogenesis (%)[b]	Inhibition of CAM vascularization (%)[c]	Interaction energy (kcal/mol)
2a	142 ± 18	2.15	100	100	−98
2b	116 ± 10	2.06	93	80	−119
2c	163 ± 16	2.21	89	62	NC
2d	390 ± 34	2.53	82	77	−83
2e	153 ± 15	2.18	80	60	−108
2f	135 ± 11	2.13	38	100	NC[d]
2g	550 ± 92	2.74	ND[e]	30	NC
2h	85 ± 18	1.93	100	83	−117
2i	109 ± 12	2.04	90	71	−84
2j	145 ± 17	2.16	94	67	−110
2k	146 ± 6	2.16	0	63	−93
2l	171 ± 14	2.23	ND	9	−90
2m	183 ± 35	2.26	ND	83	−109
2n	285 ± 40	2.45	ND	Tox[f]	−89
2o	587 ± 20	2.77	100	65	−67
2p	345 ± 20	2.54	0	Tox	NC
2q	677 ± 12	2.83	ND	44	−57
2r	857 ± 109	2.93	0	45	−32
1	91 ± 11	1.96	94	87	NC

[a]ID_{50}: dose inhibiting by 50% the binding of bFGF to its receptor determined on BALB 3T3 cells after 4 h incubation in the presence of 0.2 nM ^{125}I-bFGF and scalar concentrations of each compound.[25]

[b]Inhibition of in vivo angiogenesis induced by bFGF gelfoam implanted s.c. in C3H/He mice treated 24 h later with 200 mg/kg iv of each compound.[12] The degree of angiogenesis was measured by counting the number of vessels in control and treated mice after 15 days implantation.

[c]Inhibition of angiogenesis on the chorioallantoic membrane (CAM) assay. Results are reported as percentage of CAMs presenting an avascular zone with at least 2 mm in diameter after 48 h treatment with 350 nmol of each compound incorporated into a methylcellulose pellet.[26]

[d]NC = Not calculated.

[e]ND = Not determined.

[f]Tox = toxic at the tested dose.

All data represent mean values for at least three separate experiments.

Investigation on the mode of action of **2a** and **2b** indicated that their antitumor activity is not mediated by T-cell activity. It was hypothesized that their ability to complex bFGF may be the basis for their antitumor activity and that the compounds act by blocking the growth factor produced by the tumor cells and inhibiting their growth and/or tumor vascularization. Our results from the sequential binding assay on bFGF receptor and the bFGF-induced tyrosine phosphorylation assay[23,24] indicated that the inhibition of the binding of bFGF to its receptor was not due to a direct interaction of compounds **2a** and **2b** with the receptor itself. Moreover, the results obtained in the bFGF-induced tyrosine phosphorylation assay showed that **2a** and **2b** as well as suramin are able to dissociate the bFGF-receptor complex, suggesting that the bFGF receptor and inhibitor binding sites might be different.

Molecular modeling and ligand docking

In the absence of X-ray crystallographic coordinates and high field NMR data (no Overhauser effects were detected) concerning compounds **2**, we resorted to molecular modelling in an attempt to gain an insight into both the possible biologically active conformation(s) of these ligands and their mode of interaction with bFGF. Structures were built and optimized with the program MODEL (version KS 2.99)[29] and subjected to an extensive conformational analysis using the Monte Carlo search method of BKMDL,[29] as reported in our previous publications.[30] A *trans* conformation for all of the amide bonds was assumed on the basis of a 20 kcal mol^{-1} energy barrier (calculated with the program MOPAC[31]) for the *cis–trans* interconversion, the *trans* conformation being 4 kcal mol^{-1} more stable than the *cis*. The other torsional angles were rotated by increments of 60° (for pyrrole C_4-N) and 180° (for pyrrole C_2-CO). As a result, the molecule assumes an extended conformation which assures the maximum distance between the sulfonate groups, while the shape of the whole molecule is quasi-helicoidal with the *N*-methyl groups describing a complete turn (Figure 2).

