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Rapid Deconvolution of Combinatorial Libraries using HPLC Fractionation

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Abstract: The rapid deconvolution of combinatorial libraries prepared via solution-phase simultaneous addition of functionalities is demonstrated using HPLC. Libraries containing 25-216 members are screened for biological activity, and active libraries are fractionated into discreet pools using reversed-phase HPLC. The entire process can be completed from a single synthesis of 15-50 mg, without the need for fixed positions, serial synthesis, or tagging. The method is demonstrated for libraries with charged and uncharged functional groups, and for a library containing substituted benzyl moieties.

INTRODUCTION

The screening of combinatorial mixtures of compounds, prepared individually or simultaneously, fundamentally has changed the way drugs are discovered. The scope of combinatorial chemistry is broad, and the methods are suited for identification of unique new pharmacophores and for optimization of a lead compound against a biological target. A variety of approaches for the synthesis and deconvolution of combinatorial libraries have been described over the last 5-8 years.[1-3] The synthetic routes can be categorized based on the preparation, as solid-phase or solution-phase methods. Solid-phase synthesis methods generate single compounds from scaffolds (core structures) that can be elaborated off solid supports, such as peptides, peptide mimetics, and small heterocycles.[4-7] Solution-phase synthesis methods have been used for the preparation of mixtures of compounds from scaffolds containing several reactive sites.[8-10] The merits of these approaches have been discussed elsewhere.

Much of the initial excitement about combinatorial chemistry revolved around the ability to prepare and screen large mixtures of compounds in solution against biological targets simultaneously and rapidly. A number of deconvolution strategies have been proposed for the identification of the active species following identification of an active mixture.[11-14] The screening of large mixtures has not been pursued by most groups for many reasons. Artificial suppression of biological activity can be generated from "quasi-species" interactions among compounds (i.e. false negative). The screening of mixtures also can produce misleading high biological activity in a sample when the low individual activities of many compounds sum and are incorrectly attributed to a single very active compound (false positive). Hence, most researchers now screen

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single compounds, or a diverse group of ~5-10 compounds which has been created non-synthetically via mixing.

The iterative synthetic deconvolution of "winners" from large combinatorial libraries can require complicated protection/deprotection schemes. Several rounds of synthesis and screening may be required to fix the functional group at each position on the scaffold. The time and effort required to identify a "winner" can be significant. Other strategies have been evaluated, including positional scanning, indexing, and tagging with labels or chemical codes. These techniques place limits on the complexity of a library, the minimal biological activity which can be detected, or the types of reaction chemistry which do not degrade the label or tag.

We have examined alternative deconvolution methods to take advantage of the increased complexity which can be built into a combinatorial library using solution simultaneous addition methods, and eliminate the need for serial synthesis and biological screening. The most favorable strategy utilizes an initial HPLC separation of the library into new fractions using a reversed-phase solid support and an organic gradient with an aqueous buffer. This approach puts less stringency on the yield and purity required from the initial synthesis, since impurities can be removed during the chromatographic step. Several examples of HPLC fractionation of libraries are described. In some cases, the biological activity is distributed among many fractions, while in other cases a single fraction contains the majority of activity present in the library. A second orthogonal separation of active fractions yields single compounds, whose identity can be established using mass spectrometry and NMR spectroscopy. Hence, the library can be deconvoluted without fixing functionalities serially, and without the need for tagging or coding. Given the vast body of chromatography literature which describes the separation of similar and dissimilar molecules, we anticipate that the HPLC fractionation method should be broadly applicable to many types of combinatorial libraries prepared via solution phase synthesis.

