The Activated Core Approach to Combinatorial Chemistry

by

Kent E. Pryor
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ABSTRACT

We have devised a means by which almost any number of small molecules can be generated simultaneously using entirely solution-phase chemistry through combinatorial methods. The tactic involves taking a relatively rigid core structure with multiple functional groups and reacting it with chemical complements as a mixture. This method combines the numerical advantages of split synthesis on the solid support with the homogeneous reaction conditions of the solutions phase. We have prepared libraries of from 1 to >100,000 components and have devised deconvolution strategies to identify the most active structures for a given biological target.

Refinements to the previously-reported synthesis of polyurea libraries on 9,9-dimethyl xanthene cores is presented, as well as the development of entirely new core molecules based on glycolurils and bipyridines. These new core structures present functional groups in increasingly diverse orientations and incorporate binding motifs within the cores themselves. In each case, methodology to produce chemical libraries in a clean, one-pot reaction has been developed in conjunction with methodology to produce any arbitrary single member of a library with building blocks installed in a paricular substitution pattern.

New linkers between the core molecule and building blocks have been introduced, and libraries based on a number of different core molecules have been screened for biological activity in a number of different assays. In particular, individual potent plasminogen activator inhibitor-1 antagonists have been identified from among initial large bipyridine core molecule libraries. Additionally, a new two-phase assay system to determine residual enzyme activity in the presence of an inhibitor candidate has been developed. Its application successfully identified a new xanthene-based elastase inhibitor from a library.

Thesis Supervisor: Professor Julius Rebek, Jr.
Title: Director, The Skaggs Institute for Chemical Biology and Professor, Department of Chemistry, The Scripps Research Institute

Biographical Note

Kent E. Pryor was born in Houston, Texas on June 22, 1972. He lived there for 22 years, graduated first in his class from Bellaire High School in 1990 and attended Rice University as a National Merit Scholar. He earned a B.A. in Chemistry (*magna cum laude*) from Rice in May, 1994. While at Rice, he was a Robert A. Welch Undergraduate Research Fellow in the laboratories of Prof. John L. Margrave, where he worked on the chemical vapor deposition of thin diamond films and the development of methods to study the thermophysical properties of levitated liquid metals. In 1993 he was elected to Phi Lambda Upsilon and won the Zevi and Bertha Salzburg Memorial Award for Excellence in Chemistry. In 1994, he was elected to Phi Beta Kappa and was named the 1993–1994 NASA/Texas Space Grant Consortium Scholar of Rice University.

Mr. Pryor spent the summers of 1993 and 1994 as a summer intern at the Hoechst Celanese Technical Center in Corpus Christi, Texas, where he developed novel bidentate phosphine ligands for hydroformylation catalysts. He then moved from his native Texas to graduate school at the Massachusetts Institute of Technology, where he held a National Science Foundation Graduate Research Fellowship. He joined the research group of Prof. Julius Rebek, Jr. and started his research into the activated core approach to combinatorial chemistry. When Prof. Rebek took a new position as the Director of the Skaggs Institute for Chemical Biology at The Scripps Research Institute in La Jolla, California, Mr. Pryor followed his advisor to his new laboratories as a non-resident MIT graduate student. While living on his third coast, he continued his combinatorial chemistry research and entered into several collaborative research projects with other chemistry, vascular biology, and molecular biology research groups.

Upon the completion of his doctoral defense, Mr. Pryor will take a position as a Research Scientist at Corvas International, Inc., where he will initially work on the development of new cancer therapies based on PAI-1 antagonists.

Mr. Pryor is the author or co-author of 14 papers, patents, and patent applications and has made several public presentations of his work. The papers include "Containerless Thermophysical Property Measurements for Liquid Metals," *1994*, "Synthesis and Screening of Small Molecule Libraries Active in Binding to DNA," *1997*, "The Activated Core Approach to Combinatorial Chemistry: A Collection of New Core Molecules," *1998*, "Multifunctionalized Glycolurils," *1999*, and "A Rapid Method to Identify *Exo*-Protease Inhibition," *1999*. He has written six patents titled "Synthesis of and Hydroformylation with Fluoro-substituted Bidentate Phosphine Ligands," *issued 1996–1998*, and three patent applications titled "Activated Core Molecules for the Synthesis of Libraries of Compounds in Combinatorial Chemistry," *filed 1998*, "Glycolurils in Combinatorial Chemistry," *filed 1999*, and "Bipyridine PAI-1 Antagonists," *filed 1999*.

Acknowledgements

First of all, I would like to thank Prof. Rebek for his support and encouragement during my tenure as a graduate student in his research group. I consider myself to be very fortunate to have worked in a group where I had both the freedom to design and pursue my own research projects and the intellectual input of an exceptional chemistry mentor for the asking.

I also want to thank two research technicians who worked with me at various times during my graduate school career, Ellen Choi and Christina Cueto. Ellen has been wonderfully helpful on several different projects in a variety of roles, and I am deeply indebted to her. Christina, while working with me for less than a year, made significant contributions to the PAI project. I am glad that I had the opportunity to work with both of them.

There is a lot of effort behind the scenes that is required to keep the scientific research going, and I thank Jean Harrington, Michelle Bateman, and Janette Lundgren for all of their help in this regard. Jean worked very hard for us during the transition from MIT to Scripps. Michelle all but adopted us once we got to Scripps and took wonderful care of us. In the last several months, Janette has been enormously helpful and has become a good friend.

I appreciate the efforts of my external collaborators on our joint projects. Ed Madison has been wonderful to work with on the PAI project, and I am looking forward to working together even more closely with him in the future. I also thank Jim LaClair for working with me on the protease inhibitor project.

The Rebek group has been a great place in which to work. When I joined the group, I worked closely with Jerry Shipps, who remains my friend to this day. While I have not shared projects with any group members recently, I have shared lab space with Carina Horn, Jonathan Toker, and Doris Pupowicz, each of whom kept me entertained in different ways. My fellow graduate students Brendan O'Leary, Ron Castellano, and José Rivera, who have all been with me since my time at MIT, have not only been good friends, but have been great resources for chemistry, too. I have appreciated their advice and insight over the years.

Finally, my parents' unconditional support throughout my life in everything I have put my mind to has been instrumental in getting me to the point where I am today, and it is impossible for me to thank them enough for that.

Table of Contents

Chapter 1. Introduction

1.1 An introduction to combinatorial chemsitry

The term "combinatorial chemistry" has no single definition, but practitioners in the field seem to apply it fairly broadly to mean any methodology in which multiple compounds bearing some structural or synthetic relationship to each other are produced either together or in parallel. Scientific advances on a variety of fronts, all subsumed by the mantle of combinatorial chemistry, have been made at an ever-increasing pace; the explosive growth of the field has caused the American Chemical Society to start publishing another new journal, *The Journal of Combinatorial Chemistry*, at the beginning of this year to cover the subject in detail. A number of books on combinatorial chemistry have also been published recently.[1]

Combinatorial chemistry has primarily been used in the context of medicinal chemistry, both to help identify lead compounds and to optimize leads into better drug candidates. However, the same approaches have also been used to create libraries for a variety of other purposes, such as catalysis,[2] metal coordination,[3] optical sensing,[4] and

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[1] Some examples are: (a) Wilson, S. R.; Czarnik, A. W. *Combinatorial Chemistry: Synthesis and Application*; John Wiley & Sons: New York, 1997. (b) Obrecht, D.; Villalgordo, J. M *Solid-Supported Combinatorial and Parallel Synthesis of Small-Molecular-Weight Compound Libraries*; Pergamon Press: London, 1998. (c) Gordon, E. M.; Kerwin, J. F. *Combinatorial Chemistry and Molecular Diversity in Drug Discovery*; John Wiley & Sons: New York, 1998.

[2] (a) Cong, P. J.; Doolen, R. D.; Fan, Q.; Giaquinta, D. M.; Guan, S. H.; McFarland, E. W.; Poojary, D. M.; Self, K.; Turner, H. W.; Weinberg, W. H. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 484–488. (b) Gennari, C.; Nestler, H. P.; Piarulli, U.; Salom, B. *Liebigs Ann. Recueil* **1997**, 637–647. (c) Kobayashi, S.; Nagayama, S. *J. Am. Chem. Soc.* **1996**, *118*, 8977–8978. (d) Cole, B. M.; Shimizu, K. D.; Kureger, C. A.; Harrity, J. P. A.; Snapper, M. L.; Hoyveda, A. H. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1668–1671.

[3] (a) Albrecht, M.; Blau, O.; Frohlich, R. *Chem. Eur. J.* **1999**, *5*, 48–56. (b) Berg, T.; Simeonov, A.; Janda, K. D. *J. Comb. Chem.* **1999**, *1*, 96–100. (c) Gilbertson, S. R.; Wang, X. *Tetrahedron Lett.* **1996**, *37*, 6475–6478. (d) Francis, M. B.; Finney, N. S.; Jacobsen, E. N. *J. Am. Chem. Soc.* **1996**, *118*, 8983–8984.

[4] Michael, K. L.; Taylor, L. C.; Schultz, S. L.; Walt, D. R. *Anal. Chem.* **1998**, *70*, 1242–1248.

superconductivity,[5] just to name a few of the other fields to benefit from combinatorial techniques.

Chemical libraries can be synthesized in a variety of different ways. Very broadly, they can be divided by the phase in which the libraries are being prepared. Solid phase techniques can be used in which all library members are covalently attached to a polymer support during synthesis. Several variations on this theme exist and are discussed in Section 1.2 below. Solution phase methodologies have also been applied, and a description of the development of one particular solution phase technique constitutes the bulk of this dissertation.

1.2 Polymer-supported methods for generating libraries

Spatially separate synthesis

Perhaps the most straightforward approach to library synthesis is to keep each individual compound spatially separated from its fellows in a defined array. When the compounds are spatially separate, direct structure-activity relationships are obtained from biological evaluation. The primary disadvantage of this approach, however, is that the number of compounds that can be synthesized is more limited than with other approaches. Another disadvantage, common to all solid-phase techniques, is that all of the required chemistry to produce the desired library compounds must be adapted to work on the solid phase.

Geysen and co-workers prepared the first combinatorial library in spatially separate format in 1984.[6] They synthesized a library of peptides using functionalized pins they developed that were compatible with 96-well microtiter plates. Pin technology has been

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[5] Xiang, X.-D.; Sun, X.; Briceno, G.; Lou, T.; Wang, K.-A.; Chang, H.; Chen, S.-W.; Schultz, P. G. *Science* **1995**, *268*, 1738–1740.

[6] Geysen, H. M.; Meloen, R. H.; Barteling, S. J. *Proc. Natl. Acad. Sci.* **1984**, *81*, 3998–4002.

improved since then using other polymers and linkers and by increased loading to accommodate other chemical applications.[7]

At Affymax, Fodor and co-workers have developed methods in which large libraries (100,000 compounds) are built on a silicon wafer with photolithographic methods.[8] The application of this methodology is limited, for the most part, to DNA diagnostic tests because it requires photolabile protecting groups and support-bound biological assays. Combinatorial materials chemistry is most commonly performed on wafers as arrays. This method lends itself particularly well to applications such as the identification and optimization of new luminescent compounds, which are easy to screen because devices that measure light intensity and color are readily available. Researchers at Symyx have screened libraries of 25,000 at a time on silicon wafers and have discovered improved red phosphors[9] and a new class of blue-white phosphors.[10]

Pooling strategies

The earliest pooling strategies were independently developed by Furka,[11] Lam,[12] and Houghten.[13] They each employed a split and mix procedure to generate mixtures of peptides on solid support. In a split synthesis, equal portions of the support resin is reacted in separate reaction vessels with different monomers. After the reactions are complete, the resins are washed and combined. At this point a common transformation, such as the removal of a protecting group, can be performed. Equal portions of the well-mixed resin are

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[7] Maeji, N. J.; Valerio, R. M.; Bray, A. M.; Campbell, R. A.; Geysen, H. M. *React. Polym.* **1994**, *22*, 203–212.

[8] Fodor, S. P. A.; Read, J. L.; Pirrung, M. C.; Stryer, L.; Lu, A. T.; Solas, D. *Science* **1991**, *251*, 767–773.

[9] Sun, X. D.; Gao, C.; Wang, J. S.; Xiang, X. D. *Appl. Phys. Lett.* **1997**, *70*, 3353–3355.

[10] Wang, J.; Yoo, Y.; Gao, C.; Takeuchi, I.; Sun, X.; Chang, H.; Xiang, X. D.; Schultz, P. G. *Science* **1998**, *279*, 1712–1714.

[11] Furka, A.; Sebestyen, F.; Asgedom, M.; Dibo, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 487–493.

[12] Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82–84.

[13] Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84–86.

next charged again to separate reaction vessels, and a second monomer is attached. This cycle of splitting and pooling is continued until the desired number of monomers is attached. These combinatorial methods, while originally developed for peptides, have also been applied to the synthesis of unnatural biopolymers and small molecules.

At the end of a split synthesis, each individual bead theoretically is carrying a single product. This "one compound, one bead" approach has been used to identify the active components in a biological assay directly in some cases. With certain assays of support-bound compounds, it is possible to identify an active compound by allowing the candidate to bind to a radiolabeled or fluorescent-labelled receptor or dyes and picking out radioactive, fluorescent, or colored beads.[14] Otherwise, the compounds can be cleaved from the resin and screened in solution.

To deconvolute, or the identify actives from, a library synthesized by split and mix methods, chemical or biochemical tags can be attached to the resin during the synthesis of the library. DNA was the early choice for tagging,[15] but it is unfortunately not chemically stable under many of the reaction conditions frequently used in organic synthesis. To circumvent this problem, encoding has been performed with peptides prepared from amino acids that have relatively unreactive side chains[16] or gas chromatography-electron capture (GC-EC) tags that are inert to most of the reaction conditions typically employed.[17] Radiofreqency tagging has been developed to completely avoid any potential chemical

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[14] (a) Burger, M. T.; Still, W. C. *J. Org. Chem.* **1995**, *60*, 7382–7383. (b) Cheng, Y.; Suenga, T.; Still, W. C. *J. Am. Chem. Soc.* **1996**, *118*, 1813–1814.

[15] Needles, M. C.; Jones, D. G.; Tate, E. H.; Heinkel, G. L.; Kochersperger, L. M.; Dower, W. J.; Barrett, R. W.; Gallop, M. A. *Proc. Natl. Acad. Sci.* **1993**, *90*, 10922–10926.

[16] Vetter, D.; Tate, M. M.; Gallop, M. A. *Bioconjugate Chem.* **1995**, *6*, 319–322.

[17] Ohlmeyer, M. H.; Swanson, R. N.; Dillard, L. W.; Reader, J. C.; Asouline, G.; Kobayashi, R.; Wigler, M.; Still, W. C. *Proc. Natl. Acad. Sci.* **1993**, *90*, 10922–10926.

incompatibilities, instead depending on radio emitters embedded in each bead or reaction chamber.[18]

Soluble polymer supports

At the interface between solid phase techniques and true solution phase techniques lies the application of soluble polymer supports. Janda and co-workers[19] have built chemical libraries attached to polyethylene glycol monomethyl ether, which is soluble in a number of potential reaction media. Each reaction step is carried out under homogeneous conditions, providing the advantages of solution phase chemistry. After each step, the polymer support is precipitated with ether or ethanol and the resin is filtered, thereby eliminating non-bound reagents and reaction byproducts.

1.3 Solution phase combinatorial chemistry

A less-frequently used complement to adapting solution phase chemistry to polymer-supported combinatorial synthesis is the development of protocols for entirely solution phase combinatorial synthesis. Only a limited number of examples of solution libraries have been published so far;[20] however, there are a number of potential benefits to be derived from solution phase approaches. There is no need to develop attachment/detachment chemistry for the polymer support, and the range of chemistry available for solution phase reactions is greater than that for solid supported chemistry (although advances on this front continue to be made at an astonishing pace). Additionally, solution phase approaches are not limited in

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[18] (a) Xiao, X-Y.; Nova, M. P. *Comb. Chem.* **1997**, 135–152. (b) Nicolaou, K. C.; Xiao, X.-Y.; Parandooch, Z.; Senyei, A.; Nova, M. P. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2289–2291.

[19] (a) Han, H.; Wolfe, M. M.; Brenner, S.; Janda, K. D. *Proc. Natl. Acad. Sci.* **1995**, *92*, 6419–6423. (b) Gravert, D. J.; Janda, K. D. *Chem Rev.* **1997**, *97*, 489–509.

[20] While fewer research groups are publishing solution phase approaches, the field is still broad enough to be the subject of a recent *Tetrahedron* Symposium-in-print: Coffen, D. L., ed. *Tetrahedron* **1998**, *54*, No. 16.

scale, and thus are particularly useful for the preparation of larger samples for extensive testing or archival purposes.

The disadvantages of solution phase combinatorial chemistry relative to solid phase approaches are the relative complexity of product isolation and the potential difficulties in the identification of active compounds from among a solution phase mixture. Solution phase approaches generally depend on extractive workups or the application of scavenger resins to remove unreacted reagents.[21] In certain cases, chromatography has been used to purify solution phase mixtures,[22] although this is unfortunately not broadly applicable.

Parallel solution phase synthesis of individual compounds eliminates the problem of active compound identification by allowing direct testing of each compound individually. Multicomponent coupling reactions, such as the Ugi reaction,[23] have been used for generating solution libraries.[24] The Ugi condensation involves the reaction of four different building blocks in one pot. Upon reaction completion, the product of the Ugi condensation, often in high yield, precipitates out of solution.

Boger and co-workers have developed other parallel solution phase techniques for combinatorial synthesis. They have reported two approaches that entail the dimerization linkage of iminodiacetic acid diamides[25] using symmetrical dicarboxylic acids[26] or the olefin

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[21] (a) Kaldor, S. W.; Siegel, M. G.; Fritz, J. E.; Dressman, B. A.; Hahn, P. J. *Tetrahedron Lett.* **1996**, *37*, 7193–7196. (b) Kaldor, S. W.; Fritz, J. E.; Tang, J.; McKinney, E. R. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 3041–3044.

[22] Boger, D. L.; Chai, W.; Ozer, R. S.; Anderson, C.-M. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 463–468.

[23] Ugi, I.; Domling, A.; Horl, W. *Endeavour* **1994**, *18*, 115–122.

[24] (a) Keating, T. A.; Armstrong, R. W. *J. Am. Chem. Soc.* **1995**, *117*, 7842–7843. (b) Weber, L.; Walbaum, S.; Broger, C.; Gubernator, K. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2280–2282. (c) Keating, T. A.; Armstrong, R. W. *J. Am. Chem. Soc.* **1996**, *118*, 2574–2583.

[25] (a) Cheng, S.; Comer, D. D.; Williams, J. P.; Myers, P. L.; Boger, D. L. *J. Am. Chem. Soc.* **1996**, *118*, 2567. (b) Boger, D. L.; Tarby, C. M.; Myers, P. L.; Caporale, L. H. *J. Am. Chem. Soc.* **1996**, *118*, 2109. (c) Cheng, S.; Tarby, C. M.; Comer, D. D.; Williams, J. P.; Caporale, L. H.; Myers, P. L.; Boger, D. L. *Bioorg. Med. Chem.* **1996**, *4*, 727.

[26] Boger, D. L.; Ozer, R. S.; Andersson, C.-M. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1903.

metathesis reaction to join and randomize the length of the linking tether.[20] They have also demonstrated the preparation of higher order libraries containing up to eight variable groups by coupling iminodiacetic acid diamides with tricarboxylic acids or sequential couplings with dicarboxylic acids.[27]

One benefit of screening compounds as mixtures, however, is the much-reduced number of individual screens required to test a given number of compounds. We have identified two low-micromolar DNA intercalators from an initial body of approximately 10,000 compounds in a total of about 20 assays, thereby demonstrating the efficiency of this method.[28] Berk, Rohrer, and co-workers at Merck recently reported the solid phase synthesis of nonpeptide somatostatin agonist candidates, which they cleaved from support and screened as mixtures in solution.[29] They identified a number of subtype-selective agonists by deconvolution of their mixtures with far fewer screens than if they had attempted to synthesize and screen individual compounds, providing yet another example of the benefit to be gained by solution phase screening of mixtures, even when solid phase synthesis techniques produce the compounds.

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[27] Boger, D. L.; Ducray, P.; Chai, W.; Jiang, W.; Goldberg, J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2339–2344.

[28] Shipps, G. W., Jr.; Pryor, K. E.; Xian, J.; Skyler, D. A.; Davidson, E. H.; Rebek, J., Jr. *Proc. Natl. Acad. Sci.* **1997**, *94*, 11833–11838. See also Section 2.5 below.

[29] Rohrer, S. P.; Birzin, E. T.; Mosley, R. T.; Berk, S. C.; Hutchins, S. M.; Shen, D. M.; Xiong, Y. S.; Hayes, E. C.; Parmar, R. M.; Foor, F.; Mitra, S. W.; Degrado, S. J.; Shu, M.; Klopp, J. M.; Cai, S. J.; Blake, A.,; Chan, W. W. S.; Pasternak, A.; Yang, L. H.; Patchett, A. A.; Smith, R. G.; Chapman, K. T.; Schaeffer, J. M. *Science* **1998**, *282*, 737–740.

1.4 The activated core approach

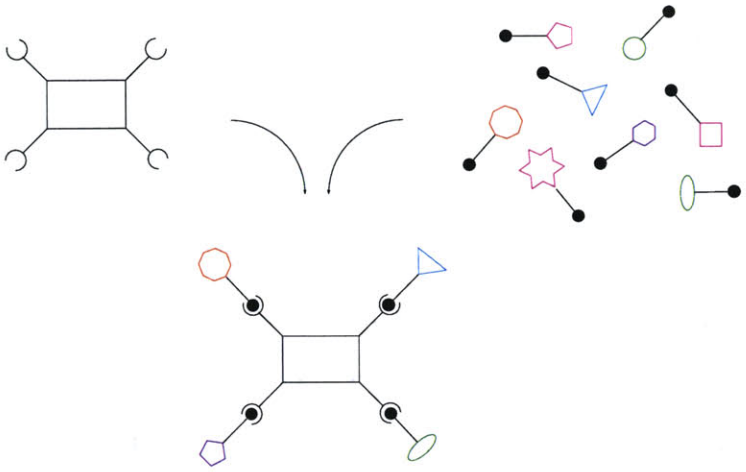

Figure 1. A schematic representation of the activated core molecule approach to combinatorial chemistry. An activated core molecule is shown on the left. It is reacted with a mixture of building blocks, and the final library is composed of a statistical mixture of all possible substitutional isomers. Covalent linker chemistry is represented by the complementary balls and sockets.

In contrast to parallel synthesis, this group has chosen to use simultaneous solution phase reactions in a method we call the "activated core" approach. The tactic involves taking a relatively rigid core structure with multiple functional groups and reacting it with chemical complements as a mixture (see Figure 1). Like combinatorial methods involving synthesis on soluble polymer supports, this method combines the numerical advantages of split synthesis on solid support with the homogeneous reaction conditions of the solution phase. We have prepared libraries of from 1 to greater than 100,000 components and have devised deconvolution strategies to identify the most active structures for a given biological target.

