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Bifunctional scaffolds as templates for synthetic combinatorial libraries

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Summary

A small-molecule synthetic combinatorial library was designed and synthesized that features potential pharmacophores attached to a variety of small cyclic scaffolds. The synthesis of the library involved randomization of three types of building blocks: 20 amino acids, 10 aromatic hydroxy acids and 21 alcohols, totaling a library complexity of 4200 compounds. Mitsunobu polymer-supported etherification was used in the last randomization. The library compounds were attached to beads via an ester-bond linkage enabling both on-bead as well as in-solution screening. When the library was tested against a model target, streptavidin, specific binders were found. The structures of the most active compounds were determined from the fragmentation pattern in MS/MS experiments.

Introduction

To create a desired chemical diversity within a synthetic combinatorial library, one designs the assembly of structures from selected suitable fragments. One strategy applied by many investigators in this field is based on the use of scaffolds that possess suitable functional groups used for attaching the set of appropriate building blocks (for recent reviews see Refs. 1–6). The scaffold can be preformed, that is, synthesized in solution, and used as one unique building block in the library synthesis (e.g. Refs. 7–10). The scaffold may contain two or three of the same groups, e.g., amino groups, that are differentially protected, or different functional groups can be present on a scaffold. In both cases the functional groups serve for attaching building blocks. The second scaffold strategy is based on building the scaffold during library synthesis and can be documented on examples of benzodiazepine [11–14], pyrrolidine [15], thiazolidine [9], benzylpiperazine [16] or diketopiperazine [17–19] library syntheses.

We have designed and synthesized a small-molecule combinatorial library that was built around a preformed scaffold. However, we have used not only one central unit, but a set of aromatic hydroxy acids, which are bifunctional building blocks with variable spacing of carboxyl and hydroxyl groups, thus increasing the structural diversity of compounds within the library. This new library serves as an example of structurally heterogeneous libraries [20].

Material and Methods

The library was synthesized on TentaGel-S-OH 130-μm resin (TG, Rapp Polymere, Tübingen, Germany). Fluorenylmethyloxycarbonyl (Fmoc) amino acids with standard side chain protecting groups were obtained from Advanced ChemTech (Louisville, KY) or Propeptide (Vertle-Petit, France). Aromatic hydroxy acids (Fig. 1) and alcohols (Fig. 2) used in randomizations, diethyl azodicarboxylate (DEAD), diisopropyl azodicarboxylate (DIAD), diisopropyl carbodiimide (DIC), *N*-hydroxybenzotriazole (HOBt), phenol, triphenylphosphine (PPh_3), piperidine, and trifluoroacetic acid (TFA) were obtained

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Abbreviations: DCM, dichloromethane; DEAD, diethyl azodicarboxylate; DIAD, diisopropyl azodicarboxylate; DIC, diisopropyl carbodiimide; DMF, dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; HOBt, *N*-hydroxybenzotriazole; MeOH, methanol; PPh_3, triphenylphosphine; *t*-Bu, *tert*-butyl; TFA, trifluoroacetic acid; TG, TentaGel-S-OH 130-μm resin; THF, tetrahydrofuran.

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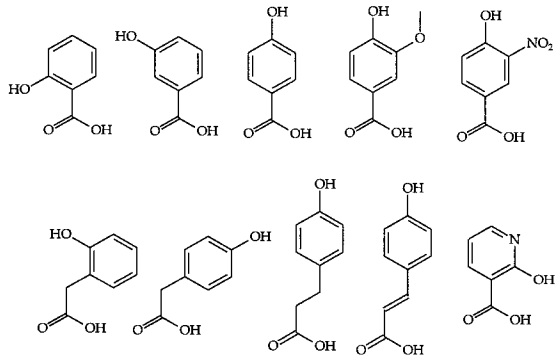

Fig. 1. Structure of aromatic hydroxy acids used in the library synthesis.

from Aldrich Chemical (Milwaukee, WI) or Sigma (St. Louis, MO). Anhydrous tetrahydrofuran (THF) was obtained from Aldrich Chemical (Milwaukee, WI). High-purity solvents (Baxter, McGaw Park, IL) were used for syntheses without further purification.