RESULTS

Initial overview of the method. Combinatorial libraries were synthesized using solution-phase simultaneous addition of functionalities, as described previously.[8] For a new library, 2-3 initial analytical HPLC separations are performed with different solid phases and solvent systems to optimize efficiency and resolution. The preparative HPLC separations are performed with a Gilson (Chicago, IL) model 215 liquid handler and pump system. Both analytical and preparative HPLC separations are performed under computer control. A 10 mm x 250 mm reversed phase C18 or C8 column is used (indicated in Figure legends), and 30 mg of material is loaded from library dissolved in the starting buffer. The elution profile is monitored at

200-280 nm. Fractions of 1-6 mL are collected into 7 mL tubes. Solvent is removed under reduced pressure to give a volume of ~200 μL, and the liquid is transferred to a pretared 1.4 mL tube (Micronics) in a 96-well rack. The fractions are taken to dryness under reduced pressure, and weights of material in each tube are determined automatically using a robotic weigh station (Bohdan Automation, Mundelien, IL). Typical first-round fractions contain 1-15 compounds, depending on the starting complexity of the library and the chemical diversity of the functional groups attached to the scaffold. Biological screening has been performed by growing *E. coli* or *S. pyogenes* to log phase in minimal media containing the fractions at 2-30 μM concentrations.[15]

HPLC fractionation of Isis 110753. A polyazapyridinocyclophane library containing 25 charged and aromatic members was prepared via simultaneous solution phase synthesis and selected for fractionation. The library was separated into 19 fractions by HPLC chromatography (Figure 1). The chromatogram contained

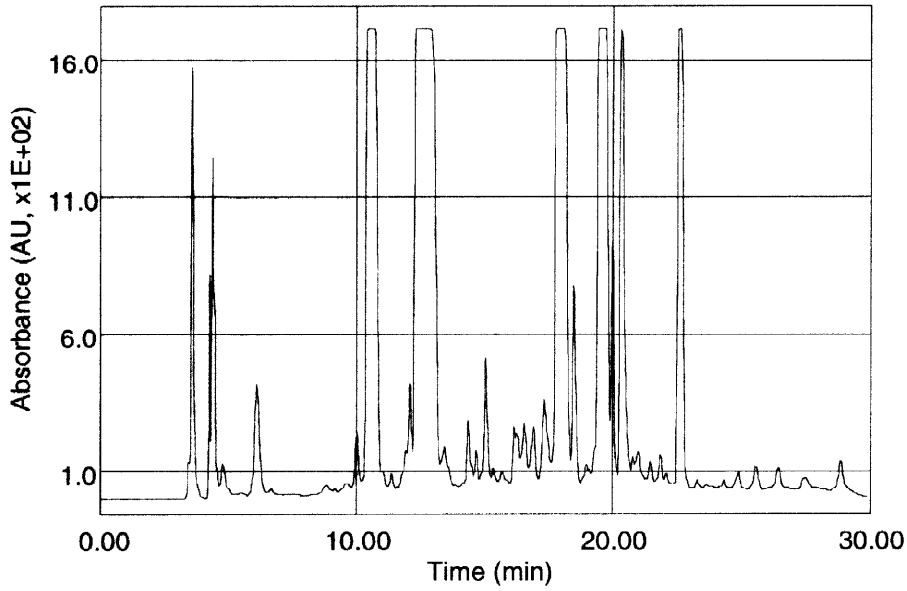

Figure 1. HPLC profile observed in a preparative separation of Isis 110753. An Altima C18 reversed-phase column (10x250 mm; 5 μM particles) was employed. The starting aqueous buffer (A) contained 50 mM ammonium acetate, pH 4.6 and the organic buffer (B) contained 50 mM ammonium acetate in 90% methanol. A linear gradient was run from 30% B to 100% B from 4 to 20 minutes.

eight major peaks and twelve lesser peaks, and fractions were pooled to generate 9 wells for screening. The contents of the individual fractions were redissolved at 5 mM based on the mass of material in each fraction and the weighted average molecular masses. Mass spectrometric analysis of the composition showed less than 10% compound overlap between adjacent fractions. The fractions were screened for their ability to inhibit the growth of *E. coli* (imp-) and *S. pyogenes*, as shown in Figure 2. A single fraction (8; 20.1-21.5 min) inhibited growth of *S. pyogenes* at 5 μM, while fractions 7 and 8 demonstrated activity against *E. coli* at 20 μM.

