

# ACCELERATING DRUG DISCOVERY BY HIGH-THROUGHPUT COMBINATORIAL SYNTHESIS

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## ABSTRACT

A fully automated organic synthesizer has been constructed to generate diverse chemical libraries for use in drug screening programs. This high-throughput chemical synthesizer is based on proprietary robotic synthesis hardware and software. The robotic synthesizer is able to transform a small set of low-cost starting materials into an equimolar mixture of tens of thousands of novel diverse chemical structures. These mixtures can then be screened against a wide variety of pharmaceutically relevant receptors. Peptoids (N-substituted glycines) as well as peptides can be synthesized in very high yields. Peptoids have the added advantage that they can be synthesized from an incredibly large pool of commercially available primary amines. Highly potent peptoid trimers have been discovered with this technology. More complex chemical structures are also being made, which has prompted significant design changes in the synthesis hardware. Recently, a new generation synthesizer has been constructed that features a high temperature heat block as well as more flexible software. This automated system has thus evolved from a peptide synthesizer into a more general purpose organic synthesizer.

## INTRODUCTION

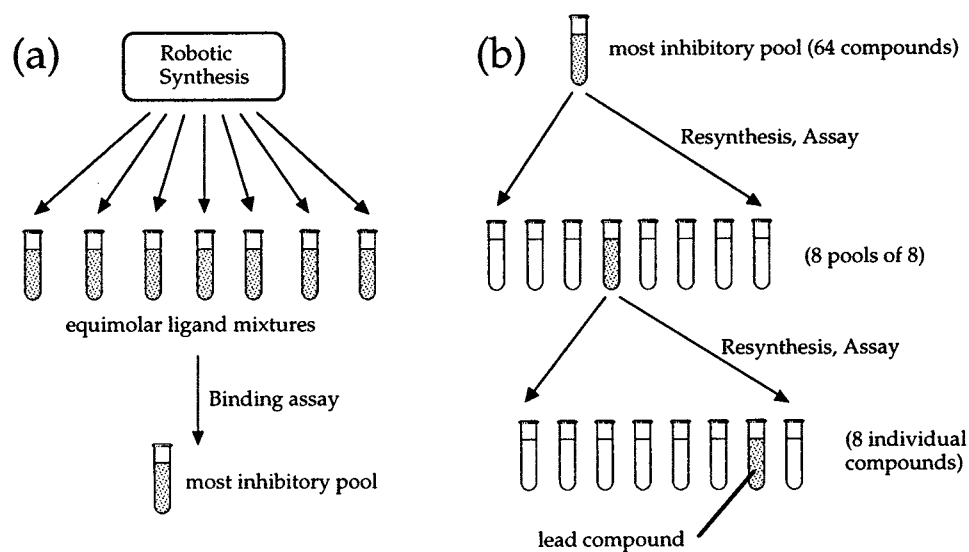
The rapid generation of a large number of chemically diverse compounds for screening can accelerate the discovery of novel lead compounds for drug discovery (1). The need to efficiently identify and optimize drug candidates has resulted in the development of two key technologies in our laboratories: multiple parallel synthesis (2) and efficient oligomerization (peptoid) chemistry (3,4). In combination these technologies have increased both the throughput of new compound synthesis and the diversity of the compounds.

We describe here the automation of multiple parallel synthesis as well as the solid-phase submonomer chemistry used to generate N-substituted glycine (peptoid) libraries. Many pools, each containing an equimolar mixture of different chemical species, are simultaneously synthesized for screening. Compounds are removed from the solid support using an automated cleavage/deprotection station so they can be assayed free in solution (5).

The operations required to synthesize chemical libraries are well defined, but labor intensive. The solid-phase synthesis of oligomers requires the performing of a small number of operations that are repeated many times. Operations that are commonly performed are: pipetting, liquid dispensing, slurry mixing, filtration and resin-splitting, all of which are amenable to automation. Since lead compounds are often identified by the iterative resynthesis of smaller pools, it is very important that the library synthesis is highly reproducible, and that the mixture components are equimolar (6). This further convinced us early on to establish an automated procedure for lead discovery.

