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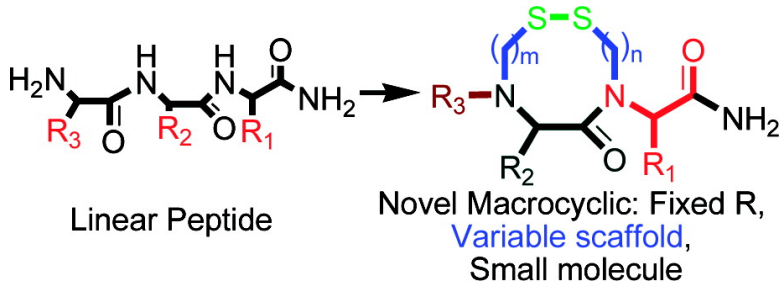

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Synthesis of a Novel Macrocyclic Library: Discovery of an IGF-1R Inhibitor

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We present a new approach for the conversion of active sequences of proteins and peptides into small molecules. A library of macrocyclic disulfide molecules was made, in which the active pharmacophores of the parent peptide are preserved while the size of the macromolecular scaffold on which the pharmacophores are arranged is varied. This enables a systematic search for macromolecules in which the pharmacophores are in an appropriate conformation for biological activity. We developed two procedures for the synthesis of such libraries from building blocks that include commercial amino acids and functionalized aldehydes. Chemical synthesis using the "tea-bag" method gave a library with higher diversity, but low yields, compared to the manual synthesis of the library, in which the compounds were synthesized in individual vessels and the yield and purity improved dramatically. As a proof of concept, we synthesized a 34-member library derived from the sequence of the activation loop of insulin-like growth factor-1 receptor. Selected compounds were screened, and one was found to be biologically active in the low micromolar range. The concept presented here may prove particularly useful in cases where the pharmacophores are known but need to be systematically screened for a spatial arrangement that will enable biological activity.

Introduction

The discovery of new drugs poses a great challenge in modern pharmaceutics. Potential drugs can be discovered by screening natural products or random chemical libraries or through rational design.[1,2] Naturally occurring materials are often used as templates for creating designed combinatorial libraries based on active sequences of peptides and proteins.[3] These libraries usually comprise heterocyclic, constrained scaffolds on which various pharmacophores are positioned.[4–8] The major limitation with such scaffolds is that their conformational space is intrinsically limited, which in many cases prevents the pharmacophores from reaching their bioactive conformation. Moreover, the rigidity of the scaffold can prevent conformational complementarity of the molecule to its target, especially in the case of highly adaptive regions in proteins.[9–11]

Known data and natural biological recognition can be used to develop small-molecule peptide-based drugs that mimic proteins or naturally occurring peptides.[12–14] The use of linear peptides as drugs is often problematic because of their conformational flexibility, susceptibility to enzymatic degradation, and low bioavailability. Cyclization can be used to overcome these limitations because it restricts the conformational freedom of peptides and improves their metabolic stability and selectivity toward receptors.[15] However, the use of cyclic peptides as a scaffold for the pharmacophoric moiety often fails because the constrained scaffold prevents the pharmacophores from achieving a bioactive conformation. Failure to achieve a bioactive conformation can be overcome by using combinatorial libraries of small, cyclic molecules, in which the scaffold is varied to achieve a library in which the pharmacophores are held in varied conformations, which are subsequently screened for bioactivity. The diversity of the library is controlled by the number and identity of the residues, their relative position on the scaffold, their chirality, and the chemical structure of the scaffold.[16,17]

The major challenge in the design of such molecules is in determining how to place the pharmacophoric side chains on the scaffold in their bioactive conformation. This can be done by construction of a structure-based library, when the structure and relative spatial orientation of the pharmacophores is known, by construction of a library based on known pharmacophores, if only the pharmacophores are known, or by construction of a random library, if no information is available.[2]

Insulin-like growth factor-1 (IGF-1) receptor (IGF-1R)[18,19] is frequently overexpressed by a broad spectrum of malignancies, among them cancers of the prostate, pancreas, breast, colon, and lung.[20,21] The activity of IGF-1R is enhanced in

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cancers because of up-regulation of its ligand IGF-1.[22] IGF-1R activates several signaling pathways, including the extracellular signal-regulated kinase (ERK) pathway, the antiapoptotic protein kinase B (PKB) pathway, and others. It signals for proliferation and protection against apoptotic stimuli, including resistance to cytotoxic drugs.[23] It is well-established that it contributes to cell transformation and to tumor development at early stages, as well as during the metastatic phase.[24] Many companies and laboratories have recognized IGF-1R as an attractive target for cancer therapy and have devoted efforts to inhibition of its activity or expression.[25]

We developed a combinatorial library of small macrocyclic molecules based on pharmacophores derived from the activation loop of IGF-1R to inhibit the activation and the tyrosine kinase activity of the IGF-1R. For a proof of concept, we screened a limited set of compounds for biological activity, and selected an active compound that was further characterized. The size of the scaffold, governed by the length of the *m* and *n* bridges, was used as a critical diversity parameter, in addition to those usually used for the preparation of scaffold-based libraries. Members of these libraries combine the advantages of peptide-based drugs with those of constrained macrocyclic scaffolding, while preserving the flexibility that enables induced fitting upon binding a target protein. Furthermore, they can comply with the Lipinski and Veber rules,[26,27] making them more promising as drug leads. Although the synthesis of this library suffers from low yields, the benefit of this approach is that the resulting library has a single set of pharmacophores of a known active site with varying scaffold sizes and chemistry. This allows systematic screening of the bioactive conformations of a given set of pharmacophores in search of drug leads. We also propose an improved synthetic methodology that may enable a wider use of the concept in the future.

Results

Synthetic Plan. The library was synthesized according to Scheme 1 to obtain members with the general structure **6A** (Scheme 1). We achieved diversity by using different natural amino acids, ω-Trt-thio-alkyl aldehydes with varying lengths of alkyl chain, and 9-fluorenylmethoxycarbonyl (Fmoc)-N^{α}(β-Acm-thioethyl)Gly-OH.[28] The S−S bond was created using iodine on-resin oxidation.[29] This cyclization was the final step of the synthesis before cleavage (Scheme 1; data for ω-Trt-thio-alkyl aldehydes are included in the Supporting Information). We obtained 34 library members with various degrees of purity according to the synthetic methodology used, as will be discussed (Figure 2 and Table S1 (Supporting Information)).

Synthetic Methods. Two methods were used for parallel synthesis of the library: the conventional Merrifield vessel method[30] and the Houghten tea bag method.[31] The tea bag method combinatorial approach was used because it was easier and faster to synthesize a very diverse library using this method. However, this method gave low yields and purity. The manual vessel method was more laborious but resulted in higher yields and purity and was preferred for the synthesis of large amounts of specific compounds (see Discussion for a comparison between the methods).

