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Identification of Inhibitors of Heparin–Growth Factor Interactions from Combinatorial Libraries of Four-Component Condensation Reactions

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Abstract—Chemical libraries based on four-component condensation (4CC) reactions of isocyanides were constructed to identify compounds capable of blocking heparin binding to vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The reaction products in the synthesized libraries contain heparin mimetic functional groups such as carbohydrates, sulfonates, carboxylates, and hydroxy groups. These libraries have been screened for the inhibition of heparin binding to growth factors such as VEGF and bFGF. Single point screening at 5.0 μM of the 18,720 reaction products generated 26 candidates. The IC_{50}s of these 26 compounds were determined using HPLC-purified products and 20 of the 26 showed significant inhibition of heparin binding to VEGF and/or bFGF. Eighteen of the 20 confirmed active compounds have a linear extended structure. Structures identified in this library revealed an initial relationship of structure and activity, thus providing direction for further investigation of this type of heparin mimetic libraries.

Introduction

Angiogenesis is involved in the development of many disease states like solid tumor growth, metastasis, and diabetic retinopathy.[1,2] A promising strategy in the fight against cancer is to inhibit the angiogenesis process of a solid tumor, therefore limiting a tumor cell's access to nutrient.[3,4] A family of growth factors, such as vascular endothelial growth factor (VEGF) and basic fibrolast growth factor (FGF), are critical for the process of neovascularization.[5,6] The promotion of neovascularization by growth factors is further facilitated by heparin, a polysulfated glycosaminoglycan. Heparin is required for the activity of these growth factors and has been hypothesized to function by facilitating the binding of growth factor and its receptor.[7] The interaction of heparin with growth factors is therefore an important target for drug discovery.

Glycosaminglycans, such as heparin and heparan sulfate, are composed of repeating disaccharides of uronic acids and glucosamines.[5] The glucosamine residue may be *N*-acetylated, *N*-sulfated, or, rarely, unsubstituted, while the uronic acid residues may be D-glucuronic acid or L-iduronic acid. Both glucosamines and uronic acids are *O*-sulfated to various degrees at the C6/C3 positions of glucosamine and C2 of uronic acids. Negative charges on the glycosaminoglycan structure, such as *N*-sulfate and *O*-sulfate, have been found to play an important role in the interactions with growth factors.[8] A space of 20 Å between two outside critical positive residues in the binding sites of growth factors was suggested.[9] The crystal structure of bFGF complexed with a heparin derived hexasaccharide indicated that there are multiple points of interaction and the functional groups of heparin, such as OSO_3^-, $NHSO_3^-$, COO^-, and OH, all participate in the specific interaction with bFGF.[10] The crystal structure also showed that the binding groove is relatively flat and long, as might be expected given the nature of the ligand, with the two outside basic residues spaced approximately 20 Å apart. The cleft is bound on either side by a basic residue leaving enough space to accommodate a glycosaminoglycan pentasacharide.

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†These authors were involved in the synthesis of combinatorial libraries and purification of active compounds.

‡These authors were involved in the biochemical screening of the chemical libraries and the purified compounds.

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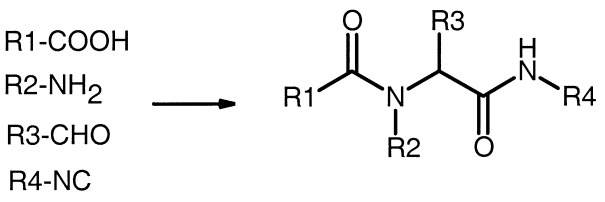
A limited number of small molecules, such as fragments of heparin (also called low molecular weight heparin), sulfated polysacharides and suramin, have been studied for their inhibitory activity towards growth factors.[11–14] Heparin analogues derived from polysaccharides are often heterogeneous, therefore it is difficult to draw SAR conclusions. However, SAR of the suramin-related structures was well studied.[2,14] Suramin is dimeric polysulfonated naphthalene linked by an amide chain of four aminobenzoic acids. The work by Manetti et al.[14] showed that both the number of sulfates and their positions on the naphthalene rings have little influence on their biological activity, whereas the length of the molecule seems to be critical. In their most active compounds, the distance between the two outside sulfonate residues is 24 Å as determined by molecular modeling, which is in good agreement with optical charge distance in the binding groove of growth factors. The biological applications of the suramin class of compounds are hampered by their strong toxicity, therefore new types of inhibitors would be desirable. To search for efficient heparin mimicking small molecules, a heparin mimetic library was constructed using Ugi 4CC reaction.[15] In an Ugi 4CC reaction (Scheme 1) an aldehyde, amine, acid and isonitrile react in one-pot fashion to form an acylamino acid amide incorporating four components into one structure. Heparin mimetic functional groups, such as carbohydrates, sulfonates, carboxylates, and hydroxyls, were introduced to the compounds in this library. Single point screening and confirmation experiments using VEGF and bFGF as representative growth factors have identified potent heparin-binding inhibitors.

Results

Solution-phase four-component condensation reactions were carried out in parallel using deep 96 well polypropylene plates. Eight rows and 10 columns of a 96-well plate were used as reaction wells, therefore each plate contains 80 reactions. Typically the four components were arranged in such a way that two of them were distributed along the rows and columns, the other two are distributed all across the plate. For components that are distributed along the rows, eight are grouped into one set. Similarly, for components that are distributed along the columns, 10 are grouped into one set. Through this kind of plate layout, each well contains one specific combination and affords one discrete product. Reactions were analyzed by reverse-phase HPLC for yield and purity, while compounds tested positive were HPLC-purified for confirmation assay and structural identification by mass spectrometry and NMR.



Scheme 1. Four-component condensation (4CC, Ugi) reaction.

Building-block selection

The four components, or building blocks, were chosen based on their functional groups. These building blocks covered a diverse set of structure features. Hydrophobicity, hydrogen-bond forming ability, particular functional groups, and size were some of the key considerations in choosing a building block. Functional groups of interest were sulfonic acid, carboxylic acid, carbohydrate, and hydroxyl groups, as these mimic groups are present in the native heparin structure.

Carboxylic acid functional groups are protected in the ester form because a carboxylic acid participates in the 4CC reaction. Esters were hydrolyzed to carboxylic acids after the Ugi reaction. Sulfonic acids do not participate in a 4CC reaction and did not need to be protected. Building blocks of carboxylic acids, primary amines and aldehydes that have sulfonic acid groups, such as 5-sulfosalicylic acid, sulfanilic acid, and 2-formylbenzenesulfonic acid, were used in this library synthesis.