This result, however, was not in accordance with the 200 and 500 MHz proton NMR spectra of **2** showing highly

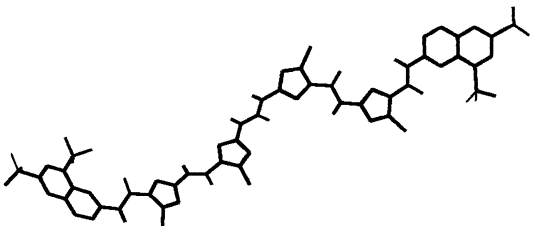


Figure 2. Minimum energy conformation of **2a** as obtained with MODEL.

symmetrical signals for the two portions of the molecules with respect to the ureido carbonyl group. Therefore, considering also the high hydrophilicity of compounds **2**, the conformational analysis was repeated using a continuum water model as implemented in BatchMin associated with the program MacroModel.[32] One of the preferred conformation of **2a** (taken as a representative of the whole class of compounds), among the 10,000 structures generated by this program ($\Delta E = 2$ kcal over the minimum energy conformation), appears as a highly symmetrical, quasi-planar arc of circumference, with all the methyl groups located in the outer part of the structure and four of the six NH groups lying in the concave face (Figure 3), in full agreement with the symmetrical pattern of the NMR spectra.

Interestingly, in this conformer of **2a** the distance between the sulfonate groups at the position 1 of the two naphthalene units is 24–26 Å, which is in full agreement with the distance (25 Å) calculated between the heparin and the receptor binding sites on bFGF.[11] It is noteworthy that, when the extended conformation obtained in vacuum with BKMDL was subjected to reminimization with MacroModel (considering the water contribution), such a structure evolved to the previously described symmetrical conformation. Conformational search on the other ligands **2b–r** led to analogous results. These findings are particularly interesting considering that our compounds have a highly hydrophilic character and exert their biological effects in an aqueous environment. All these considerations led us to choose the symmetrical, planar structure as the putative bioactive conformation of **2a–r**.

Ligands **2a–r** clearly possess the appropriate chemical functionalities that allow them to establish either ionic or hydrogen-bonding interactions with bFGF, which is rich in basic amino acid residues. However, no information to date has addressed the issue of how such compounds can selectively and productively dock into their target. To begin to broach this issue, we performed docking experiments using the program Sybyl[33] with the goal to produce a complex between ligand and target that optimizes geometric and chemical complementarity. For this purpose we used the three-dimensional structure of bFGF determined by X-ray crystallography and refined at 1.6 Å resolution[10b] (Brookhaven Protein Data Bank). The key amino acids which comprise the heparin binding domain on bFGF constitute a discontinuous binding epitope and include some basic residues such as Lys26, Asn27, Arg81, Lys119, Arg120, Gln123, Lys125, Lys129, Gln134, and Lys135. A sulfate ion is bound within this region on the surface of the molecule, its four oxygens being hydrogen-bonded by the side chains of Asn27, Arg120, and Lys125 as well as the main-chain amide of Arg120. These residues are thought to be the heparin binding site. Residues 106–115, which are presumed to bind to the bFGF receptor, include an irregular loop that extends somewhat from the surface of the protein and is about 25 Å from the presumed heparin binding site. For simplicity, we first focused our attention on these two domains of the protein as possible ligand attachment sites. When compound **2a** in its preferred conformation (MacroModel) was docked into the X-ray structure of bFGF, a very good interaction of the sulfonate groups of the