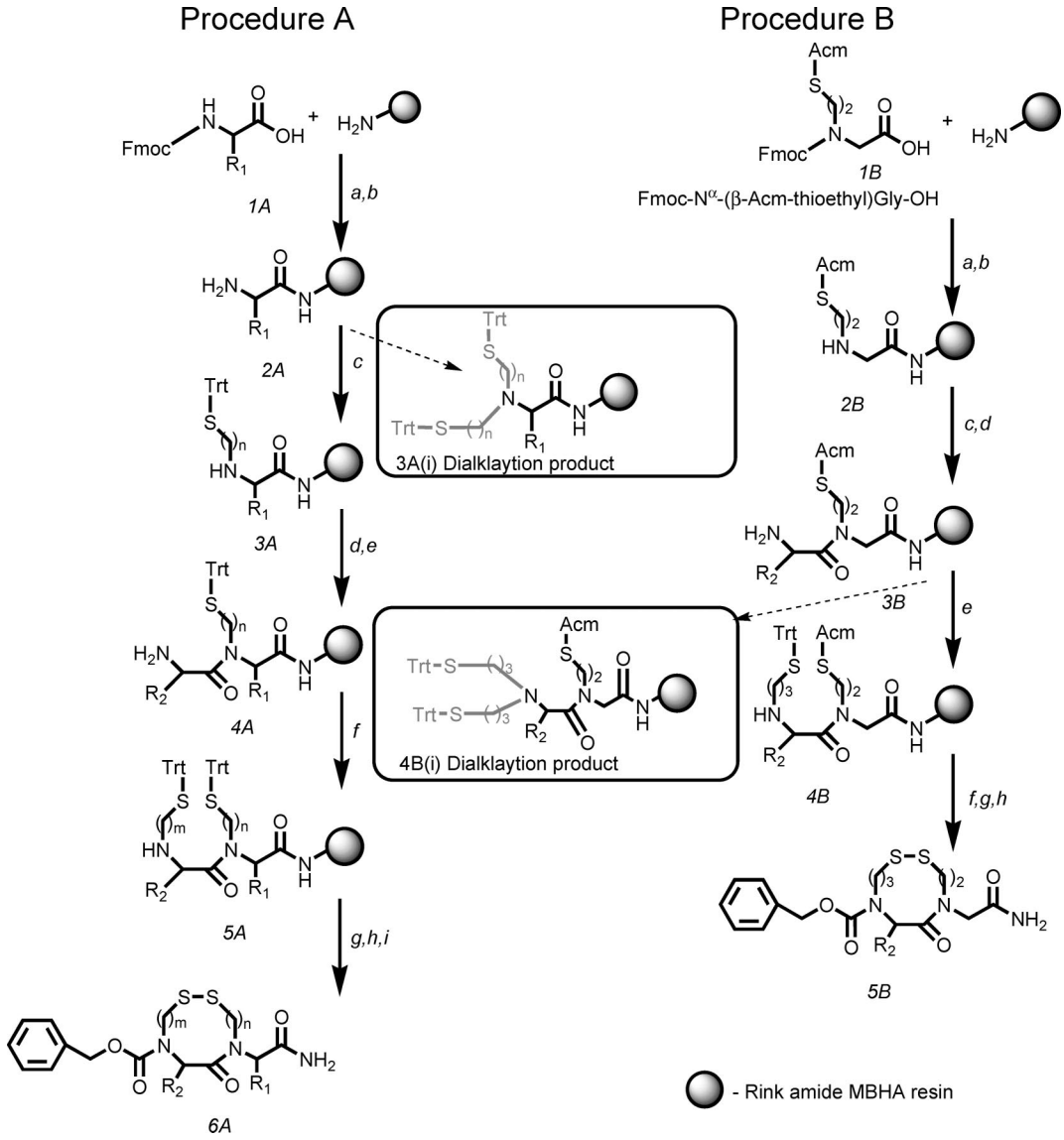
Synthetic Procedures. We explored two synthetic procedures, termed Procedures A and B (Scheme 1), for the synthesis of the library based on multiple parallel solid-phase synthesis. In Procedure A, the two functionalized *N*-alkyl chains were introduced by two cycles of in situ reductive alkylation, while in Procedure B, the first functionalized *N*-alkyl chain was introduced by coupling a premade Fmoc-N^{α}(β-Acm-thioethyl)Gly-OH building block, and the second functionalized *N*-alkyl chain was introduced by in situ reductive alkylation (Scheme 1). Reductive alkylation steps are known to reduce yields and purity.[35–38] Six members with various R groups (members 1–6, Figure 2 and Table S1 (Supporting Information)) were synthesized by Procedure B to establish the synthetic procedures.

Synthetic Difficulties. With standard coupling reagents, the coupling step to the secondary sterically hindered amines originating from the functionalized, protected *N*-alkyl amino acid residue (Procedure A, step d, and Procedure B, step c, Scheme 1) gave low yields even at elevated temperatures. Bis(trichloromethyl)carbonate (BTC) is an efficient activating agent for coupling Fmoc-protected amino acids to non-Gly-functionalized *N*-alkyl amino acid residues[32] and was used for the total solid-phase synthesis of cyclosporine analogs.[33] We successfully coupled Fmoc-Xaa-OH to products **3A** and **2B** (Procedure A and B, Scheme 1) using two cycles of BTC-mediated couplings, one of 2 h and one overnight at 60 °C, using 1,2-dibromoethane (DBE, bp 131 °C) instead of the usual DCM (bp 39.8 °C) to achieve products **4A** and **3B** (Procedure A and B, Scheme 1). We found that BTC raised all yields of the coupling steps of the secondary amines in the syntheses, including coupling of the Fmoc-N^{α}(β-Acm-thioethyl)Gly-OH to the resin (product **2B**, Procedure B, Scheme 1).

The subsequent reductive alkylation was performed twice for 3 h.[34] The most significant product was the undesired dialkylation product (products **3A(i)**, Procedure A, and **4B(i)**, Procedure B, Scheme 1), rather than the monoalkylation product. We optimized the experimental conditions to increase the monoreductive alkylation product on a model aldehyde system, 6-(tritylthio)hexanal, (Trt-S-$(CH_2)_5$-CHO), which is the least sterically hindered aldehyde in the series. Conditions studied included the number of equivalents of added aldehyde, the type of reducing reagent ($NaBH_3CN$, $NaBH_4$ or sodium triacetoxyborohydride), the number of equivalents of reducing reagent, the reaction time, and the number of reaction cycles. We found that a cycle of 2 h using 2–5 equiv of $NaBH_3CN$ at RT gave the monoalkylation product as the major product although there was still a minor dialkylation byproduct.[35–38] We subsequently used 1–3 equiv of the respective aldehyde and obtained the monoalkylation product as the major product.

The library was analyzed using mass spectrometry (MS) and the results are shown in Table S1 (Supporting Information). In some cases, experimental MW values of MW+2 or +3 were found, a phenomenon that has been shown previously for cyclic disulfide peptides.[28,39,40] To confirm that these members were indeed cyclic, we characterized the

Scheme 1[a]



[a] Procedure A: (a) BTC, collidine, RT; (b) 20% piperidine in NMP; (c) Trt-S-$(CH_2)_n$-CHO, $NaBH_3CN$; (d) Fmoc-Xaa_2-OH, BTC, collidine, 60°C; (e) 20% piperidine in NMP; (f) Trt-S-$(CH_2)_m$-CHO, $NaBH_3CN$; (g) benzylchloroformate, DIEA, 60°C; (h) (i) I_2, (ii) ascorbic acid; (i) TFA-TIS-TDW. Product **3A(i)**: A dialkylation side product obtained from step c in Procedure A. Procedure B: (a) BTC, collidine, RT; (b) 20% piperidine in NMP; (c) Fmoc-Xaa_2-OH, BTC, collidine, 60°C; (d) 20% piperidine in NMP; (e) Trt-S-$(CH_2)_2$-CHO, $NaBH_3CN$; (f) benzylchloroformate, DIEA, 60°C; (g) (i) I_2, (ii) ascorbic acid; (h) TFA-TIS-TDW. Product **4B(i)**: A dialkylation side product obtained from step e in Procedure B.