Building blocks of carboxylic acids, primary amines and aldehydes that contain unprotected hydroxyl groups and carbohydrates were directly used in the condensation reactions. The hydroxyl groups of isocyanides were protected as esters, like in the case of 2,3,4,5,6-pentaacetoxyhexaneisocyanide, because these hydroxyl groups had to be protected during the synthesis of isocyanides. These hydroxyl groups were regenerated by hydrolysis. In addition to the selection of functional groups that are potentially important for activity, building blocks with protected reactive groups, such as Boc-protected amines and ester-protected acids, were also included since deprotection of these functional groups provide a point for further chemical modifications.

Validation of the building blocks

The reactivity of the building blocks was initially tested in a small number of reactions with other components that have excellent 4CC reactivity. Building blocks that passed this initial reactivity test were used in a pilot library synthesis, carried out under library synthesis conditions. The criteria for a pass are that the yield should be higher than 50% and the reaction should be clean, meaning that there should be no significant amount of side reactions. Those building blocks that passed both the initial test and pilot library synthesis were used in the final library synthesis.

Library synthesis

We initiated our research by carrying out parallel solution-phase synthesis using 20 carboxylic acids, 16 primary amines, 12 aldehydes, and five isocyanides as building blocks. The full combination of these building blocks should produce $20\times16\times12\times5=19{,}200$ products. But as an initial screening library, half the combination may be sufficient to meet the diversity requirement. The combination sets with building block structures are shown in Figures 1–4, respectively. For each combination set, all possible reaction products were synthesized. These four

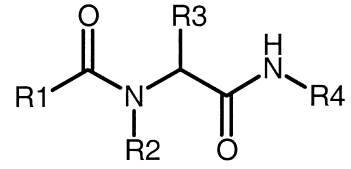
combination sets yielded 9600 small molecules for screening.

Reactions with one of the isocyanides used in the library synthesis, 1,6-diisocyanohexane, provided extended structures of symmetric dimers (Fig. 5) which represent 20% of the products. This library was biased toward extended structures since structural studies have shown these have the best potential for interaction with the growth-factor binding site. To increase the number and diversity of extended structures, one bisacid, adipic acid, and one bisamine, 1,6-hexanediamine, were also used as building blocks in the library synthesis. The combination sets for the bisacid and the bisamine are shown in Figures 6 and 7. Each combination set represents 640 reactions.

To generate a carboxylic acid or an alcohol, the 4CC products with esters were transferred to new deep 96 well plates and treated with 0.1 N NaOH. The pH of the reaction was brought to neutral by acetic acid after the reaction was done. For instance, the full plate constructed with methyl 4-formylbenzoate ($R_3$5, Fig. 1) was aliquoted and treated with 0.1 N NaOH. There were five such plates with five different isocyanides. Similarly full plates that were made from ethyl 2-isocyanoethylthiopropionate ($R_4$2) were hydrolyzed to carboxylic acids and the plates that were made from 2,3,4,5,6-pentaacetoxyhexaneisocyanide ($R_4$5) were hydrolyzed to polyalcohols. For an ester acid (R_1), or ester amine (R_2) the corresponding row and column was transferred to a new plate and subsequently hydrolyzed. This sub-library, also called a carboxylate library, represents 7840 reactions. The sum of initial library reactions, bisacid and bisamine compounds, and carboxylate libraries resulted in a total of 18,720 compounds available for initial screening. 30% of the library was analyzed by HPLC and the average yield found to be 56% calculated by the absorption area at 215.

Library screening

Initial library screening to identify a growth-factor heparin antagonist was carried out using a filter binding



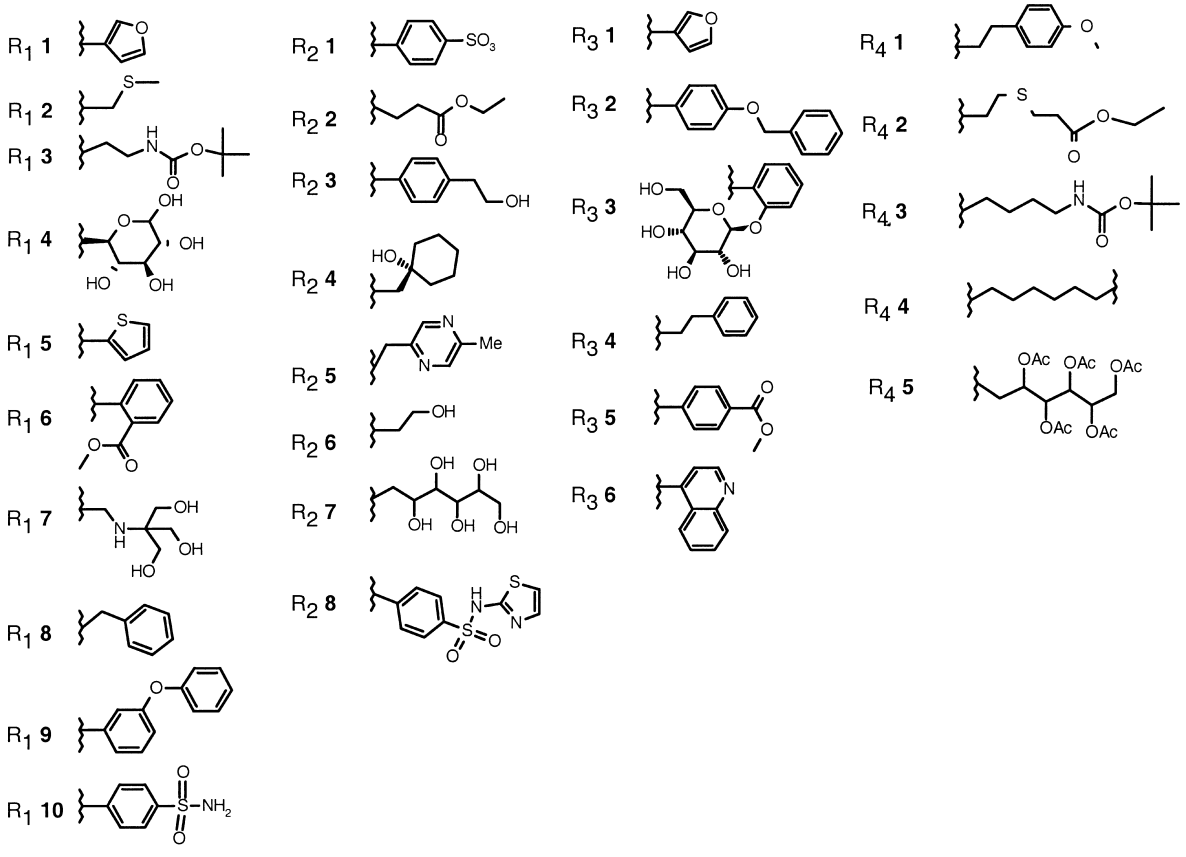
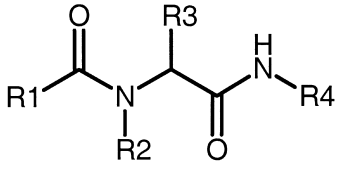