Other groups have also reported single-step solution phase synthesis of combinatorial libraries. Smith and co-workers prepared a library of potentially 1600 amides

by reacting 40 acid chlorides with 40 nucleophiles.[30] The library was screened as 80 sample mixtures in a matrix format, allowing immediate deconvolution. A similar sublibrary format was used by Pirrung and Chen, who prepared a series of carbamate mixtures which were screened for acetylcholinesterase inhibitory activity.[31]

Even more similar is the SPSAF (Solution Phase Simultaneous Addition of Functionalities) approach taken by An, Cook, and co-workers at Isis Pharmaceuticals. They have synthesized unsymmetric polyazacyclophane[32] and linear[33] scaffolds and utilized them for the generation of a variety of mixture libraries, and they have evaluated the libraries' biological activity in antibacterial and HIV-1 tat/TAR protein–RNA disruption assays.

1.5 Design considerations for the activated core approach

The core

The activated core approach can be divided into three components: the core, the linker, and the building blocks. Each can be designed to give the desired level of diversity (numbers of compounds) and/or the desired physical property (solubility, shape space, chemical functionality). A selection of core molecules developed in this group is shown below in Figure 2.

In initial studies in this research group, xanthene and cubane tetraacid chlorides were reacted with mixtures of amino acid esters to form polyamide libraries. These libraries were subsequently successfully screened for activity against trypsin, and a deconvolution scheme

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[30] Smith, P. W.; Lai, J. Y. Q.; Whittington, A. R.; Cox, B.; Houston, J. G. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2821–2824.

[31] (a) Pirrung, M. C.; Chen, J. *J. Amer. Chem. Soc.* **1995**, *117*, 1240–1245. (b) Pirrung, M. C.; Chau, J. H. L.; Chen J. *Chem. Biol.* **1995**, *2*, 621–626.

[32] (a) An, H.; Cook, P. D. *Tetrahedron Lett.* **1996**, *37*, 7233–7236. (b) An, H.; Haly, B. D.; Cook, P. D. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2345–2350. (c) An, H. Y.; Wang, T.; Mohan, V.; Griffey, R. H.; Cook, P. D. *Tetrahedron* **1998**, *54*, 3999–4012. (d) An, H. Y.; Haly, B. D.; Cook, P. D. *J. Med. Chem.* **1998**, *41*, 706–716.

[33] An, H.; Haly, B. D.; Fraser, A. S.; Guinosso, C. J.; Cook, P. D. *J. Org. Chem.* **1997**, *62*, 5156–5164.

was applied to identify the active compounds.[34] One drawback to the cubane core molecule is its high degree of symmetry, which disallows differentiation of the four reactions sites; this drawback is shared by the adamante core molecule, as well.

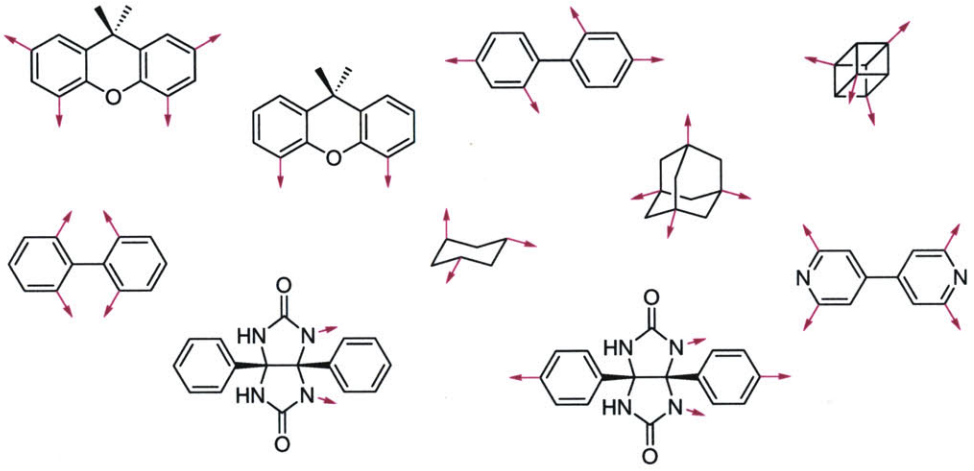

Figure 2. A selection of core molecules for combinatorial chemistry developed in the Rebek group.

Other core molecules were subsequently introduced,[35] and were chosen to provide different orientations of the attached building blocks and new functionalities within the core. The properties of the core molecule have (in several assays) been critical to activity, as libraries made with the same building blocks and linkages have had very different activity levels.[36]

The symmetry of the core molecule, the number of reaction sites, and the number of building blocks used in the diversity-forming reaction all determine the total number of compounds produced in the final library. This is illustrated in Figure 3. Three different core

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[34] (a) Carell, T.; Wintner, E. A.; Rebek, J., Jr. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2061–2064. (b) Carell, T.; Wintner, E. A.; Bashir-Hashemi, A.; Rebek, J., Jr. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2059–2061.

[35] (a) Pryor, K. E.; Shipps, G. W., Jr.; Skyler, D. A.; Rebek, J., Jr. *Tetrahedron* **1998**, *54*, 4107–4124. (b) Pryor, K. E.; Rebek, J., Jr. *Org. Lett.* **1999**, in press. See also Chapters 3 and 5 below.

[36] Carell, T.; Wintner, E. A.; Sutherland, A. J.; Rebek, J., Jr.; Dunayevskiy, Y. M.; Vouros, P. *Chem. Biol.* **1995**, *2*, 171–183. See also Chapter 4 below.

molecules A, B, and C are given, and the library size when each is reacted with a particular number of building blocks is graphed. In all cases, the combinatorial rule, $x = m!/n!(m-n)!$, is applied to determine the number of combinations x in which m building blocks can be taken in n groups. This number of combinations is then scaled by a "symmetry multiplier," which is the number of different structural isomers that can be formed with m building blocks. This number obviously varies according to the symmetry of the core molecule. Compound A, incorporating only two reaction sites, produces far smaller libraries than either of the tetrasubstituted compounds B or C. Between the tetrasubstituted compounds, the freedom of rotation about the bipyridyl bond in compound B increases its symmetry relative to compound C, thereby decreasing the size of libraries produced with core B. The calculations of library sizes for several core molecules can be found in the Appendix.

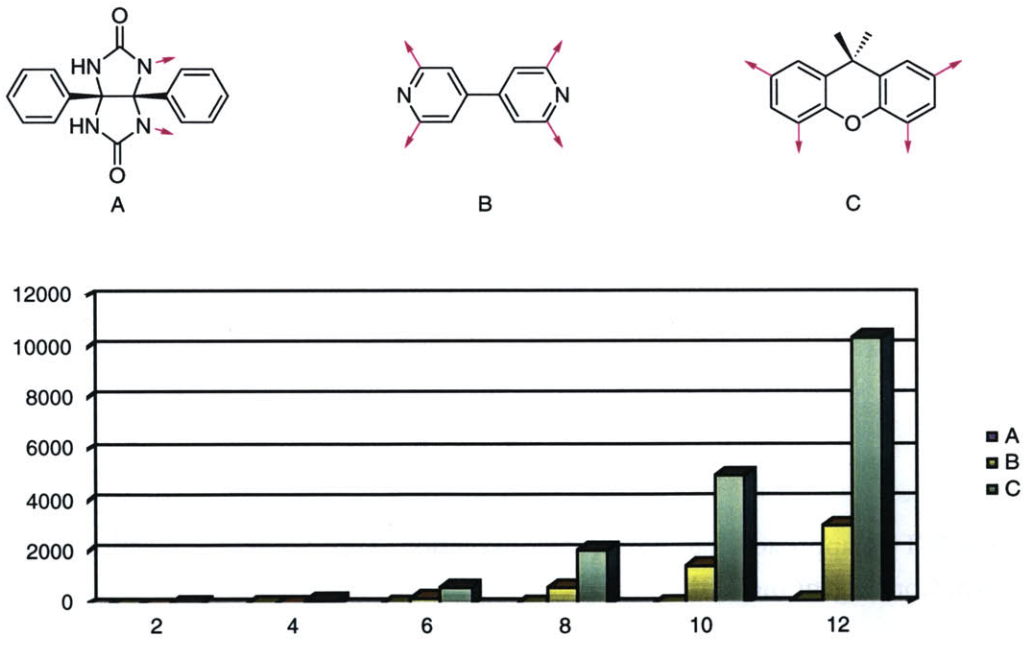

Figure 3. A graph of library size vs. number of building blocks for three different core molecules.

For any given core molecule, methodology to produce chemical libraries in a clean, one-pot reaction must be developed in conjunction with methodology to produce any arbitrary single member of a library with building blocks installed in a particular substitution pattern. Two different approaches to this problem suggest themselves (see Figure 4). One method is by the incorporation of orthogonal protecting groups at the various substiution points and building up individual compounds in a stepwise reaction/deprotection scheme. Another method is to take a convergent approach wherein the building blocks are installed on a partial core molecule and in a final step the core molecule is assembled with all of its building blocks already attached in their proper positions. Examples of both of these methods will be described in subsequent chapters.

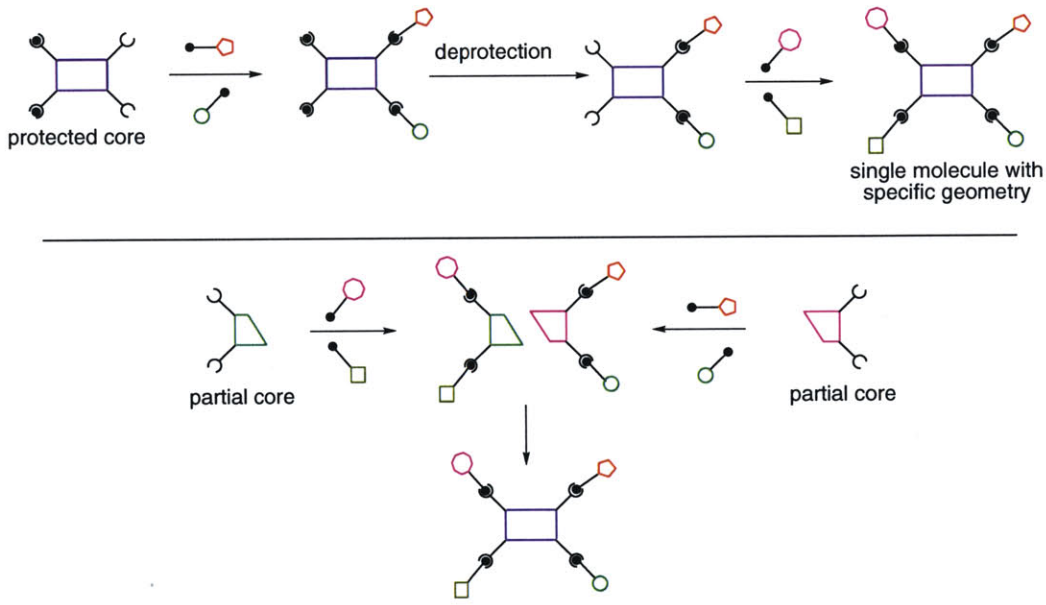

Figure 4. Two approaches to library deconvolution. Top: Sequential reaction/deprotection. Bottom: Convergent method.

A recent new direction in core molecule design within this research group has been the development of large platforms with convergent functional groups (see Figure 5).[37] The advantage of such systems lies in presenting the diversity on a well-defined surface.

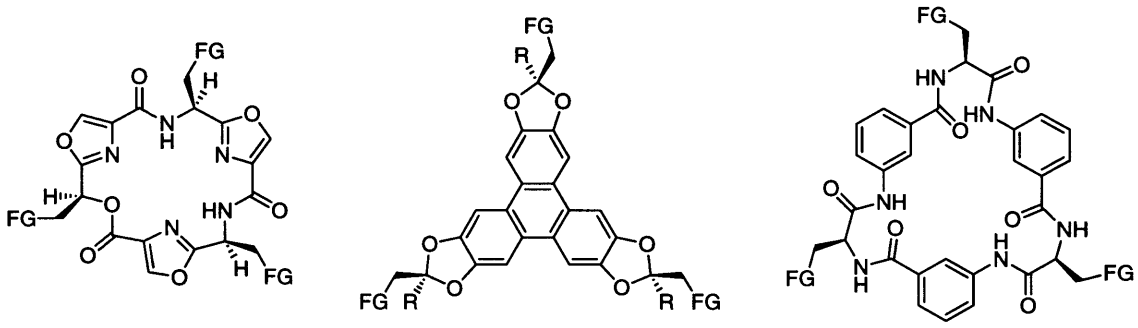

Figure 5. C_3 and C_{3v} symmetric platforms developed with potential application as core molecules for combinatorial chemistry.

The linker

The linker chemistry between the core and the building blocks is another key consideration for designing libraries using the activated core approach. In a perfect world, the linker chemistry would provide linkages between building blocks and core in 100% yield and absolute purity, as well as being robust under building block deprotection conditions, any conceivable screening conditions, and prolonged storage. We have tried to approximate this as closely as possible, and have made use of three types of linker chemistry in particular, as shown in Figure 6.

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[37] (a) Mink, D.; Mecozzi, S.; Rebek, J., Jr. *Tetrahedron Lett.* **1998**, *39*, 5709–5712. (b) Waldvogel, S. R.; Wartini, A. R.; Rasmussen, P. H.; Rebek, J., Jr. *Tetrahedron Lett.* **1999**, *40*, 3515–3518. (c) Rasmussen, P. H.; Rebek, J., Jr. *Tetrahedron Lett.* **1999**, *40*, 3511–3514.

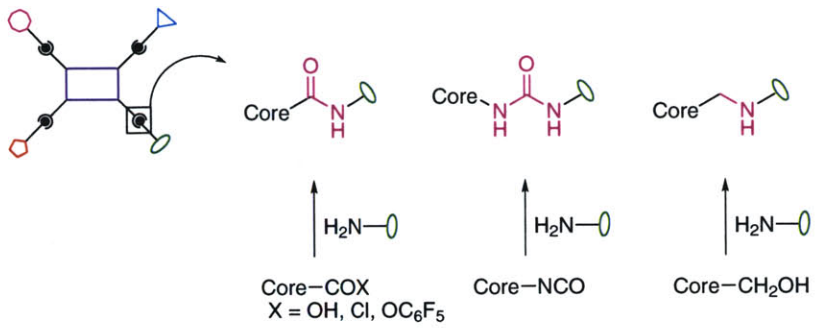

Figure 6. The choice of core molecule functionality provides different linker possibilities. Amides, ureas, and amines are accessible by the reaction of amines with activated acids, isocyanates, or methyl alcohols, respectively.

The formation of amides by the reaction of amines with core acid chlorides has already been discussed. We have since extended amide formation to make use of core pentafluorophenyl esters rather than acid chlorides in cases where core functionality would itself be incompatible with acid chlorides (see Chapter 5). The formation of ureas was first introduced by the dealcoholation of core carbamates followed by addition of amine building blocks,[38] but a more recent refinement involves the direct synthesis, isolation, and reaction of core isocyanates (see Chapter 2). Finally, methyl amines, or reduced amides, are available through the reaction of core methyl alcohols with amines protected as sulfonamides[39] in a Mitsunobu reaction[40] (see Chapter 6).

The building blocks

The building blocks we use are chosen for a variety of reasons. Availability, compatibility with intended linker chemistry, and diversity in structure are perhaps the most important of these. In general, we have stayed with variously-substituted primary amines (see Figure 7), which satisfy all of the above requirements. A wide variety of amines, including variously-protected amino acids, are commercially available, providing us with a ready supply

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[38] Shipps, G. W., Jr.; Spitz, U. P.; Rebek, J., Jr. *Bioorg. Med. Chem.* **1996**, *4*, 655–657.

[39] Fukuyama, T.; Cheung, M.; Jow, C.-K.; Hidai, Y.; Kan, T. *Tetrahedron Lett.* **1997**, *38*, 5831–5834.

[40] (a) Mitsunobu, O.; Wada, M.; Sano, T. *J. Am. Chem. Soc.* **1972**, *94*, 679. (b) Mitsunobu, O. *Synthesis* **1981**, 1.

of different chemical structures. Additionally, the strong nucleophilicity of amines facilitates amide and urea formation, and the ready conversion of amines to sulfonamides suitable for use in the Mitsunobu reaction allows an extension of the amine building block utility. In certain cases, ethanol has been used as a building block to provide an unsubstituted position in the final molecules (see Chapters 3 and 4).

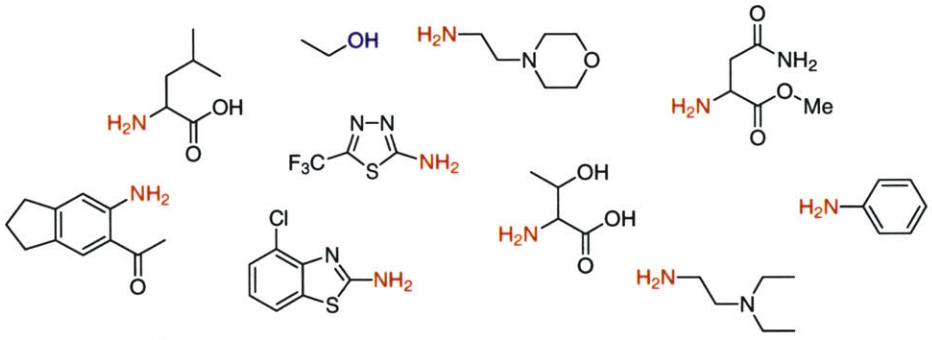

Figure 7. A small sampling of building blocks applied in the activated core approach.

1.6 The screening of libraries

While the development of chemistry related to the production of libraries by the activated core approach has been a primary focus of our research efforts, the synthesis of libraries and the evaluation of their biological activity has been an important second step. Libraries based on the bipyridine core molecule with four acid chloride reaction points have shown activity in inhibiting the protein-protein interaction between tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) (see Chapter 4). Several rounds of screens identified certain crucial building blocks, and we subsequently discovered that we could get increased activity by making use of a partially-hydrolyzed tetraacid chloride core molecule, thereby introducing free carboxylic acid groups into our polyamide libraries. To date, we have found two compounds which have been shown to be among the most active compounds ever identified for the inhibition of the t-PA/PAI-1 interaction.

Other current projects include the development of new methods to screen libraries of small molecules against RNA targets (see Chapter 3) and to rapidly identify small molecule *exo*-protease inhibitors (see Chapter 7). Using an LC-MS system to identify the individual components of the library, our collaborators intend to use columns with the RNA bound to beads to separate compounds of interest from the library. We will attempt to show that by using multiple columns with different RNA molecules attached to the beads, we can select molecules form a library that bind to the RNA of interest both tightly and specifically.

On the protease front, we have developed a two-phase assay system to determine the residual enzyme activity in the presence of an inhibitor candidate by following the migration of an organic-soluble fluorescent dye from the aqueous phase, where it is covalently bound to an enzyme substrate and does not fluoresce, into a layer of mineral oil upon enzymatic cleavage from the substrate. By using a very small amount of mineral oil relative to the volume of the aqueous phase, the dye can be concentrated many-fold, substantially increasing the sensitivity of the assay system. The result is an assay that can easily identify inhibitors by visual inspection, and simple extensions of the process allow quantitation of the inhibitory activity by fluorescence spectroscopy.

Chapter 2. Xanthene Di- and Tetraureas *via* isocyanates [1]

2.1 Introduction to xanthene tetraureas

A number of considerations, including synthetic access, versatility,[2] lack of apparent toxicity[3] and attractive physical properties make the tetrasubstituted xanthene (Figure 1) a desirable core molecule. In the latter regard, xanthenes have a strong UV chromophore for easy detection and are soluble in chlorinated organic solvents but nearly insoluble in hexanes/ether mixtures. This differential solubility allows for crude purifications following library synthesis using liquid-liquid extractions and, after deprotection, by precipitation from ether/hexane. Finally, the four sites for reaction on the xanthene core allow a large number of compounds to be formed using only a limited number of building blocks (eight building blocks give 2,080 compounds while 16 building blocks give 32,896 compounds).

The xanthene tetraureas (Figure 1) present an electron-rich aromatic scaffold capable of intercalation into DNA[4] and forming π–π stacks with aromatic side chains of proteins. The thickness of the 9,9-dimethyl center may disfavor some of these binding modes, but the ureas at the 4 and 5 positions provide a cavity (see Figures 1 and 2) which is known to bind carboxylates strongly.[5] In addition, all four ureas provide numerous hydrogen bonding donor and acceptor sites for molecular recognition. Molecular modeling calculations[6] were used to

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[1] Portions of this chapter have been previously published and are being used with permission from the National Academy of Sciences. Shipps, G. W., Jr.; Pryor, K. E.; Xian, J.; Skyler, D. A.; Davidson, E. H.; Rebek, J., Jr. *Proc. Natl. Acad. Sci.* **1997**, *94*, 11833–11838. Much of the work in this chapter was undertaken in collaboration with Dr. Gerald Shipps, and also appears in his Ph.D. thesis. Shipps, G. W., Jr. *Ph. D. Thesis* **1996**, Massachusetts Institute of Technology, Cambridge.

[2] Shimizu, K. D.; Dewey, T. M.; Rebek, J., Jr. *J. Am. Chem. Soc.* **1994**, *116*, 5145–5149.

[3] Birman, V. B.; Chopra, A.; Ogle, C. A. *Tetrahedron Lett.* **1996**, *37*, 5073–5076.

[4] Dugas, H. *Bioorganic Chemistry* **1996**, Springer, New York.

[5] Hamann, B. C.; Branda, N. R.; Rebek, J., Jr. *Tetrahedron Lett.* **1993**, *34*, 6837–6840.

[6] Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hedrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440–467.

minimize a few tetraurea structures and revealed a planar, disc-shaped presentation of the building blocks (see Figure 1).

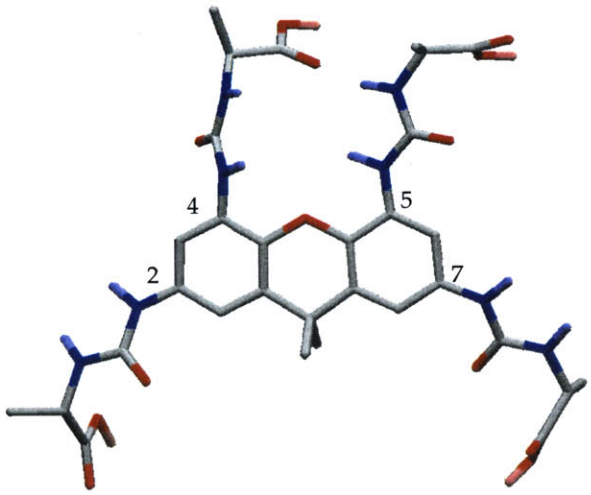

Figure 1. A polytube rendering of an MM2* minimized tetraalanine tetraurea. Several contact surfaces are available, including the 2,4; 4,5; 5,7; and 2,7.

Finally, the use of the urea linkage conformationally limits the building blocks, as rotation about the urea N—C bond and the core-carbonyl bonds at the 4 and 5 positions are restricted. For example, the solution and solid-state structure of a 4,5-diurea xanthene has been determined (Figure 2).[7] These studies showed that the urea N—H groups are directed inward (toward the xanthene oxygen) and create a binding pocket between them. The carbonyl oxygens are directed outward and are available for intermolecular hydrogen bonding. This preorganization reduces the entropic cost of binding to target molecules.

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[7] Hamann, B. C. *Ph. D. Thesis* **1996**, Massachusetts Institute of Technology, Cambridge.