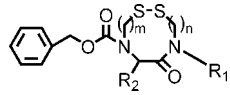


Figure 1. The model compound. R_1 and R_2 are pharmacophores derived from the active regions of peptides or proteins and were occupied almost exclusively by benzyl and various 4-hydroxybenzyl moieties; *n* and *m* are the lengths of the alkyl chains varied among 2–6.

S−S bond using MS−MS analyses that yielded fragments that contained (S−S)-dialkyl chains. This confirmed the cyclic nature of the main product and eliminated the possibility of a reduced noncyclic product (see, for example, the fragment in compound **9**, Figure 2 in gray). Furthermore, Ellman tests performed on a number of products were negative for thiols indicating the disulfide bond.[41]

Designing the IGF-1R Model Library. IGF-1R was chosen as a model system because it is an attractive target for cancer therapy and its pharmacophores are known and spatially defined (PDB accession code 1K3A).[19] One of the initial and necessary events in IGF-1R activation is the autophosphorylation of its activation loop. Therefore, we assumed that mimics of this loop should inhibit IGF-1R activation, as well as IGF-1R downstream signaling.[18] We based the library on the tyrosine-rich motif of the IGF-1R activation loop.[42] The autophosphorylation of the activation loop occurs on a motif of three active tyrosine residues: Tyr 1131, Tyr 1135, and Tyr 1136.[19] In this library, the active residues were mimicked by positions R_1 and R_2, which were mainly aromatic benzyl, various 4-hydroxybenzyl moieties, and the benzyloxycarbonyl group (Figure 1, Figure 2, and Table S1 (Supporting Information)).

Biological Screening of the Library. Selected compounds (**9**–**17**) were screened in crude form for their inhibitory action by two independent assays: inhibition of IGF1-induced IGF-1R phosphorylation in breast cancer MCF7 cells at 20 μM

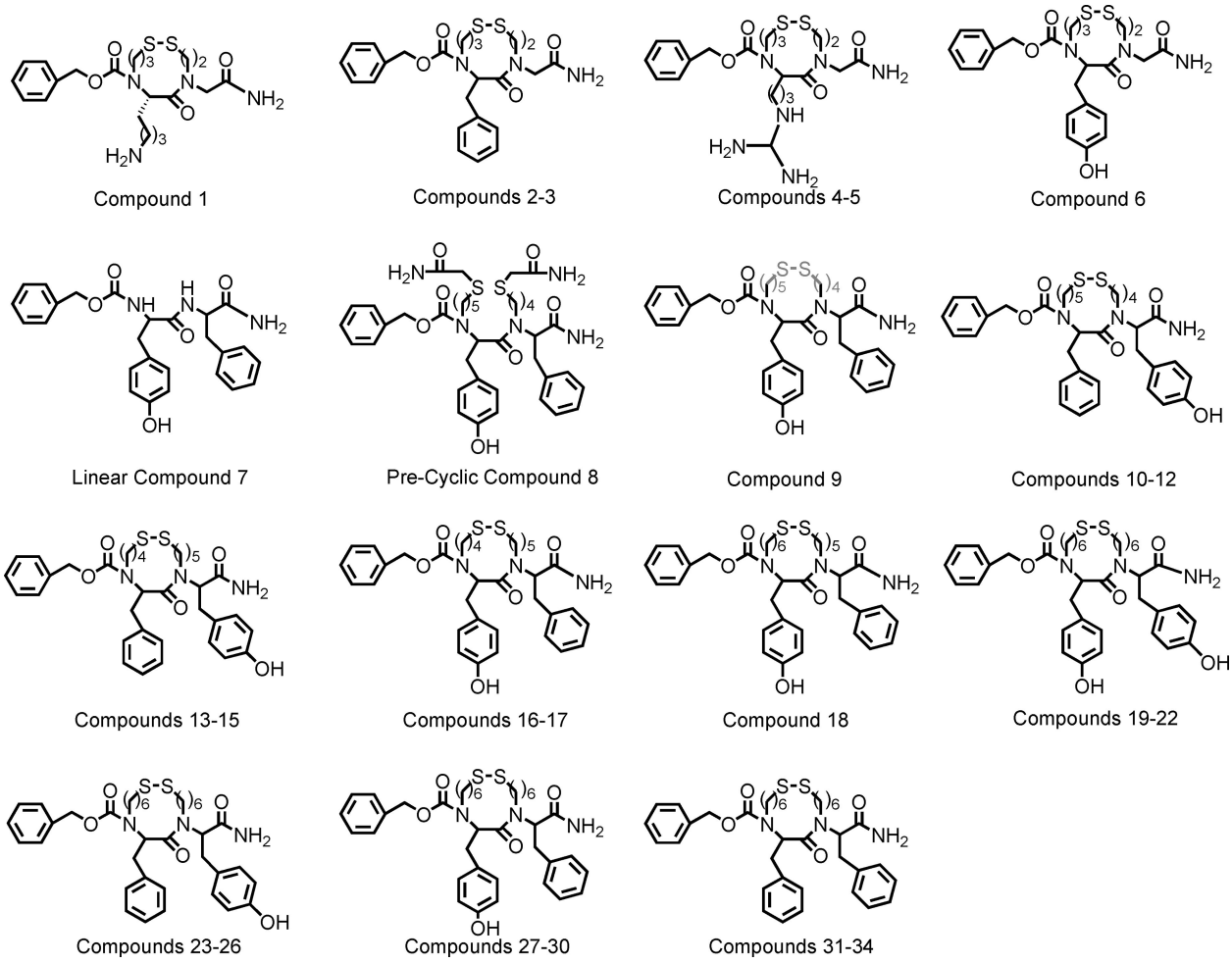


Figure 2. The library. General structures of compounds **1**–**34** are shown. For detailed information about the individual compounds including L and D configuration, see Table S1 (Supporting Information). In compound **9**, the MS fragmentation of the disulfide bridge is marked in gray.

concentration and inhibition of anchorage-dependent growth of MCF7 cells at various concentrations of each compound (Table S2 (Supporting Information)). Compound **9**, which was the most active inhibitor in both assays (Figure 2 and Table S1 (Supporting Information)), was synthesized in larger amounts by method A using a vessel, purified, and characterized (Figure 3). Pure compound **9** was tested for its inhibitory action on IGF-1R in a dose-dependent manner, as well as for its selectivity toward another tyrosine kinase receptor. Compound **9** inhibited the IGF-1-dependent phosphorylation of both the IGF-1R itself and its downstream element ERK in MCF7 breast cancer cells (Figure 4A) in a dose-dependent manner, showing IC_{50} values of 6 and 8 μM, respectively. Compound **9** had no inhibitory effect on epidermal growth factor (EGF)-induced phosphorylation of the EGF receptor (EGFR), or on EGF-induced ERK phosphorylation (Figure 4B). Each biological assay was repeated at least twice. In the case of purified compound **9**, the assay was repeated on different synthetic batches.