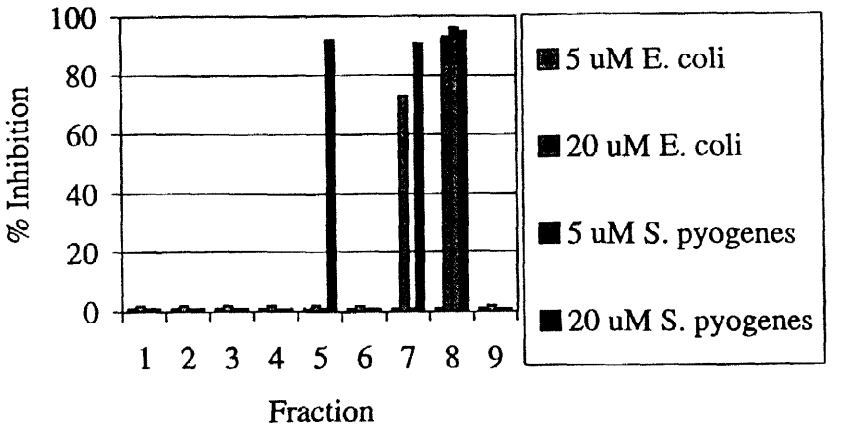

Figure 2. Inhibition of growth of *E. coli* observed for fractions obtained from Isis 110753.

Mass spectrometric analysis demonstrated that fraction 8 contained a single compound with a molecular mass of 537.3 Da. The structure could be established as the dibenzoyl derivative immediately, given the limited number of potential masses and the symmetry of the compound.

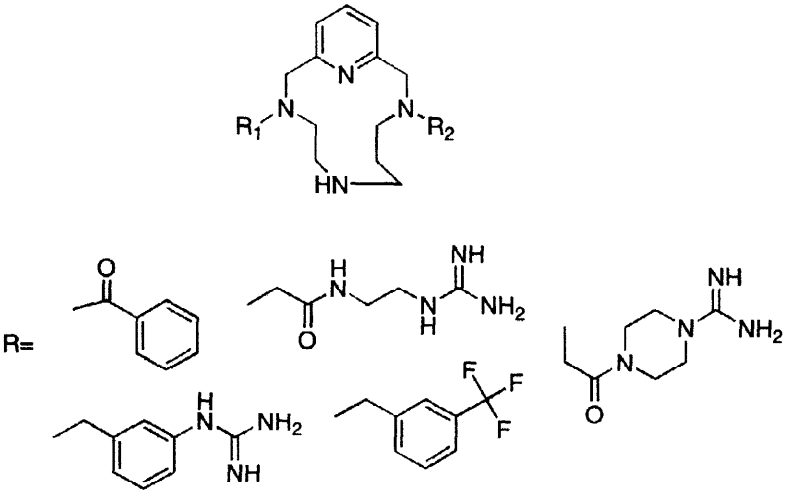

Figure 3. Structure of Isis 110753.

HPLC fractionation of Isis 100302. A second polyazapyridinocyclophane library containing 100 members was prepared via simultaneous solution phase synthesis of 10 meta-substituted benzyl groups and selected for study (Figure 4). This library was chosen as a test example where the properties of the functional groups are similar. The HPLC trace is presented in Figure 5. The separation was adequate to resolve 26 peaks, and 25 fractions were collected. The majority of the fractions failed to inhibit growth of *E. coli* (imp-). However, activity was observed for fractions 12-15 (Figure 6). In addition, fractions 12-15 inhibited the growth of *S.*

pyogenes at 5 μM. Mass spectrometric analysis showed that fraction 12 contained 11 compounds. Three HPLC conditions were compared in attempts to resolve the compounds. Separation with a Fluofix C18