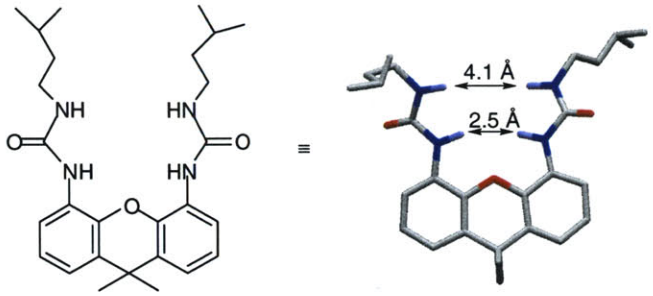

Figure 2. A polytube rendering of the x-ray crystal structure of a xanthene diurea.[7] Note the orientation of the ureas. Nuclear Overhauser effect NMR studies suggest a similar solution phase structure.[5]

2.2 Background—B-chlorocatecholborane usage in urea formation

Xanthene tetraacid **1**, previously used as its acid chloride as a core molecule for combinatorial polyamide synthesis,[8] was taken as the starting point for building combinatorial urea libraries, as well. In an initial approach, Shipps, *et al.*,[9] made use of a procedure introduced by Valli and Alper[10] in which a carbamate (urethane) is subjected to B–chlorocatecholborane-induced dealcoholysis to yield an isocyanate *in situ*, followed by addition of an amine to provide the final urea. This scheme required a xanthene tetracarbamate as the core molecule, and thus xanthene tetraethylcarbamate **2** was synthesized in a two-step, one-pot reaction from tetraacid **1** (Scheme 2).

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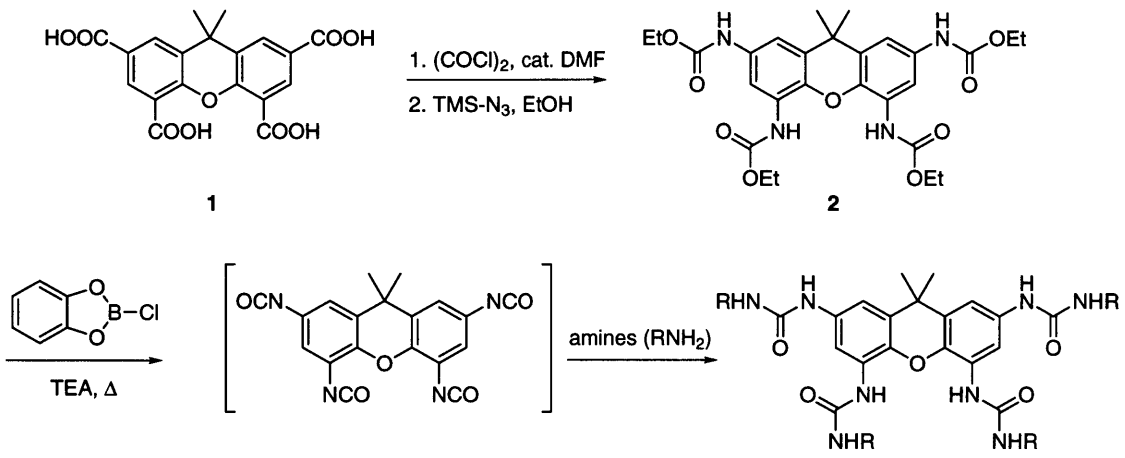
[8] Carell, T.; Wintner, E. A.; Rebek, J., Jr. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2061–2064.

[9] Shipps, G. W., Jr.; Spitz, U. P.; Rebek, J., Jr. *Bioorg. Med. Chem.* **1996**, *4*, 655–657.

[10] Valli, V. L. K.; Alper, H. *J. Org. Chem.* **1995**, *60*, 257–258.



Scheme 1. Synthesis of tetraurea xanthenes using B-chlorocatecholborane.

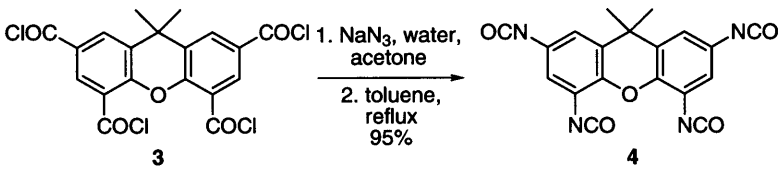
2.3 Isolation of isocyanates

Xanthene tetraisocyanate

While the B-chlorocatecholborane approach to the synthesis of xanthene polyureas had its utility, it also suffered from a number of drawbacks, including sensitivity of the key reagent to air and moisture, difficulty in reaction workup, and dependence on multiple reaction steps for each point of diversity in the core. Isolation of the core isocyanates was advantageous for a number of reasons. The condensation reaction would be reduced merely to the addition of free-based amines directly to the activated core, and a simple aqueous acid wash would be sufficient workup. Additionally, if the polyisocyanates could be isolated and purified, the final purity of the libraries could be improved since the total number of reactions per site would be limited to one.

Given these considerations, we undertook studies to try and isolate the xanthene tetraisocyanate. A successful synthesis thereof is shown in Scheme 2. We introduced an alternative method to synthesize the requisite xanthene tetraacyl azide from tetraacid

chloride **3**,[8] making use of aqueous sodium azide in acetone,[11] which allowed the intermediate to be cleaned by extraction prior to the Curtius rearrangement. This next step was carried out in refluxing toluene without isolating the potentially-explosive tetraacyl azide, yielding tetraisocyanate **4** in overall 95% yield.



Scheme 2. Synthesis of the xanthene tetraisocyanate core molecule.

The tetraurea libraries were then synthesized by reacting the tetraisocyanate core molecule simultaneously with a mixture of amines in a single reaction vessel. Simple liquid–liquid extraction (dichloromethane–1 N citric acid) eliminated excess amines after the reaction, then the libraries were treated with neat TFA to cleave the acid-labile protecting groups. This deprotection liberates hydrophilic functions and enhances solubility in aqueous solutions for screening. Following deprotection, the libraries could be precipitated by the addition of ether/hexanes (1:1); filtration allowed removal of the soluble remnants of the amino acid side-chain protecting groups and isolation of the tetraureas.

Xanthene diisocyanate

Because the xanthene 4 and 5 positions are close in space, steric interactions between a building block attached at one of these positions and a nucleophile attacking at the other could introduce concentration biases during library synthesis. Previously-published methods[9] were used to verify that statistical mixtures of compounds were indeed formed in the libraries. Accordingly, the 4,5-xanthenediisocyanate **7** was synthesized (Scheme 3), and was used to synthesize diureas for HPLC and CE-MS analysis. The results of these diurea studies

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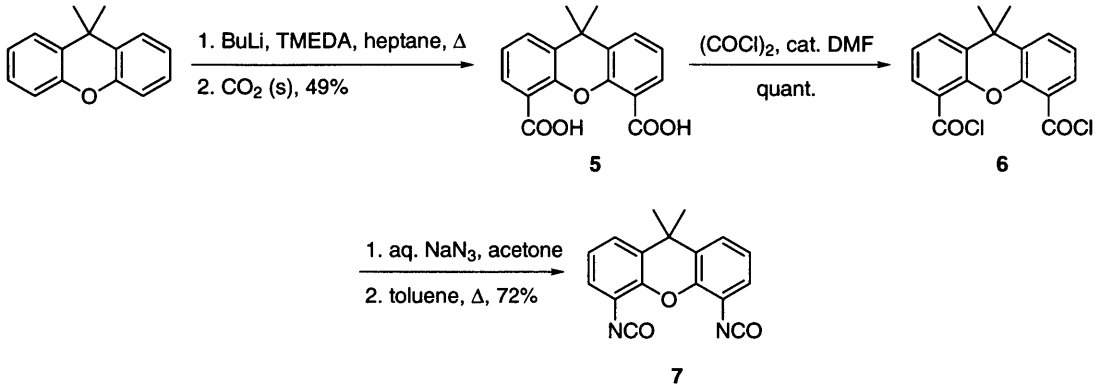
[11] Capson, T. L.; Poulter, C. D. *Tetrahedron Lett.* **1984**, *25*, 3515–3518.

supported the claims of predictable, approximately statistical product distributions. Diisocyanate **7** proved to be a such a versatile intermediate that it was used to synthesize diureas for studies in molecular recognition reported elsewhere.[7]



Scheme 3. Synthesis of the xanthene diisocyanate core molecule.

Xanthene diacid **5** was prepared by the dilithiation of 9,9-dimethylxanthene[12] followed by quenching the dilithio species with dry ice, and was then converted into diacid chloride **6** with oxalyl chloride. The diacyl azide was formed *in situ* from the diacid chloride with aqueous NaN_3 in acetone, and was converted to the diisocyanate **7** in a manner analogous to tetraisocyanate **4** above.

Adamantane tetraisocyante

While the study of the xanthene polyisocyanates was underway, a revisiting of the tetrasubstituted adamantane core[13] was also considered. Fellow graduate student Gerald Shipps had previously made use of adamantane tetraacid chloride **8** as a core molecule for tetraamide libraries. An extension of this core to tetraisocyanate **9** for the production of tetraurea compounds was successful by the application of similar methodology to the xanthene case (Scheme 4).

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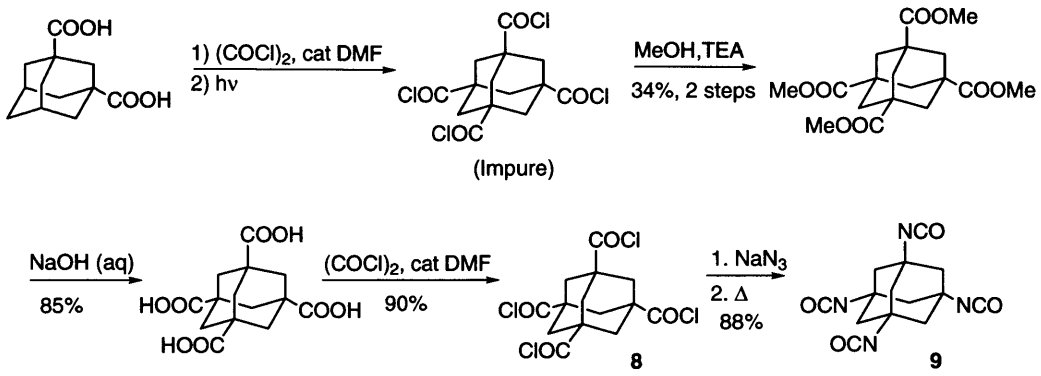
[12] Hillebrand, S.; Bruckmann, J.; Krüger, C.; Haenel, M. W. *Tetrahedron Lett.* **1995**, *36*, 75–78.

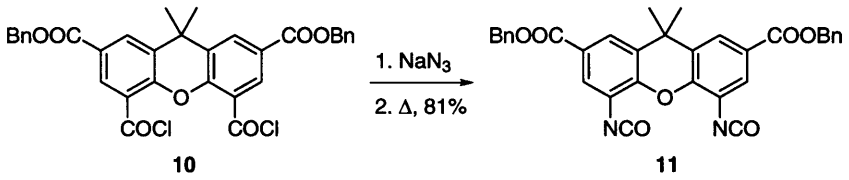
[13] Shipps, G. W., Jr. *Ph. D. Thesis* **1997**, Massachusetts Institute of Technology, Cambridge.



Scheme 4. Synthesis of adamantane tetraisocyanate **9**.

While the adamantane tetraisocyanate was well-behaved chemically, disadvantages to tetrasubstituted adamantane libraries, such as the large volume of the tetrafunctionalized core and particularly the inaccesibility of differently-substituted derivatives suitable for deconvolution, discouraged further use of the core.

2.4 Deconvolution of xanthene tetraurea libraries



Scheme 5. Synthesis of a site-differentiated xanthene intermediate for the deconvolution of tetraurea libraries.

The development of a method for obtaining unsymmetrical tetraureas is shown in Scheme 5. The dibenzylester diacid chloride **10**[14] served as a precursor to a urea-based core structure which distinguished the "top" two sites from those at the bottom. The benzyl ester protecting groups are easily removed using hydrogenolysis,[15] a procedure compatible with most peptide protecting groups. The diacid chloride **10** was converted to the diacyl azide

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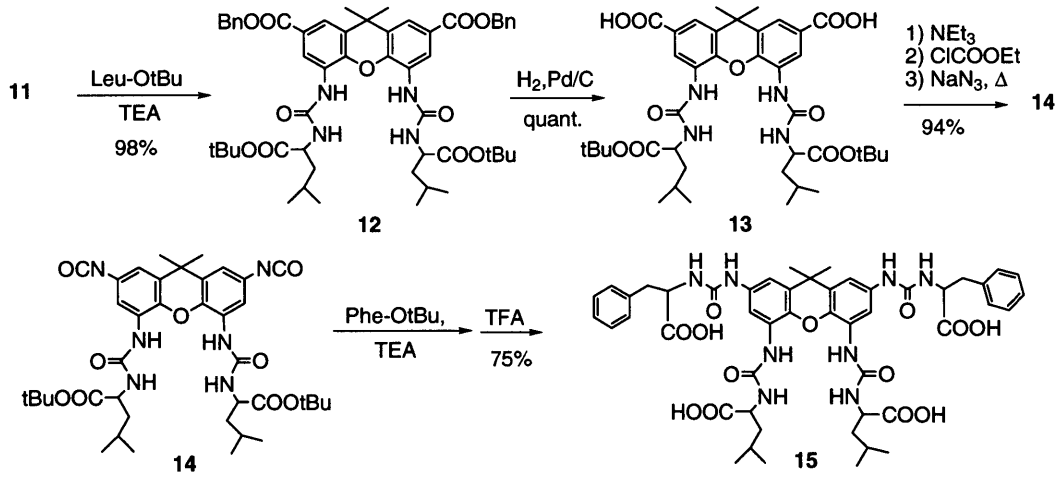
[14] Carell, T.; Wintner, E. A.; Sutherland, A. J.; Rebek, J. Jr.; Dunayevskiy, Y. M.; Vouros, P. *Chem. Biol.* **1995**, *2*, 171–183.

[15] Greene, T. W.; Wuts, P. G. M. *Protective Groups in Organic Synthesis* **1991**, Wiley, New York.

using sodium azide in acetone as above and then heated to give the diisocyanate dibenzyl ester **11**.

Xanthene **11** could then be reacted with one or more amines, depending upon the stage of the deconvolution sequence (Scheme 6). If one (in the case shown, Leu *t*-butyl ester) or two different amines are condensed with **11** a separation can be performed to give purified diureas **12**. Hydrogenolysis cleaves the benzyl ester groups and gives the diacid diurea **13**. Activation of the acid groups as acid chlorides proved, unfortunately, to be incompatible with acid-sensitive functionality on the protected amino acids (i.e., the *t*-butyl esters), or the urea function itself, for that matter. Instead, the diacid was converted to the mixed anhydride with ethyl chloroformate using triethylamine as the base in acetone (Scheme 6). Without purification, the mixed anhydride was treated with sodium azide to furnish the diacyl azide (22). Rearrangement was effected by heating the diacyl azide in toluene, and the diisocyanate dileucine diurea **14** was obtained. Then, one (Phe *t*-butyl ester in the case of **15**) or more amines could be reacted with **14** to give the desired tetraurea(s).



Scheme 6. Synthesis of a tetraurea using the deconvolution protocol.

2.5 Identification of xanthenes active in binding to DNA

Xanthene libraries prepared both with the B-chlorocatecholborane method and, after its discovery, the xanthene tetraisocyanate method were assayed for activity in the inhibition of DNA/Zn-finger transcription factor complex formation. The assays were performed by collaborators in the Division of Biology at CalTech, and the library preparation and deconvolution was performed primarily by coworkers of the author. A detailed description of this particular project can be found elsewhere,[1] and the results are summarized here. From five initial libraries of 2080 compounds each, two compounds (Figure 3) were shown to be active in binding to DNA at 5- to 10-μM concentration. The selection process that identified two active compounds from a pool of approximately 10,000 required only about 20 assays, and is therefore one of the most efficient procedures in that regard.

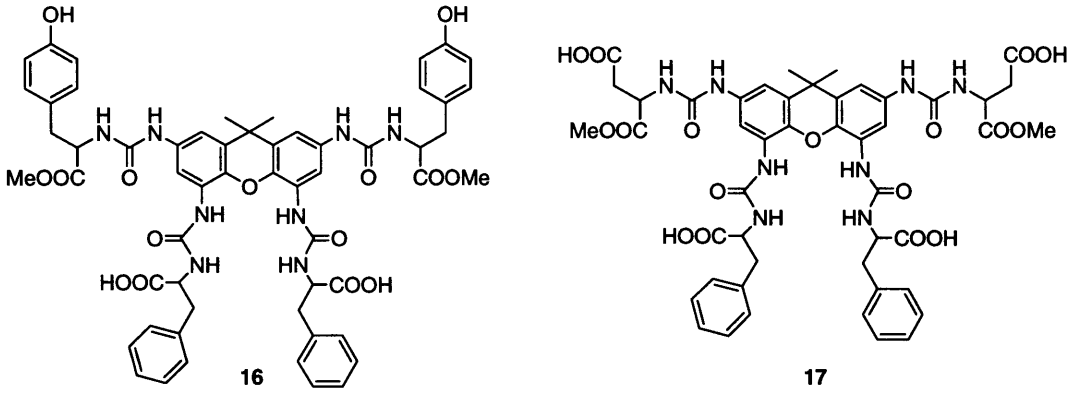


Figure 3. Structures of the two xanthene tetraureas active in binding to DNA.

2.6 Experimental

General

All reagents were purchased from Aldrich Chemical Company and were used without further purification except as noted. Amino acid esters, PyBOP, and EDC•MeI were acquired from Novabiochem (San Diego, CA). Deuterated solvents were obtained from

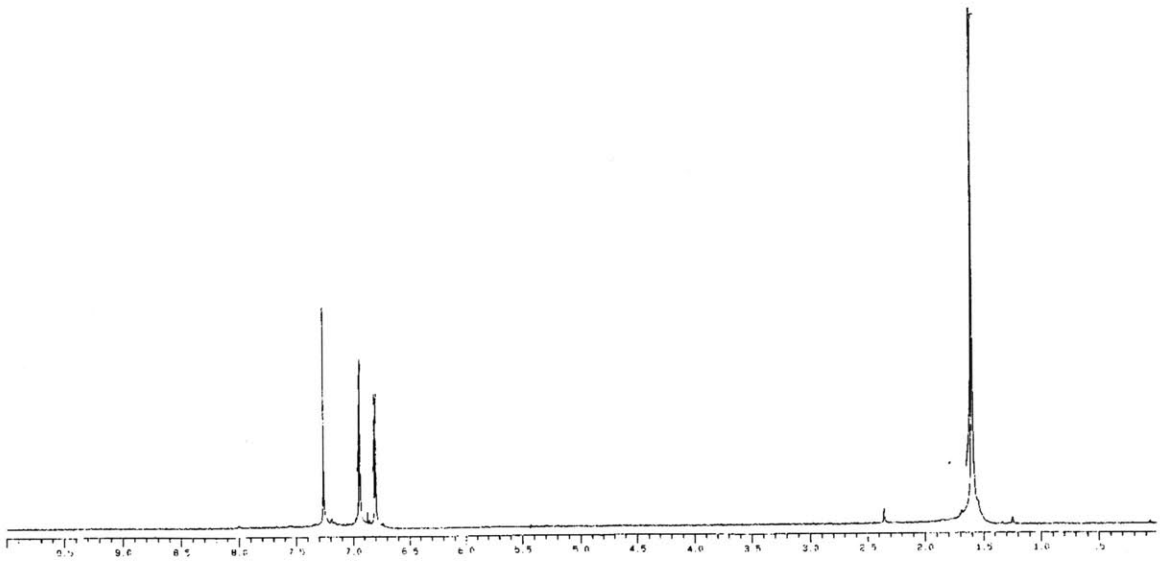
Cambridge Isotopes Laboratories and deuterated chloroform was dried over 4Å molecular sieves. HCl refers to a 1N stock solution. NMR spectra were recorded on either a Bruker AC-250, a Bruker AM-300, or a Bruker DRX-600; TMS was used as a reference in some chloroform-*d* spectra, otherwise residual solvent was used as a reference. Either a Finnegan Mat 8200 (for EI) or a VG ZAB-VSE (for FAB) mass spectrometer was used to ascertain masses. FT-IR spectra were obtained on a Perkin Elmer Paragon 1000 PC FT-IR Spectrometer. Silica gel chromatography was performed with Silica Gel 60 (EM Science or Bodman, 230–400 mesh). TLC analysis was performed using glass-bound Silica Gel 60 (F254) plates. DMAP = *N*,*N*-dimethyl-4-aminopyridine, DPPA = diphenylphosphoryl azide, EDC•MeI = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide, PyBOP = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate.

HPLC

For tetraester tetraureas (before deprotection) a C18 reverse-phase column (Rainin Microsorb-MV, 4.6 x 250 mm, 5 μm, 100 Å) was used for HPLC analysis with a flow rate of 1 mL/min. A linear gradient of 100% water to 100% MeCN over 30 min with a total run time of 45-50 min was used for most runs. The detection wavelength was 268 nm (λ_{max} for the xanthene tetraurea fragment), and the extinction coefficients of the tetraureas were assumed to be equal. After deprotection a C8 reverse-phase column (Waters Symmetry, 3.9 x 150 mm, 5 μm, 100 Å) was used employing similar gradients and a 1 mL/min flow rate.

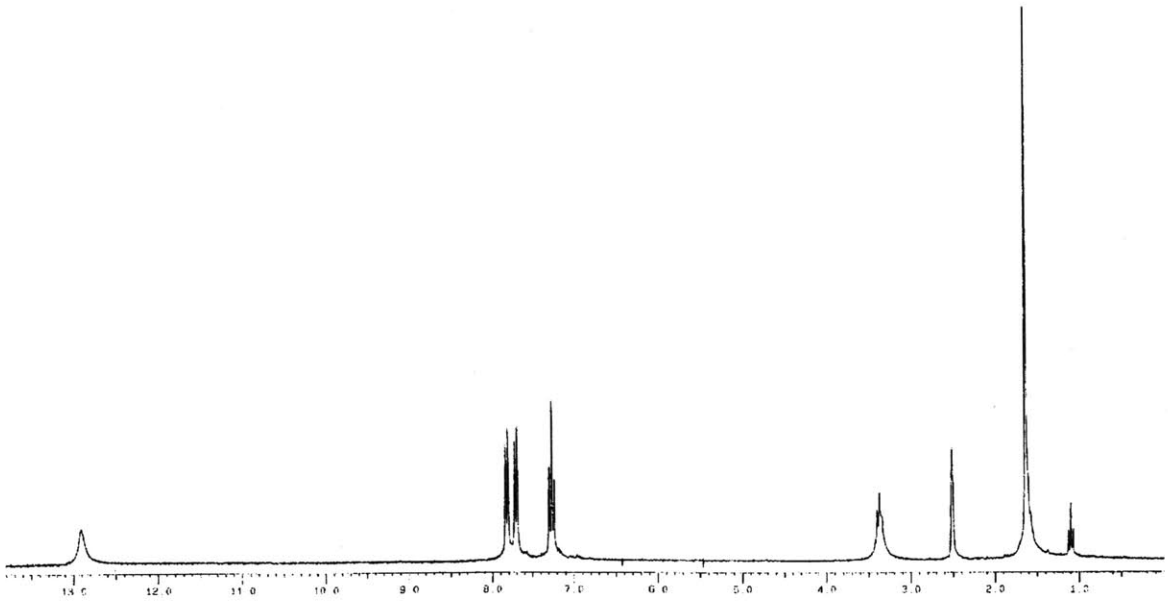
2,4,5,7-Tetraisocyanato-9,9-dimethylxanthene (4).