## COMBINATORIAL DRUG DISCOVERY

The screening of large collections of organic compounds should have an increased probability of finding molecules that bind to a specific pharmaceutical target like an enzyme or a receptor. By screening pools of equimolar mixtures in a binding assay, the most inhibitory pool can be selected (6). The compounds in this pool can then be resynthesized into less complex mixtures and re-assayed. This procedure can be repeated until it is reasonable to synthesize the individual compounds in a pool. For example, eight pools of equimolar ligand mixtures are assayed and the most inhibitory is chosen (Figure 1). This pool of 64 compounds can be resynthesized as 8 pools of 8 equimolar mixtures and assayed. At the last step the pool of 8 compounds is resynthesized as 8 individual compounds and assayed. In the above example it required only two steps to reduce 512 compounds to 1 lead compound.

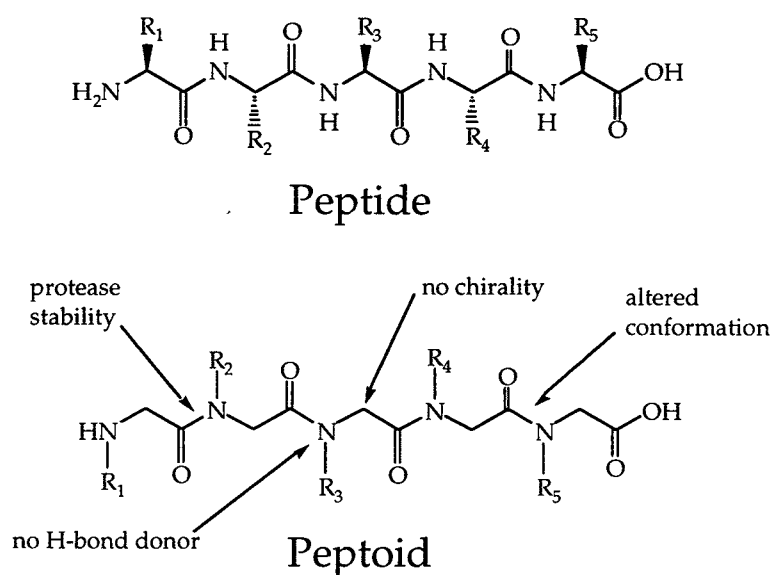


**Figure 1:** The deconvolution of a library of compounds is done by the repetitive synthesis and assaying of the most inhibitory pool.

## PEPTOIDS VS. PEPTIDES

As a consequence of the availability of optimized protocols for solid phase peptide and nucleotide synthesis, all the initial work on synthetic molecular diversity was accomplished with peptide libraries (7). In general, however, peptides suffer from poor oral bioavailability and rapid metabolic inactivation (8). Therefore, nonnatural analogs of peptides that are stable to proteases and have good oral bioavailability appear promising.

Peptoids are nonnatural oligomers that contain N-substituted glycines (NSGs) as their structural motif (9). Peptoid oligomers are achiral and possess comparable spacing of the sidechains (and the amide bonds) as their natural analogs. In addition, the nature of the peptoid backbone is very similar to the nature of the peptide backbone. However, peptoids are devoid of amide protons, which decreases their overall polarity and should increase their oral bioavailability. Since they contain only tertiary amide bonds, they are not subject to degradation by common proteases. A direct comparison of the structural features of peptides and peptoids is shown in Figure 2.



**Figure 2:** Peptoid oligomers are achiral and possess comparable spacing of the side-chains as their natural analogs. In addition, the nature of the peptoid backbone is very similar to the nature of the peptide backbone.