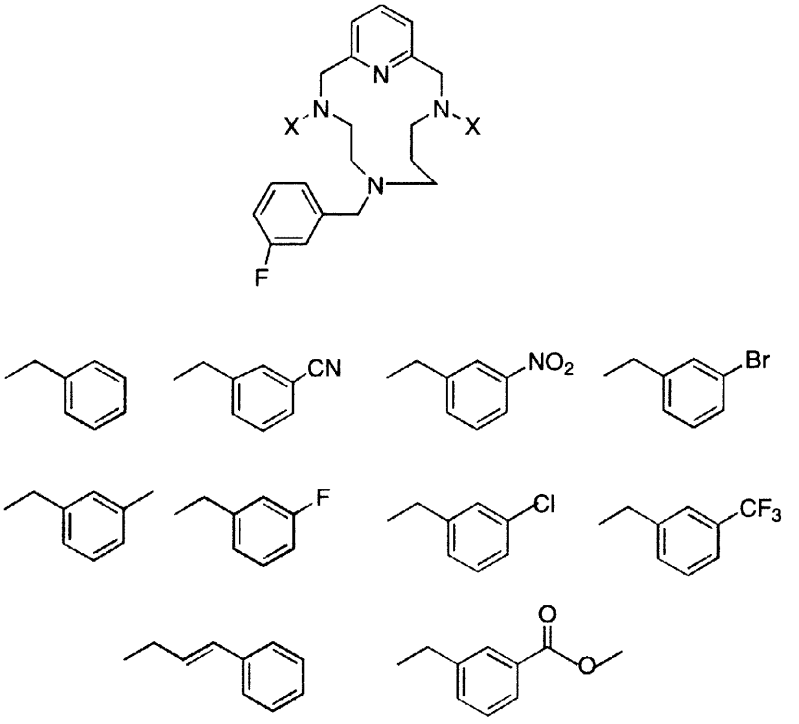

Figure 4. Structure of Isis 100302 combinatorial library.

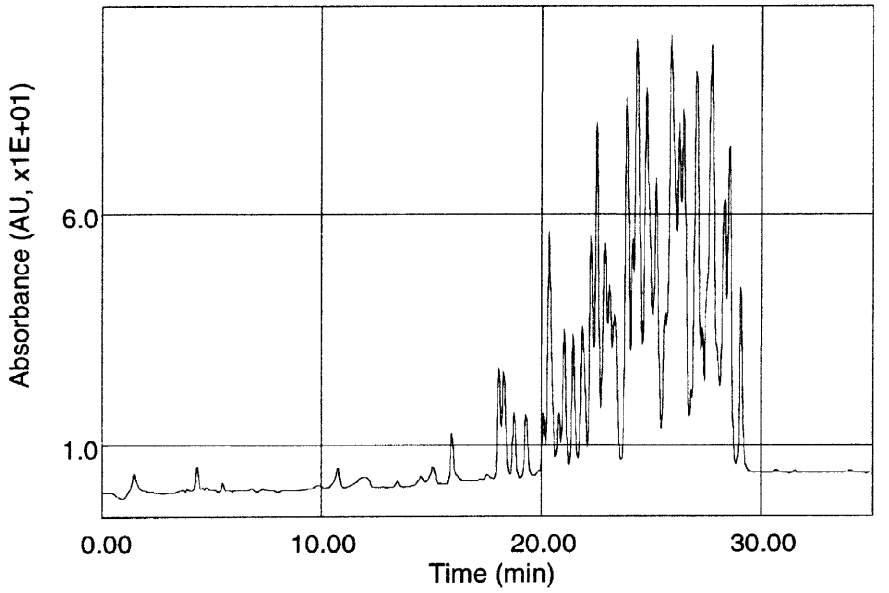

Figure 5. HPLC profile observed for Isis 100302. An Altima C18 reversed-phase column (10x250 mm; 5 μM particles) was employed. The starting aqueous buffer (A) contained 50 mM ammonium acetate, pH 4.6 and the organic buffer (B) contained 50 mM ammonium acetate in 90% methanol. A linear gradient was run from 60% B to 100% B from 4 to 30 minutes.