To a solution of tetraacid chloride **3** (0.40 g, 0.76 mmol) in dry acetone (20 mL) at 0 °C was added a solution of sodium azide (0.81 g, 12.5 mmol) in 2 mL water. A precipitate immediately formed, and the mixture was stirred for 30 min. The resulting slurry was extracted with methylene chloride (2 x 100 mL), and the organic layer was dried over $MgSO_4$, filtered, and concentrated to about one-tenth its original volume. Toluene (20 mL) was added, and the remaining methylene chloride was evaporated. The toluene solution was refluxed for 1.5 h under argon, cooled to rt, then filtered through a fine frit to remove traces of suspended solids. Concentration of the solution afforded **4** (0.270 g, 95%) as a beige solid. mp 173–176°C. ^{1}H NMR (250 MHz, $CDCl_3$): δ 6.93 (d, J = 2.4 Hz, 2H), 6.79 (d, J = 2.4 Hz, 2H), 1.59 (s, 6H). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 141.54, 131.25, 129.16, 126.98, 124.72, 123.26, 119.82, 119.46, 35.13, 31.04. HRMS (EI) m/z calcd for $[C_{19}H_{10}N_4O_5]^{+\bullet}$ 374.0651 found 374.0643.



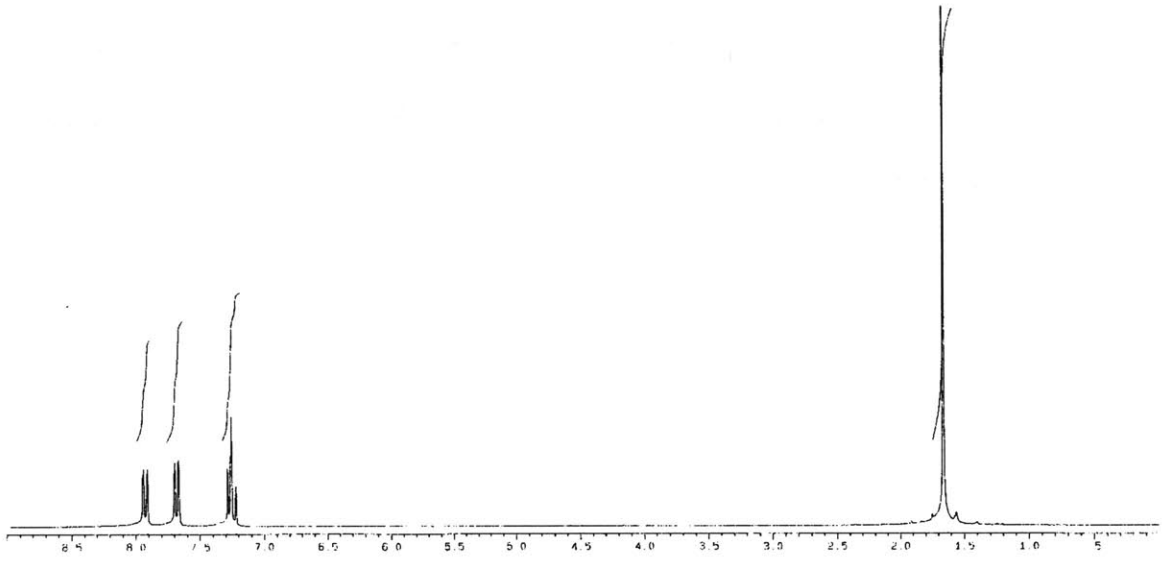
9,9-Dimethyl-4,5-xanthene dicarboxylic acid (**5**).

9,9-Dimethylxanthene (2.00 g, 9.5 mmol), dry heptane (100 mL), and TMEDA (3.5 mL, 24 mmol) were combined and degassed with Ar for 15 min before *n*-BuLi (1.6 M in hexane, 15 mL, 24 mmol) was added. The solution was warmed to reflux under Ar for 15 min, then allowed to cool to rt. The reddish solution was canulated onto a large excess of dry ice, allowed to stand for 1 h, then EtOAc and 2 M HCl were added until most of the solids dissolved and the pH = 1. The layers were separated and the aqueous layer was extracted twice with EtOAc:THF (20:1). The combined organic layers were rinsed once with brine and dried over Na_2SO_4. Upon concentration a white solid precipitated. The flask was then cooled to 0 °C, and **5** (1.39 g, 49%) was isolated asa slightly yellow powder upon filtration. Additional material (~0.2 g) could be recovered from the orange filtrate but contained about 15% of an impurity. m.p. 248–249 °C. ^{1}H NMR (250 MHz, DMSO-d_6): δ 12.9 (br. s, 2H), 7.80 (dd, J = 7.8, 1.7 Hz, 2H), 7.69 (dd, J = 7.7, 1.5 Hz, 2H), 7.23 (t, J = 7.8 Hz, 2H), 1.61 (s, 6H). ^{13}C NMR (62.9 MHz, DMSO-d_6): δ 166.19, 147.72, 130.72, 130.40, 129.45, 123.57, 119.93, 33.85, 31.94. HRMS (EI) m/z calcd for $[C_{17}H_{14}O_5]^{+\bullet}$ 298.0841 found 298.0839.



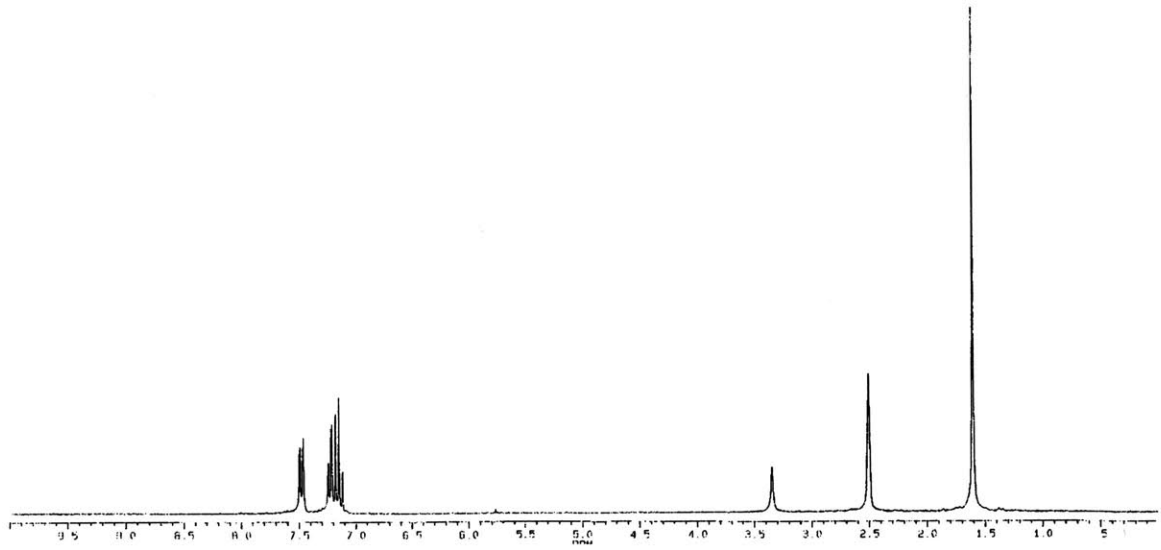
9,9-Dimethyl-4,5-xanthene dicarboxylic acid chloride (**6**).

To the suspended diacid **5** (0.75 g, 0.25 mmol) at 0 °C in ethanol-free chloroform (100 mL) was added oxalyl chloride (4.0 mL, 46 mmol) and DMF (5 μL). Within 15 min a clear solution was evident and the contents were warmed to reflux under a drying tube for 1 h. The solution was evaporated to dryness, 5 mL dry toluene was added, and the mixture was again evaporated, yielding **6** (0.84 g, quant.) as a pale yellow solid. m.p. 144–146 °C. ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.92 (dd, J = 7.7, 1.5 Hz, 2H), 7.68 (dd, J = 7.9, 1.5 Hz, 2H), 7.25 (t, J = 7.8 Hz, 2H), 1.66 (s, 6H). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 163.55, 163.34, 131.57, 131.22, 131.11, 123.65, 34.41, 31.99. HRMS (EI) m/z calcd for $[C_{17}H_{12}Cl_2O_3]^{+\bullet}$ 334.0163 found 334.0160.



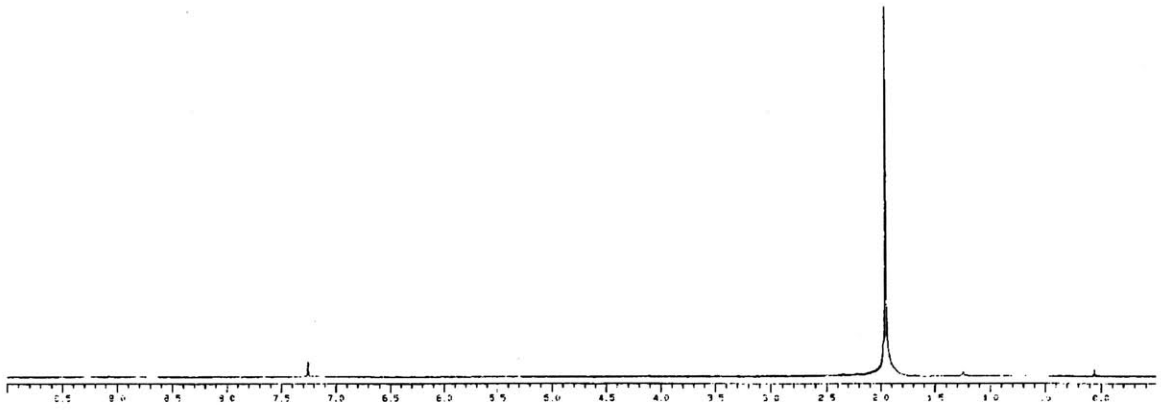
4,5-Diisocyanato-9,9-dimethylxanthene (7).

To a solution of **8** (0.594 g, 1.77 mmol) in dry acetone (25 mL) at 0 °C was added aqueous sodium azide (0.61 g, 9.38 mmol in 1.5 mL water). A fine precipitate formed immediately, and the mixture was stirred for 30 min. The resulting slurry was extracted with methylene chloride (2 x 100 mL) and the organic layer was dried over $MgSO_4$, filtered, and concentrated. Toluene (15 mL) was added, and the remaining methylene chloride was removed *in vacuo*. The toluene solution was refluxed for 1 h under argon before the solvent was removed. The residual oil was taken up in methylene chloride, and the solvent was again removed to yield a crystalline product (0.374 g, 72%). m.p. 83–86 °C ^{1}H NMR (250 MHz, DMSO-d_6, unstable for more than 1 h): δ 7.48 (dd, J = 7.8, 1.7 Hz, 2H), 7.23 (dd, J = 7.8, 1.7 Hz, 2H), 7.14 (t, J = 7.8 Hz, 2H), 1.60 (s, 6H). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 143.85, 130.95, 126.72, 123.67, 123.30, 123.30, 122.13, 34.63, 33.29. HRMS (EI) m/z calcd for $[C_{17}H_{12}N_2O_3]^{+\bullet}$ 292.0848 found 292.0852.



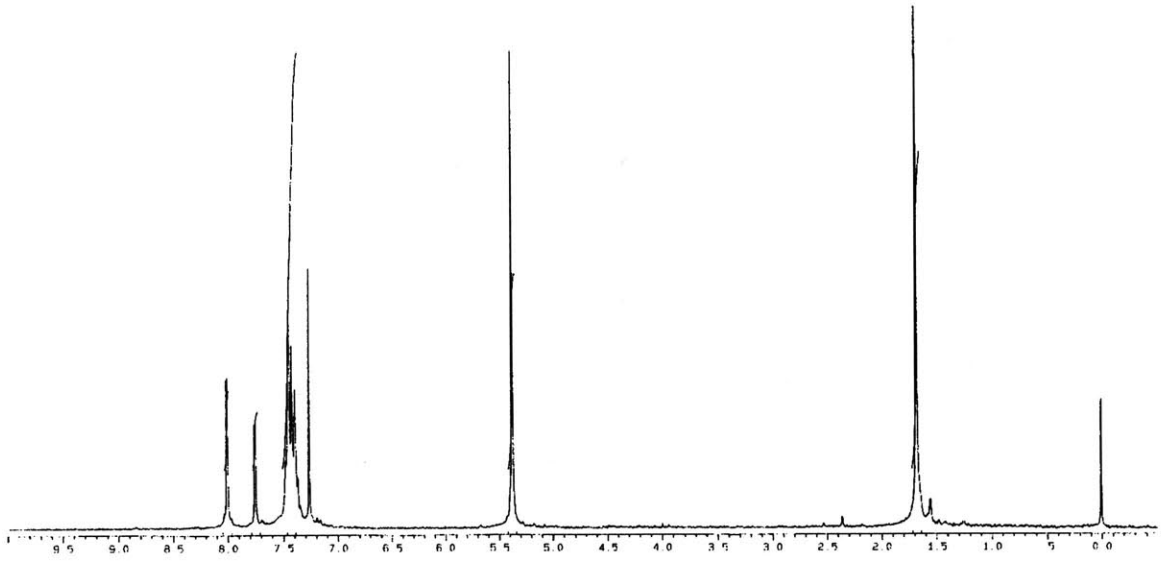
1,3,5,7-Tetraisocyanato adamantane (9).

Acetone (7 mL) was used to dissolve adamantane tetraacid chloride **9** (120 mg, 0.31 mmol), and the flask was cooled to 0 °C. Sodium azide (400 mg in 1 mL water, 6.2 mmol) was added dropwise and the suspension was stirred for 30 min at 0 °C. Methylene chloride (25 mL) and water were added, the layers were separated and the aqueous layer was extracted with more methylene chloride (3 x 25 mL). The organic layer was dried over $MgSO_4$ and concentrated to near dryness. Toluene (10 mL) was added and the solution was concentrated until the toluene began to evaporate. The solution was warmed to reflux for 1 h and bubbling was observed at 60 °C. Evaporation yielded a solid which was taken up in methylene chloride, filtered through glass wool, and concentrated to yield a **9** (83 mg, 88%) as a slightly yellow powder. ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.95 (s). ^{13}C NMR (62.9 MHz, CDCl3): δ 49.98, 55.30, 124.22. HRMS (EI) m/z calcd for $[C_{14}H_{12}N_4O_4]^{+\bullet}$ 300.0859 found 300.0858.



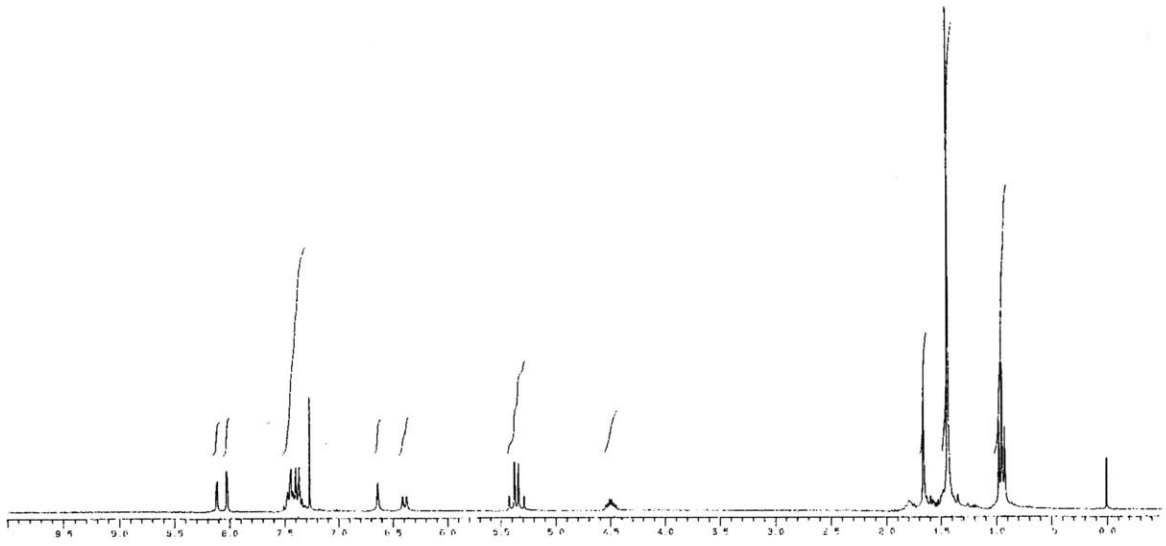
Dibenzyl 4,5-diisocyanato-9,9-dimethyl-2,7-xanthene dicarboxylate (11).

Dibenzyl ester diacid chloride **10** (325 mg, 0.539 mmol) was added to acetone (20 mL) and sonicated for 2 min. After cooling to 0 °C, sodium azide (285 mg, 4.39 mmol, in 0.8 mL water) was added dropwise with brisk stirring. A precipitate formed immediately and the suspension was stirred for 30 min before being poured into a separatory funnel containing methylene chloride (125 mL) and water (25 mL). The layers were separated, the water layer extracted with methylene chloride, and the hazy organic layer rinsed once with brine, dried over $MgSO_4$, and partially concentrated. Toluene was added and the remaining methylene chloride was evaporated. The solution was then warmed to near reflux for 1 h. The solution was concentrated to a yellow foam, yielding **11** (375 mg, 81%). ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.00 (d, J = 1.8 Hz, 2H), 7.75 (d, J = 1.7 Hz, 2H), 7.44-7.39 (m, 10 H), 5.37 (s, 4H), 1.68 (s, 6H). ^{13}C NMR (62.9 MHz, CDCl3): δ 164.91, 146.51, 135.71, 130.62, 128.66, 128.42, 128.31, 126.88, 126.37, 125.29, 125.05, 122.68, 67.10, 34.88, 32.65. HRMS (EI) m/z calcd for $[C_{33}H_{24}N_2O_7]^+$ 560.15835 found 560.15822.



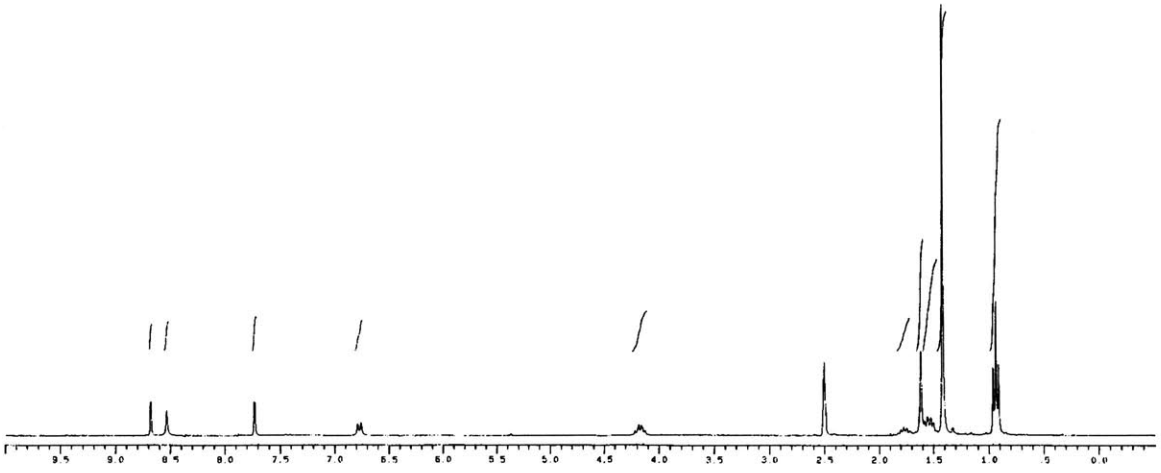
Dibenzyl 4,5-dileucine-OtBu diurea-9,9-dimethyl-2,7-xanthene dicarboxylate (12).

To a solution of dibenzyl ester diisocyanate **11** (0.302 g, 0.539 mmol) in methylene chloride (10 mL) was added Leu-O*t*Bu•HCl (0.265 g, 1.19 mmol) in methylene chloride (5 mL) containing TEA (0.20 mL, 1.4 mmol). After 1.5 h the reaction was diluted with methylene chloride (25 mL) and rinsed with citric acid. Concentration of the methylene chloride solution produced a **12** (0.481 g, 98%) as a yellow solid. ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.15 (s, 2H), 7.97 (d, J = 2 Hz, 2H), 7.47-7.27 (m, 12H), 6.45 (d, J = 7 Hz, 2H), 5.34 (appar q, J = 12 Hz, 4H), 4.47 (m, 2H), 1.75–1.65 (m, 4H), 1.62 (s, 6H), 1.43 (s, 18H), 1.42–1.33 (m, 2H), 0.93 (m, 12H).



4,5-Dileucine-OtBu diurea-9,9-dimethyl-2,7-xanthene dicarboxylic acid (13).

The dibenzyl ester diLeu **12** (350 mg, 0.47 mmol) was taken up in 4:1 EtOAc: ethanol (20 mL) and stirred under a H_2 (atm) for 12 h in the presence of 10% Pd/C (10 mg, cat). The solution was filtered through celite using ethyl acetate and concentrated to give **13** in quantitative yield as a yellow solid. ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.65 (d, J =1.8 Hz, 2H), 8.63 (br s, 2H), 7.71 (d, J = 1.8 Hz, 2H), 6.92 (d, J = 6.8 Hz, 2H), 4.14 (m, 2H), 1.8–1.44 (m, 6H), 1.66 (s, 6H), 0.95–0.89 (m, 12H). HRMS (FAB in NBA/CsI) m/z calcd for $[C_{39}H_{54}O_{11}N_4 + Cs]^+$ 887.2843 found 887.2853.



2,7-Diisocyanato-4,5-dileucine-OtBu diurea xanthene (14).

The diacid diLeu urea **13** (130 mg, 0.17 mmol) was dissolved in acetone (0.8 mL) and cooled to 0 °C; triethylamine (54 μL, 0.381 mmol) was added, followed by ethylchloroformate (40 μL, 0.38 mmol). After 20 min, sodium azide (50 mg, 0.76 mmol, in 0.2 mL water) was added and the emulsion was stirred for 25 min. Methylene chloride (20 mL) and water (10 mL) were added and the layers were separated. The organic layer was extracted (2 x 20 mL methylene chloride), rinsed with brine, and dried over $MgSO_4$. The organic layer was concentrated to near dryness, toluene (5 mL) was added, the remaining methylene chloride stripped off, and the solution refluxed for 1.5 h, with gas evolution beginning at 72 °C. Concentration of the solution gave **14** (122 mg, 94%) as a brown solid. ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.59 (d, J = 2 Hz, 2H), 7.50 (br. s, 2H), 6.79 (d, J = 2 Hz, 2H), 6.43 (d, J = 8.5 Hz, 2H), 4.44 (appar q, J = 5.5 Hz, 2H), 1.54 (s, 6H), 1.44 (s, 18H), 1.41–1.2 (m, 6H), 0.93, (d, J = 6.4 Hz, 12H).

^{1}H NMR spectrum not available.