Figure 1.

assay in a competition format. Briefly, growth factor was incubated with a ^{125}I-tyraminated 16-mer heparin fragment in the presence or absence of 5 μM compounds. Inhibition of radiolabeled heparin binding was quantitated as a loss of signal compared to control after filtration of solution through a nitrocellulose membrane. This assay was carried out in μL volumes in a 96-well filter plate, making it compatible with 96-well plate library synthesis. Primary screening of library compounds in single point at 5 μM generated 26 active compounds using a cut off value of 50% inhibition, giving a hit rate of 0.14% for VEGF and bFGF. Reactions demonstrating inhibition at this level were purified by reverse-phase HPLC and re-assayed to obtain IC_{50} values against each of the two growth factors. Of the initial 26 compounds purified, 20 of them had IC_{50} values less than 100 μM. The 20 confirmed active products with their structures and IC_{50} values are shown in Table 1.

The active compounds can be divided into four major groups. The first group is generated by a single round of 4CC reaction represented by two structures, compounds **1** and **2**. The two compounds of the first group are smaller compared to the rest of the active compounds. The second group is composed of extended structures formed by 1,6-bisisocyanohexane. There are nine compounds in this category, compounds **3**–**11**. The third group consists of extended structures formed by 1,6-hexanediamine, represented by five compounds, **12**–**16**. There is a sulfonate moiety in all of the active compounds in groups 1–3, which can be at the acid, the amine, or the aldehyde position. Another characteristic of these active compounds is the presence of a large hydrophobic moiety. The fourth group contains structures that have a carboxylic acid instead of a sulfonic acid at various locations. The four compounds in this group, **17**–**20**, also have extended structures and bulky hydrophobic substituents.



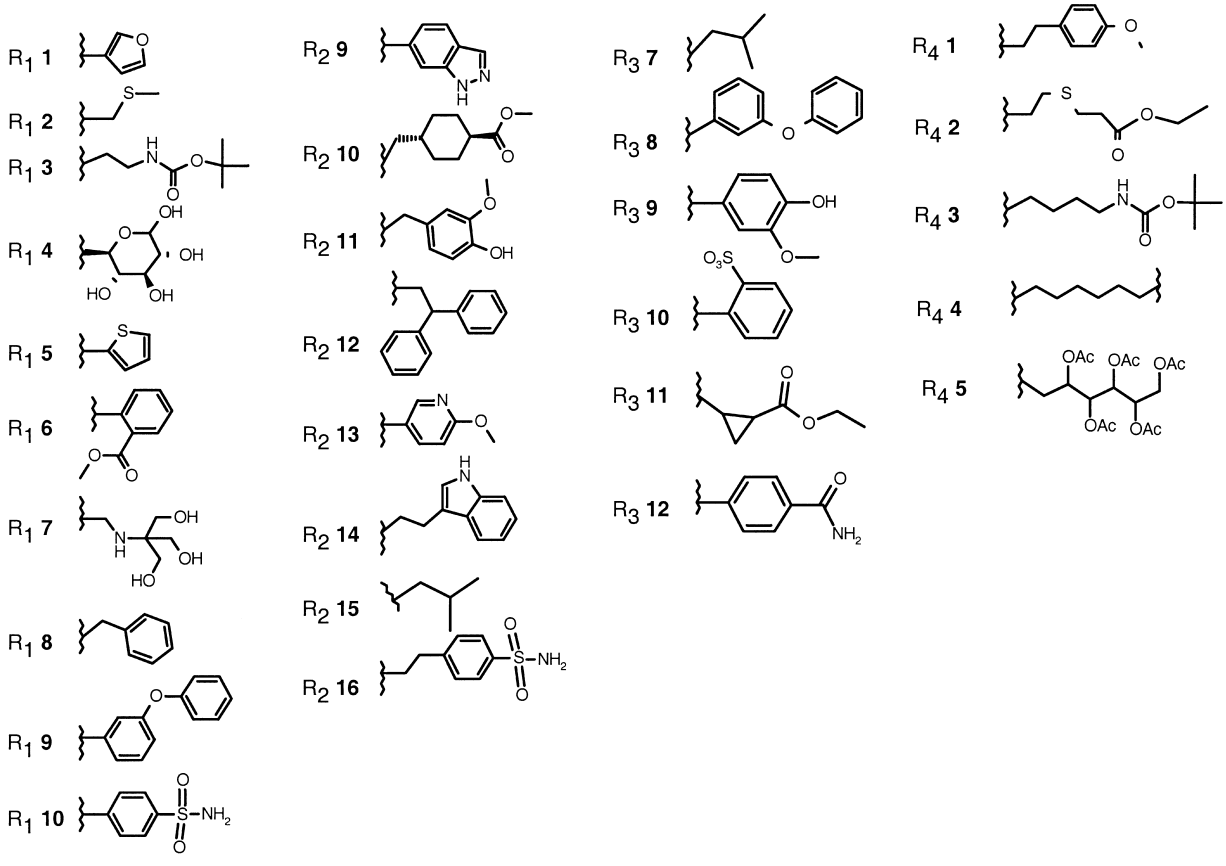
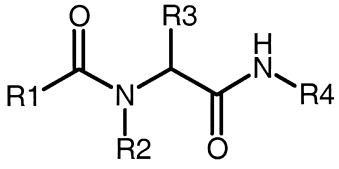


Figure 2.

Discussion

To identify small molecules that can inhibit VEGF–heparin and bFGF–heparin interactions, heparin mimetic combinatorial libraries of the 4CC reaction were constructed. Since heparin is a glycosaminoglycan decorated with sulfates and carboxylates along the polysaccharide chain, heparin mimetic functional groups such as carbohydrates, sulfonates, carboxylates, and hydroxy groups were incorporated into the final products. These libraries have advantages in that non-peptide, nonsaccharide compounds of defined and diverse structures can be synthesized and tested rather than relying on natural glycosaminoglycan molecules which are heterogenous and easily degraded *in vivo*.