Analytical HPLC was carried out on a Hitachi gradient system equipped with a L-7450 diode array detector, L-7100 pump and L-7200 autosampler (Hitachi, Tokyo, Japan) using a Vydac Peptide and Protein C_{18} analytical column (4.6 × 250 mm, 5 μm, 1 ml/min; The Separation Group, Hesperia, CA). The analytical gradient was run from water containing 0.07% TFA to 60% of acetonitrile/water in 30 min. UV/VIS Absorption spectra were recorded on a Hewlett Packard HP 8452A Diode-Array spectrophotometer (Palo Alto, CA) using a 1-cm quartz cuvette. Ion-spray mass spectra were obtained on a triple quadrupole PE-Sciex API III$^+$ mass spectrometer (Perkin-Elmer/Sciex, Thornhill, ON) with an articulated ion spray sample inlet system.

Synthesis of the library

The library was synthesized on 5 g of hydroxy TentaGel (substitution level 0.25 mmol per g). All reactions were carried out in a plastic syringe equipped with a sintered polypropylene disc at the bottom [21]. The syringe was charged with resin, the solvent to be used in the following reaction was added to make a slurry, and the resin was washed with this solvent. The synthesis followed the split/mix technique [22–24] and consisted of the following steps:

(1) dividing the resin beads into 20 parts;
(2) esterification with Fmoc amino acids;
(3) recombining the resin beads;
(4) removing the Fmoc group;
(5) dividing the resin beads into 10 parts;
(6) coupling of aromatic hydroxy acids;
(7) recombining the resin beads;
(8) dividing the resin beads into 21 parts; and
(9) etherification with alcohols.

After finishing the library synthesis, the resin beads were washed with DMF (three times), DCM (three times), treated twice with mixture K [25] for 1 min and 2 h. The resin was washed with TFA (three times), DCM (five times), MeOH (three times) and freeze-dried.

Esterification of hydroxy-TentaGel by amino acids

The syringe was charged with TentaGel-S-OH (0.25 g, 0.25 mmol OH/g). The resin was washed three times with dry THF, and 0.4 ml of a 0.5 M solution of Fmoc-protected amino acid in THF and 0.8 ml of a 0.5-M solution of PPh_3 in THF was added and the syringe was cooled to −10 °C in a DMF bath [26]. Then 0.6 ml of a 0.5-M solution of DEAD in THF was added and the syringe was rotated on a tumbler for 2 h. After esterification the resin was washed five times with DMF.

Fmoc deprotection

The resin was washed three times with DMF, repeatedly treated with 50% piperidine in DMF for 5 min and 20 min, and washed six times with DMF. All washes were collected, the absorbance at 302 nm was measured and the Fmoc release calculated ($\varepsilon_{302} = 8100$).

Condensation of aromatic hydroxy acids

Aromatic hydroxy acid and HOBt were dissolved in DMF (0.5 mmol in 1 ml), DIC was added, the solution transferred into the syringe, and the mixture was shaken for 1 h. The presence of free amino groups was checked [27]. If the test was positive, the resin was washed with DMF (three times) and this coupling step repeated.

Etherification of polymer-supported phenols by alcohols

The resin (ca 0.8 ml per syringe) was washed five times with dry THF. A solution of PPh_3 in THF (1.5 ml, 1 M) and 3 mmol of alcohol were added and the slurry was