2,7-Diphenylalanine-4,5-dileucine tetraurea xanthene (15).

To the diisocyanate **14** (30 mg, 0.040 mmol) in methylene chloride (5 mL) was added Phe-O*t*Bu•HCl (26 mg, 0.10 mmol) in methylene chloride (3 mL) with TEA (15 μL, 0.11 mmol). After 1 h at rt the clear solution was diluted with methylene chloride and rinsed with citric acid; concentration gave the tetra *t*-butyl ester of tetraacid **15** (40 mg, 83%) as a white solid. ^{1}H NMR (broad, 250 MHz, DMSO-d_6): δ 8.69 (s, 2H), 8.50 (br. s, 2H), 7.80 (d, J = 2 Hz, 2H), 7.31–7.19 (m, 12H), 6.85 (br. s, 2H), 6.21 (d, J = 8.4 Hz, 2H), 4.41 (appar q, J = 6 Hz, 2H), 4.15 (m, 2H), 2.96 (d, J = 6.3 Hz, 4H), 1.51–1.45 (m, 10 H), 1.39 (s, 18H), 1.35 (s, 18H), 1.28-1.22 (m, 2H), 0.91 (appar t, J = 6.5 Hz, 12H).

To the above tetra *t*-butyl ester (35 mg, 0.029 mmol) was added TFA (2 mL) and small bubbles evolved immediately. After stirring for 6 h under a drying tube the TFA was

evaporated to yield a yellow oil. Addition of a 1:1 solution of Et_2O:hexanes caused immediate precipitation. Drying of this solid *in vacuo* yielded **15** (25 mg, 90%) as a slightly yellow residue. ^{1}H NMR (broad, 250 MHz, DMSO-d_6): δ 8.67 (br. d, 2H), 8.38–8.33 (m, 2H) 7.93 (br. d, 2H), 7.31–7.19 (m, 10 H) 6.73 (m, 4H), 6.15 (d, J = 7.2 Hz, 2H), 4.44–4.42 (m, 2H), 4.18 (m, 2H), 2.95 (m, 2H), 1.75–1.51 (m, 6H), 1.47 (s, 6H), 0.92–0.85 (m, 12H). HRMS (EI) m/z calcd for $[C_{49}H_{58}N_8O_{13}]^{+\bullet}$ 966.4123 found 966.4114.

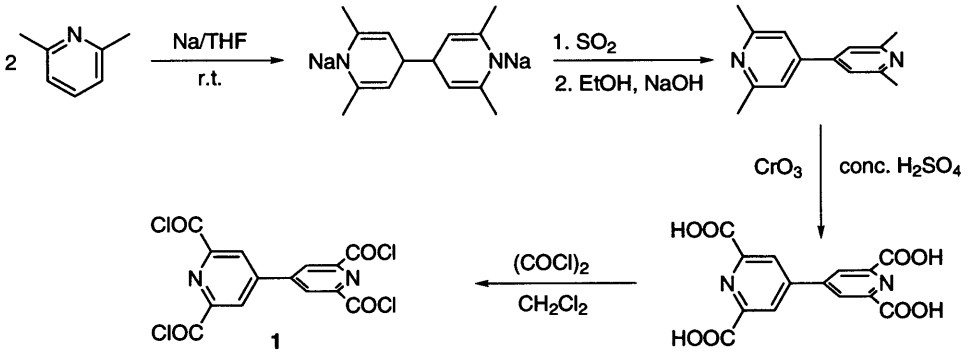
^{1}H NMR spectrum not available.

Chapter 3. The Bipyridine Core Molecule[1]

3.1 Synthesis and validation of the bipyridine core

While the substituents attached to the core molecule are intended to be the main sources of binding interactions, the incorporation of additional binding groups on the core molecule itself should increase the maximal activity of strongly-binding library components. One way this can be accomplished is to use core molecules that contain basic nitrogens that can be protonated or act as good hydrogen-bond acceptors in a complex with macromolecular targets. A review of relevant literature reveals that Hünig and co-workers synthesized a compound, 2,2',6,6'-(4,4'-bipyridine)tetraacid chloride **1**,[2] that meets this criterion; it was synthesized in this laboratory by similar procedures (Scheme 1). The ring nitrogens provide two more sites that can potentially increase the affinity of library members for binding partners.

Additionally, the ring nitrogens can be used to rigidify pendant groups by hydrogen bonding to amide protons in the linker. That amides in the 2- and 6-positions on the pyridine skeleton are involved in strong hydrogen bonding is evidenced by the fact that, in $CDCl_3$, the chemical shifts of the amide protons are routinely found between δ 8 and 9.



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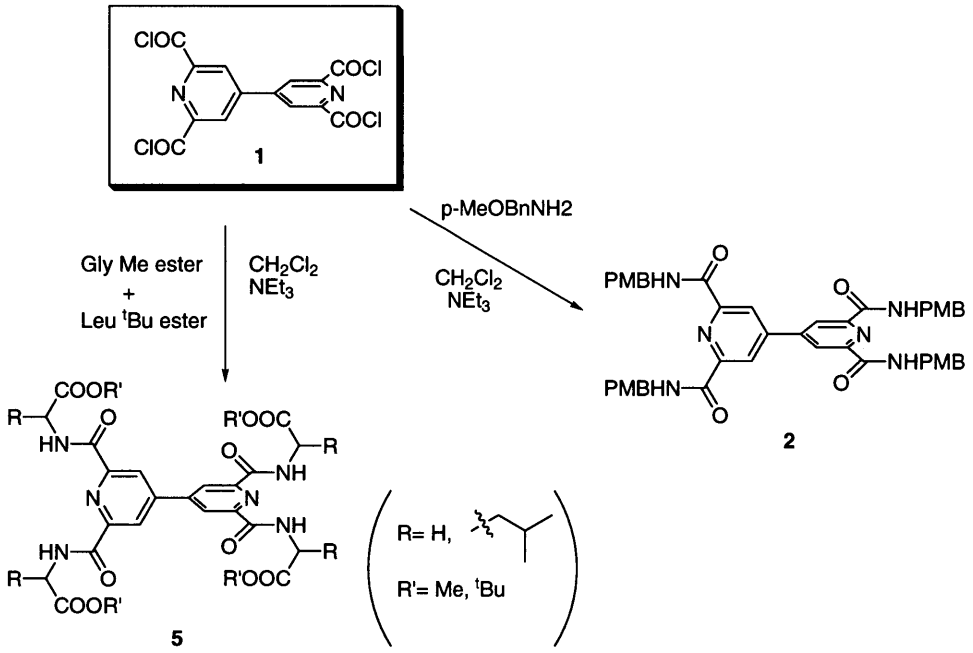
[1] Portions of this chapter have been previously published and are being used with permission from Elsevier Science. Pryor, K. E.; Shipps, G. W., Jr.; Skyler, D. A.; Rebek, J., Jr. *Tetrahedron* **1998**, *54*, 4107.

[2] Hünig, S.; Wehner, I. *Synthesis* **1989**, 552–554.

Scheme 1. Synthesis of 2,2',6,6'-(4,4'-bipyridine)tetraacid chloride.

To demonstrate the utility of the tetraacid chloride as a core for combinatorial synthesis, it was reacted with a variety of amines, both singly and in small mixtures (see Scheme 1, Experimentals **2–5** for examples). In mixtures with two components, such as glycine and leucine, six products are expected to form (Figure 2). HPLC analysis of the products resulting from such a reaction produced six peaks of comparable, if not theoretically-perfect, intensities.



Scheme 2. Synthesis of a homosubstituted adduct and a small test library.

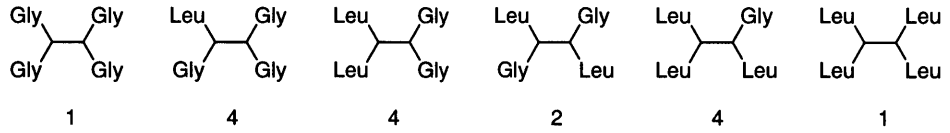


Figure 1. The components of a library produced with two building blocks, with their theoretical relative concentrations.

3.2 Attempts to incorporate isocyanates in the bipyridine core

Since this project was being started at the same time that refinement of urea linkages on the xanthene cores was underway, some effort was made to introduce urea precursor functionalitites in this core. Literature precedent for similiar systems was discouraging; isocyanates of nitrogen-containing heterocycles are notoriously unstable.[3] For instance, 2-isocyanatopyridine undergoes cyclodimerization immediately upon formation,[4] and 2,6-diisocyanatopyridine has been reported,[5] but undergoes homopolymerization upon standing. In this laboratory, it was found that, while the tetraacyl azide of the bipyridine **6** could be obtained by chemistry similar to that which was successful for the xanthene core, attempts to isolate the tetraisocyanate resulted only in the formation of orange polymeric material. Analog studies starting with pyridine-2,6-carboxylic acid were successful in producing pyridine-2,6-diethylcarbamate **8** by the reaction of ethanol with the diacyl azide **7** as it was warmed to induce a Curtius arrangement in situ, but subsequent attempts to make use of B-chlorocatecholborane-induced dealcoholation of the carbamate to regenerate the isocyanate[6] in the presence of benzylamine yielded a mixture of products as shown by ^{1}H NMR.

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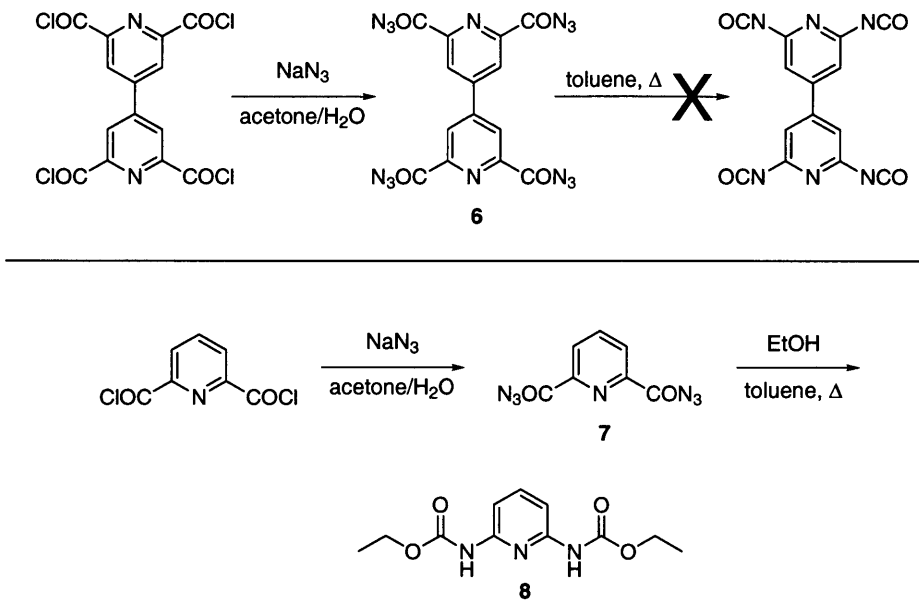
[3] Richter, R.; Ulrich, H. "The Chemistry of Cyanates and Their Thio Derivatives, Part 2" Patai, S., Ed., John Wiley & Sons: New York, **1977**, pp. 652–654.

[4] Gizycki, U. V.; Oertel, G. *Angew. Chem. Intl. Ed. Engl.* **1968**, *7*, 381.

[5] Hyden, S.; Wilbert, G. *Chem. & Ind.* **1967**, 1406.

[6] Valli, V. L. K.; Alper, H. *J. Org. Chem.* **1995**, *60*, 257.



Scheme 3. Attempted synthesis of bipyridine tetraisocyanate and test reactions on pyridine diisocyanate.

3.3 Deconvolution of the bipyridine core molecule

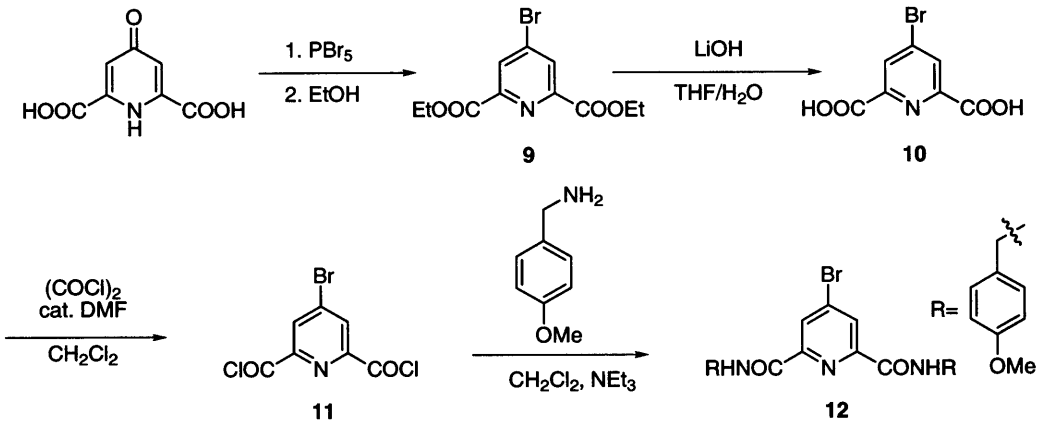
The route used to deconvolute libraries made with this core differs from those developed for the other core molecules described previously. Rather than building the entire core with different protecting groups and attaching building blocks in a linear synthesis, we use a convergent synthesis that makes use of a Stille coupling[7] to create the bipyridyl bond once all substituents are already attached.[8] A common intermediate, 4-bromo-2,6-pyridine diacid chloride **11** is used in the synthesis of each half of the final bipyridine molecule.

[7] Echavarren, A. M.; Stille, J. K. *J. Am. Chem. Soc.*, **1987**, *109*, 5478–5486.

[8] A very recent publication describes another method for the formation of 2,2',6,6'-tetraamide-4,4'-biaryls by the condensation of corresponding aryl iodides in the presence of 0.1 eq 10% Pd/C. Boger, D. L.; Goldberg, J.; Andersson, C.-M. *J. Org. Chem.* **1999**, *64*, 2422–2427.

4-Bromo-2,6-pyridinediamides—intermediates for deconvolution

The synthesis of bromopyridine diacid chloride **11** is shown in Scheme 4. In an adaptation of a reported procedure,[9] chelidamic acid monohydrate is converted to bromopyridine diethyl ester **9** upon reaction with PBr_5 followed by treatment of the intermediate acid bromide with EtOH. Diester **9** is saponified with LiOH to yield diacid **10**, which is in turn converted to diacid chloride **11** with oxalyl chloride. A test reaction using the diacid chloride half-core and *p*-methoxybenzylamine yielded the expected diamide **12**.



Scheme 4. Synthesis of 4-bromo-2,6-pyridine diacid chloride **11** and its use in making a test diamide.

Stannane test reactions

Our next step was to determine the optimal conditions for synthesizing the requisite aryl tin reagents and coupling conditions. There are several methods detailed in the literature for producing aryltins.[10] Broadly, they involve either transmetallation from an aryllithium or aryl Grignard with a tin halide or oxide, or palladium catalysis for the substitution of a halide with a tin using a ditin reagent. Using a sonochemical Barbier reaction outlined by Lee and

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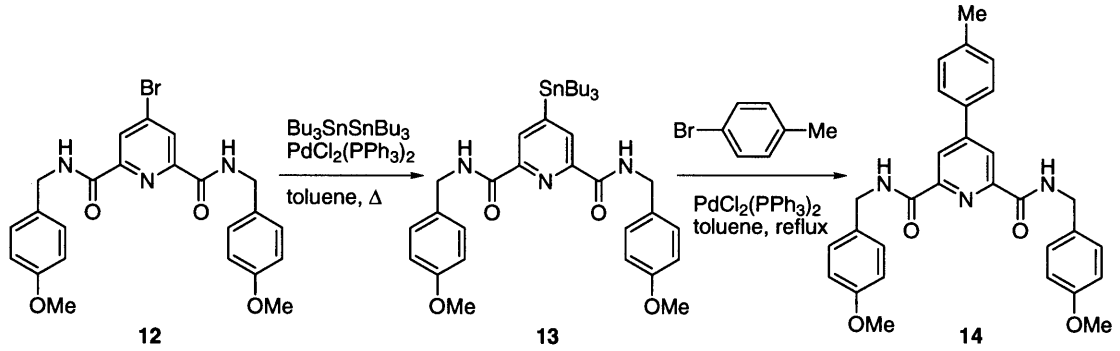
[9] Takalo, H.; Kankare, J. *Acta Chem. Scand.* **1987**, 219–221.

[10] (a) Eaborn, C.; Waters, J. A. *J. Chem. Soc.* **1962**, 1131–1132. (b) Trost, B. M.; Tanigawa, Y. J. *J. Am. Chem. Soc.* **1979**, *101*, 4743–4745. (c) Kashin, A. N.; Bumagina, I. G.; Bumagin, N. A.; Bakunin, V. N.; Beletskaya, I. P. *Zhur. Org. Khim.* **1981**, *17*, 905–911. (d) Iddon, B.; Lim, B.-L. *J. Chem Soc. Perkin I* **1983**, 271–277.

Dai,[11] 4-(tributylstannyl)toluene was synthesized as a model compound. However, the same conditions failed repeatedly using 4-bromopyrimidine as the substrate, seemingly because the Grignard reagent was not formed under the reaction conditions. Since some manner of protecting the amides or esters on the real substrates from the Grignard intermediate would be necessary if this method were used anyway, it was quickly abandoned.

The preferred method for the case at hand involves the reaction of hexabutylditin with aryl bromides in the presence of a catalytic amount of a palladium catalyst.[12] This mild method does not require the use of active organometallic species and so is particularly useful for synthesizing aryltins that bear reactive functional substituents, thus obviating the need for protection of the substrate. The transformation of bromo compound **12** into tributylstannane **13** (Scheme 5) was accomplished in 70% yield, while the coupling of **13** with *p*-bromotoluene to give **14** went in only 18% purified yield. The yield of the coupling was disappointing, but the success of this initial test reaction provided enough encouragement to take on a more complex test system.

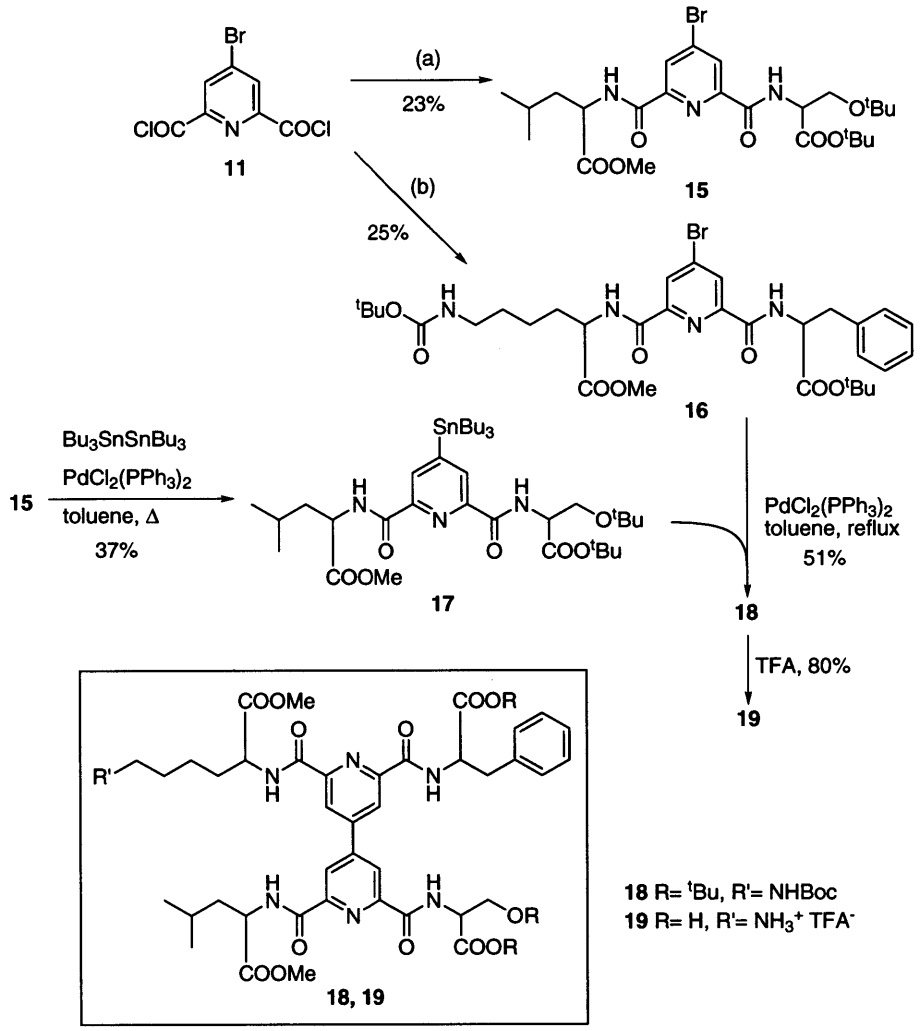


Scheme 5. Initial test stannane reactions.

[11] Lee, A. S.-Y.; Dai, W.-C. *Tetrahedron Letters*, **1996**, *37*, 495–498.

[12] Kosugi, M.; Shimizu, K.; Ohtani, A.; Migita, T. *Chem. Letters* **1981**, 829–830.

Synthesis of a tetraheterosubstituted bipyridine



Scheme 6. Deconvolution protocol for 2,2',6,6'-(4,4'-bipyridine)tetraamide libraries. (a) L-leucine methyl ester hydrochloride, *O*-*t*-butyl-L-serine *t*-butyl ester hydrochloride, Et_3N, CH_2Cl_2. (b) N-Boc-L-lysine methyl ester hydrochloride, L-phenylalanine *t*-butyl ester hydrochloride, Et_3N, CH_2Cl_2.

In order to demonstrate the method's general utility, we undertook a synthesis of the hypothetical target molecue **19**. Compound **19** was chosen because it contains a wide variety of amino acid substituents, including both free acids and methyl esters, and aliphatic, aromatic, heteroatomic, and charged side chains. Starting with bromopyridinedicarboxylic acid chloride **11**, two heterosubstituted 4-bromo-2,6-pyridinediamides **15** and **16** were

synthesized and purified by silica gel column chromatography from the two homodisubstituted compounds concurrently produced. Then, by using the palladium-catalyzed stannyllation reaction with bis(tributyltin) pyridylstannane **17** is produced. The subsequent Stille coupling and deprotection of the tetraamide proceed smoothly, thereby demonstrating a convergent method to deconvolute bipyridine tetraamide libraries.

Alternative routes to heterosubstituted bromopyridines

In the case above, the two heterosubstituted bromopyridines **16** and **17** were sufficiently different from the homosubstituted compounds also formed in their respective reactions to allow straightforward chromatographic separations. However, other cases arose in which the two building blocks were so similar to each other that chromatographic separation of the heterosubstituted pyridine diamides from the two homosubstituted variants was prohibited. We therefore devised two alternative routes to access these heterosubstituted compounds with highly similar building blocks (Scheme 7). In the first case, excess ethanol was used as one of the building blocks in the reaction and a mixture comprised of a diester, monoester/monoamide **20**, and a diamide was the result. These three dissimilar compounds could then be separated by chromatographic techniques, and the monoester could be subsequently saponified. The resulting carboxylic acid **21** could be substituted with the remaining desired amine building block under standard PyBOP coupling conditions.