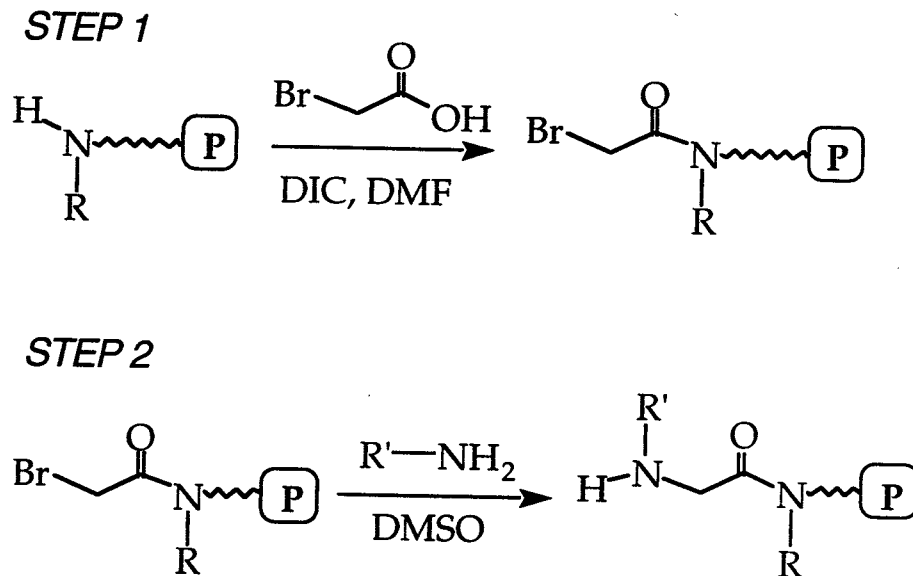
Since this novel class of molecules is based on an oligomeric glycine backbone, peptoids were expected to have a similar conformational profile as glycine. In addition, both cis- and trans- conformers of the amide bond should be accessible at room temperature. However, the substituent on the nitrogen is expected to confer different structural properties on peptoids that might limit their flexibility.

## SYNTHESIS OF N-SUBSTITUTED GLYCINES

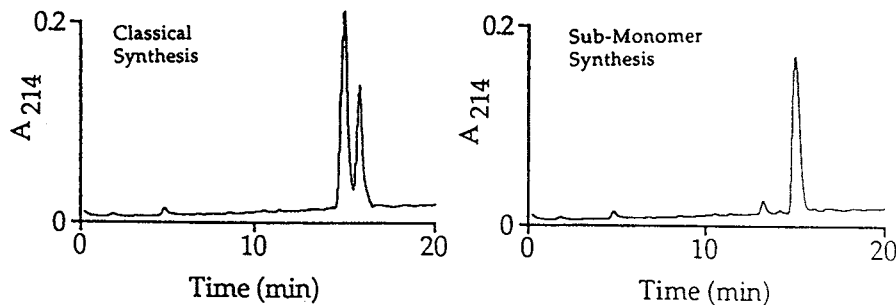
Our initial approach for the synthesis of NSG peptoids was analogous to conventional solid phase peptide synthesis and based on the condensation of N-Fmoc-protected, N-substituted glycines (9). Using the standard Fmoc-protocol and PyBOP or PyBroP-activation of the monomers, oligomeric peptoids were assembled in good yields and high purity. The NSG monomers had to be synthesized prior to assembly of our libraries and were obtained by alkylation of primary amines with electrophiles (e.g. haloacetic acids and acrylamides) or by reductive amination of primary amines with aldehydes (e.g. glyoxylic acid). Since the chemist had to make protected monomers as well as purify them, this was a rate limiting step.

A synthetic advance made it possible to improve the efficiency of the synthesis for a large variety of NSG peptoid oligomers. Regarding NSG peptoids as controlled copolymers of primary amines and acetate units, we developed a protocol that allows for the assembly of peptoids from the readily available building blocks ("submonomers") bromoacetic acid and primary amines (3,4). As shown in Figure 3, NSG peptoid oligomers can be synthesized using acylation reactions and  $S_N2$ -reactions in an alternating fashion, without the need to synthesize N-Fmoc-protected monomers.

Penta-(N-benzyl)glycine was robotically synthesized by both the classical method and the submonomer method. The submonomer method produced a cleaner product as shown in the HPLC traces in Figure 4.



**Figure 3:** Peptoid oligomers can be synthesized in a two step process: acylation reactions and  $S_N2$ -reactions are performed in an alternating fashion.



**Figure 4:** Penta-(N-benzyl)glycine was robotically synthesized by both the classical method and the submonomer method. The submonomer method produced a cleaner product.