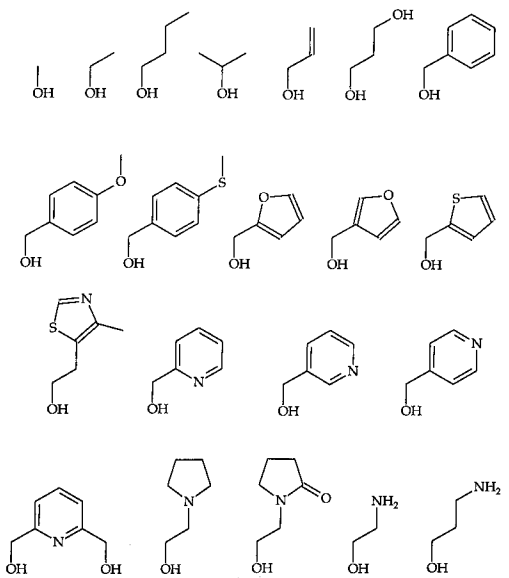

Fig. 2. Structure of alcohols used in the library synthesis.

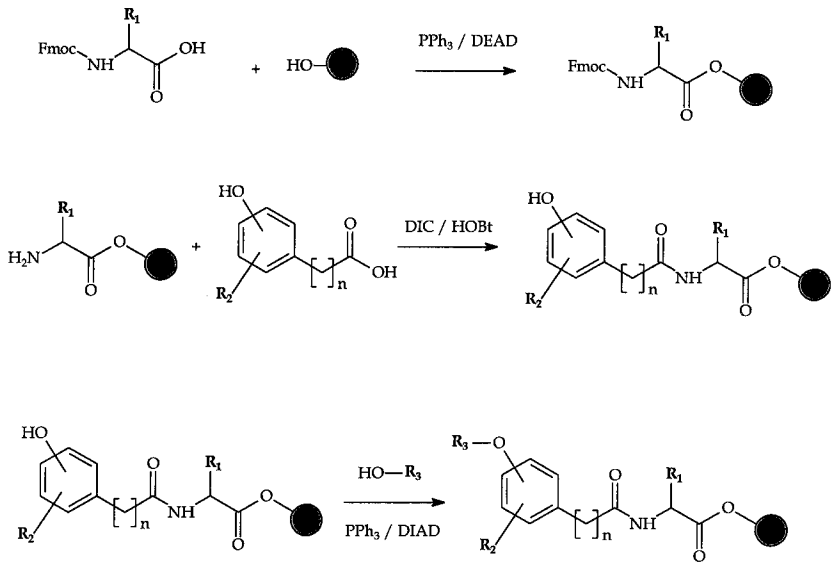

Fig. 3. Synthetic scheme of the library.

shaken. Then, a solution of 1.5 mmol of DIAD in 0.2 ml of THF was added to the resin in four portions at 5-min intervals. The mixture was shaken overnight and then washed five times with DMF.

Results and Discussion

We have designed and synthesized a combinatorial library composed of compounds that present three potential pharmacophores on a small rigid scaffold (six-membered aromatic or heteroaromatic ring). The library was designed for the Selectide one-bead–one-compound strategy [24]. The synthesis of the library consisted of three randomization steps (Fig. 3):

(i) amino acid randomization by attaching a variety of amino acids to solid support;

(ii) aromatic hydroxy acid randomization by coupling a set of aromatic hydroxy acids to the amino group of the amino acid;

(iii) alcohol randomization by etherification of phenolic hydroxyl groups by alcohols using the Mitsunobu redox condensation procedure [28–30].