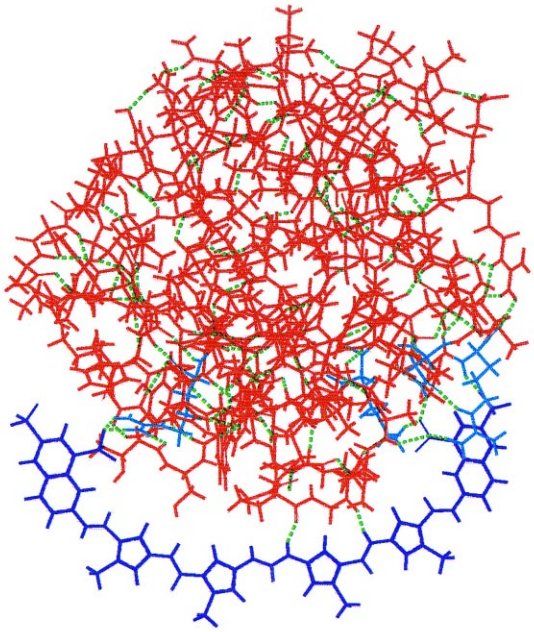


Figure 3. **2a**–bFGF complex: blue, energy-minimized (MacroModel) conformation of **2a**; red, three-dimensional structure of bFGF from X-ray crystallography;[10b] cyan, basic amino acids Arg121, Lys126, and Asn28 of the heparin binding site (right side) and Arg108 (left side) of the receptor binding domain interacting with the naphthysulfonic moieties of **2a**. Green dotted lines represent interactions among bFGF amino acids themselves and between Pro133 and Gly134 of the growth factor and the ligand.

ligand with Arg120, Lys 125 and Asn27 at the heparin binding site and with Arg108 at the receptor binding site of the target was found (Figure 3). Moreover, once the complex structure was optimized, the electrostatic isopotential maps were computed,[33] using the atomic point charges obtained by the MNDO method of MOPAC[31] through a Mulliken electron population analysis.

Excellent complementarity was found between the positive surfaces corresponding to the above cited amino acids and the negative ones on the sulfonate groups (Figure 4). Finally, examination of the van der Waals volumes calculated[33] for each of the complexes between bFGF and **2a**, **2c**, and **2g** (differing for the number of pyrrole spacers) shows the best fit in the case of **2a**, whose naphthylsulfonate moieties lie at a distance of about 25 Å (Figure 5).[34]

In order to further test this binding model, we performed a complete docking of **2a** to the whole bFGF molecule, aimed at finding other possible binding ways. Among the 20 basic amino acids present on the surface of bFGF, that could in principle interact with the sulfonate groups of the ligands, only three (Arg81, Arg22, and Lys21), in addition to Arg108 previously considered, lie at a distance of approximately 25 Å from the heparin binding domain, assumed to be in all the experiments the first attachment point of the ligand to the protein. However, since Arg22 and Lys21 define the same binding domain, the possible interaction sites could be only two. Manual docking experiments followed by extensive minimization of the two new complexes showed strong steric interactions when the ligand was docked to the site defined by Arg22 and Lys21. Better results were obtained for the interaction of the

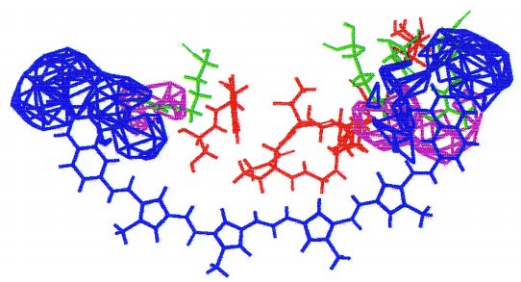


Figure 4. **2a**–bFGF complex: blue, energy-minimized (MacroModel) conformation of **2a**; red, three-dimensional structure of bFGF from X-ray crystallography;[10b] green, basic amino acids of the heparin (right side) and the receptor (left side) binding sites. Good complementarity can be noticed between the negative electrostatic isopotential maps corresponding to the sulfonate groups of the ligand (blue) and the positive ones corresponding to the basic amino acids (magenta). For sake of clarity only part of the full three-dimensional structure of bFGF is shown.