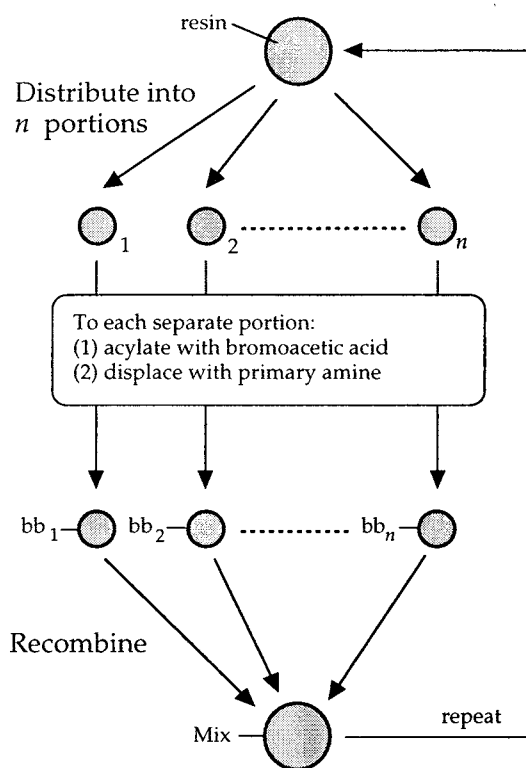
In summary, the submonomer protocol allows for the efficient synthesis of equimolar mixtures of NSG peptoid oligomers with a wide variety of sidechain functionalities. The high-yielding, reproducible linking chemistry, combined with the structural and functional diversity of the monomers that can be incorporated, renders the peptoid approach ideally suited for the automated generation of molecular diversity. More than 13,000 primary amines are listed in the Available Chemicals Database (10). Over 1,000 of these are inexpensive (priced less than \$10.00/g) and suitable for submonomer synthesis. In a set of peptoid trimers, more than  $10^9$  different combinations can be generated with these inexpensive amines alone.

## RESIN SPLITTING

We use the “resin splitting” approach to synthesize combinatorial libraries on a solid support (polystyrene resin). The distribution and recombining of the solid support is performed after generating a free-flowing, isopycnic slurry of the resin in 65% 1,2-dichloroethane/DMF (2). Resin splitting allows for precise control over the composition of the libraries (Figure 5). The resin starts out in a large mixing vessel as a slurry. Equal aliquots are distributed into individual reaction vessels (up to 36). To each portion an acylation and displacement is performed. All portions are then recombined into the mixing vessel and the process repeats. After the last building block is added to the individual vessels, the portions are not recombined. A syringe is used to measure the equal volumes of the slurry into the reaction vessels so that equimolar amounts of resin are transferred. By repeating each of the resin transfer steps three times, virtually quantitative transfer of the resin particles is achieved.

Equimolarity can be achieved despite some differences in the reactivity of the individual substrates and reagents since the reaction times are long enough to drive the reactions to completion. Excess reagents or even the

repetition of each reaction step also contribute to driving reactions to completion.



**Figure 5:** Resin-splitting scheme for the generation of equimolar peptoid mixtures.

## ROBOTIC SYNTHESIZER

The high-throughput synthesis of diverse peptoid libraries is performed by a robotic workstation of our own design (Figure 6) (2). The apparatus is capable of performing all the required synthetic and resin-splitting manipulations. The key features of the instrument have been described in detail (2); some of the most significant characteristics can be summarized as follows:

- The workstation consists of a Zymate XP robot (Zymark Corporation, Hopkinton, MA) that is interfaced with a Macintosh computer (Apple Computer, Cupertino, CA) (11). The robotic arm



- delivers solvents and reagents from pressurized lines into a 6 x 6 array of reaction vessels (Figure 7) as well as the mixing vessel.
- The reaction scale is between 0.05 and 0.60 mmol per vessel.
  - There are 62 monomer positions.
  - Supports both Fmoc or peptoid chemistry.
  - Active mixing is done by bubbling argon and draining is done by applying vacuum. Each row of reaction vessels shares a valve that can be opened either to argon or vacuum (the plumbing is configured in such a way to prevent cross contamination due to sharing a valve).
  - Temperature control up to 100°C.
  - Automated cleavage station.