Characterization by NMR. The NMR spectrum of compound **9** is given in Table 1 (the alkyl chains are enumerated starting at the tertiary nitrogens). The assignment was done on the basis of considerations given in the Experimental Section. All the protons in all spin systems were present. The three aromatic groups were identified: The Tyr ring showed a TOCSY and NOESY interaction with the water peak and only two interacting aromatic peaks. The Phe ring showed internal interactions as expected and an additional two ROESY interactions with the carbobenzoxy group (carbobenzoxy-H_{ortho} and -H_{meta} to Phe-H_{ortho} and -H_{meta}, respectively). The carbobenzoxy methylene was identified according to its chemical shift, integration, and a NOESY interaction with the Tyr Hβ methylene protons, which in turn, were interacting with the Tyr-Hα proton. The Tyr-H_{ortho} protons interacted with the *n*-alkyl chain-Hα protons, giving this connection, which was followed throughout this portion of the bridge, in which the Hβ and Hδ protons overlapped. The *m*-alkyl system was identified as the remaining aliphatic system that also showed overlap in the Hβ/Hϵ and Hγ/Hδ resonances and also interacted with the Phe-Hα protons via the bridge-Hα protons. The Phe-Hα−Hβ interactions were evident, as well. There was an unresolved ROESY peak that included the *n*-alkyl-Hβ−Hγ interaction and another interaction attributed to the *n*-alkyl-Hδ−*m*-alkyl-Hϵ interaction. The Tyr-Hβs are resolved as we expected for a residue in a cyclic molecule, and the Phe methylene is also resolved, although it is a terminal residue.

Necessity for Ring Closure. Actual ring closure was required for activity as demonstrated by comparison of compound **9** to linear and precyclic analogs. Compound **7**

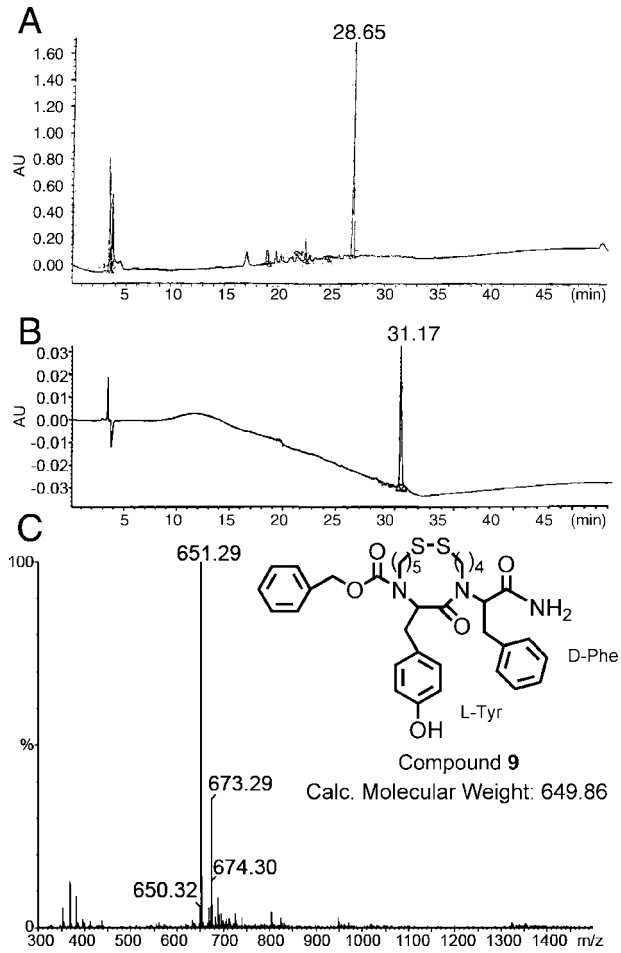


Figure 3. Characterization of compound **9**: (A) analytical HPLC at 220 nm, (B) analytical HPLC at 254 nm, and (C) MS. The peaks in the MS are 651.29 *m*/*z*, MW of compound **9** + H, and 673.29 *m*/*z*, MW of compound **9** + Na.

is a linear compound with the same amino acids as compound **9**. The precyclic analog, compound **8**, has the same amino acids and two alkyl chains as compound **9**, but the final cyclization step was not performed (Figure 2). Compounds **7**, **8**, and **9** are identical in the number of their pharmacophores, their chemical structures, pharmacophore positioning, and chirality. Compounds **7** and **8** were about four times less active than the cyclic compound **9**.

Discussion

In the current study, we developed a new type of macrocyclic library with a variable scaffold. We explored various synthetic procedures for preparation of such libraries, some of which resulted in respectable yields (85%) and some in low yields (20%). The strength of the design proposed here is in the ability to introduce diversity into the scaffold of the library. We present this concept as a general methodology for solid-phase synthesis of novel small molecule libraries, in which the pharmacophores are embedded in a scaffold of extendable size. We prepared a library with the following diversity parameters: the chemical structure of the scaffold, its ring size, the chemical character of the pharmacophores, their position on the scaffold, and their chirality.

We believe that the scaffolds in the different library members are conformationally diverse. The most important factors in determination of the conformation of the scaffold are the order of the alkyl chains in the aldehyde and the ring size, manipulated by changing the length of the two alkyl chains ($n = 2$–6 and $m = 2$–6, see Figure 1, Figure 2, and Table S1 (Supporting Information)). For example, a compound in which $n = 4$ and $m = 5$ is different from a compound in which $n = 5$ and $m = 4$, even though the scaffold sizes are equal, as seen by the different biological activity of compounds **9** and **17** (Table S2 (Supporting Information)). These factors may be crucial in the determination of the location and topology of the pharmacophores on the cyclic scaffold and result in molecules with differing biological activity.

Synthetic Considerations. The two major factors that affect the yields and purity of the crude library members are the choice of synthetic procedure (A vs B, see Scheme 1) and the synthetic method (vessels vs tea bag). Procedure A is more general and allows a more combinatorial approach because higher diversity can be achieved using fewer synthetic steps. The first coupling is of any amino acid of choice, and the subsequent reductive alkylation steps can be performed with ω-functionalized aldehydes containing alkyl chains of any length. In Procedure B, the first coupling incorporates a building block that determines the diversity of R_1 and the alkyl chain length. The use of these building blocks is limited by a lack of commercial availability and the fact that they are synthetically challenging to prepare, giving low yields and purity. The Tyr and Phe building blocks have been shown to be synthetically feasible but were obtained at low yields (10% and 5%, respectively).[23] However, when starting from purified building blocks, Procedure B gives yields (~40%) and purity (20–45%) that are about twice those of Procedure A (~20 and 10–20%, respectively). The difference in the purity of the crude products of the two procedures is most likely caused by the additional reductive alkylation step performed in Procedure A.

We demonstrate the feasibility of synthesizing a library with a single sequence of pharmacophores by both Procedures A and B. When the tea bag method was used, the products were obtained in low yield and purity, making it a less attractive procedure for general use. The use of a vessel instead of the tea bag method dramatically increases the purity and yield: Compounds prepared in vessels were over 85% pure, compared to a maximum of 45% using the tea bag method. Moreover, we showed that the library members can be purified to a degree sufficient for biological assays and structural studies. We conclude that given adequate building blocks, the optimal method for making a combinatorial library of many members in parallel is to start the synthesis in a manual reaction vessel using Procedure B and then to split the resin before the reductive alkylation step, using different protected functionalized alkyl aldehydes.