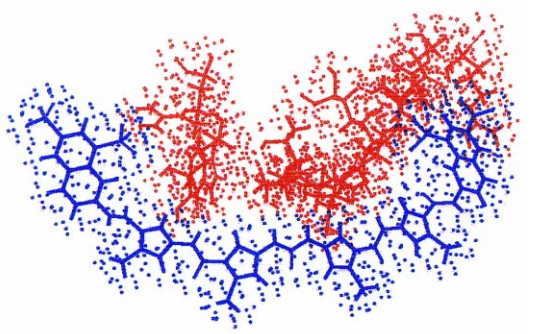


Figure 5. Van der Waals surfaces representation of **2a** (blue)-bFGF (red) complex, obtained employing the vdW Dot Surface command in the View category within Sybyl.[33] For sake of clarity only part of the full three-dimensional structure of bFGF is shown.

ligand with Arg81: the energy of this complex was quite similar to that of the complex derived by docking the ligand to the bFGF receptor domain. Nevertheless, only the formation of this last complex seems to explain the results of the sequential binding assay, that is the ability of the ligands to dissociate the bFGF–receptor complex.[23] Although we can not definitely rule out the possibility that such results may be due to conformational changes of the bFGF molecule caused by the interaction with the ligand, no evidence has been to date provided supporting such a hypothesis. To the contrary, the currently held view is that the binding of heparin-like polysaccharides to bFGF is not associated to any significant conformational change in the polypeptide backbone.[35]

With a plausible model of interaction in our hands, we sought a possible correlation between the interaction energy of the complexes and the biological activity of the involved ligands. The biological activity of the compounds we took into consideration is bFGF-binding affinity expressed as $\log(ID_{50})$, that is the logarithm of the compound concentration (μM) inhibiting by 50% the binding of bFGF to its receptor (see Table 2). Of the 18 compounds **2** of Table 2, 14 compounds (**2a**, **2b**, **2d**, **2e**, **2h–o**, **2q**, and **2r**), showing $\log(ID_{50})$ values ranging from 1.93 to 2.93 log unit, were used in this study. IE (kcal mol^{-1}) corresponds to the sum of van der Waals and electrostatic interactions between bFGF and ligand in their minimized complex, and was computed according to the following equation:

$$IE = E_{complex} - E_{receptor} - E_{ligand}$$

where $E_{complex}$ represents the total energy of the complex as obtained by docking analysis, while $E_{receptor}$ and E_{ligand} represent the energy of the receptor and the

ligand, respectively, when extracted from the complex itself.[36] The proposed correlation between binding affinity value of the ligands and IE of the corresponding complexes is premised on two assumptions: (1) that IE is proportional to the enthalpy of binding (ΔH_{bind}) and (2) that the entropy of binding (ΔS_{bind}) is small or more likely constant. Thus, IE would be proportional to the free energy of binding (ΔG_{bind}) and correlate with the experimental observation (i.e. ID_{50}).[37]

As can be seen in Figure 6, a good linear correlation (RSQ = 0.844, $RSQ^2 = 0.710$) was found between IE (actually reported as −IE in order to avoid negative numbers) and log(ID_{50}) values for the test compounds. This result leads to assume that all compounds **2** show the same binding mode to bFGF.