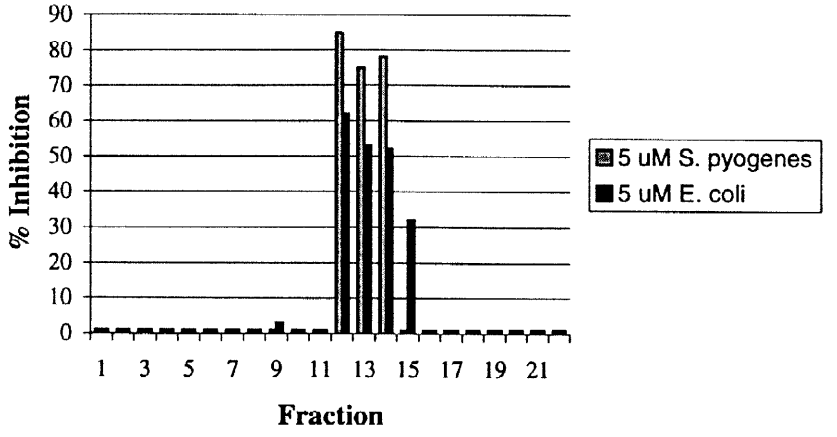

Figure 6. Biological activity of fractions of 100302

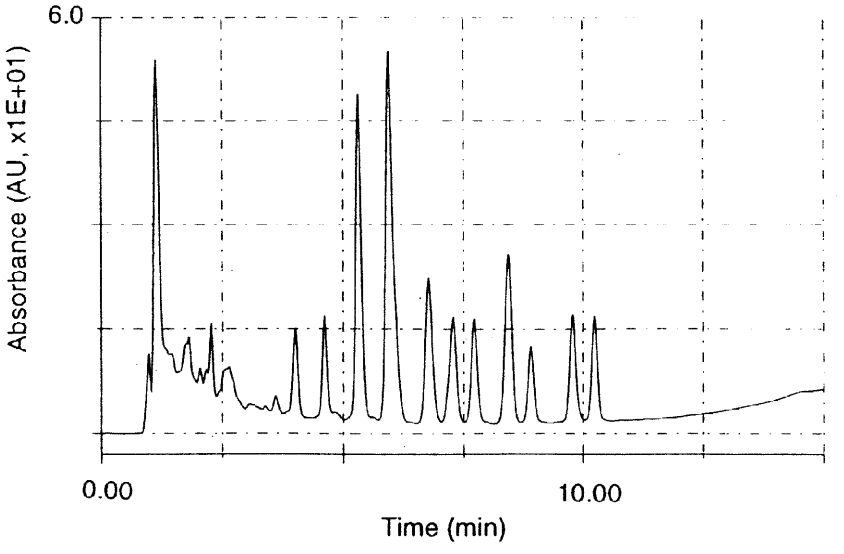

Figure 7. HPLC profile observed for fraction 12. A Zorbax C8 reversed-phase column (4.6x150 mm; 5 μM particles) was employed. A 200 μL portion of a 2 mM stock solution was loaded onto the column. The starting aqueous buffer (A) contained 50 mM triethylammonium acetate, pH 6.8, and the organic buffer (B) contained 50 mM triethylammonium acetate, pH 6.8 in 95% acetonitrile. A linear gradient was used to effect the separation.

column and an Intertsil C8 column failed to resolve all of the compounds. However, a second HPLC separation could be performed on fraction 12 using a Zorbax C8 solid support, with a flow rate of 1 mL/min. Eleven individual compounds were resolved (Figure 7) in the second chromatogram. Peak 5 of the second round fractionation (from original fraction 12) produced 95% inhibition of growth for *S. pyogenes* at 10 μM concentrations, and the major component had a molecular mass of 581.3 Da. The activities of the individual fractions summed to give 75-88% of the biological activity found in the initial screening of the library. These results suggest that the observed inhibition of microbial growth does not result from synergistic activity among the compounds in the starting fraction.

HPLC fractionation of Isis 113067. A third polyazapyridinocyclophane library containing 216 members was prepared via simultaneous solution phase synthesis of six substituted benzyl groups and selected for study (Figure 8). An HPLC purification was performed on 22 mg of material, using a gradient of ammonium acetate/acetonitrile and a C8 reversed phase silica column. The HPLC trace is presented in Figure 9. Over sixty peaks are resolved, and 34 fractions were collected for screening. As shown in Figure 10, fractions 14-16 and 21-22 inhibited the growth of *E. coli* and *S. pyogenes* at 20 μM.