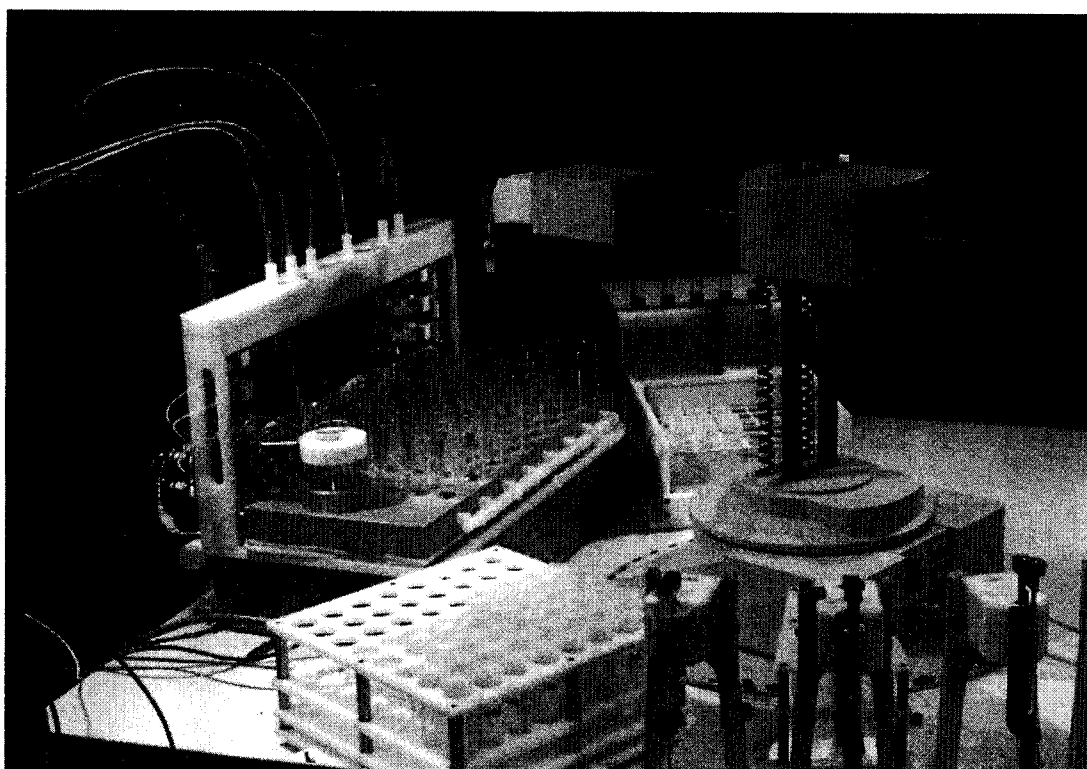
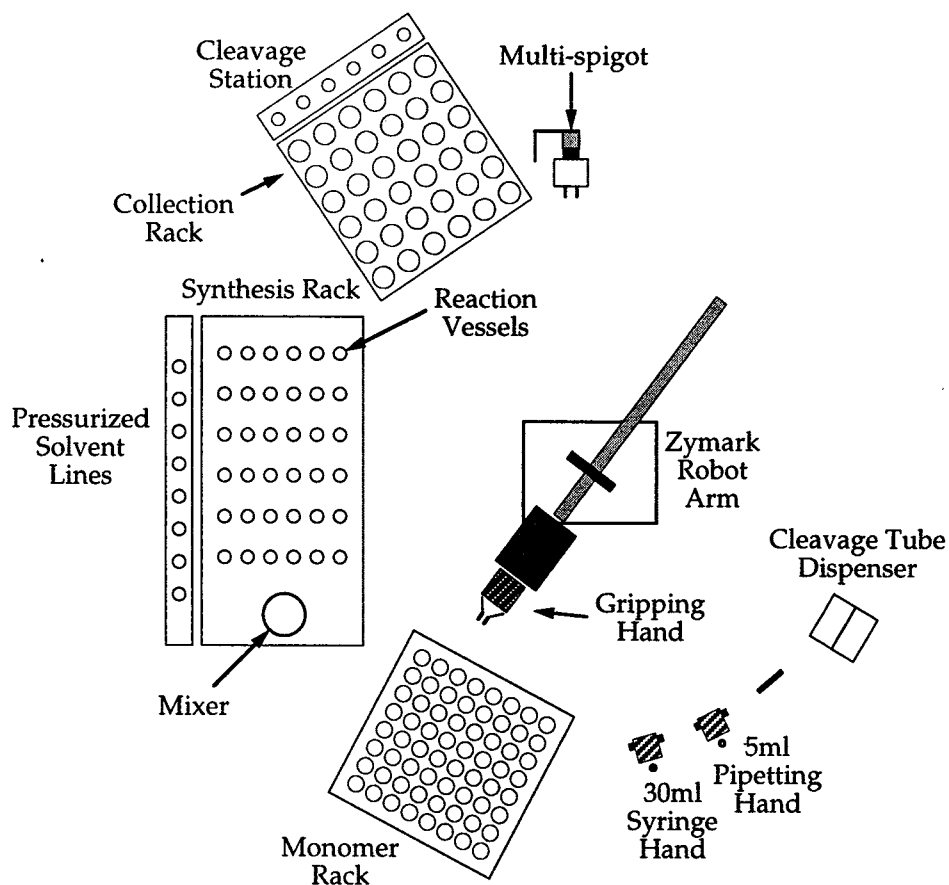