Discussion

It has been recently reported in the literature[38] that suramin can form with some growth factors, such as bFGF, human recombinant acidic fibroblast growth factor (aFGF), and human recombinant platelet-derived growth factor (PDGF), multiple GF-containing aggregates by acting through a cross-linking mechanism, due to the potential bifunctionality of suramin and to the presence of more than one suramin binding site on the growth factors. It is interesting to note, however, that the relationship, if any, between aggregation and inhibition of FGFs is not clearly understood, since both sulfated β-cyclodextrine and heparin, which can form multiple aFGF-containing complexes, actually stimulate growth factor activity. Therefore the crucial question as to why suramin inhibits FGFs has not been yet conclusively addressed. Anyway, a model of interaction based on a cross-linking mechanism, in analogy to that reported for suramin, can be ruled out in our situation on the basis of SAR of compounds **2**. Thus, while the cross-linking model is expected to have no dependency on the length of the whole molecule nor on the nature of the backbone, we found on the contrary that such elements deeply influence the biological properties of compounds **2** and hence must be considered for assessing the mode of interaction between **2** and bFGF. In particular, while no substantial difference in affinity is observed for **2c**, a compound having the same naphthyl moieties as **2a** but two more spacers in the backbone, a marked decrease in the binding inhibiting activity is clearly observed in the case of a shorter molecule such as **2g**. Examination of the van der Waals volumes calculated for each of the complexes between bFGF and **2a**, **2c**, and **2g** well accounts for this different binding ability. Compound **2a**, having the sulfonate groups at the 1-position of the two naphthalene moieties at the proper distance of 24–26 Å fits in an excellent way the growth factor (Figures 3–5). A quite good complementarity is still observed for compound **2c**, while **2g** is too short for a profitable (i.e. bidentate) interaction without steric repulsion. Conversely, no affinity is observed for compounds having the same naphthyl moieties at the same relative distance as in **2a**, but differing in the nature of the spacers (γ-aminobutyric acid).[39] A possible explanation for the lack of affinity in this case could be found in the greater conformational freedom of these molecules, which might prefer an extended conformation that makes difficult the binding to a single bFGF molecule. Therefore, the presence of a rigid, planar backbone which assures the proper distance between the sulfonate groups and the correct geometry for binding seems to be necessary for a molecule to show high affinity. The lack of affinity of pyrazole derivatives **2p,q** with respect to **2l** is difficult to rationalize in these terms and would deserve further investigation, that has been avoided at this stage due to the biological inactivity of these compounds. On the other hand, the total inactivity of compounds **2** possessing only one naphthylsulphonic moiety[39] leads us to exclude a model of interaction which implies a 1:1 monodentate binding of the drug to the growth factor. Conversely, in light of the biological and computational results reported above, we infer that these compounds might interact with the growth factor in a unique manner, by the bidentate attachment of their naphthylsulfonic moieties to both the heparin and receptor binding sites of bFGF, thus preventing the interaction of the growth factor with the corresponding receptor. The specificity of this mechanism, while justifying the low toxicity of these molecules,[40] can explain their activity as inhibitors of in vitro and in vivo bFGF-induced angiogenesis and the observed reduction in tumor, such as the murine reticulosarcoma M5076, growth.

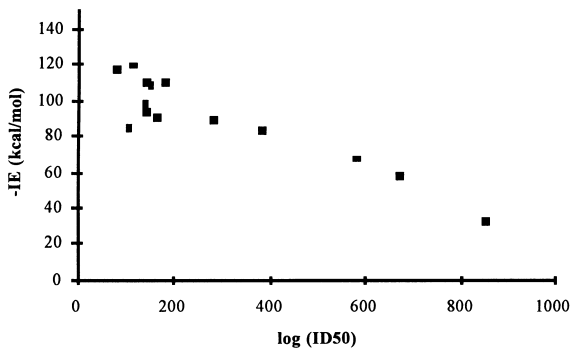


Figure 6. Correlation between log(ID_{50}) and interaction energy values for 14 compounds.

In vitro experiments based on steady-state fluorescence anisotropy at 22 °C of **2** as a function of bFGF concentration showed a saturable trend that fits with the

model of 1:1 reversible complex formation.[41] Moreover, they confirmed the hypothesis of a direct binding of **2** to bFGF and allowed the calculation of the dissociation constants of the complexes.[42] Attempted determination of the dissociation constant and stoichiometry of the suramin–bFGF complex by fluorescence was frustrated by light-scattering and turbidity artifacts due to the extensive aggregation of bFGF, with precipitation of the complex, caused by suramin. However, when a chemically modified protein, containing two carboxymethylated cystein residues (cm-FGF), was utilized in these experiments, its interaction with suramin occurred without precipitation and a 1:1 stoichiometry of this complex could be determined.[43]

Considering the difficulty of obtaining single crystals of the putative suramin–bFGF complex, suitable for X-ray analysis, the model described herein will hopefully lead to a better understanding of the molecular mechanisms implied in the inhibition of bFGF by suramin and related compounds.

Experimental

Chemistry

Materials. IR spectra were recorded on a Perkin–Elmer 297 spectrometer. ^{1}H NMR were measured with a Varian VXR 200 (200 MHz) spectrometer. Microanalyses (within ±0.4% of the theoretical values) were performed in house (Pharmacia & Upjohn). All the compounds were analyzed for C, H, N, and S.

Syntheses. Specific examples presented below illustrate general synthetic methods A–C. In general, samples prepared for biological studies were dried in vacuum over P_2O_5 for 20 h at temperatures ranging from 20 to 110 °C, depending on the sample melting point.