A second method makes use of monosaponification of the bromopyridine diethylester **9** to yield monoacid **23**[13] and stepwise amide coupling, saponification of the second ester, and a second amide coupling to install the two different building blocks. An advantage to this route is that only one compound is expected at each step, obviating the need to isolate the correct compound from a mixture in a reaction that statistically has a maximum yield of 50%.

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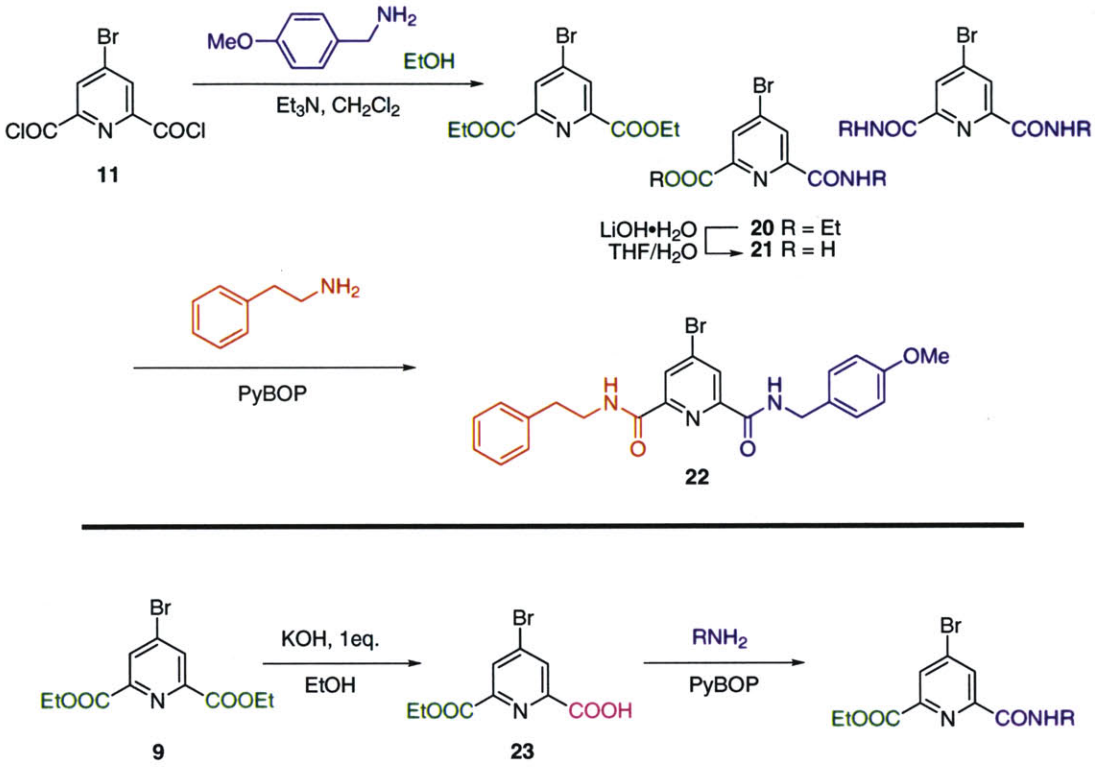
[13] Takalo, H.; Hemmilä *Helv. Chim. Acta* **1996**, *79*, 789–802.



Scheme 7. Alternative routes to heterosubstituted bromopyridinediamides.

3.4 Applications of bipyridine libraries in binding to TAR RNA[14]

The goal of this project is to develop a method of screening libraries of small molecules against RNA targets. Using an LC-MS system to identify the individual components of the library, our collaborators intend to use columns with RNA bound beads to separate compounds of interest from the library. We will attempt to show that by using multiple columns with different RNA molecules attached to the beads, we can select molecules form a library that bind to the RNA of interest both tightly and specifically.

[14] This work was carried out in collaboration with Prof. Jamie Williamson and his graduate student Ivan Baxter. Technical assistance was provided by Ellen Choi.

The human immunodeficiency virus (HIV) is a retrovirus, which stores its genetic information in the form of RNA. The 5' end of the viral RNA transcript includes a conserved stem loop structure named the *trans*-activation responsive element (TAR). The secondary structure of TAR is composed of an RNA hairpin with a small bulge containing two or three nucleotides. The HIV protein Tat forms a complex with TAR and the cellular protein Cyclin-T and activates transcription of the viral genome. The nucleotides in the loop appear to be required for the binding of Cyclin-T while U23 in the bulge and other residues in the bulge region are required for the binding of Tat.[15]

The specificity of TAR for the Tat peptide is mediated by the interaction of the RNA with a single arginine residue. Arginine is able to inhibit complex formation at least 12-fold better than lysine.[16] A high resolution NMR structure of TAR with argininamide (an arginine analog) shows that the U23 forms a base triplet with the A27-U38 base pair which helps form a binding pocket of the guanidinium group with the G26-U39 and A22-U40 base pairs.[17] Studies of the interactions of peptides derived from Tat and a cellular protein thought to bind to TAR appear to demonstrate that there are additional binding sites for arginine, although the disassociation constant for these binding sites are probably quite high.[18]

Several small molecule inhibitors of the Tat-TAR interaction have recently been reported.[19] While they all interact with the bulge region of TAR, none of them includes a guanidinium group to specifically mimic the binding of Tat.

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[15] (a) Frankel, A. D. *Development* **1992**, *2*, 293–298. (b) Wei, P.; Garber, M. E.; Fang, S.-M.; Fisher, W. H.; Jones, K. A. *Cell* **1998**, *92*, 451–462.

[16] Tao, J.; Frankel, A. D. *Proc. Natl. Acad. Sci.* **1992**, *89*, 2723–2726.

[17] Brodsky, A. S.; Williamson, J. R. *J. Mol. Bio.* **1997**, *267*, 624–639.

[18] Erard, M.; Barker, D. G.; Amalric, F.; Jeang, K.-T.; Gatignol, A. *J. Mol. Bio.* **1998**, *279*, 1085–1099.

[19] Hamy, F.; Brondani, V.; Florsheimer, A.; Stark, W.; Blommers, M. J. J.; Klimkait, T. *Biochem.* **1998**, *37*, 5086–5095.

If this strategy is successful it could be an excellent technique for screening libraries for specific binding to many biological macromolecular targets. Compounds that are selected could be excellent leads for structure-based drug design. Finding a specific binder for TAR RNA would be of particular interest for two main reasons: 1. Acquisition of the high-resolution structure of the identified binder with the RNA could be done rapidly, and 2. The Tat-TAR interaction is critical in the lifecycle of HIV and to date there are no drugs on the market that inhibit this interaction.

Our collaborators are presently in the process of developing an affinity column by binding biotinylated RNA to streptavidin linked to agarose beads. Our input in the project comes in the synthesis of specialized libraries for testing. Our starting libraries were composed using the bipyridine core molecule and a variety of building blocks which are shown in Figure 2. In order to determine the maximum number of compounds that can be effectively screened by the proposed combination affinity chromatography/LC-MS system, a series of nested libraries was prepared. All libraries included arginine methyl ester to ensure that guanidinium groups would be present in a certain number of the library components. Our prediction is that arginine-containing compounds might have the highest likelihood for success given the known binding mode of Tat. Once the affinity column is demonstrated to work and the maximum library complexity that can be studied has been established, additional libraries can be produced to explore a greater diversity of chemical structures.

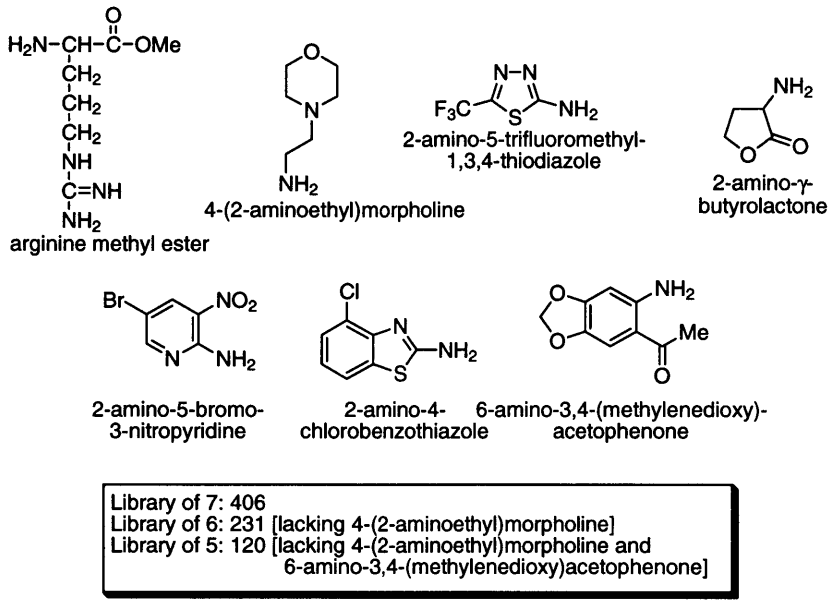

Figure 2. Building blocks used for initial TAR RNA binding libraries based on the bipyridine tetraacid chloride core molecule.

3.5 Experimental

General

All reagents were purchased from Aldrich Chemical Company and were used without further purification except as noted. Amino acid esters, PyBOP, and EDC•MeI were acquired from Novabiochem (San Diego, CA). Deuterated solvents were obtained from Cambridge Isotopes Laboratories and deuterated chloroform was dried over 4Å molecular sieves. HCl refers to a 1N stock solution. NMR spectra were recorded on either a Bruker AC-250, a Bruker AM-300, or a Bruker DRX-600; TMS was used as a reference in some chloroform-*d* spectra, otherwise residual solvent was used as a reference. Either a Finnegan Mat 8200 (for EI) or a VG ZAB-VSE (for FAB) mass spectrometer was used to ascertain masses. FT-IR spectra were obtained on a Perkin Elmer Paragon 1000 PC FT-IR Spectrometer. Silica gel chromatography was performed with Silica Gel 60 (EM Science or Bodman, 230–400 mesh). TLC analysis was performed using glass-bound Silica Gel 60

(F254) plates. DMAP = *N,N*-dimethyl-4-aminopyridine, DPPA = diphenylphosphoryl azide, EDC•MeI = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide, PyBOP = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate.

HPLC Analysis of Test Libraries

Library synthesis is described below. The library, after workup, was taken up in methanol and injected into the HPLC. The separation was accomplished using a Waters Symmetry C8 (3.9 x 150 mm) column, running a linear gradient of 100% water to 100% MeCN over 50 min. The effluent was analyzed at a wavelength of 254 nm.

General procedure for library synthesis

Note: The solvent system and scale of the synthesis varied slightly in some cases, and several different core molecules were used; the procedure below exemplifies the procedure and can be considered fairly general.

In a 25 mL round bottom flask was stirred 0.072 mmoles of each of 15 amine building blocks (4.4 equivalents total, 1.1 equivalents per core reactive site), added neat or in DMF solution. DMF was added to bring the total DMF volume to 2 mL. To this solution was added 1 mL triethylamine. A solution of 2,2',6,6'-(4,4'-bipyridine)tetracarboxylic acid chloride (100 mg, 0.246 mmole) in 4 mL warm methylene chloride was added to the chilled (0 °C) amine solution with stirring under nitrogen and the solution was diluted with methylene chloride to a total volume of 10 mL. This solution was left to stir for 1.5 hours under nitrogen. The solution was diluted with methylene chloride to ~30 mL and was washed twice with water and once with brine. The organic layer was dried over $MgSO_4$ and was filtered. The solution was concentrated by rotary evaporation to an oil, then was further dried under high vacuum for 3 hours.

This oil was then stirred in 10 mL TFA overnight to effect the deprotection of tert-butyl ester functionalities.[20] Removal of TFA by rotary evaporation yielded a yellow oil which was lyophillized to yield a pale yellow powder.

2,2',6,6'-(4,4'-Bipyridine)tetracarboxylic acid chloride (1).[2]

All intermediates were prepared according to Ref. 2. A modification of the synthesis of **1** was used as follows. A CH_2Cl_2 (70 mL) suspension of 2,2',6,6'-(4,4'-bipyridine) tetracarboxylic acid (1.00 g, 3.01 mmol), oxalyl chloride (2.1 mL, 24 mmol) and six drops of a 4% solution of DMF in CH_2Cl_2 was refluxed under nitrogen for 17 h. The solution was filtered through Celite was and concentrated to yellow solids, which were recrystallized from hot toluene to yield **1** (0.796 g, 65%) as a white powder.

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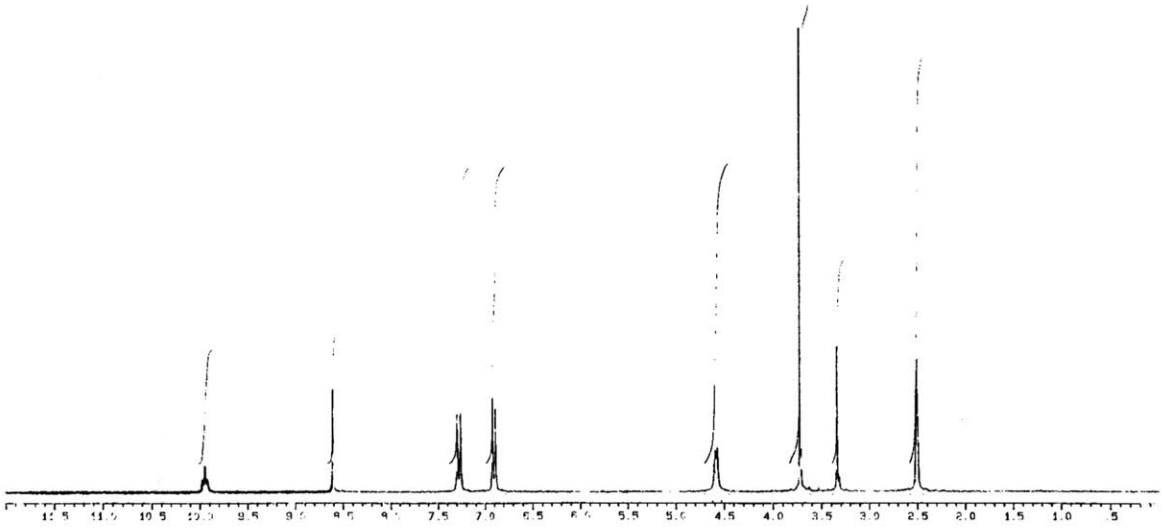
[20] In cases where no removable protecting groups were present among the building blocks, the TFA deprotection step was skipped.

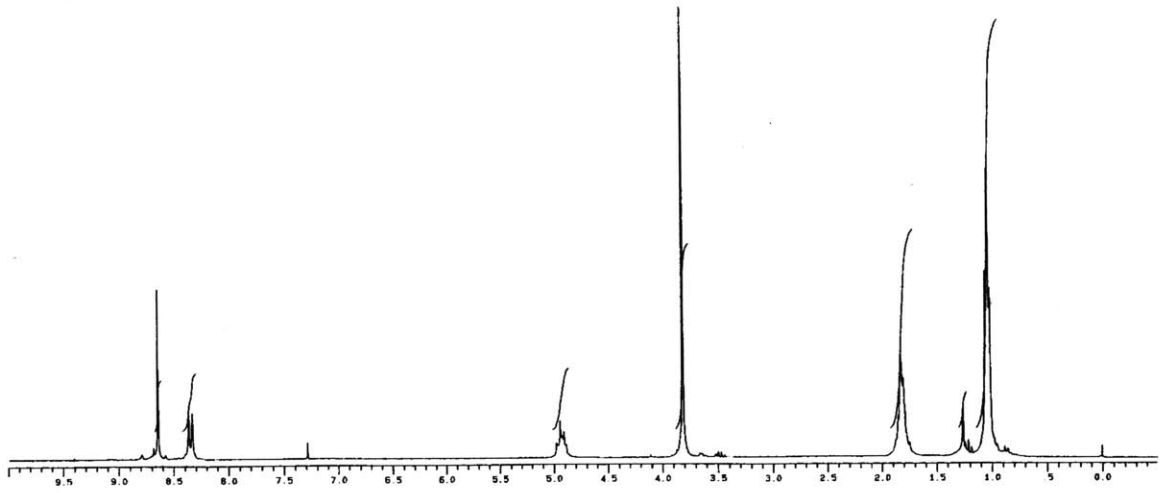
4,4'-Bipyridine-2,2',6,6'-tetrakis(*p*-methoxybenzyl)amide (**2**).

To 10 mL methylene chloride was added 2,2',6,6'-bipyridinetetracarboxylic acid chloride **1** (104 mg, 0.256 mmol), *p*-methoxybenzylamine (145 μL, 152 mg, 1.11 mmol), and four drops of triethylamine and the solution was stirred overnight under a drying tube. The precipitate that formed was removed by filtration, yielding **2** as a white powder. ^{1}H NMR (250 MHz, DMSO-d_6): δ 9.94 (t, J = 6 Hz, 4H), 8.50 (s, 4H), 7.28 (d, J = 9 Hz, 8H), 6.89 (d, J = 9 Hz, 8H), 4.58 (d, J = 6 Hz, 8H), 3.72 (s, 12H).



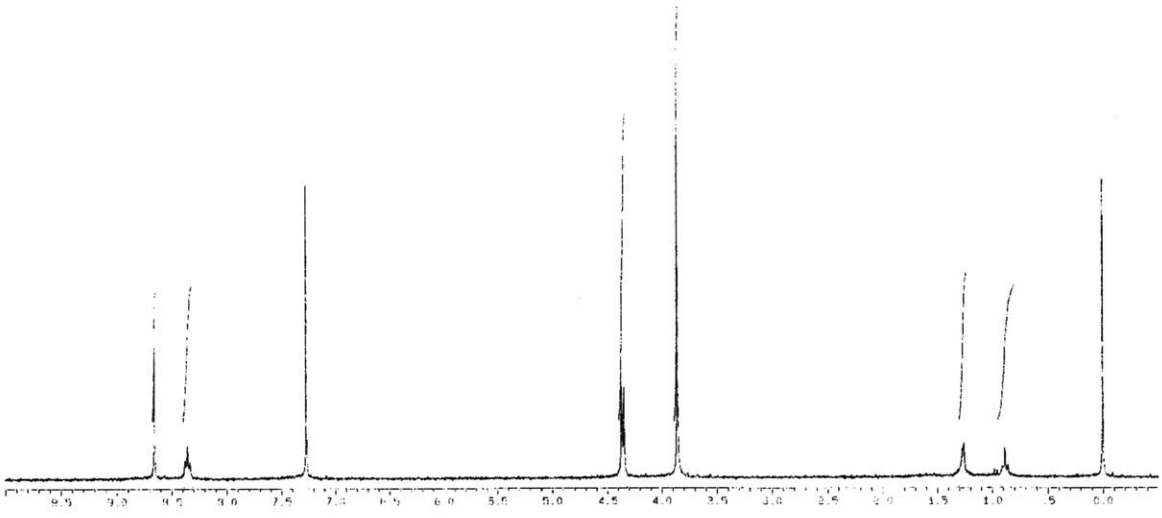
4,4'-Bipyridine-2,2',6,6'-tetrakis(leucine methyl ester amide) (3).

To 10 mL methylene chloride was added 2,2',6,6'-bipyridinetetracarboxylic acid chloride **1** (101 mg, 0.249 mmol), leucine methyl ester hydrochloride (224 mg, 1.23 mmol) and triethylamine (205 μL, 149 mg, 1.47 mmol). The solution was stirred under a drying tube for 15 h, then was diluted to 75 mL with methylene chloride and was washed twice with 1 M citric acid, once with saturated sodium bicarbonate, and once with water. The organic layer was dried over magnesium sulfate, filtered, and the solution was concentrated by rotary evaporation.Hexane was added to precipitate the product, then all solvent was removed by rotary evaporation, yielding **3** as a pale pink powder. ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.64 (s, 4H), 8.33 (d, J = 9 Hz, 4H), 4.93 (m, 4H), 3.81 (s, 12H), 1.82 (m, 12H), 1.03 (m, 24H).



4,4'-Bipyridine-2,2',6,6'-tetrakis(glycine methyl ester amide) (4).

To 5 mL methylene chloride was added glycine methyl ester hydrochloride (69 mg, 0.55 mmol) and triethylamine (100 μL, 73 mg, 0.72 mmol). Next, 4,4'-bipyridine-2,2',6,6'-tetracarboxylic acid chloride **1** (50 mg, 0.12 mmol) was added after the glycine was dissolved. The solution was stirred under a drying tube for 17 h, then was diluted to 50 mL with methylene chloride and was washed twice with 1 M citric acid, once with saturated sodium bicarbonate, and once with water. The organic layer was dried over magnesium sulfate, filtered, and all solvent was removed by rotary evaporation, yielding **4** as a white powder. ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.65 (s, 1H), 8.35 (t, J = 6 Hz, 4H), 4.35 (d, J = 6 Hz, 8H), 3.85 (s, 12H).

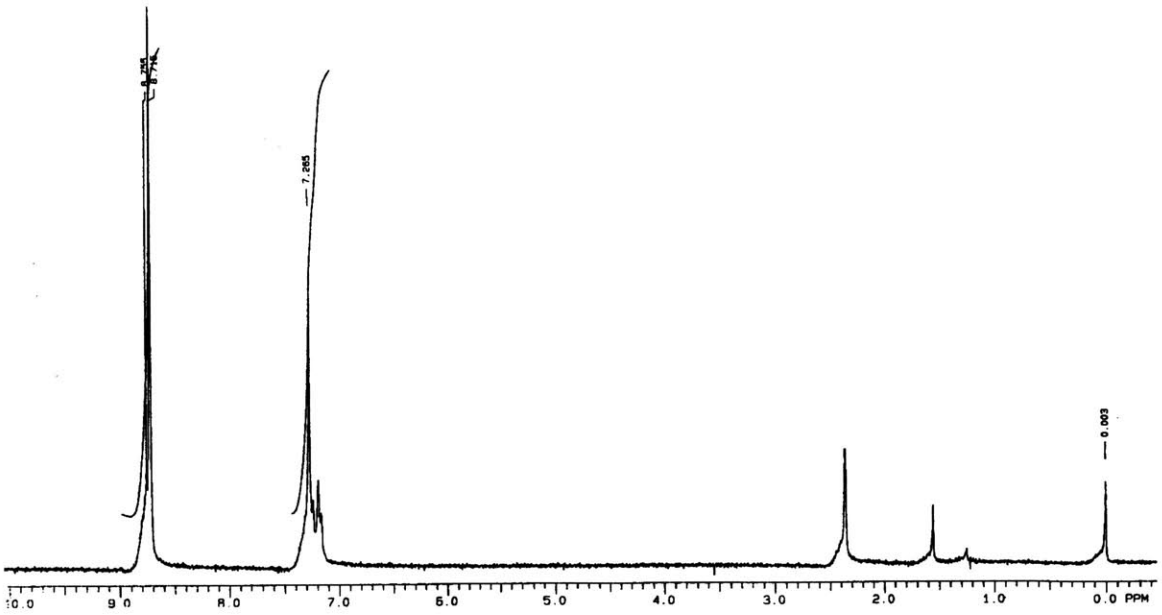


Bipyridine glycine/leucine library (5).