Figure 6: Robotic equimolar mixture synthesizer and cleavage station

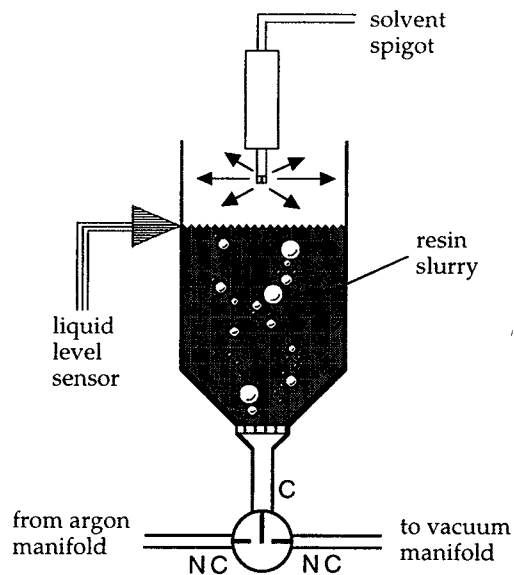
The mixing vessel has a level sensor that is used to obtain a pre-determined volume of resin slurry despite the changes in the resin volume throughout the synthesis. The mixing vessel is filled with an excess of solvent, using the pressurized solvent spigot, and the solvent is then drained to the sensor level by opening the bottom valve to vacuum (Figure 8). The

resin slurry is mixed by opening the bottom valve to argon. This is done prior to the removal of each aliquot of resin. The pressurized solvent spigot contain radial holes which spray the walls of the vessel to rinse down any resin beads that may have stuck to the wall.