Method A example. 8-(1-Methyl-4-nitro-1*H*-pyrrole-2-carbonylamino)naphthalene-1,3,5-trisulfonic acid trisodium salt (5d). A solution of 1-methyl-4-nitropyrrole-2-carbonyl chloride (**4a**) (0.94 g, 5 mmol) in 1,4-dioxane (45 mL) was added dropwise during 1 h to a cold (5 °C) solution of 8-aminonaphthalene-1,3,5-trisulfonic acid (**3d**) trisodium salt (1.35 g, 3 mmol) and sodium acetate (0.49 g, 6 mmol) in water (45 mL). After stirring for 3 h at 5 °C, the solution was acidified to pH 4 with 1N HCl and evaporated to dryness. The residue was treated with ethyl acetate (300 mL), stirred for 1 h and filtered to give 1.70 g (94%) of **5d**, which was used in the next step without further purification: IR 3340, 1650, 1530, 1305, 1200, 1030 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.96 (s, 3H), 7.84 (d, 1H), 8.06 (m, 2H), 8.15 (d, 1H), 8.63 (d, 1H), 9.40 (d, 1H), 12.55 (br s, 1H).

Method B example. 8-(4-Amino-1-methyl-1*H*-pyrrole-2-carbonylamino)naphthalene-1,3,5-trisulfonic acid trisodium salt hydrochloride (6d). A solution of **5d** (1.803 g, 3 mmol) in water (120 mL) and 1 N HCl (3 mL) was hydrogenated (50 psi) over 10% palladium on carbon (0.9 g) for 4 h. The catalyst was filtered off and the resulting solution was concentrated to afford 1.802 g (99%) of the title compound **6d**: IR 3440, 1640, 1520, 1190, 1030 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.90 (s, 3H), 7.11 (d, 1H), 7.29 (d, 1H), 8.04 (m, 2H), 8.60 (d, 1H), 9.88 (d, 1H), 10.04 (br s, 3H), 12.39 (br s, 1H).

Method C example. Carbonyl-bis[8-[4-(4-amino-1-methyl-1*H*-pyrrole-2-carbonylamino)-1-methyl-1*H*-pyrrole-2-carbonylamino]naphthalene-1,5-disulfonic acid] tetrasodium salt (2o). A 20% solution of phosgene in toluene (1 mL, 2 mmol), diluted with 1,4-dioxane (3 mL), was added slowly to a cold (5 °C) solution of **8m** (0.6 g, 1.02 mmol) and sodium acetate (0.33 g, 4 mmol) in water (20 mL) and dioxane (5 mL). After stirring for 2 h at 5 °C solvents were evaporated under vacuum and the residue was taken up in methanol. Precipitated salts were filtered off, the filtrate was evaporated and the residue was chromatographed on a silica gel column with dichloromethane:methanol:water (60:40:4) as eluent to give 0.16 g (26%) of the title compound: IR 3440, 1660, 1640, 1585, 1180, 1030 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.84 (s, 3H), 3.85 (s, 3H); 6.80 (d, 1H), 7.07 (m, 2H), 7.41 (m, 2H), 7.92 (dd, 2H), 8.12 (s, 1H), 8.27 (dd, 1H), 9.07 (dd, 1H), 9.90 (br s, 1H), 12.27 (br s, 1H).