To a solution of glycine methyl ester hydrochloride (34 mg, 0.27 mmol), leucine *t*-butyl ester hydrochloride (61 mg, 0.27 mmol), and triethylamine (130 μL, 94 mg, 0.93 mmol) in 10 mL methylene chloride was added a solution of 4,4'-bipyridine-2,2',6,6'-tetracarboxylic acid chloride **1** (50 mg, 0.123 mmol) in 2 mL methylene chloride. The resulting solution was stirred under a drying tube for 16 h, then was diluted to ~50 mL and washed twice with 1 M citric acid, once with saturated sodium bicarbonate, and once with water. The organic phase was dried over magnesium sulfate, filtered, and all solvent was removed by rotary evaporation, yielding the library as a white foam. ^{1}H NMR (250 MHz, $CDCl_3$) showed clusters of peaks in expected positions. HPLC showed 6 peaks: 5.57 min (area: 1730), 21.13 min (area: 12300), 35.88 min (area: 8520), 37.41 min (area: 5330), 45.59 min (area: 10700), 46.49 min (area: 3130).

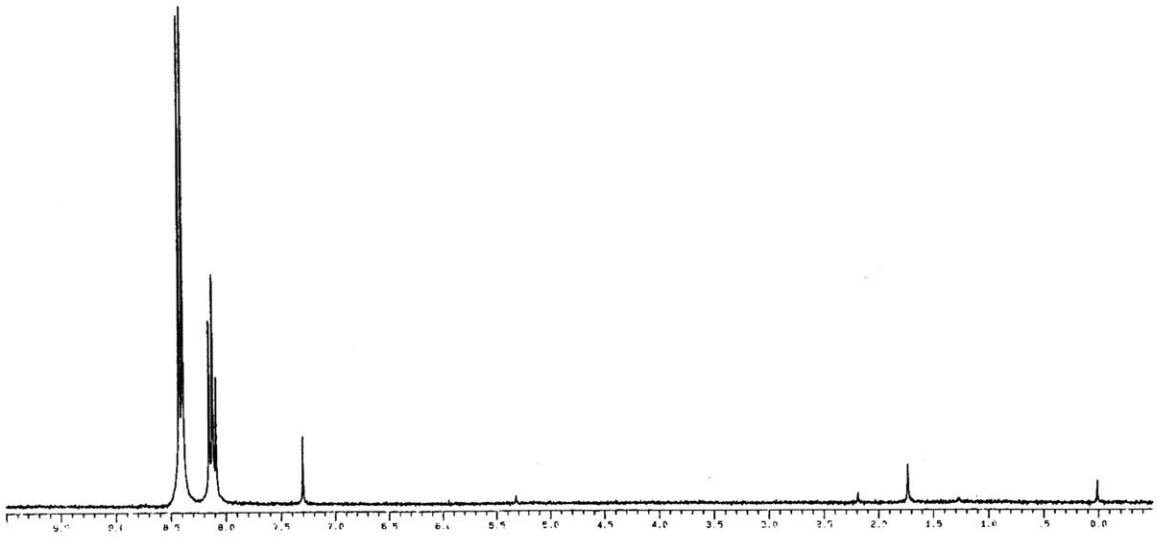
4,4'-Bipyridine-2,2',6,6'-tetraacyl azide (6).

To 25 mL HPLC-grade acetone was added 2,2',6,6'-(4,4'-bipyridine) tetraacid chloride **1** (0.301 g, 0.741 mmol). This was chilled in an ice bath to 0 °C. A solution of sodium azide (0.73 g, 11.2 mmol) in 4 mL water was added, and an immediate precipitate formed and an orange color was noted. The mixture was stirred for 1 h at 0 °C, then the solution was diluted with water and the acetone was removed by rotary evaporation. The resulting aqueous solution contained white solids, and was extracted with methylene chloride. The organic phase was dried over magnesium sulfate, filtered, and the solvent was removed by rotary evaporation, yielding **6** as white crystals. A portion was removed before final workup for continued reaction, so overall yield could not be calculated, and some toluene remained in the sample. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.72 (s, 2H). FT-IR (neat) 3067.4, 2146.4 ($-N_3$), 1685.7, 1304.8, 1232.4, 1198.1, 1009.5 cm^{-1}.



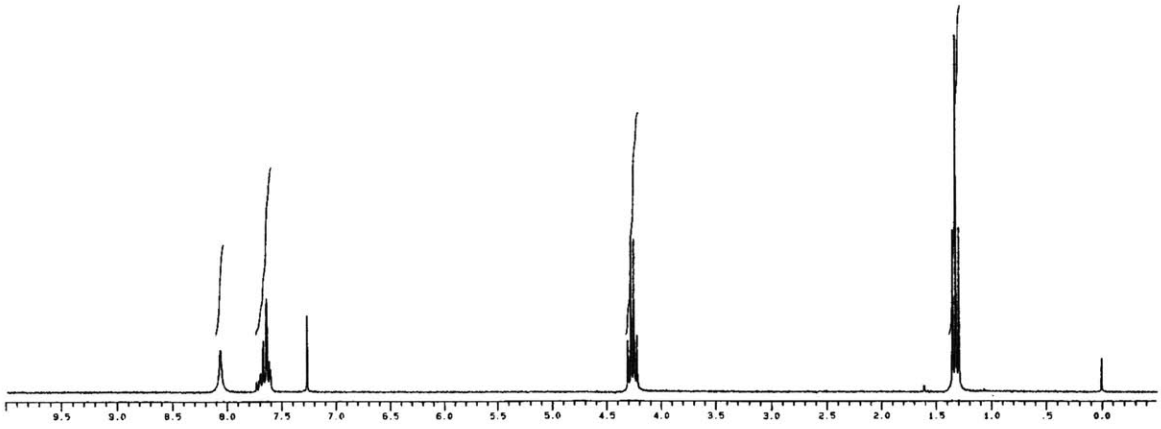
Pyridine-2,6-diacyl azide (**7**).

To 75 mL HPLC-grade acetone was added 2,6-pyridine diacid chloride (1.50 g, 7.35 mmol). This was chilled in an ice bath to 0 °C. A solution of sodium azide (4.00 g, 61.5 mmol) in 15 mL water was added, and an immediate precipitate formed and an orange color was noted. The mixture was stirred for 1 h at 0 °C, then the solution was diluted with water and the acetone was removed by rotary evaporation. The resulting orange aqueous solution contained white solids, and was extracted with methylene chloride (3 x 25 mL). The organic phase was dried over magnesium sulfate, filtered, and the solvent was removed by rotary evaporation, yielding **7** (1.416 g, 89%) as white crystals. ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.40 (d, J = 8 Hz, 1H), 8.11 (t, J = 8 Hz, 2H).



Pyridine-2,6-diethylcarbamate (**8**).

To 10 mL toluene and 10 mL ethanol was added 2,6-pyridine diacyl azide **7** (0.47 g, 2.16 mmol). The solution was refluxed for 1.5 h, after which time the solvent was removed by rotary evaporation. The crude product was recrystallized from warm ethanol, yielding **8** (0.276 g, 50% yield) as white crystals. ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.06 (br. s, 2H), 7.65 (m, 3H), 4.26 (q, J = 7 Hz, 4H), 1.32 (t, J = 7 Hz, 6H).

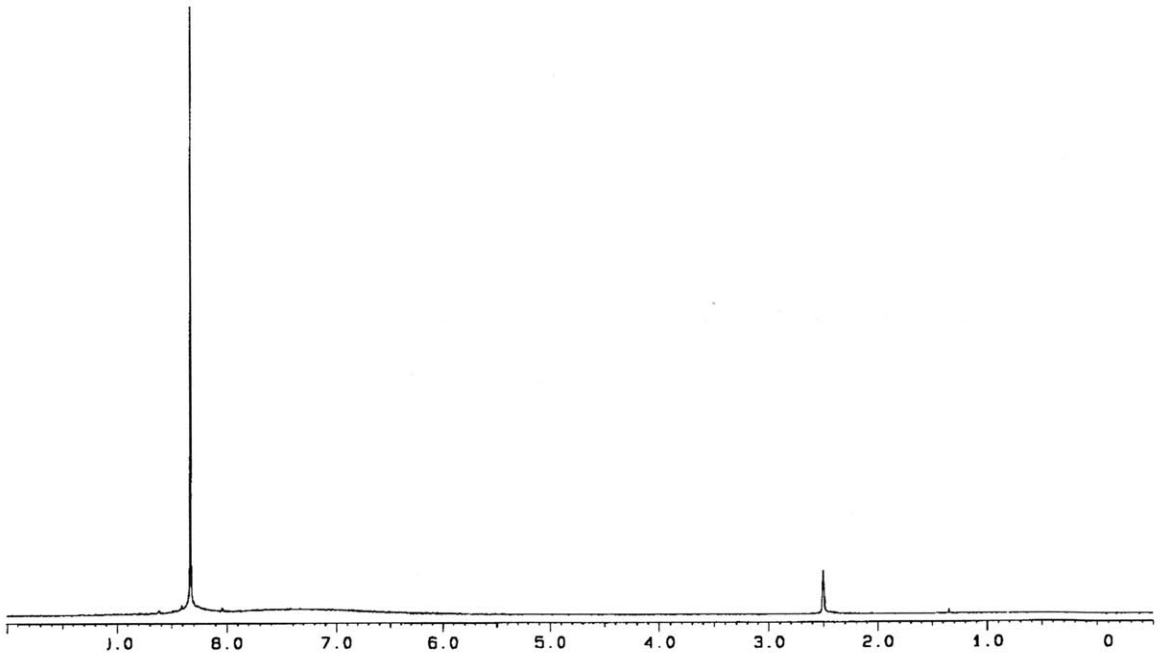


Diethyl 4-bromo-2,6-pyridinedicarboxylate (**9**).[9]

Procedure adapted from Ref. 9. Chelidamic acid monohydrate (6.32 g, 31.4 mmol) and phosphorus pentabromide (63 g, 150 mmol) were heated to 90 °C, whereupon the solids formed a melt. This was stirred at 90 °C for 1 h 45 min. After the solution cooled, 45 mL chloroform was added and the solution was filtered. The filtrate was chilled in an ice bath as 250 mL ethanol was slowly added to the solution. All solvent was removed by rotary evaporation, as well as a high boiling, oily, clear liquid, yielding dirty crystals. Recrystallization from ethanol yielded **9** (6.56 g, 69%) as pale yellow crystals.

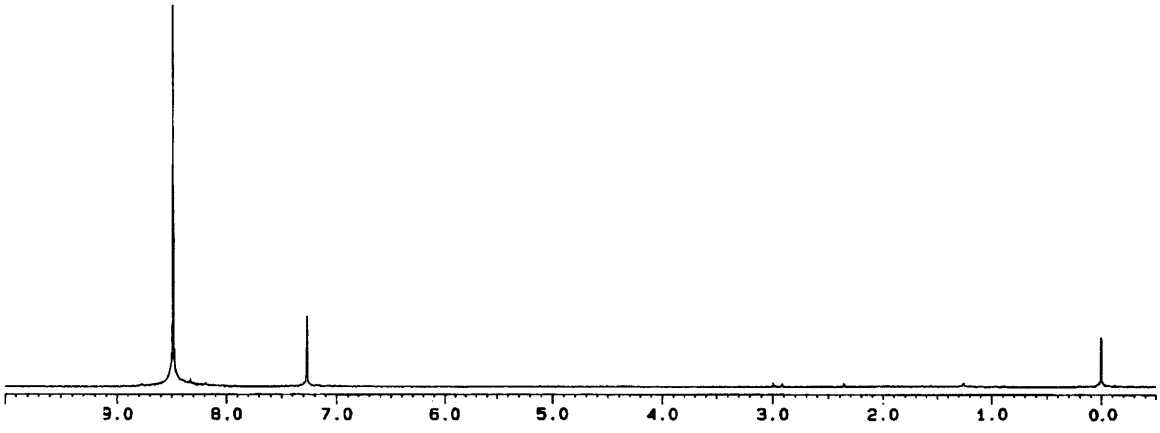
4-Bromo-2,6-pyridinedicarboxylic acid (10).

To a solution of **9** (8.6 g, 28 mmol) in 200 mL THF was added a solution of lithium hydroxide monohydrate (2.9 g, 69 mmol) in 40 mL water. This was stirred together for 2 h 45 min, then the solution was acidified to pH 0–1 with concentrated hydrochloric acid. The acidified solution solidified upon standing; with the addition of more water and sonication the solids broke up and were filtered, then dried on a steam bath, yielding **10** (7.07 g, 91%) as a white powder. m.p. 205–209 °C (dec.). ^{1}H NMR (DMSO-d_6, 600 MHz): δ 8.35 (s, 2H). ^{13}C NMR (DMSO-d_6, 151 MHz): δ 164.89, 150.18, 134.45, 130.37.HRMS (FAB in NBA/NaI) m/z calcd for $[C_7H_4BrNO_4]^+$ 245.9402 found 245.9404. FT-IR (neat): 684, 719, 805, 897, 1174, 1207, 1314, 1400, 1477, 1573, 1732, 3093, 3385, 3489 cm^{-1}.



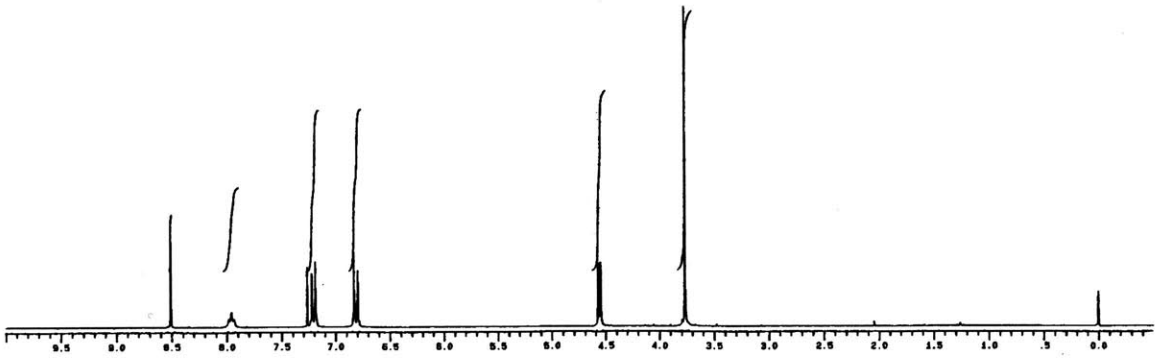
4-Bromo-2,6-pyridinedicarboxylic acid chloride (11).

To a suspension of diacid **10** (7.06 g, 25.8 mmol) in 290 mL CH_2Cl_2 was added oxalyl chloride (8.6 mL, 13 g, 99 mmol) and catalytic DMF (6 drops 4% solution in CH_2Cl_2). The stirred suspension was refluxed for 20 h. The cooled solution was filtered through Celite to remove hazy undissolved material and the solution was concentrated. The residue was dissolved in toluene and again the solvent was removed by rotary evaporation, yielding **11** (7.41 g, 92%) as salmon-colored micro crystals. m.p. 105–108 °C. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.49 (s, 2H). ^{13}C NMR ($CDCl_3$, 151 MHz): δ 168.90, 150.10, 136.35, 132.21. LRMS (ESI +) *m/z* calcd for $[C_7H_3BrCl_2NO_2 + H]^+$ 274/276, found 274/276. FT-IR (neat): 3077.4, 1759.8, 1554.3, 1427.9, 1251.8, 987.9, 937.7, 926.8, 908.0, 710.9 cm^{-1}.



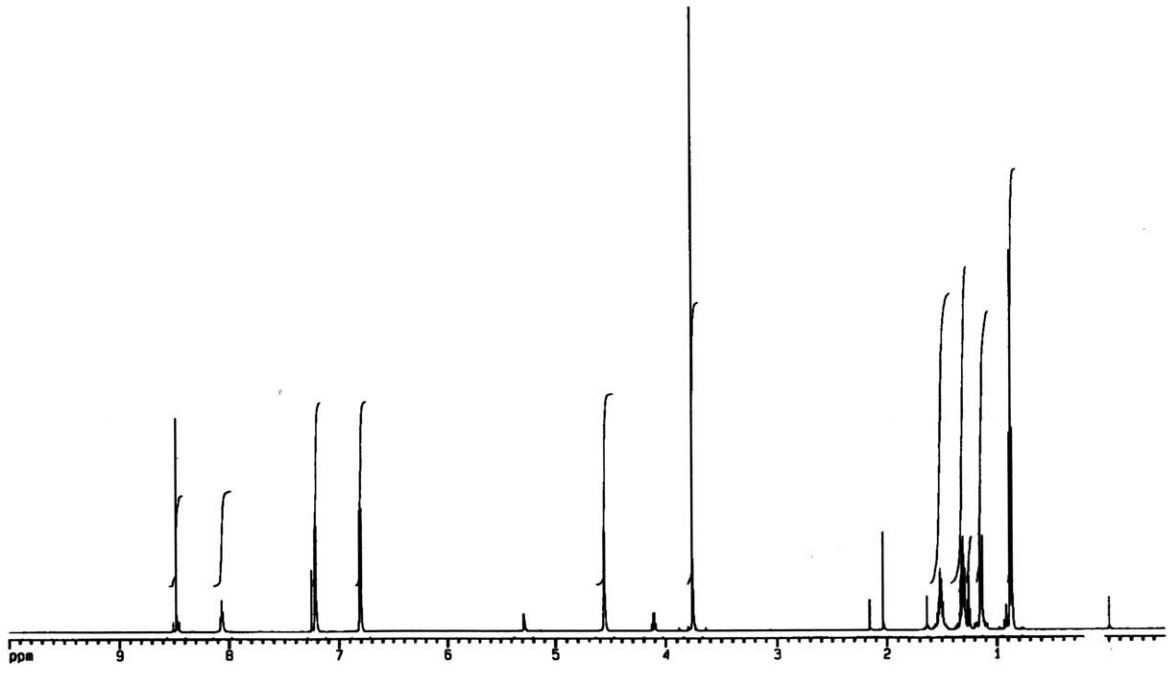
4-Bromo-2,6-pyridine-bis(*p*-methoxybenzylamide) (12).

To 175 mL methylene chloride chilled in an ice bath was added diacid chloride **11** (7.40 g, 23.8 mmol). While stirring, *p*-methoxybenzylamine (6.65 mL, 50.9 mmol) was added dropwise, followed by dropwise addition of triethylamine (7.3 mL, 52.4 mmol). The ice bath was removed and the mixture was allowed to warm to room temperature with stirring. After 2 h, the solution was washed with 1 M citric acid (3 x), saturated sodium bicarbonate (2 x), and brine (1 x). The solution was then dried over magnesium sulfate and filtered. An oil was left by the removal of solvent by rotary evaporation; upon exposure of this oil to high vacuum **12** (10.46 g, 91% yield) was isolated as a tan foam. ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.51 (s, 2H), 7.96 (br. t, J = 10.0 Hz, 2H), 7.20 (m, 4H), 6.81 (m, 4H), 4.56 (d, J = 10.0 Hz, 4H), 3.77 (s, 6H). ^{13}C NMR (76 MHz, $CDCl_3$): δ 162.84, 158.68, 149.53, 135.87, 129.68, 128.79, 128.21, 113.71, 55.08, 42.89. HRMS (FAB, NBA/NaI) m/z calcd for $[C_{23}H_{22}BrN_3O_4 + Na]^+$ 506.0691/508 found 506.0675/508.



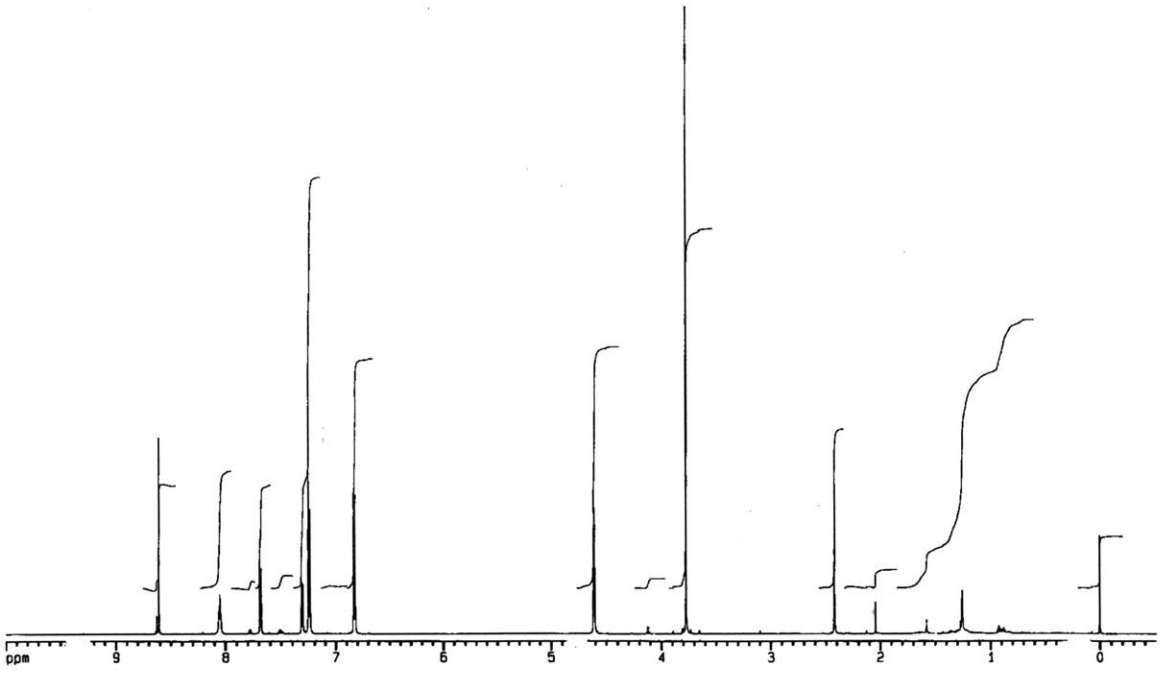
4-(Tributyltin)-2,6-pyridine-bis(4-methoxybenzylamide) (13).

To 12 mL toluene was added **12** (503 mg, 1.04 mmol), bis(tributyltin) (1.70 mL, 3.36 mmol), and dichlorobis(triphenylphosphine)palladium (II) (11 mg, 0.016 mmol). The solution was stirred and heated to 80 °C for 20 h, after which time the color turned brown. After cooling overnight, a fine precipitate was removed by filtration. Silica gel column chromatography (8:1→3:1 hexane/EtOAc) of the residue from the evaporation of solvent yielded **13** (502 mg, 70%) as a thick clear oil that crystallized upon standing overnight. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.48 (s, 2H), 8.07 (t, J = 6.1 Hz, 2H), 7.22 (d, J = 8.7 Hz, 4H), 6.81 (d, J = 8.7 Hz, 4H), 4.57 (d, J = 6.1 Hz, 4H), 3.77 (s, 6H), 1.51 (m, 6H), 1.30 (m, 6H), 1.15 (m, 6H), 0.88 (t, J = 7.2 Hz, 9H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 164.61, 158.26, 158.65, 146.53, 133.31, 130.53, 129.33, 114.23, 55.35, 43.03, 29.00, 27.33, 13.67, 9.87. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{35}H_{49}N_3O_4Sn + Cs]^+$ 828.1799 found 828.1822. FT-IR (neat): 3315 (br.), 2956, 2927, 2851, 1674, 1651, 1612, 1513, 1464, 1357, 1303, 1250, 1174, 1110, 1074, 1036, 820, 683 cm^{-1}.