The condensation reactions with equal molar amounts of acid, amine, aldehyde and isocyanide were carried out at 45 °C overnight. Reactions with 2,3,4,5,6-penta-acetoxyhexaneisocyanide were incubated at room temperature for 4 days since the reactivity of this isocyanide is weaker. It was observed that methanol is the preferred solvent for the condensation reactions. Inhibitory effect was noticed when DMSO was composed of more than 50% of the solvent. Of the 18,720 reactions in the combined libraries, 70% represent products from a single 4CC reaction and 30% are homodimers of extended structures. Although extended structures in this library only represent 30% of the total products, they occupy 90% of the confirmed active compounds, suggesting VEGF and bFGF prefer extended ligands. This is in agreement with the extended binding site for heparin found in the crystal structure.[10] A ligand with a distance of about 20 Å between two negative charges was suggested to be important for binding to the growth factors.[14] Although the exact distance between charges of the identified compounds in their active conformation is difficult to determine, the number of atoms between the charges may provide an estimate. The number of atoms between the two charges for confirmed



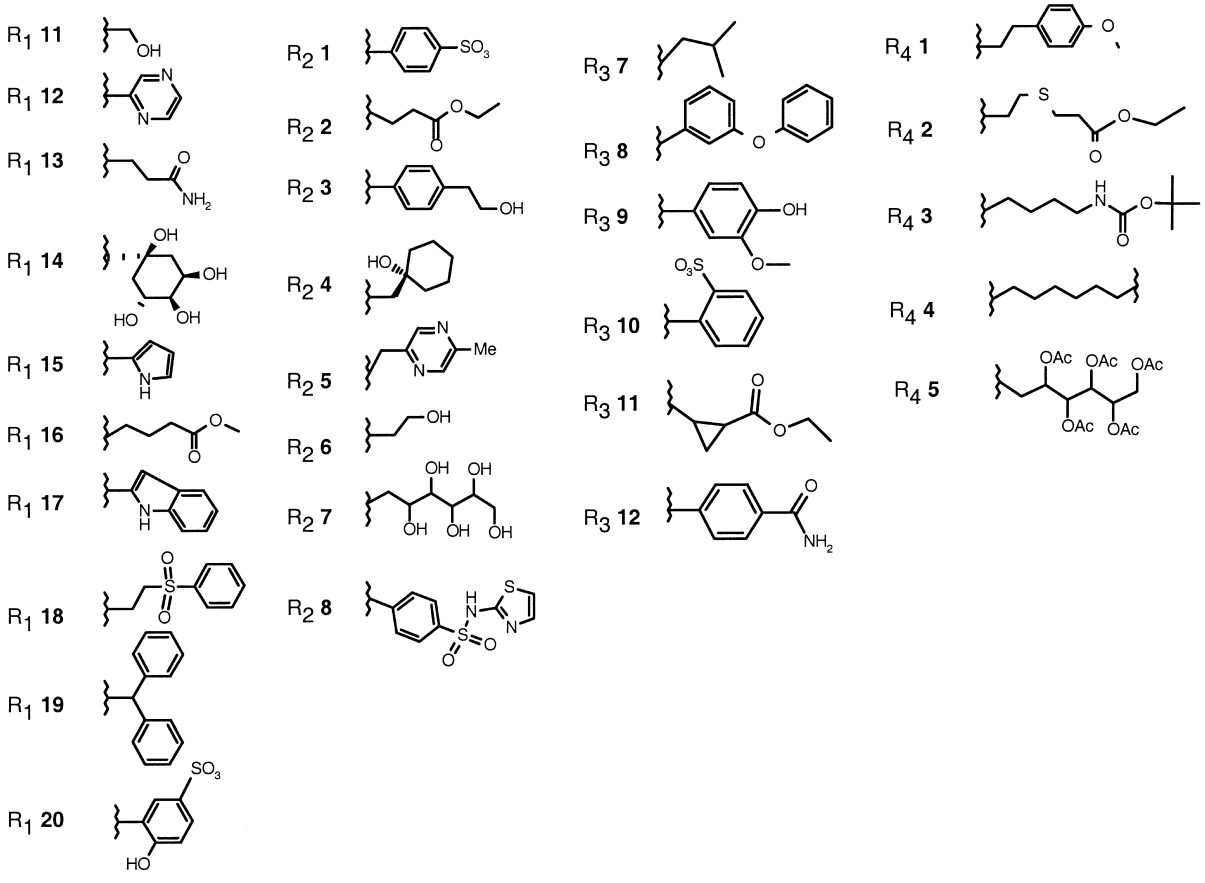
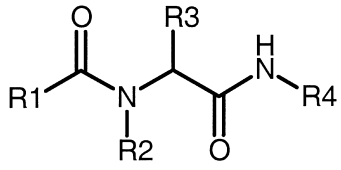


Figure 3.

active compounds is listed in Table 1. The number of atoms ranges from 14 to 24. In comparison, the number of atoms between two outside sulfates of a pentasaccharide that can fit into the binding groove of bFGF is 20. For a peptide in a β-sheet conformation, 17 atoms are required to generate a distance of 20 Å. It seems reasonable to suggest that the distance between the two negative charges in the identified active compounds is in the neighborhood of 20 Å.

In support of the charge and distance hypotheses a much lower hit rate, 0.094% was seen with extended compounds incorporating a four-carbon space contributed by adipic acid than with six carbon spaces contributed by 1,6-diisocyanohexane and 1,6-hexanediamine. Of the 1064 reactions of adipic acids only one active compound, **20**, was identified. The two carboxylates of compound **20** are located at the far end of the isocyanide group (R_4 substitution), presumably to achieve