Method D example. Carbonyl-bis[7-[4-(4-amino-1-methyl-1*H*-pyrrole-2-carbonylamino)-1-methyl-1*H*-pyrrole-2-carbonylamino]naphthalene-1,5-diphosphonic acid] tetrasodium salt (2n). A solution of trimethylsilyl bromide (10 mL) in dry dichloromethane (10 mL) was added dropwise, under a nitrogen atmosphere, to an ice-cooled solution of **2s** (1.00 g, 0.74 mmol) in dry dichloromethane (100 mL). After stirring for 24 h at room temperature, the volatiles were evaporated under reduced pressure. The residue was taken up with acetone and a solution of water (0.5 g) in acetone (10 mL) was added. After stirring for 4 h, the separated microcrystalline solid was filtered, washed with acetone, methanol, ether, and vacuum dried. The acid thus obtained (0.65 g, 0.58 mmol) was dissolved in water (50 mL) and neutralized with sodium hydrogen carbonate (0.195 g, 2.32 mmol) to pH 6.5–7. The solution was filtered, concentrated under reduced pressure to a small volume, and freeze-dried to give 0.98 g (99%) of the title compound: IR 3450, 1650, 1580, 1190, 1030 cm^{-1}; ^{1}H NMR (DMSO-d_6 + CF_3CO_2H) δ 3.85 (s, 3H), 3.89 (s, 3H), 6.83 (1H, d), 7.04 (1H, d), 7.27 (1H, d), 7.34 (1H, d), 7.51 (1H, ddd), 8.05 (1H, ddd), 8.40 (1H, s), 8.48 (1H, dd), 8.68 (1H, d), 9.10 (1H, s), 9.89 (1H, s), 10.34 (1H, s).

Molecular modeling

Calculations and graphics manipulations were performed on a Silicon Graphics Iris 4D/35 and Iris Indigo R4000. Starting geometries of compounds **2** were generated and energy-minimized using the program MacroModel-BatchMin (version 4.0)[32] equipped with the OPLS force field. Compounds **2** were subjected to a statistical conformational search with Monte Carlo Multiple Minimum (MCMM) method, which causes an input structure to be modified by random changes in its torsion angles as given by the TORS command. The values of 0 and 180 degrees are, respectively, the minimum and maximum angular increments to be added or subtracted from the current dihedral angles. This particular pair of values specifies a completely random resetting of each torsion selected at each MC step. The GB/SA solvation method, considering water as the solvent, was used. The program BatchMin uses a continuum approach to represent solvent, unlike other programs which use large number of explicit solvent molecules. A cluster of atoms representatives of the whole molecule was selected in order to compare each new conformer with all the minimum-energy conformers previously found. Structures are considered the same unless the least square superimposition of the compared atoms finds one or more pair of equivalent atoms separated by more than 0.25 Å.

Structures were minimized using the Polak–Ribiere Conjugate Gradient method until the derivative convergence was 0.01 kcal/Å mol. Every structure which is more than 6 kcal mol^{-1} above the minimum-energy conformation was rejected and the search was continued until each conformer in this energy range was located at least three times.

After conversion of coordinates to the appropriate format, the output conformers of compounds **2** were transferred to the program Sybyl (version 6.1)[33] and manually aligned. Atomic coordinates of bFGF were obtained from the Brookhaven Protein Data Bank. Hydrogen atoms were added in their idealized positions and the structure of the protein was refined using the AMBER all-atom force field.

The DOCK command of Sybyl provides a useful tool for interactively identifying possible binding interactions between a pair of molecules, one of which is called the ligand (compounds **2**) and the other the site (the protein). During the docking operation, a lattice of points is overlaid on the initial position of the ligand. Distance beyond greatest extent of any ligand atoms, along X, Y, and Z axes in both directions, where lattice was built was set to 1.5 Å, while the distances between lattice points were set to 0.25 Å (default conditions) and the maximum number of lattice points was set to 600,000. Possible non-bond interactions are calculated on the lattice. The key assumption is that the actual coordinates of each ligand atom may be approximated by those of its nearest lattice point. The energies of interaction are based on steric contributions from the Tripos force field and electrostatic contributions from any atomic charges present (calculated using Pullman method). During the docking operation, the ligand molecule can be moved freely relative to the site. The current configuration can be minimized at any time, with any combination of ligand or site held rigid. In our case, the molecule whose internal geometry was kept rigid was the site.

Complexes between bFGF and ligands **2** were built in the following manner. The minimum-energy conformers of **2** were interactively docked into the bFGF molecule. Unfavorable contacts were removed using the translation–rotation feature of Sybyl. The resultant complexes were then optimized through an automatic, interactive procedure which minimizes both steric and electrostatic interactions until the derivative convergence was 0.05 kcal/Å mol.

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