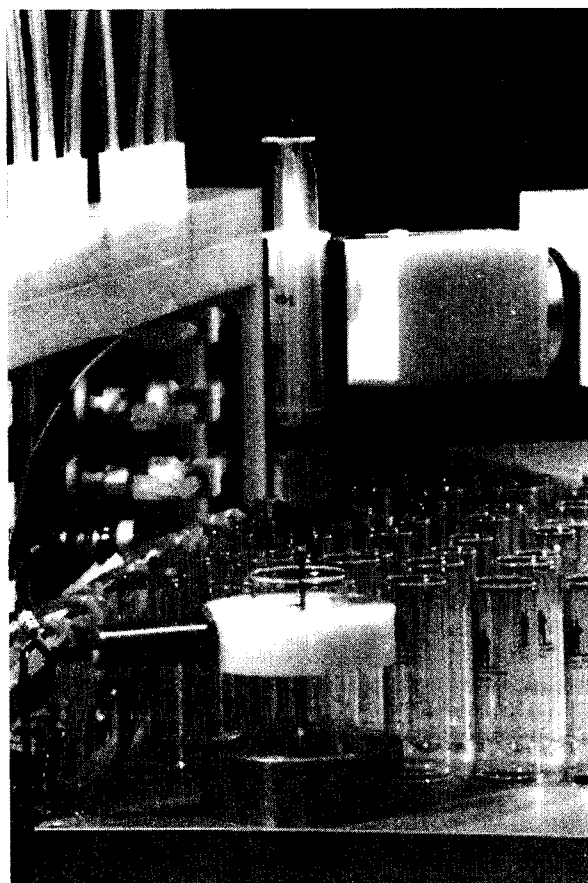


**Figure 7:** The robotic synthesizer consists of a 6 x 6 array of reaction vessels, a rack to store monomer solutions, pressurized solvent lines, a robotic arm, a 5mL pipette hand, a 30mL syringe hand, a gripping hand and a cleavage/deprotection station.

Resin aliquots are transferred to and from the mixer using a 30mL syringe hand (Figure 9). The hand is a modified 5mL syringe hand that is fitted with a disposable 30mL syringe.



**Figure 8:** A level sensor is used to obtain a pre-determined volume of resin slurry despite the changes in the resin volume throughout the synthesis.



**Figure 9:** Resin aliquots are transferred to and from the mixer as an isopycnic slurry using a 30mL syringe hand.

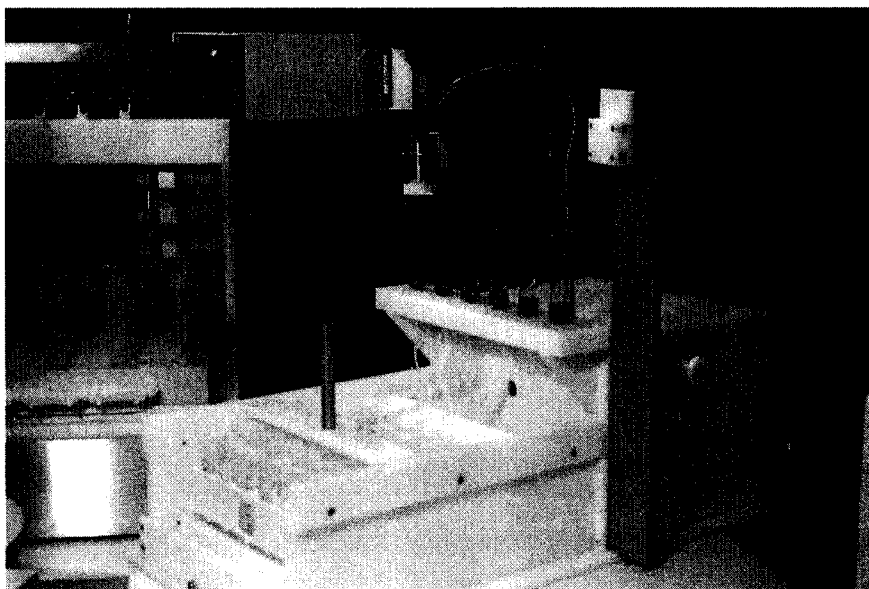
## SOFTWARE FEATURES

In order to run the robotic synthesizer, software had to be written that could control solenoid valves, read sensors, and control the robot. The software runs on a Macintosh and provides a graphical user interface. The valves and the sensors are accessed via digital I/O boards in the Mac and the robot communications are through the RS-232 port (modem port). Both peptide and peptoid chemistries are supported with the current software which is written in C/C++. Up to 36 individual compounds or equimolar mixtures of compounds can be synthesized.

As the complexity of the compounds we want to synthesize increases, we need to increase the flexibility of the synthesis software. To accommodate more general organic chemistries the synthesis program will have to take a more modular approach. The current software is rigid since it was only designed to handle oligomeric chemistries. Currently under development is a scheme where the user has a list of operations to choose from (such as a dispense operation, a drain operation etc.). By selecting a sequence of operations from the list, new automated chemical procedures can be constructed. Designing or modifying a synthesis procedure would be performed by the chemist.