A novel method is presented for the synthesis of a cyclic scaffold with diversity on the resin via a disulfide bridge. The cyclization step in other libraries has been done in our laboratory via amide or urea bonds (Hurevich, M., Barda, Y., Gilon, C. *Heterocycles* (2007), in press), and similar

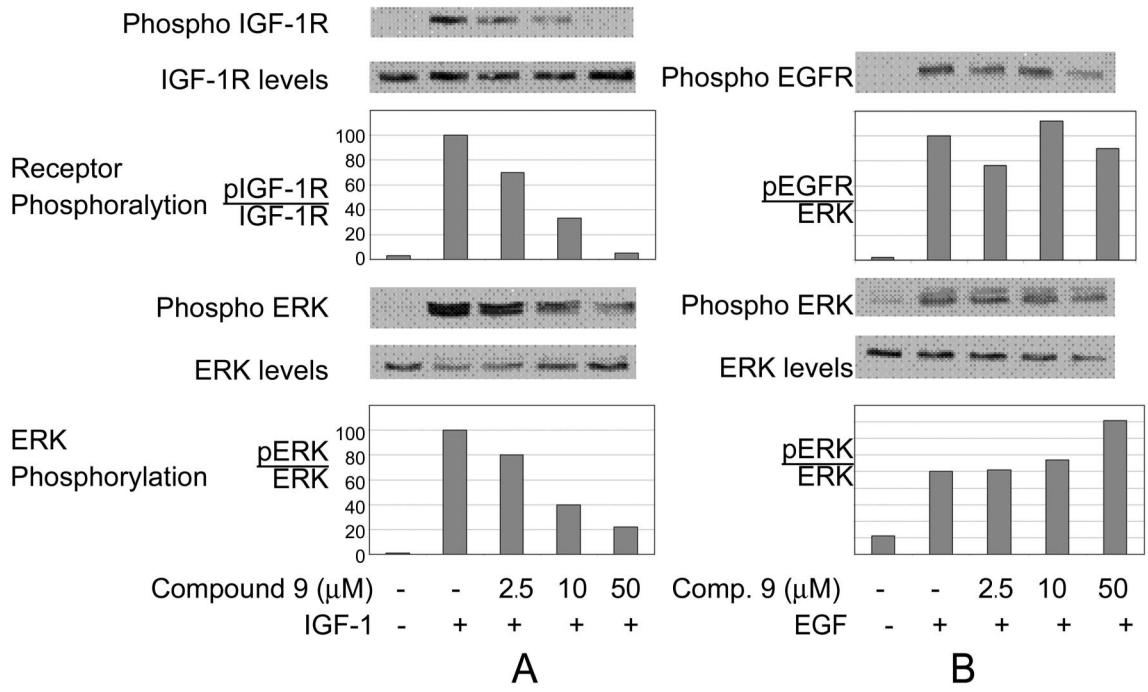


Figure 4. Biological results. (A) Compound **9** inhibited the IGF-1-induced IGF-1R autophosphorylation in MCF-7 breast cancer cells (IC_{50} = 6 μM), as well as the IGF-1-induced ERK phosphorylation (IC_{50} = 8 μM). (B) Compound 9 did not inhibit the activity of a related receptor tyrosine kinase EGFR in intact cells.

Table 1. ^{1}H NMR Chemical Shift Values for Compound **9** in CD_3CN (ppm, Relative to the Acetonitrile Peak at 1.94 ppm)

carbobenzoxy	Hβ 5.54; H_{ortho} 7.04; H_{meta} 7.32; H_{para} 7.30
tyrosine	Hα 5.16; Hβ 3.01, 3.17; H_{ortho} 6.62; H_{meta} 6.86
phenylalanine	Hα 4.69; Hβ 2.70, 2.94; H_{ortho} 7.22; H_{meta} 7.48; H_{para} 7.42
m-alkyl	Hα 3.92; Hβ 1.56; Hγ 0.52; Hδ 0.52; Hϵ 1.56
n-alkyl	Hα 4.14; Hβ 2.76; Hγ 1.54; Hδ 2.76

scaffolds with different chemistry of cyclization have been previously reported.[43,44]

A Biologically Active Compound. We exemplify the power of these new macrocyclic libraries to find novel active compounds by showing that an initial screen of a small library yielded a selective and active compound. The library was based on the tyrosine residues of the IGF-1R activation loop and aimed at inhibition of the IGF-1R tyrosine kinase. We found that compound **9** (Figure 2, Figure 3, and Table S1 (Supporting Information)) inhibited the IGF-1R activity with an IC_{50} value of 6 μM, while no inhibitory effect was detected at concentrations of up to 50 μM (the highest tested concentration) on a closely related tyrosine kinase receptor, EGFR. We further showed that compound **9** selectively blocked IGF-1-induced and not EGF-induced activation of the ERK pathway (Figure 4). These results suggest that compound **9** may be a lead compound, showing promising features of inhibitory activity and selectivity toward an attractive target in cancer therapy. This example demonstrates the potential of the macrocyclic disulfide library method for generating active compounds by a short and powerful process.

Conclusion

We believe that the new compounds presented in this study incorporate properties of cyclic peptides in terms of modularity, flexibility, and metabolic stability but are drug-like in the sense that they are small molecules and comply with most of the Veber and Lipinski rules. Their scaffold is constrained but enables sufficient flexibility to allow conformational complementarity when binding to their target protein. We demonstrated that the chemical synthesis is feasible, although challenging and far from trivial, and gives biologically active products. The diversity of the library allowed compound **9** to be selectively active. This concept should be particularly useful in cases where the pharmacophores are known but need to be systematically screened for a spatial arrangement that will enable biological activity.

Experimental Section

Hazards. *Bis(trichloromethyl)carbonate (BTC) is very toxic and may cause death by inhalation. When this material is used, it should be handled in the hood with extreme caution.*

Reagents. All starting materials were purchased from commercial suppliers and were used without further purification. Rink amide MBHA resin was purchased from Novabiochem (catalog no. A28529, lot no. 01-64-0037). Solid-phase reactions were run at RT, except for couplings to the *N*-alkyl residue, and were performed in a manual SPS vessel or in a tea bag by the SMS methodology.[30,31] The SPS vessels were equipped with sintered glass bottoms. When tea bag SMS was implemented, the resin was sealed in 5.5 $\times$ 6 cm polypropylene bags, which were placed in polypropylene boxes. The SPS vessel and SMS tea bag were shaken with a MilliGen 504 Big Bill shaker. All amino acids were Fmoc-protected on their N^{α} and *t*-Bu protected on their side-chain functional groups.

General Methods. Mass Spectrometry. Mass spectra were acquired on a ThermoQuest Finnigan LCQ-Duo spec-

trmeter, a Q-TOF II mass spectrometer (Micromass, U.K.), and a Voyger-DE PRO Biospectrometry workstation, all in the positive mode unless otherwise noted.

Thin Layer Chromatography. Thin layer chromatography plates were Merck silica gel 60 F_{254} on aluminum. Visualization was achieved either with UV light at 254 nm or by spraying a solution of 1% ninhydrin in methanol and heating in an oven at 100 °C.