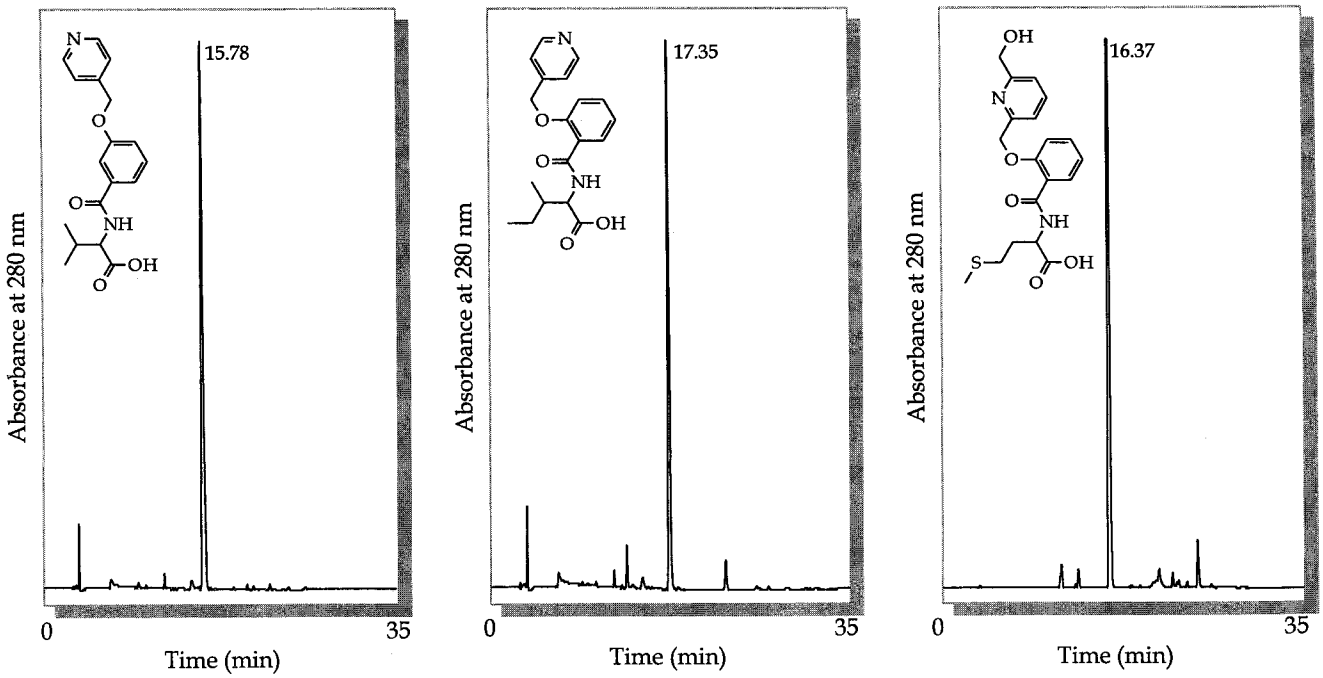

Fig. 4. Analytical HPLC traces of three model compounds.

To esterify the polymer-supported hydroxyl groups of TentaGel in the first randomization step we used the Mitsunobu esterification [29]. The esterification proceeds fast, it is racemization-free and the yield is comparable with other methods [26]. There is a variety of protected amino acids that are commercially available and the selection is not limited to α-amino acids only. The α-amino groups were Fmoc-protected while *t*-Bu-protecting groups were used for side-chain functionality. Twenty proteinogenic amino acids were used in the library synthesis.

We used aromatic hydroxy acids to introduce a central scaffold. The aromatic hydroxy acids can be considered as bifunctional building blocks, similarly to amino acids that are building blocks for the synthesis of peptides. The concept of creating structurally heterogeneous combinatorial libraries based on combinations of different bifunctional building blocks will be described elsewhere [20].

More than hundred of aromatic hydroxy acids are commercially available and, if necessary, dedicated building blocks can be synthesized relatively easily. The structures of the 10 hydroxy acids used in the library are shown in Fig. 1. Since the activated carboxyl group can acylate the unprotected hydroxy group, we prepared 4-hydroxybenzoyl-Gly-OH as a model, using both unprotected and *t*-Bu-protected 4-hydroxybenzoic acid (DIC/HOBt activation and 1 h reaction time). Both products provided the same HPLC gradient profile after deprotection, showing no sign of undesirable acylation of unprotected hydroxyl groups under these conditions. However, only mild activation of carboxyl should be used here, to avoid the undesirable acylation of phenolic hydroxyl.

The last randomization takes advantage of the Mitsunobu ether formation. The polymer-supported etherification using the Mitsunobu redox coupling procedure has recently been described by us and others [30,31]. The reaction proceeds under mild conditions and the yield and purity of aryl ethers are good to excellent [30]. We have tested more than 50 alcohols for their potential use in this library format out of which we selected only 21 (Fig. 2) for the first library. The whole set of applicable alcohols will be published elsewhere [20].