## CLEAVAGE

After completion of the synthesis, the peptoid libraries are detached from the solid support using standard, trifluoroacetic acid based cleavage protocols. An automated cleavage station has been developed that greatly increases the library throughput (Figure 10) (5). The resin is transferred to cleavage vessels in the cleavage station as a resin slurry. After cleavage, the compounds are filtered into 50mL tubes and lyophilized.



**Figure 10:** An automated cleavage station is used to remove the oligomers from the solid support. This station supports trifluoroacetic acid based cleavage protocols.

## LIBRARY DESIGN AND SYNTHESIS

The process of creating an equimolar library of diverse compounds starts by choosing a diverse set of amines. By choosing from thousands of amines listed in the Fine Chemicals Database and sorting by cost, a diverse set of inexpensive amines can be chosen (1). To decide which amines to use from this set, we use computational tools to determine and measure similarity and dissimilarity between monomers by computing a variety of structural and functional properties.

The sidechains of the selected amines are protected if necessary prior to synthesis. The library of equimolar mixtures is then synthesized and cleaved from the resin.

## DISCOVERY OF A PEPTOID LIGAND WITH NANOMOLAR AFFINITY

Using the library design and synthesis method just described, a biased library was designed to target 7-Transmembrane/G-protein coupled receptors (1). From the set of monomers that were chosen a library of ~5000 peptoid dimers and trimers were synthesized. In total 18 pools of ~250 compounds per pool were synthesized.

The equimolar peptoid mixtures were screened for their ability to competitively inhibit binding of high-affinity radioligands to 7-transmembrane/ G-protein coupled receptors. Several of the pools shared a high degree of inhibition of the opiate receptor. After deconvolution and individual resynthesis of the three most potent compounds CHIR 4531 was shown to inhibit binding the best with a  $K_i = 7\text{nM}$ . Figure 11 is a comparison of CHIR 4531 with a known opiate ligand.

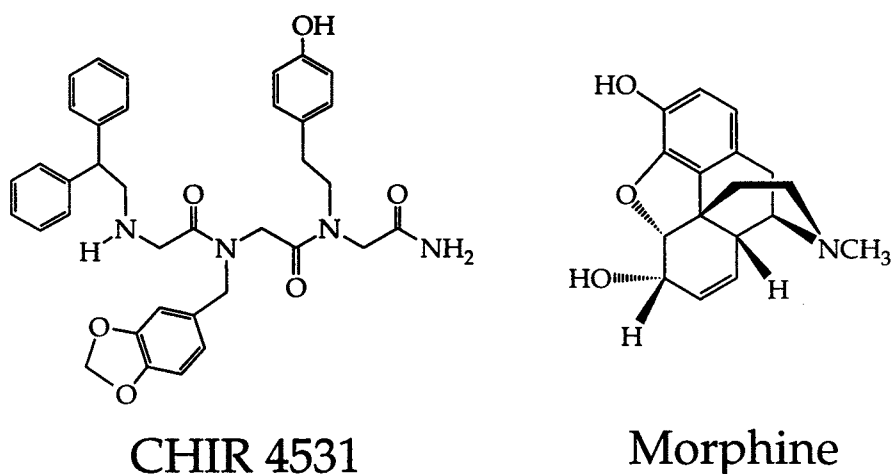


Figure 11: Comparison of CHIR 4531 with a known opiate ligand.

## SUMMARY

Combining multiple parallel synthesis with solid-phase submonomer chemistry has made the generation of diverse synthetic libraries and screening a highly effective drug discovery tool. The automation of these technologies has accelerated the process of finding lead compounds. Choosing from a large set of commercially available primary amines, the robotic synthesizer is able to transform a small sub-set of low-cost starting materials into thousands of novel diverse chemical structures.

The robotic synthesis and deconvolution of an equimolar peptoid library has led to the discovery of a new class of compounds (peptoids). A peptoid has been found that binds to a  $\mu$ -opiate receptor. Other lead compounds have also been discovered with these technologies (1).

## ACKNOWLEDGMENTS

The authors would like to thank our colleagues in the Drug Discovery Research group at Chiron for their valuable contributions.

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