High Performance Liquid Chromatography. Analytical high performance liquid chromatography was performed on a Waters 2695 system (equipped with a 2996 diode array detector) at 220 and 254 nm, as well as on a Merck-Hitachi LaChrom D7000 system (equipped with L-7400 UV–vis detector) at 220 or 254 nm, with an L-7100 pump and L-7200 autosampler. In all cases, the samples were analyzed on an xTerra RP-8, 3.5 μm, 4 × 150 mm column at 30 °C. The elution program on both systems used eluents A (0.1% TFA in TDW) and B (0.1% TFA in ACN) in a linear gradient, starting with 5% B and 95% A with a slope of 2.25%/min and a flow of 1 mL/min. Retention times refer to the designated system.

Purification. Preparative high performance liquid chromatography was performed on a Merck-Hitachi 665A system equipped with a Jasco Uvidec-100-v UV–vis detector set at 220 or 254 nm, a preparative L-6200A intelligent pump, and a D-2500 chromato integrator. All the compounds were purified on an xTerra RP-18, 10 μm, 19 × 300 mm column at 30 °C. Eluents used were A (0.1% TFA in TDW) and B (0.1% TFA in ACN) in a linear gradient, starting with 5% B and 95% A with a slope of 2.25%/min and a flow of 9 mL/min.

Kaiser (Ninhydrin) Test. A sample containing 1–3 mg of resin was transferred to a glass test tube, and 3 drops of a solution of 2.5 g of ninhydrin in 50 mL of ethanol, 3 drops of a solution of 40 g of phenol in 10 mL of ethanol, and 3 drops of a solution of 1 mL of 0.001 M aqueous KCN in 49 mL of pyridine were added. The solution was mixed well and placed on a heating block preheated to 110 °C for 5 min. A positive reaction, giving the beads a blue color, indicated incomplete coupling of an amino acid or deprotection of the Fmoc protecting group.[46]

Chloranil Test. A sample containing 1–3 mg of resin was transferred to a test tube; 3 drops of 2% acetaldehyde in DMF and 3 drops of 2% chloranil in DMF were added. The solution was mixed well and left at RT for 5 min, and the beads were inspected. A positive reaction, giving the beads a blue color, indicated incomplete coupling of an amino acid or deprotection of the Fmoc protecting group.[47]

Small Cleavage. A small amount of peptidyl resin was treated with a precooled mixture of 95% TFA, 2.5% TDW, and 2.5% TIS. The mixture was shaken for 0.5 h at RT. The resin was removed by filtration, and the solvents were evaporated by a stream of nitrogen. The residue was dissolved in ACN−TDW 1:1. The filtered solution was analyzed by HPLC, MS, or both.

Chemistry. The synthesis of all the Trt-S-$(CH_2)_n$-CHO or Trt-S-$(CH_2)_m$-CHO aldehydes was carried out as published previously[28] via a hydroxamate intermediate. Here, we demonstrate the synthesis of one aldehyde 4-(tritylthio)-butanal. The ^{1}H NMR data of all the products and intermediates are in the Supporting Information.

4-(Tritylthio)butyric Acid. Trityl mercaptan (36.13 g, 0.131 mol) was added stepwise to a suspension of NaH (11.5 g, 60% in mineral oil 0.288 mol) in 100 mL of DMF under cooling and a nitrogen atmosphere; the reaction mixture was stirred for 30 min after the addition was completed. A solution of bromobutyric acid (21.95 g, 0.131 mol) dissolved in 150 mL DMF was added stepwise. After the addition was completed, the reaction mixture was stirred for 30 min; then the cooling and nitrogen atmosphere were stopped, and the reaction mixture was sealed and left overnight. Then, 500 mL of chloroform was added, and the mixture was washed with 4 × 200 mL of a saturated solution of $KHSO_4$ and 4 × 300 mL of water. The organic layer was evaporated, and the oily product was precipitated by addition of 300 mL of TDW and stirring for few minutes. The product was collected by filtration, washed by TDW, and dried by suction. The crude product was purified as follows: 200 mL of petroleum ether was added to the white solid, and the mixture was stirred for few minutes. The solid was collected by filtration to give a white powder (77% yield). ^{1}H NMR ($CDCl_3$, 300 MHz, 298 K): δ_{1H} 1.67 (m, 2H), 2.22 (t, 2H), 2.30 (t, 2H) 7.10–7.45 (m, 15H). MS: m/z 362 $(M + H)^+$; calcd 362.

***N*-Methoxy-*N*-methyl-4-(tritylthio)butanamide.** A solution of *N*,*O* dimethylhydroxylamine hydrochloride (0.83 g, 0.0084 mol, 1.1 equiv) in 20 mL of DMF was added to a mixture of 2.77 g (0.0076 mol, 1 equiv) of 4-(tritylthio)butyric acid and PyBoP (4.39 g, 0.0084 mol, 1.1 equiv). DIEA (4 mL, 0.023 mol) was added, and the clear solution was stirred for 3 h. The pH should be monitored and kept basic. Ethyl acetate (50 mL) was added to the stirred solution, followed by the addition of 90 mL of saturated bicarbonate solution. The organic layer was collected, washed with additional portions (two) of 40 mL of saturated bicarbonate solution, 40 mL of water, 2 × 40 mL of $KHSO_4$ (1M), dried over Na_2SO_4, and evaporated to dryness to give a brown oil (quantitative yield). ^{1}H NMR ($CDCl_3$, 300 MHz, 298 K): δ_{1H} 1.72 (m, 2H), 2.23 (t, 2H), 2.38 (t, 2H), 3.13 (s, 3H), 3.63 (s, 3H), 7.10–7.50 (m, 15H).

4-(Tritylthio)butanal. $LiAlH_4$ (0.574 g, 0.0151 mol) was added portionwise to a solution of 3.06 g (0.0075 mol) of the *N*-methoxy-*N*-methyl-4-(tritylthio)butanamide in 100 mL of dry diethyl ether under cooling in an ice bath and argon atmosphere. The reaction mixture was stirred for 1 h (monitored by TLC, PE-EtOAc 1:1); 150 mL of ethyl acetate was added, followed by the addition of 150 mL of $KHSO_4$ (1 M). The mixture was stirred for an additional 30 min. The organic layer was collected, washed with 100 mL of $KHSO_4$ (1 M) and 100 mL of saturated NaCl, dried over Na_2SO_4, and evaporated to give 4-(tritylthio)butanal (73% yield). ^{1}H NMR ($CDCl_3$, 300 MHz, 298 K): δ_{1H} 1.66 (m, 2H), 2.22 (t, 2H), 2.38 (t, 2H), 7.15–7.50 (m, 15H), 9.61 (t, 1H).