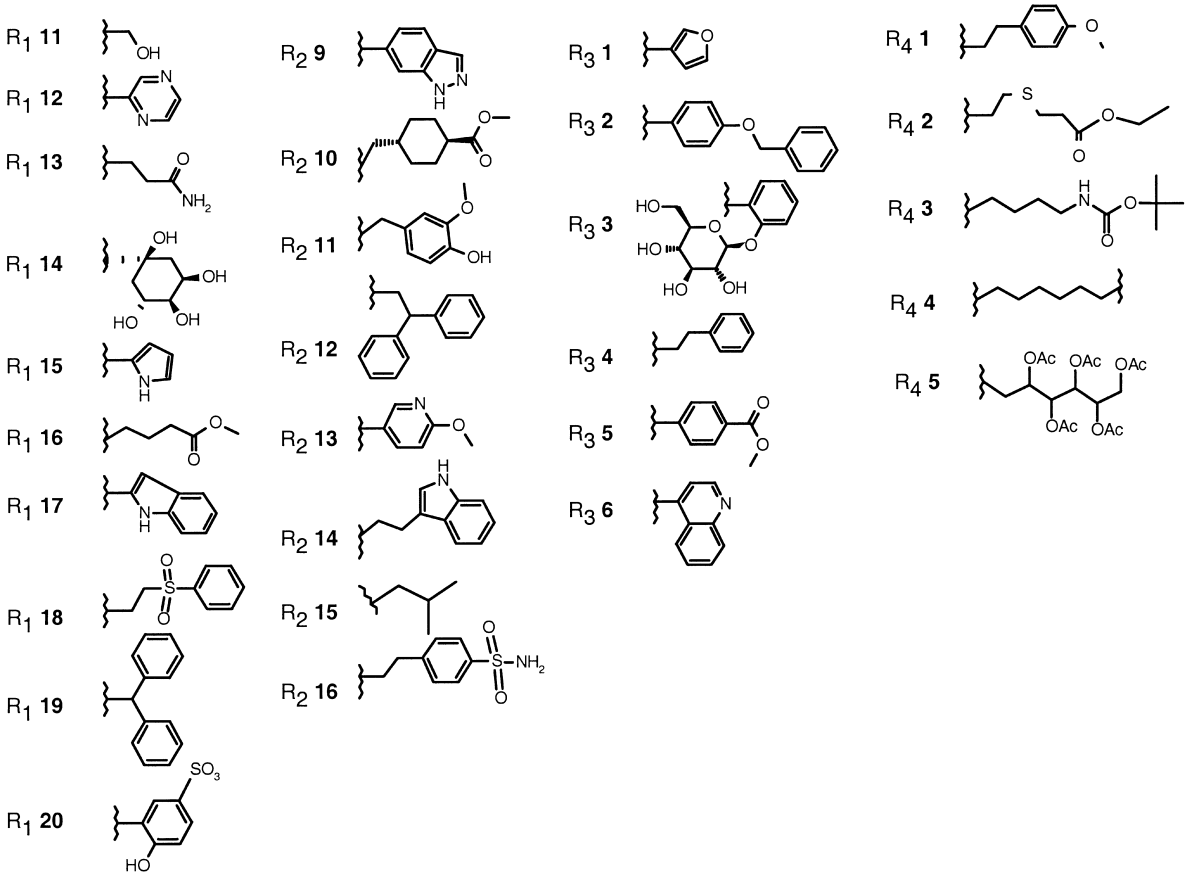


Figure 4.

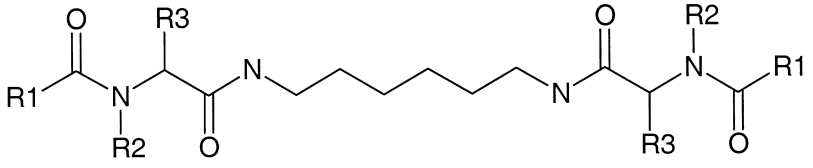


Figure 5. Dimeric structures generated by 1,6-diisocyanohexane.

the optimal charge separation. Compounds with charges at the aldehyde group (R_3 substitution) and amine group (R_2 substitution) were not identified as active inhibitors in the adipic acid reactions, presumably due to the charge separation being shorter than the expected 20 Å. However, of the 1064 reactions of 1,6-hexanediamine-five active inhibitors were identified, a rate of 0.47%. The charges of this series of compounds are located on the aldehyde group or the amine group. Of the 2672 reactions of 1,6-diisocyanohexane 12 compounds were identified as active inhibitors, a rate of 0.45%. The sulfonate or carboxylate groups are located on all three possible substitutions. The rate of identified active compound with single charge is even smaller, two out of 13,920 reactions or 0.015%, indicating two negative charges are important for activity.

The structures identified from this initial library clearly suggest that two negative charges, either sulfonate or carboxylate, are important for activity. The exact location of these charges does not seem to be critical, possibly due to compound flexibility, but the distance between the two charges is important since extended structures that cover a longer space are strongly preferred. It was also noticed that bulky hydrophobic moieties, such as phenoxyphenyl, benzyloxyphenyl, diphenylethyl, and indolyl, are present in most of the structures. Since the heparin-binding site of bFGF as seen in the crystal structure is flat and positively charged with no indication of a hydrophobic pocket, the function of these hydrophobic groups is unclear and requires further investigation. Most of the active compounds showed moderate selectivity towards VEGF versus bFGF. The cause of this selectivity is presumably due to the differences in the structure of VEGF and bFGF heparin-binding sites. In summary, inhibitors of heparin binding to VEGF and bFGF with IC_{50} at low micromolar concentrations were identified from heparin mimetic libraries of 4CC reactions. These SAR observations provide direction to obtain a more