The last step of the library synthesis involves cleavage of side-chain protecting groups using TFA with scavengers (mixture K [25]). Since some ethers are acid-sensitive, we divided the library into two portions and treated only half of the library beads with TFA.

Before the library was synthesized, we tested the polymer-supported chemistry intended to be used in the library synthesis and prepared library model compounds. Each

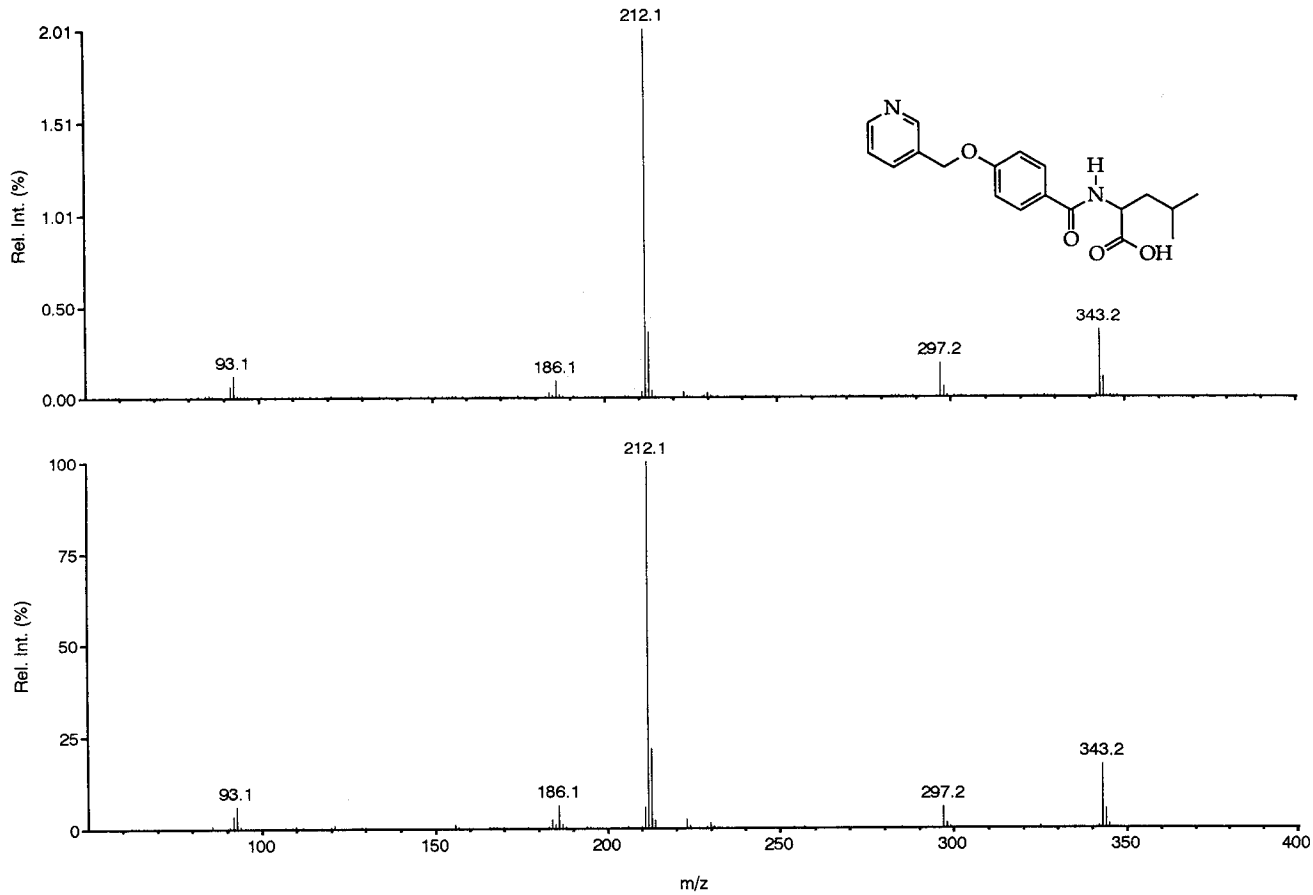

Fig. 5. MS/MS spectra of a compound found in the library (upper trace) compared to a resynthesized compound (lower trace).