Solid-Phase Synthesis. Procedure A. A Rink amide MBHA resin (loading 0.6 mmol/g) was preswollen for 4 h in NMP. The Fmoc protecting groups were removed from the resin with 20% piperidine in NMP (2 × 30 min). The resin was washed with NMP (5 × 2 min), and the reaction

was monitored by qualitative Kaiser and chloranil tests. The first Fmoc amino acid coupling cycles were carried out with BTC activation as follows: Fmoc-protected amino acid (5 equiv) was dissolved in DCM, and BTC (1.65 equiv) was added. The mixture was cooled in an ice bath, and 2,4,6-collidine (14 equiv) was slowly added. The mixture was preactivated by mixing for 1 min, added to the resin, and shaken for 2 h. The reagents and solvents were aspirated, replenished, and shaken overnight. The resin was washed with DCM (5 × 2 min) and NMP (2 × 2 min). The reaction was monitored by qualitative Kaiser and chloranil tests and small cleavage. Capping was performed with a 20 mL mixture of acetic anhydride (0.5 M), DIEA (0.125 M), and HOBT (0.015 M) in DMF for 1 h, twice. The resin was washed with NMP (5 × 2 min) and DCM (2 × 2 min). The Fmoc group was removed; the resin was washed, and Fmoc removal was monitored by qualitative Kaiser and chloranil tests (product **2A**, Procedure A, Scheme 1). Reductive alkylation was carried out first by washing the peptidyl resin with a mixture of NMP−MeOH−AcOH 49:49:1 (2 × 2 min). A solution of the aldehyde (1–3 equiv) in NMP−MeOH−AcOH 49:49:1 was then added, and the mixture was shaken for 5 min. $NaBH_3CN$ (2–5 equiv) was added, and the reaction mixture was shaken for 2 h (product **3A**, Procedure A, Scheme 1). The resin was washed as follows: DCM (5 × 2 min), EtOH (2 × 2 min), NMP (2 × 2 min), and DCM (3 × 2 min). The chloranil test gave a green-blue color instantly. The second amino acid was then coupled using two cycles of BTC activation as described above in DBE using the tea bag method or in THF with a manual vessel at 60 °C, and this was repeated if necessary. The resin was washed with DCM (5 × 2 min). Reaction completion was determined by qualitative Kaiser and chloranil tests and small cleavage. The Fmoc group was removed; the resin was washed, and the Fmoc removal was monitored by qualitative Kaiser and chloranil tests (product **4A**, Procedure A, Scheme 1). Reductive alkylation with Trt-S-$(CH_2)_2$-CHO was performed as described above (product **5A**, Procedure A, Scheme 1). Benzyloxycarbonyl was introduced with a solution of benzylchloroformate (6 equiv) and DIEA (12 equiv) in DMF. The solution was added to the resin, and the mixture was shaken for 1 h at 60 °C. The reagents and solvents were aspirated, replenished, and shaken for 1 h. The resin was washed with NMP (5 × 2 min) and DCM (2 × 2 min). Reaction completion was determined by qualitative Kaiser and chloranil tests. The disulfide bridge was formed by oxidization of the Trt-protected compounds for 3 h by iodine treatment (10 equiv) in DMF. The resin was washed as follows: DMF (2 × 2 min), 2% ascorbic acid in DMF (2 × 2 min), NMP (5 × 2 min), and DCM (4 × 2 min). The polymer-bound molecules were cleaved by addition of a precooled mixture of 95% TFA, 2.5% TDW, and 2.5% TIS. The mixture was kept in an ice bath for an additional 0.5 h and then shaken for 2.5 h at RT. The resin was removed by filtration and washed three times with small portions of TFA. The combined TFA fractions were evaporated to dryness with a stream of nitrogen, and the oily residue was dissolved in ACN−TDW 1:1 and lyophilized (product **6A**, Procedure A, Scheme 1). The crude members were analyzed by MS and HPLC.

Procedure B. A Rink amide MBHA resin (loading 0.6 mmol/g) was preswollen for 4 h in NMP. The Fmoc protecting groups were removed from the resin with 20% piperidine in NMP (2 × 30 min). The resin was washed with NMP (5 × 2 min), and the reaction was monitored by qualitative Kaiser and chloranil tests. The Fmoc-N^{α}–(β-Acm-thioethyl)Gly-OH was coupled using BTC-activation as follows: Fmoc-N^{α}-(β-Acm-thioethyl)Gly-OH (5 equiv) was dissolved in DBE, and BTC (1.65 equiv) was added. The mixture was cooled in an ice bath, and 2,4,6-collidine (14 equiv) was slowly added. The mixture was preactivated by mixing for 1 min, added to the resin, and shaken for 2 h. The reagents and solvents were aspirated, replenished, and shaken overnight. The resin was washed with DCM (5 × 2 min) and NMP (2 × 2 min). The reaction was monitored by qualitative Kaiser and chloranil tests and small cleavage. Capping was performed with a 20 mL mixture of acetic anhydride (0.5 M), DIEA (0.125 M) and HOBT (0.015 M) in DMF for 1 h, twice. The resin was washed with NMP (5 × 2 min) and DCM (2 × 2 min). The Fmoc group was removed; the resin was washed, and the Fmoc removal was monitored by qualitative Kaiser and chloranil tests (product **2B**, Procedure B, Scheme 1). The second amino acid was then coupled using two cycles of BTC-activation as described above in DBE using the tea bag method or in THF with a manual vessel at 60 °C, and this was repeated if necessary. The resin was washed with DCM (5 × 2 min). Reaction completion was determined by qualitative Kaiser and chloranil tests and small cleavage. The Fmoc group was removed; the resin was washed, and the Fmoc removal was monitored by qualitative Kaiser and chloranil tests (product **3B**, Procedure B, Scheme 1). Reductive alkylation with Trt-S-$(CH_2)_m$-CHO was performed as described above (product **4B**, Procedure B, Scheme 1): Benzyloxycarbonyl was introduced with a solution of benzylchloroformate (6 equiv) and DIEA (12 equiv) in DMF. The solution was added to the resin, and the mixture was shaken for 1 h at 60 °C. The reagents and solvents were aspirated, replenished, and shaken for 1 h. The resin was washed with NMP (5 × 2 min) and DCM (2 × 2 min). Reaction completion was determined by qualitative Kaiser and chloranil tests. The disulfide bridge was formed by oxidization of the Trt or Acm-protected members for 3 h by iodine treatment (10 equiv) in DMF. The resin was washed as follows: DMF (2 × 2 min), 2% ascorbic acid in DMF (2 × 2 min), NMP (5 × 2 min), and DCM (4 × 2 min). The polymer-bound molecules were cleaved by addition of a precooled mixture of 95% TFA, 2.5% TDW, and 2.5% TIS. The mixture was kept in an ice bath for an additional 0.5 h and then shaken for 2.5 h at RT. The resin was removed by filtration and washed three times with small portions of TFA. The combined TFA fractions were evaporated to dryness with a stream of nitrogen, and the oily residue was dissolved in ACN−TDW 1:1 and lyophilized (product **5B**, Procedure B, Scheme 1). The crude members were analyzed by MS and HPLC, and selected compounds were sent for biological screening; the active compounds

were purified by preparative HPLC and checked by analytical HPLC to determine their degree of purity.

NMR Experiments. The samples for the NMR experiments were dissolved in $CDCl_3$ or CD_3CN (Aldrich Chemicals Co., U.S.A.) from their lyophilized form. 1D NMR data were acquired on a Bruker AMX 300 MHz spectrometer, and 2D data were acquired on a Bruker Avance 600 MHz DMX spectrometer. Chemical shifts are in parts per million relative to the internal solvent peak (7.26 ppm for $CDCl_3$, 1.94 ppm for CD_3CN) at 25 °C for 1D and 10 °C for 2D experiments. TOSCY,[48] COSY,[49,50] NOESY,[51] and ROESY[48]experiments were acquired for assignment of the spectra: TOCSY spectra were recorded using the MLEV-17 pulse scheme for the spin lock,[52] and ROESY experiments, optimized to a mixing time of 200 ms, were used because the NOESY signals, using a mixing time of 150 ms, were small. Spectra were processed and analyzed with the XWINNMR (Bruker Analytische Messtechnik GmbH) and SPARKY (version 3, provided by T.D. Goddard and D. G. Kneller, University of California, San Francisco) programs.