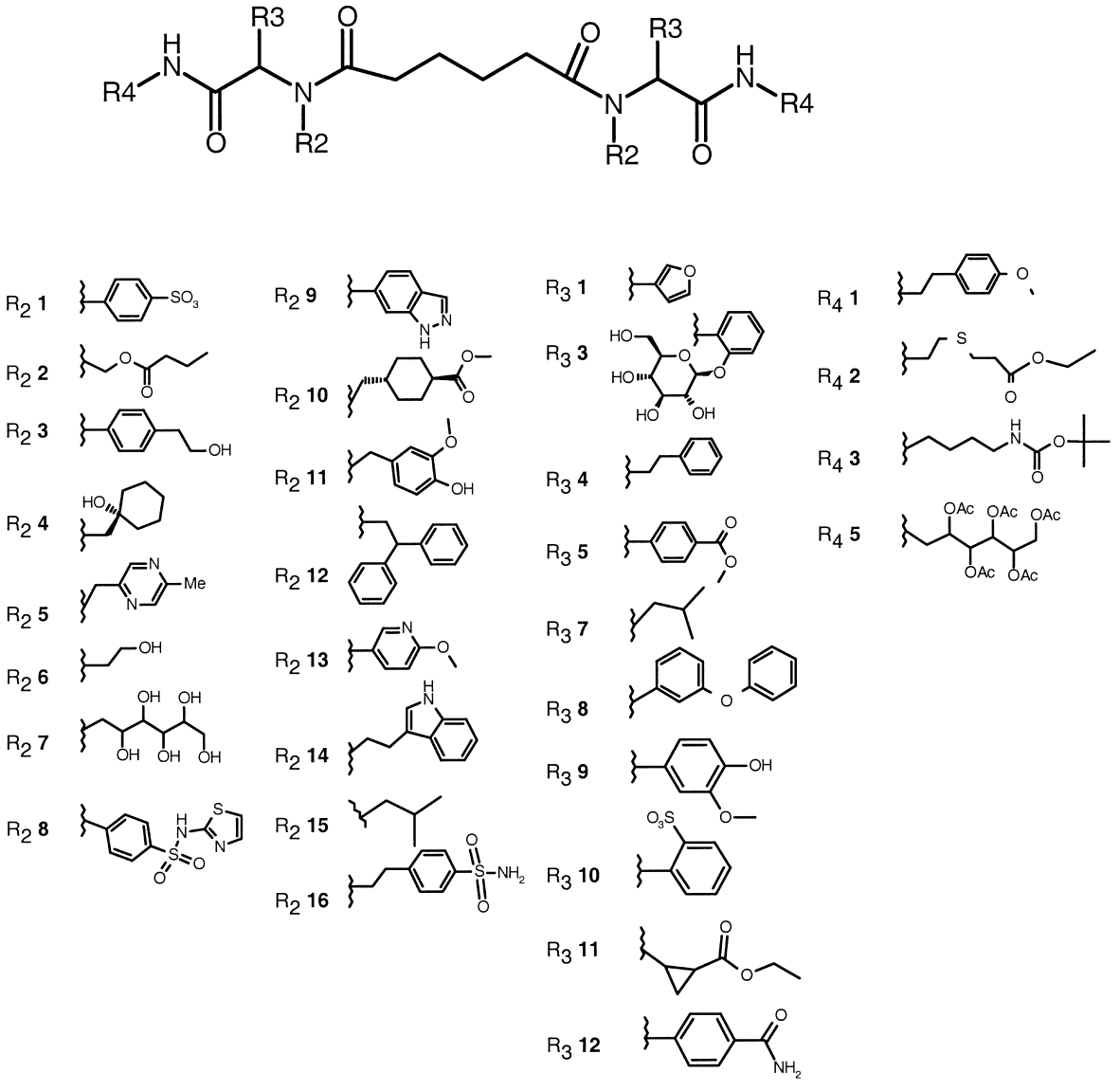


Figure 6.

specific SAR profile of this type of compounds and hopefully to obtain more potent and more selective inhibitors.

Experimental

^{1}H NMR spectra were recorded using 60 or 200 MHz NMR spectrometers. Chemical shifts were referenced with TMS as the internal standard. HPLC was run on a 0.4×10 cm C18 column with 0.1% TFA water as buffer A and 0.1% TFA acetonitrile as buffer B at 2 mL per min on a HP1090 system. All chemicals were purchased from commercial sources except four of the isocyanides. 4-Methoxyphenylethylisocyanide and ethyl 3-(2′-isocyanoethyl)thiopropionate were prepared using the reported procedure.[16] 4-(*tert*-Butoxycarbonylamino)-butylisocyanide and 2,3,4,5,6-pentaacetoxyhexaneisocyanide were prepared through dehydration of formamides, which were formed by reacting primary amines with ethyl formate.

General procedure for the synthesis of formamides

One equivalent of primary amine and 1.5 equiv of ethyl formate were stirred at room temperature in a round bottom flask equipped with a condenser. In general, the heat generated by the reaction kept the reaction at reflux; if not, gentle heat was applied to hold the reaction at reflux for 1 h. The volatile solvent was then removed and the remaining residue was used directly in the dehydration reaction.

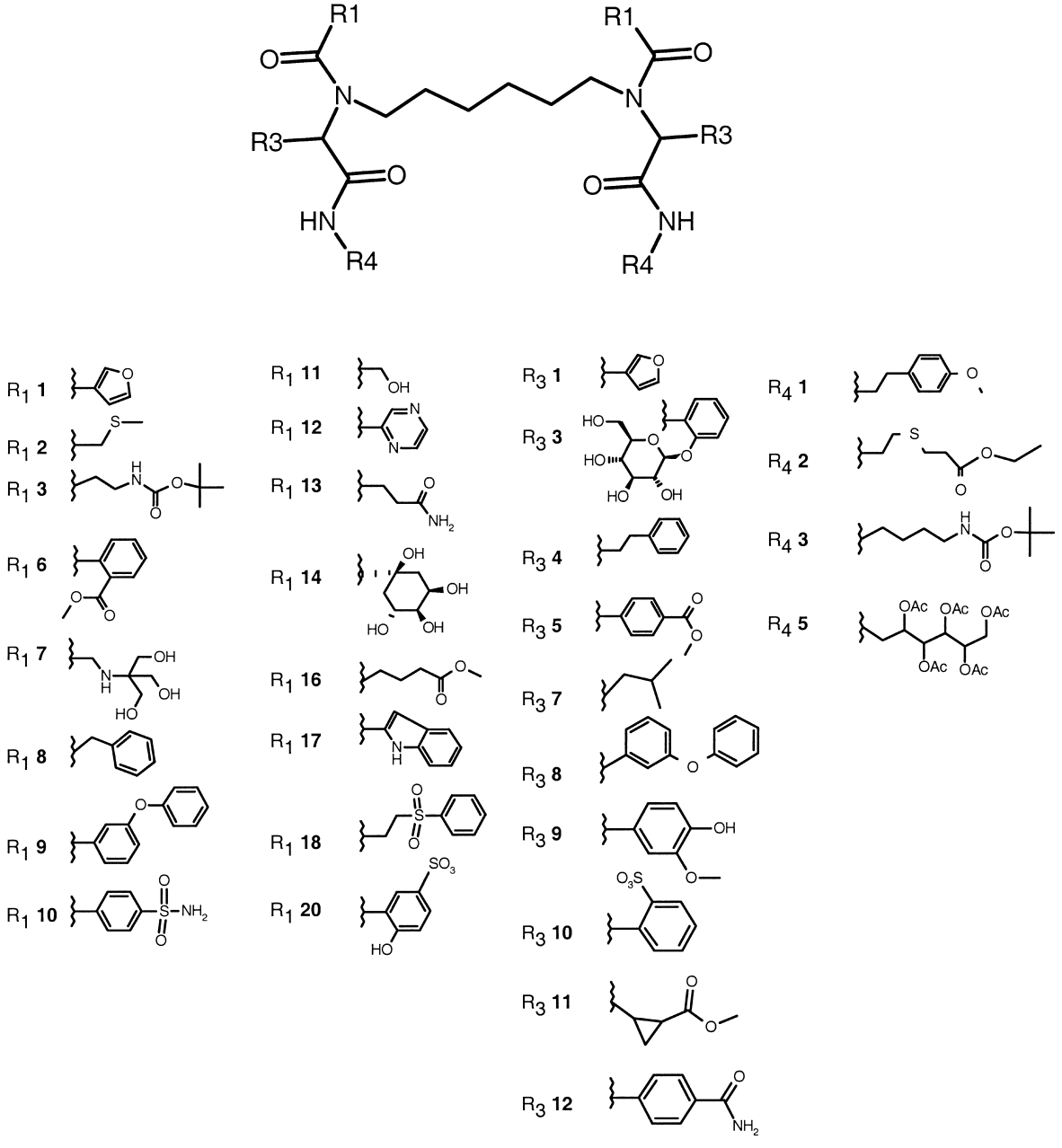
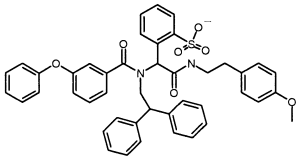
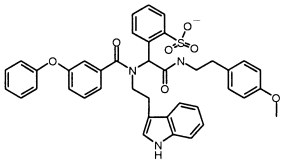
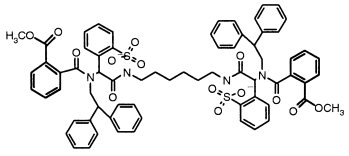
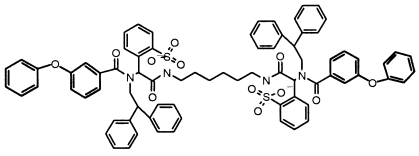
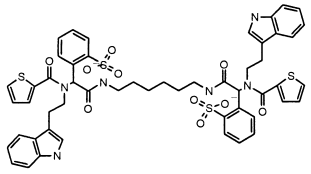
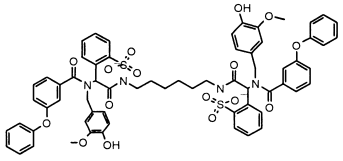
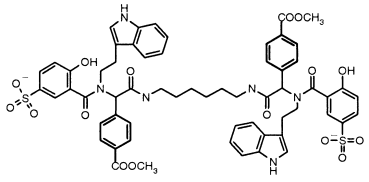
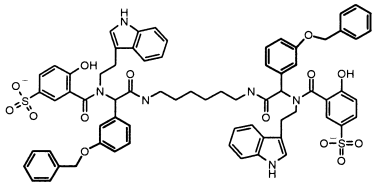
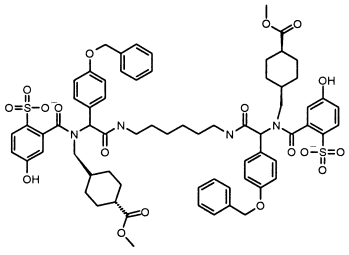