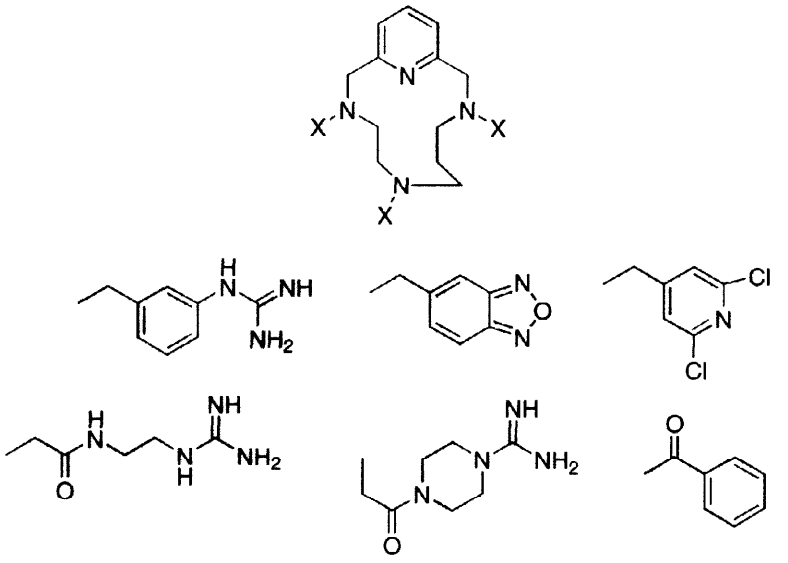

Figure 8. Structure of Isis combinatorial library 110367.

DISCUSSION

The HPLC fractionation of combinatorial libraries prepared via solution-phase simultaneous addition of functionalities offers many advantages as a deconvolution strategy. Enough material can be fractionated and

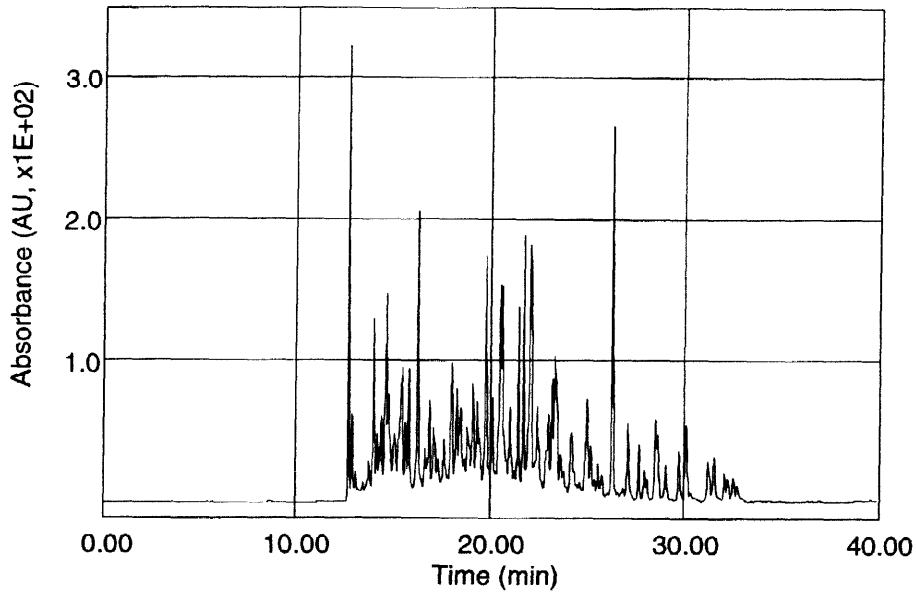

Figure 9. HPLC profile observed for Isis 113067. An Altima C18 reversed-phase column (10x250 mm; 5 μM particles) was employed. The starting aqueous buffer (A) contained 25 mM ammonium formate, pH 3.5 and the organic buffer (B) contained 25 mM ammonium formate in 85% acetonitrile. A linear gradient was run from 0% B to 100% B over 40 minutes.