Despite the high degree of purity by HPLC, the NMR spectra showed additional peaks attributed to multiple conformations. Resonance assignment was based on COSY, TOCSY, NOESY, and ROESY spectra measured under identical experimental conditions for three samples from different synthetic batches, in which the product could not be separated from different co-eluted compounds. The set of common peaks was attributed to the compound **9** molecule, and peaks that were absent from any of the spectra were not considered in the assignment process. Strong apodization was applied in the form of a shifted squared sine bell window function to enhance resolution and allow careful integration and that was also used to differentiate among the peaks. The final assignment is given for interconnected spin systems that are both relevant chemically and also have correct integration values. We discounted spin systems whose integration was within the correct range of values but were not interconnected to the rest of the molecule.

Biological Assays. Cell Culture. The mammary gland breast carcinoma cell lines MCF-7 and MDA MB-468 were obtained from the American Type Culture Collection (ATCC). The cells were maintained in growth medium (Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin) in a humidified atmosphere of 94% air and 6% CO_2 at 37 °C. All reagents for cell culture were purchased from Biological Industries Bet-Haemek Ltd., Israel.

Anchorage-Dependent Cell Growth Assay. The cell proliferation assay was conducted as previously described.[53] MCF-7 cells were seeded in 96-well tissue culture plates (180 μL of 4×10^3 cells/well), and the inhibitors were added 3 h later, following cell adhesion to the plates. The compounds were dissolved in DMSO to a 100 mM stock concentration and diluted to 1 mM concentration in PBS containing 0.1% BSA. Further 1:1 dilutions were made, while retaining a constant vehicle concentration. Aliquots (20 μl) were dispersed in triplicate into the cell-containing wells, either of compound dilutions, vehicle, or nontreated (BSA-PBS). Plates were incubated at 37 °C in a 5% CO_2 humidified incubator for 4 days, fixed in 4% buffered formaldehyde solution for 2 h, washed with 0.1 M sodium borate buffer pH 8.5, and stained with 1% methylene blue dissolved in 0.1 M borate buffer solution for 10 min. Excess dye was washed out, and cell-bound dye was eluted with 200 μL/well of 0.1 M HCl. The optical density value was read at 595 nm in the Labsystems Multiscan RC Elisa plate reader. The data was analyzed in Microsoft Excel, using the vehicle control as 100% proliferation. The vehicle had no significant effect on cell proliferation.

Effects of Compound 9 Treatment on Cellular Signaling. For the Western blot analysis, MCF-7 cells were starved in a serum-free medium for 14 h and exposed to various concentrations of compound **9** or the vehicle control (0.1% DMSO in growth medium) for the last 5 h of starvation. Cells were stimulated with IGF-1 (50 ng/ml) for 10 min and lysed with lysis buffer containing 20 mM Tris HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1% Triton X 100, 0.5 mM sodium vanadate, 10 mM β-glycerophosphate, 5 mM sodium pirophosphate, 50 mM sodium fluoride, 1 mM benzamidine, and protease inhibitor cocktail (Sigma). Equal amounts of protein were resolved on an 8% SDS−polyacrylamide gel electrophotesis and electrotransferred onto nitrocellulose membrane. Membranes were blocked with 3% BSA in TBST (25 mM Tris-HCl (pH 7.4), 0.17 M NaCl, and 0.2% Tween 20) for 1 h at RT and incubated with primary antibodies (see below) at 4 °C overnight. Following extensive washing, the membranes were probed with HRP-conjugated secondary antibodies at room temperature for 1 h, and the proteins of interest were visualized using an enhanced chemiluminescence kit (Super Signal, Pierce).

The respective phosphorylation of IGF-1R and ERK was determined by immuno-blotting with antibodies against phosphorylated IGF-1R (Tyr 1131)/ insulin receptor (Tyr 1146) (cell signaling) and diphosphorylated (Thr 183/Tyr 185) ERK1 and ERK2 (Sigma). Blots were stripped and reprobed with anti-IGF-1R and anti-ERK (Santa Cruz Biotechnology), respectively. Quantification of the blots was performed using the ImageJ analysis system. Histograms represent the ratio between the levels of the phosphorylated protein (pIGF-1R and pERK) and the levels of the total proteins (IGF-1R and ERK), respectively.

A similar procedure was performed to detect the effects of compound **9** treatment on the IGF-1R related kinase, EGFR. Prostate cancer DU-145 cells expressing significant levels of EGFR were incubated with compound **9** or vehicle, and EGF (20 ng/mL, 10 min) was used as a stimulant. The levels of phosphorylated EGFR were detected using antiphosphotyrosine antibodies (4G10), and the histogram represents the ratio between the levels of the phosphorylated EGFR and the levels of total protein (ERK). The EGF-induced phosphorylation of ERK was detected as described above.

Abbreviations. The following abbreviations are used throughout the text: AcOH, Acetic acid; ACN, acetonitrile; ATCC, American Type Culture Collection; Boc, *t*-butoxycarbonyl; Acm, acetamidomethyl; bp, boiling point; BSA, bovine serum albumin; BTC, bis(trichloromethyl)carbonate;

COSY, correlation spectroscopy; DBE, 1,2-dibromoethane; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMEM, Dulbecco's modified eagles medium; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetracetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EGTA, glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EtOH, ethyl alcohol; EtOAc, ethyl acetate; ERK, extracellular regulated kinase; Fmoc, 9-fluorenylmethoxycarbonyl; Gly, glycine; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; HRP, horse-radish peroxidase; IGF-1, insulin-like growth factor-1; IGF-1R, insulin-like growth factor-1 receptor; MBHA, methylbenzhydrylamine; MeOH, methyl alcohol; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiosol-2-yl)-2,5-diphenyltetrazolium bromide; MW, molecular weight; NMP, 1-methyl-2-pyrrolidinone; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PBS, phosphate-buffered saline; PE, petroleum ether (40-60); Phe, phenylalanine; PKB, protein kinase B; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; ROESY, rotational nuclear Overhauser effect spectroscopy; RT, room temperature; SDS, sodium dodecyl sulphate; SMS, simultaneous multiple synthesis; SPS, solid-phase synthesis; TBST, tris-buffered saline tween; *t*-Bu, *tert*-butyl; TDW, triple distilled water; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TIS, triisopropylsilane; TLC, thin layer chromatography; TOCSY, total correlation spectrometry; Trt, trityl; Tyr, tyrosine; UV, ultraviolet; Z, benzyloxycarbonyl. The abbreviations for amino acids are according to the IUPAC-IUB Commission of Biochemical Nomenclature http://www.chem.qmul.ac.uk/iupac/AminoAcid.

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Supporting Information Available. Molecular structure, HPLC, and MS characterization of the IGF-1R combinatorial library and biological screening results. This material is available free of charge via the Internet at http://pubs.acs.org.

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