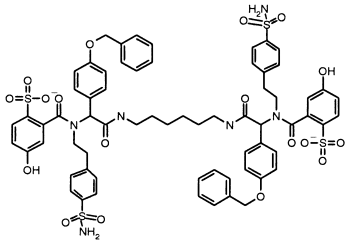
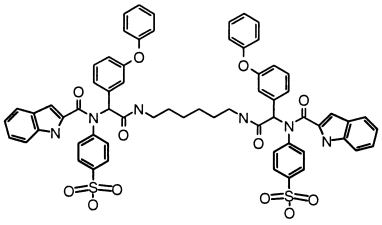
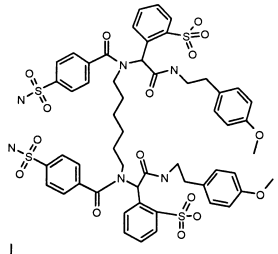
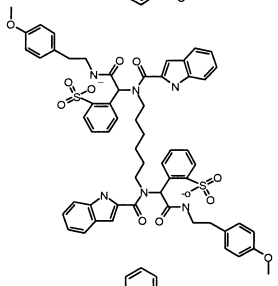
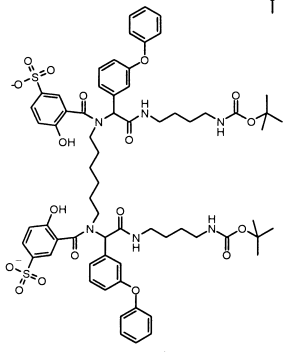
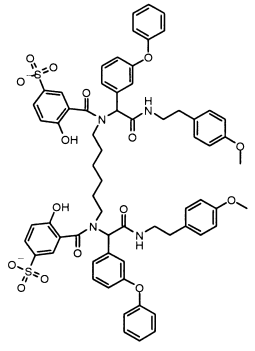
Figure 7.

Table 1. A list of identified active compounds

Entry	Structure	Formula	Exact mass	FAB–MS	IC_{50} μM VEGF	IC_{50} μM bFGF	Number of atoms between two charges
1		$C_{44}H_{40}N_2O_7S$	740	739 (M−H, FAB−)	5.5±0.5	35.5±2.5	N/A
2		$C_{40}H_{37}N_3O_7S$	703	702 (M−H, FAB−)	13.5±2.5	> 50	N/A
3		$C_{68}H_{66}N_4O_{14}S_2$	1226	1225 (M−H, FAB−)	7.95±0.6	45±3.2	16
4		$C_{76}H_{70}N_4O_{12}S_2$	1294	1293 (M−H, FAB−)	1.65±0.15	10.2±2.8	16
5		$C_{52}H_{52}N_6O_{10}S_4$	1048	1047 (M−H, FAB−)	5.3±0.20	27.5±2.5	16
6		$C_{64}H_{62}N_4O_{16}S_2$	1206	1205 (M−H, FAB−)	14.5±0.50	> 50	16
7		$C_{60}H_{60}N_6O_{16}S_2$	1184	1183 (M−H, FAB−)	4.8±0.00	> 50	22
8		$C_{70}H_{68}N_6O_{14}S_2$	1280	1279 (M−H, FAB−)	5.75±0.45	25±2.0	22
9		$C_{68}H_{78}N_4O_{18}S_2$	1302	1301 (M−H, FAB−)	5.75±0.75	16.5±7.5	22

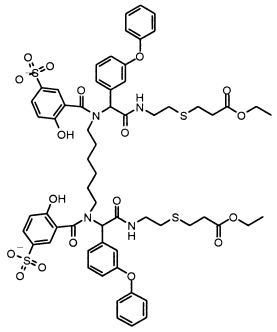
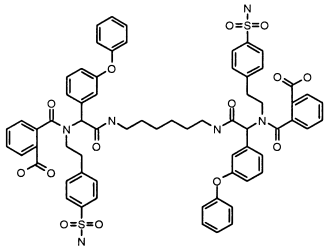
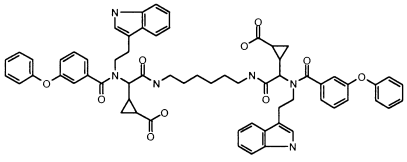
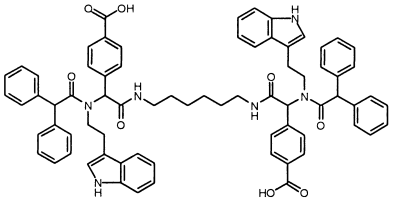
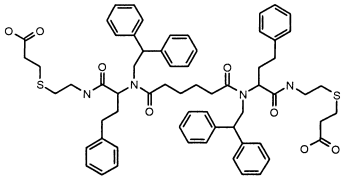
(*continued on next page*)