TABLE 1
POSSIBLE STRUCTURES OF STREPTAVIDIN BINDERS AS DETERMINED FROM THE MS/MS FRAGMENTATION PATTERNS AND THE INTENSITY OF STAINING IN THE STREPTAVIDIN ON-BEAD BINDING ASSAY

Pool number	Amino acid	Hydroxybenzoic group		
		ortho-	*meta-*	*para-*
16	Val	+++	–	(+)
16	Leu	++++	–	(+)
16	Ile	++	++	–
15	Pro	+	+++	(+)
17	Met	+++	–	–

Relative color intensities: '–' denotes no color reaction; '++++' denotes the strongest color reaction.

compound was analyzed on HPLC and its molecular weight was confirmed by mass spectrometry. The HPLC traces of three representative compounds are shown in Fig. 4.

Lam's one-bead–one-compound technique [24] does not track the chemical history of library compounds during its synthesis, and therefore a structure determination is necessary once a bead has been identified. We used mass spectroscopy for elucidation of the structure. We found that the fragmentation pattern of library compounds is informative and provided sufficient information for determination of the structure. In some instances, however, the molecular weight of building blocks is identical and then all possible compounds have to be synthesized. Nevertheless, this technique eliminates the need to synthesize a coding arm and thus simplifies substantially the synthesis of the library.

The library was tested in a model on-bead binding assay, as previously described [32], to find specific streptavidin binders. Since the library beads were not combined after the last randomization, 21 sublibraries could be tested. Positive beads in pools 15, 16, and 17, i.e. 3-hydroxymethyl pyridine, 4-hydroxymethyl pyridine, and 2,6-dihydroxymethyl pyridine, were identified as an alcohol component in the etherification. The most intensely colored beads were taken for structure determination.

Compounds were cleaved from the beads by alkaline hydrolysis and each solution was divided in two parts. The first half of the releasate was analyzed by LC/MS spectrometry to obtain the molecular weight. The second half was used for LC/MS/MS experiments where the ions were fragmented by collision with argon in a low-energy collision cell. Analysis of MS/MS spectra enabled us to determine the structure of compounds from all beads submitted for analysis. Verification of the analysis was confirmed by synthesis. An example is presented in Fig. 5 showing the MS/MS spectrum of a synthesized compound (lower trace), matching the daughter spectrum of parent compound $MH^+ = 343$ found in the library (upper trace).

We synthesized 15 compounds to find out which hydroxy and amino acids were present in the specific binders to streptavidin. The results are shown in Table 1. Hydroxybenzoic acid was present in all hits. Since all three isomers have been used in the library synthesis and mass spectrometry cannot distinguish among them, all combinations have been synthesized. In the hit from pool 16 only compounds prepared from *o*-hydroxybenzoic acid showed binding to streptavidin. Three hydrophobic amino acids were tolerated in the first randomization position: Val, Leu, and Ile. The best binding compound from pool 15 contained *m*-hydroxybenzoic acid and proline, and the one from pool 17 contained *o*-hydroxybenzoic acid and methionine.

Conclusion

We have designed and synthesized a small-molecule combinatorial library characterized by a variable central scaffold unit that displays potential pharmacophores. A new type of bifunctional building blocks, the aromatic hydroxy acids, have been used in the library synthesis. Testing the library in a model streptavidin assay yielded specific binders. Their structures have been elucidated from the fragmentation patterns of the MS/MS spectra. The complexity of this small library comprised 4200 compounds (20 amino acids, 10 hydroxy acids, 21 alcohols); larger libraries of the same or similar format have been reported [20].

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