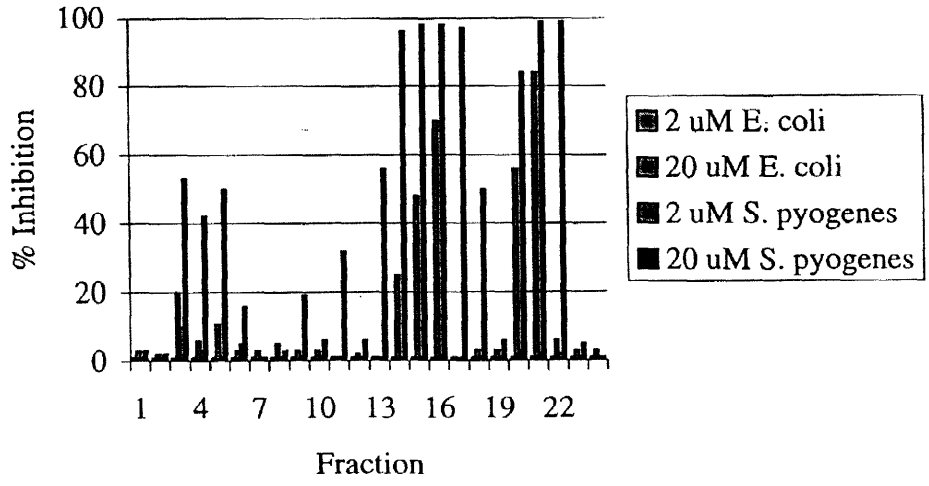

Figure 10. Biological activity observed for fractions of Isis 113067.

purified from one HPLC run for evaluation in 10-20 biological assays. If biological activity is observed from a fraction, individual compounds can be isolated in a second round of HPLC purification *without the need for resynthesis.* The fractionation process can be used to grade compounds on hydrophobicity, charge, or other physical properties. This information is of value when the biological activity tracks with a characteristic of the separation process.

The fractionation process provides insight into the quality of potential "winners" in the library. For example, when all of the fractions from a library demonstrate low levels of biological activity, the library may be dropped from further consideration since the probability of finding a single "winner" is low. In cases where the biological activity is observed in a single first round fraction, additional fractionation is warranted. In the second round fractions, the biological activity may again be distributed among several individual compounds. In favorable cases, a single active compound can be isolated in a single secondary fraction, whose identity can be established using methods described below. As with other combinatorial strategies, the biological activity of a fraction and the compound identities can be used to perform QSAR analyses. The conditions developed for HPLC separation of libraries or sublibraries can be transferred to larger scales as required for toxicology or animal pharmacology.

Strategies for determination of structure. Electrospray and atmospheric pressure chemical ionization mass spectrometry can be used to determine the molecular masses of compounds present in each fraction. Given the known masses of the starting scaffold and the functionalities, the composition of individual compounds can be determined rapidly. In ambiguous cases, MS/MS could be employed to assign the regiochemistry of a functional group. If required, sufficient material can be isolated to perform a multidimensional NMR analysis of structure. We have observed differences in the HPLC retention times for regioisomers having the same groups and molecular masses, which simplifies the determination of structure.

CONCLUSIONS

Solution-phase combinatorial synthesis can be used to prepare large quantities (20-100 mg) of libraries containing 25-1000 members. These large libraries can be fractionated into smaller subsets of compounds using HPLC techniques, which are well-suited for biological evaluation. The fractions which demonstrate biological activity can be separated further using an orthogonal HPLC method. In many cases, single compounds can be isolated and identified without the need for iterative synthesis and deconvolution or tagging. Hence, this strategy provides a rapid route to identification of novel pharmacophores from large combinatorial libraries prepared via the efficient simultaneous addition of functionalities in solution.

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15. *S. pyogenes* (ATCC# 14289) and *E. coli* (imp-) are grown for 6 h in minimal media at 37°C, then re-inoculated into fresh media and grown overnight. The bacterial cells are collected by centrifugation, diluted, and the absorbance read at 595 nm. Bacteria are diluted into broth at $1x10^4$ colony forming units per well, and mixed with the compounds under investigation in a 96-well format. The plates are incubated at 37°C for 24 h, and optical density is read using a BioRad 3550 UV microplate reader. Concentrations of material in each fraction were estimated based on the weighted molecular masses of all library members. This calculation can introduce an error of $\pm$10% in the actual concentration submitted for testing.

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