Table 1 (*continued*)

Entry	Structure	Formula	Exact mass	FAB–MS	IC_{50} μM VEGF	IC_{50} μM bFGF	Number of atoms between two charges
10		$C_{66}H_{68}N_6O_{18}S_4$	1360	1359 (M−H, FAB−)	6.0±1.0	36.0	22
11		$C_{64}H_{56}N_6O_{12}S_2$	1164	1163 (M−H, FAB−)	2.1±0.6	5.2±1.2	22
12		$C_{54}H_{60}N_6O_{16}S_4$	1176	1175 (M−H, FAB−)	11.5±1.5	> 50	14
13		$C_{58}H_{60}N_6O_{12}S_2$	1096	1095 (M−H, FAB−)	5.7±0.6	42.0±8.0	14
14		$C_{66}H_{80}N_6O_{18}S_2$	1308	1307 (M−H, FAB−)	6.7±1.1	> 50	16
15		$C_{66}H_{66}N_4O_{16}S_2$	1234	1233 (M−H, FAB−)	9.5±0.5	47.5±2.5	16

(*continued on next page*)

Table 1 (*continued*)

Entry	Structure	Formula	Exact mass	FAB–MS	IC_{50} μM VEGF	IC_{50} μM bFGF	Number of atoms between two charges
16		$C_{62}H_{70}N_4O_{18}S_4$	1286	1285 (M−H, FAB−)	9.9±0.1	> 50	16
17		$C_{66}H_{63}N_6O_{14}S_2$ Na	1250	1251 (M + H, FAB+)	2.25±0.4	25.5±1.8	20
18		$C_{64}H_{64}N_6O_{10}$	1076	1075 (M−H, FAB−)	3.2±0.6	21.5±0.5	16
19		$C_{72}H_{68}N_6O_8$	1144	1145 (M + H, FAB+)	4.7±0.1	18.5±1.5	20
20		$C_{64}H_{74}N_4O_8S_2$	1090	1089 (M−H, FAB−)	6.0±1.0	9.3±1.6	24

4-(*tert*-Butoxycarbonylamino)butylformamide. ^{1}H NMR (CD_3OD) δ 8.24 (s, 1H), 3.43 (m, 4H), 1.86 (m, 2H), 1.62 (m, 2H), 1.50 (s, 9H).

2,3,4,5,6-Pentaacetoxyhexylformamide. ^{1}H NMR (CD_3-OD) δ 8.25 (s, 1H), 5.45 (m, 2H), 5.10 (m, 2H). 4.08 (m, 2H), 3.52 (m, 2H), 1.98 (s, 15H).

General procedure for the synthesis of isocyanides

To a solution of 1 equiv formamide and 3.3 equiv triethylamine in dry methylene chloride on an ice bath was added dropwise 1.1 equiv phosphorus oxychloride over 1 h. The reaction was stirred for another hour followed by adding 1.1 equiv sodium carbonate in water solution. The organic layer was then separated from the water layer, which was extracted again with methylene chloride. The combined methylene chloride solutions were dried with sodium sulfate and concentrated in vacuo. Isocyanides were purified by flash chromatography eluted with hexane and ethylacetate.

4-(*tert*-Butoxycarbonylamino)butylisocyanide. ^{1}H NMR ($CDCl_3$) δ 3.34 (m, 4H), 1.82 (m, 2H), 1.60 (m, 2H), 1.51 (s, 9H). IR cm^{-1} 2141.0 (CN-).

2,3,4,5,6-Pentaacetoxyhexaneisocyanide. ^{1}H NMR ($CDCl_3$) δ 5.50 (m, 2H), 5.18 (m, 2H). 4.23 (m, 2H), 3.78 (m, 2H), 2.09 (s, 15H).). IR cm^{-1} 2141.2 (CN-).

Library synthesis

One molar solution of the starting materials were prepared in either methanol, a mixture of methanol and DMSO, or DMSO. A SAIGEN multichannel pipettor was used to dispense 1.0 M stock solution of amines, aldehydes, acids, and isonitriles into a deep 96-well plate (Polyfiltronics). Equivalent amounts of amines and aldehydes were mixed and allowed to incubate at room temperature 1–2 h before addition of equivalent amounts of acids and isonitriles. The plates were then securely capped followed by an overnight incubation at 45 °C. For reactions containing 2,3,4,5,6-pentaacetoxyhexaneisocyanide, plates were incubated at room temperature for 4 days. Daughter plates at 5.0 mM concentration were made by dilution of the master plates with DMSO for HPLC analysis of the library. A portion of the products was HPLC purified and structures were confirmed by FAB–MS.

General synthesis of discrete compounds

Fifty microliters of 1.0 M appropriate amines, aldehydes, acids and isonitriles were added to a glass vial and capped. The glass vial was then incubated at 45 °C overnight. The solution was centrifuged and directly loaded onto a C18 column for purification. The peak corresponding to the product was collected, pooled, and dried under speed vacuum, after which the product was weighed and characterized by FAB–MS.

Screening assay

^{125}I heparin 16-mer was prepared by mixing 10 μg of purified tyraminated heparin 16 mer with 2 mCi ^{125}I (Dupont) in an Iodogen tube (Pierce) in the presence of 50 mM acetate, pH 6.0 for 5 min. After completion of the reaction, excess free iodine was removed via passage over a PD-10 desalting column (Pharmacia). Iodinated 16 mer was stored at −80 °C.

VEGF-165 was produced in a transfected insect cell line and purified from conditioned media using heparin-agarose with elution at 250 mM NaCl. Each lot of purified protein was assayed for its ability to bind heparin and stored at −80 °C until used. Basic FGF was obtained from Reprotech (Rocky Hill, NJ) and stored at −80 °C until use.

For the library screening against VEGF, mixtures of 50 nM VEGF, 50 nM ^{125}I-heparin and 5 μM library compounds were incubated in phosphate-buffered saline (PBS) containing 0.1% ovalbumin pH 7.4 for 1 h at room temperature. Incubation was carried out in a Millipore nitrocellulose filter-bottom plate. Library compounds were assumed to be present in 56% yield after synthesis, which was an average value obtained from analyzing 30% of the reactions. After incubation for 1 h at room temperature, the solution was removed by vacuum filtration and the plates washed with 2×100 μL PBS. After washing, the plates were left dry for 1 h. Scintillation cocktail mix was added to each well and plates were counted in a Wallac Microbeta plate reader. Each plate set was accepted based on the IC_{50} of a known heparin-binding inhibitor, suramin, as well as positive and negative controls.

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