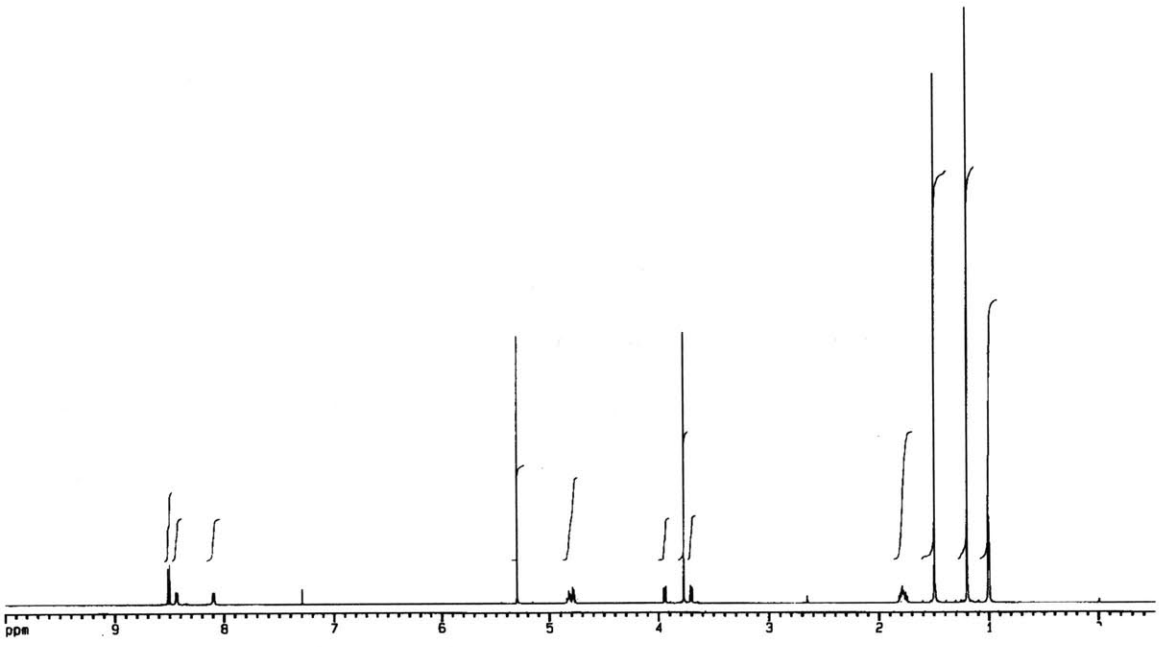
4-(4-Toluyl)-2,6-pyridine-bis(4-methoxybenzylamide) (14).

A solution of **13** (0.299 g, 0.431 mmol), 4-bromotoluene (55 μL, 0.076 g, 0.44 mmol), and dichlorobis(triphenylphosphine)palladium (II) (0.0038 g, 0.0054 mmol) in 10 mL toluene was refluxed under nitrogen for 20 h. The solution was diluted with methylene chloride and was filtered through a plug of silica gel. All solvent was removed and the residue was purified by silica gel column chromatography (4:1→3:1 gradient hexane/ethyl acetate eluent), yielding **14** (0.0391 g, 18% yield) as a white crystalline solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.62 (s, 2H), 8.05 (t, J = 6.0 Hz, 2H), 7.69 (d, J = 8.1 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.25 (d, J = 8.6 Hz, 4H), 6.83 (d, J = 8.6 Hz, 4H), 4.62 (d, J = 6.1 Hz, 4H), 3.78 (s, 6H), 2.05 (s, 3H). HRMS (FAB, NBA/CsI) m/z calcd for [$C_{30}H_{29}N_3O_4$ + Cs^+] 628.1212 found 628.1224.



4-Bromo-2-(leucine methyl ester)-6-(O-t-butylserine t-butyl ester)pyridine (15).

To a chilled (0 °C) solution of leucine methyl ester hydrochloride (1.26 g, 6.93 mmol) and *O*-*t*-butylserine *t*-butyl ester hydrochloride (1.76 g, 6.93 mmol) in CH_2Cl_2 (25 mL) was added Et_3N (3 mL) and a solution of **11** (1.96 g, 6.30 mmol) in CH_2Cl_2 (25 mL). This was stirred under nitrogen for 13 h, warming to rt. The solution was diluted to 100 mL with CH_2Cl_2 and was washed with HCl, saturated $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated to off-white solids. Compound **15** was separated from the other two diamides formed by silica gel column chromatography (8:1 hexane/EtOAc) and was isolated as a pale yellow oil (0.843 g, 23%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.52 (d, J= 1.4 Hz, 1H), 8.50 (d, J = 1.5 Hz, 1H), 8.44 (d, J = 8.5 Hz, 1H), 8.10 (d, J = 8.3 Hz, 1H), 4.84–4.81 (m, 1H), 4.79 (dt, J = 8.4 Hz, 3.0 Hz, 1H), 3.95 (dd, J = 2.7 Hz, 9.0 Hz, 1H), 3.78 (s, 3H), 3.71 (dd, J = 3.1 Hz, 9.0 Hz, 1H), 1.83–1.74 (m, 3H), 1.50 (s, 9H), 1.20 (s, 9H), 1.01 (d, J = 4.5 Hz, 3H), 1.00 (d, J = 4.5 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 173.10, 169.32, 162.44, 162.28, 149.96, 149.61, 136.39, 128.93, 128.81, 82.29, 73.45, 62.24, 53.64, 52.49, 51.18, 41.56, 28.06, 27.40, 24.96, 22.90, 21.89. HRMS (FAB in NBA/NaI) m/z calcd for $[C_{25}H_{38}BrN_3O_7Na + Na]^+$ 594.1791 found 594.1766.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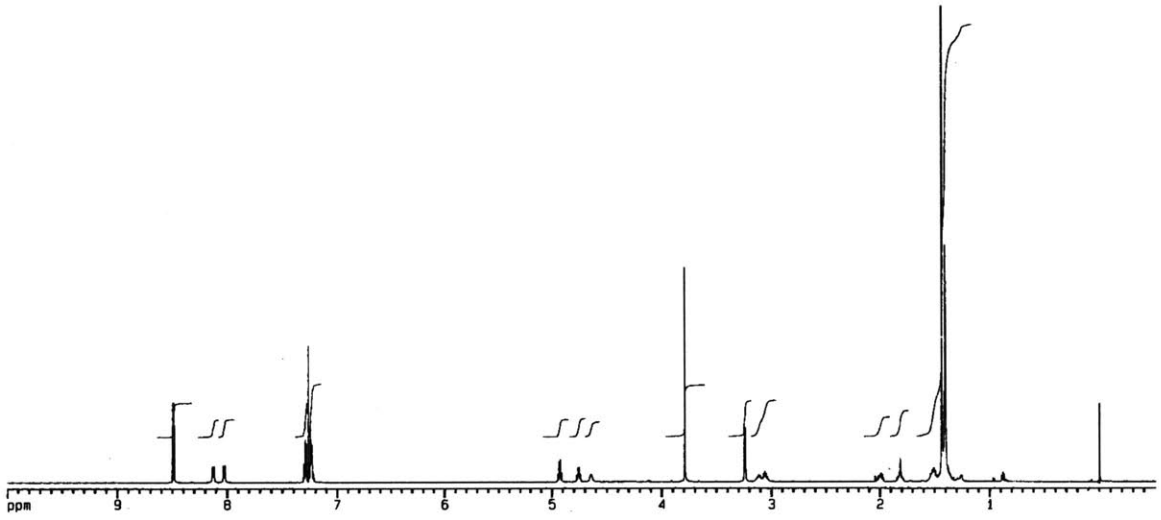
4-Bromo-2-(N-Boc-lysine methyl ester)-6-(phenylalanine t-butyl ester)pyridine (16).

To a chilled (0 °C) solution of *N*-Boc-lysine methyl ester hydrochloride (1.06 g, 3.57 mmol) and phenylalanine *t*-butyl ester hydrochloride (0.92 g, 3.6 mmol) in CH_2Cl_2 (25 mL) was added Et_3N (3 mL) and **11** (1.01 g, 3.25 mmol). The solution was stirred for 17 h loosely capped, warming to rt. The solution was diluted to 50 mL with CH_2Cl_2 and was washed with HCl, saturated $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated to a yellow oil. Compound **16** was separated from the other two diamides formed by silica gel column chromatography (4:1 hexane/EtOAC) and was isolated as a white solid (0.565 g, 25%). m.p. 50–54 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.50 (d, J = 2.0 Hz, 1H), 8.48 (d, J = 1.9 Hz, 1H), 8.13 (d, J = 7.9 Hz, 1H), 8.03 (d, J = 8.1 Hz, 1H), 7.31–7.29 (m, 2H), 7.25–7.22 (m, 3H), 4.95–4.92 (m, 1H), 4.78–4.74 (m, 1H), 4.65 (br. t, 1H), 3.80 (s, 3H), 3.24 (d, J = 6.0 Hz, 2H), 3.14–3.04 (m, 2H), 2.04–1.97 (m, 1H), 1.85–1.79 (m, 1H), 1.54–1.47 (m, 2H), 1.43–1.36 (m, 20H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 172.54, 170.61, 162.43, 162.00, 156.30, 149.83, 149.67, 136.64, 136.31, 129.85, 128.98, 128.84, 127.42, 82.92, 54.09, 52.74, 52.54, 40.33, 38.34, 32.23, 29.55, 28.51, 28.10, 22.76. HRMS (FAB in NBA/CsI) m/z calcd for $[C_{32}H_{43}BrN_4O_8 + Cs]^+$ 823.1319/825 found 823.1344/825. FT-IR (neat): 3341.7, 2976.2, 2932.1, 1736.3, 1676.7, 1523.0, 1454.7, 1365.8, 1249.2, 1155.2, 912.4, 842.6, 730.2 cm^{-1}.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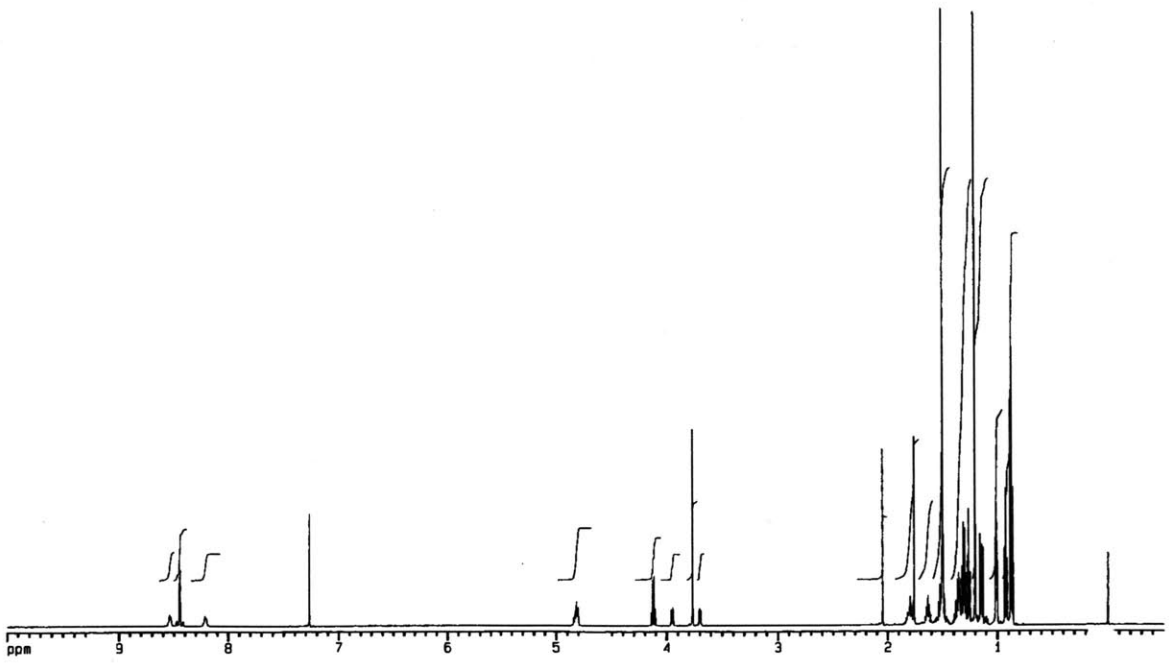
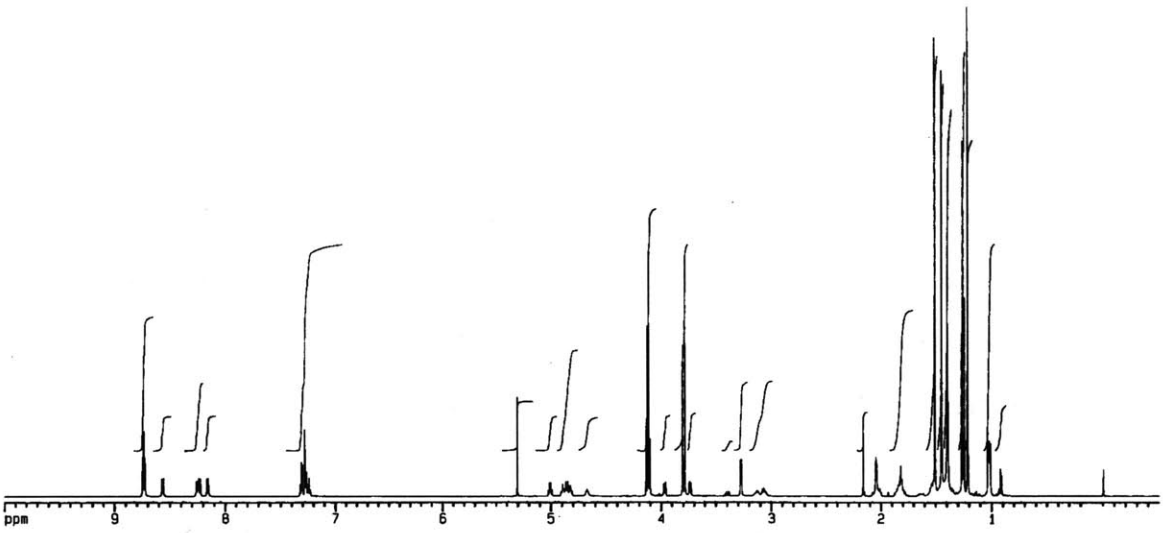
4-(Tri-*n*-butylstannyl)-2-(leucine methyl ester)-6-(O-*t*-butylserine *t*-butyl ester)pyridine (**17**).

A solution of **15** (0.843 g, 1.47 mmol), bis(tributyltin) (2.3 mL, 4.5 mmol), and dichlorobis(triphenylphosphine) palladium (II) (11.4 mg, 0.0162 mmol) in toluene (10 mL) was stirred and heated to 80 °C. A brown color developed as the reaction progressed. The solution was heated for 2 h 40 min, then after cooling it was concentrated to a dark brown oil. Column chromatography (8:1 hexane/EtOAc), after elution of excess bis(tributyltin), yielded **17** (0.430 g, 37%) as a clear oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.55 (br. t, J = 7.8 Hz, 1H), 8.45 (d, J = 5.4 Hz, 2H), 8.22 (br. t, J = 8.3 Hz, 1H), 4.84–4.80 (m, 2H), 3.95 (dd, J = 2.7, 8.7 Hz, 1H), 3.77 (s, 3H), 3.70 (dd, J = 2.8, 8.7 Hz, 1H), 1.82–1.77 (m, 3H), 1.53–1.46 (m, 15H), 1.38–1.35 (m, 6H), 1.20 (s, 12H), 1.18–1.10 (m, 6H), 1.02–1.00 (m, 6H), 0.87 (t, J = 7.3 Hz, 9H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 173.66, 169.81, 164.90, 164.64, 158.42, 146.18, 146.13, 133.33, 133.25, 82.40, 73.78, 53.50, 52.38, 50.41, 40.98, 28.91, 27.99, 27.30, 27.25, 24.92, 22.89, 21.71, 13.55, 9.76. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{37}H_{65}N_3O_7Sn + Cs]^+$ 915.5332 found 915.5368.



2-(Leucine methyl ester)-6-(O-*t*-butylserine *t*-butyl ester)-2'-(N-Boc-lysine methyl ester)-6'-(phenylalanine *t*-butyl ester)-4,4'-bipyridine (**18**).

A toluene (5 mL) solution of **16** (0.389 g, 0.562 mmol), **17** (0.420 g, 0.537 mmol), and dichlorobis(triphenylphosphine) palladium (II) (18.9 mg, 0.0269 mmol) was refluxed for 17 h, turning from yellow to brown in color over that period. The solution was concentrated to brown solids and column chromatography (4:1→3:1 hexane/EtOAc gradient) yielded **18** (0.305 g, 51%) as a thick, clear oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.75–8.73 (m, 4H), 8.57 (d, J = 8.5 Hz, 1H), 8.26 (d, J = 8.1 Hz, 1H), 8.23 (d, J = 8.2 Hz, 1H), 7.32–7.24 (m, 5H), 5.01 (dt, J = 2.7, 8.9 Hz, 1H), 4.91–4.81 (m, 3H), 4.68 (br. t, 1H), 3.97 (dd, J = 2.7, 8.9 Hz, 1H), 3.81 (s, 3H), 3.79 (s, 3H), 3.75 (dd, J = 2.8, 8.9 Hz, 1H), 3.28 (d, J = 6.0 Hz, 2H), 3.29–3.05 (m, 2H), 2.03–2.00 (m, 1H), 1.86–1.81 (m, 4H), 1.55–1.52 (m, 11H), 1.45 (br. s, 11H), 1.40 (s, 9H), 1.22 (s, 9H), 1.03 (d, J = 5.7 Hz, 3H), 1.02 (d, J = 5.7 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 173.34, 172.68, 170.78, 169.55, 163.11, 163.00, 162.91, 162.57, 156.28, 150.44, 150.27, 150.17, 150.13, 147.92, 147.66, 136.36, 129.87, 128.79, 127.37, 123.25 (3 overlapping peaks), 123.18, 82.83, 82.34, 79.16, 73.57, 62.50, 54.03, 53.65, 52.66, 52.50 (2 overlapping peaks), 51.20, 41.61, 40.30, 38.41, 32.20, 29.60, 28.46, 28.11, 28.07, 27.45, 25.02, 22.98, 22.76, 21.93. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{57}H_{81}N_7O_{15} + Cs]^+$ 1236.4845 found 1236.4913. FT-IR (neat): 3335.5, 2974.7, 2933.7, 2249.6, 1741.0, 1677.5, 1522.3, 1456.1, 1366.6, 1248.9, 1158.1, 903.5, 847.2, 733.0 cm^{-1}.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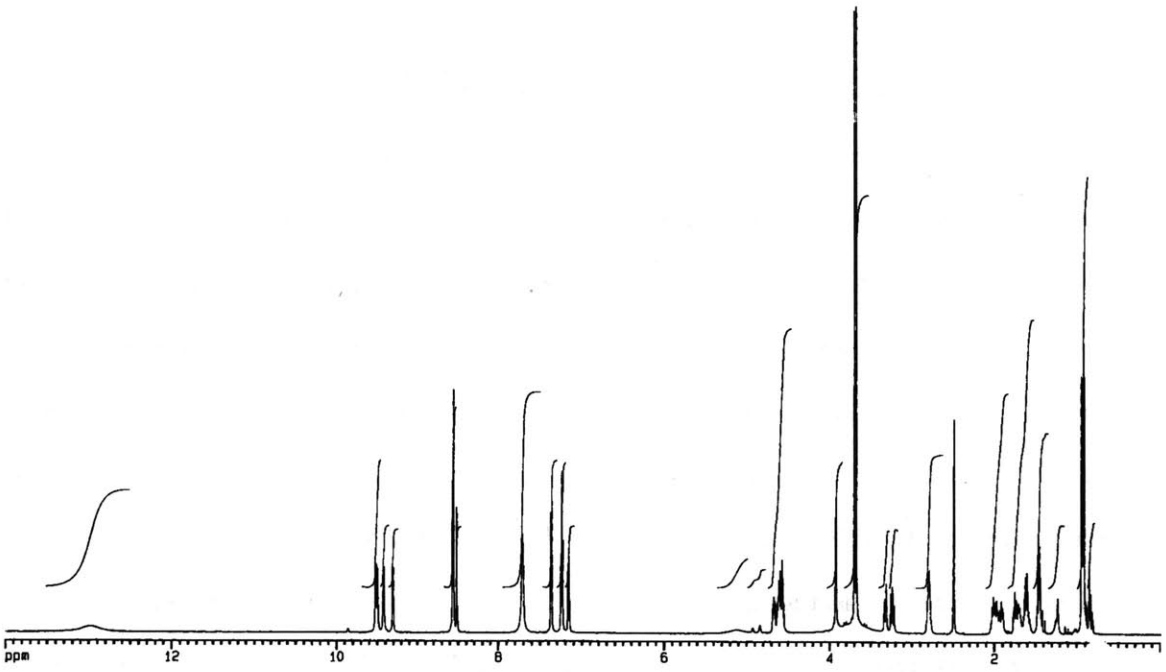
2-(Leucine methyl ester)-6-serine-2'-(lysine methyl ester, trifluoroacetate salt)-6'-(phenylalanine)-4,4'-bipyridine (**19**).

Compound **18** (0.208 g, 0.230 mmol) was stirred at rt in TFA for 19 h. The TFA was removed by rotary evaporation, and the resulting oil was precipitated by sonication with 1:1 Et_2O/hexane. Filtration yielded a white powder (0.143 g, 80%). m.p. 153–158 °C (dec.). ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.0 (br. s), 9.53 (d, J = 7.9 Hz, 1H), 9.51 (d, J = 8.1 Hz, 1H), 9.43 (d, J = 7.8 Hz, 1H), 9.32 (d, J = 8.1 Hz, 1H), 8.58–8.52 (m, 4H), 7.72 (br. s, 3H), 7.38 (d, J = 7.4 Hz, 2H), 7.25 (t, J = 7.5 Hz, 2H), 7.17 (t, J = 7.4 Hz, 1H), 5.11 (br. s, 1H), 4.68–4.57 (m, 4H), 3.93 (d, J = 5.3 Hz, 2H), 3.71 (s, 3H), 3.69 (s, 3H), 3.33 (dd, J = 4.3, 13.8 Hz, 1H), 3.24 (dd, J = 10.8, 13.5 Hz, 1H), 2.82–2.78 (m, 2H), 2.03–1.91 (m, 3H), 1.77–1.60 (m, 4H), 1.48–1.42 (m, 2H), 0.95 (d, J = 6.3 Hz, 3H), 0.92 (d, J = 6.3 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 172.67, 172.51, 171.93, 171.46, 163.20, 163.04, 162.88, 162.84, 149.83, 149.60 (2 overlapping peaks), 149.42, 137.97, 129.17, 128.22, 126.43, 122.65, 122.60, 122.52 (2 overlapping peaks), 60.80, 55.44, 54.42, 52.26, 52.11, 52.07, 50.96, 39.28, 38.64, 36.11, 30.05, 26.59, 24.56, 22.77, 22.52, 21.41. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{40}H_{49}N_7O_{13} + Cs]^+$ 968.2443 found 968.2410. FT-IR (neat): 3351.1, 2959.9, 1734.2, 1653.3, 1534.0, 1200.0, 900.1, 651.1 cm^{-1}.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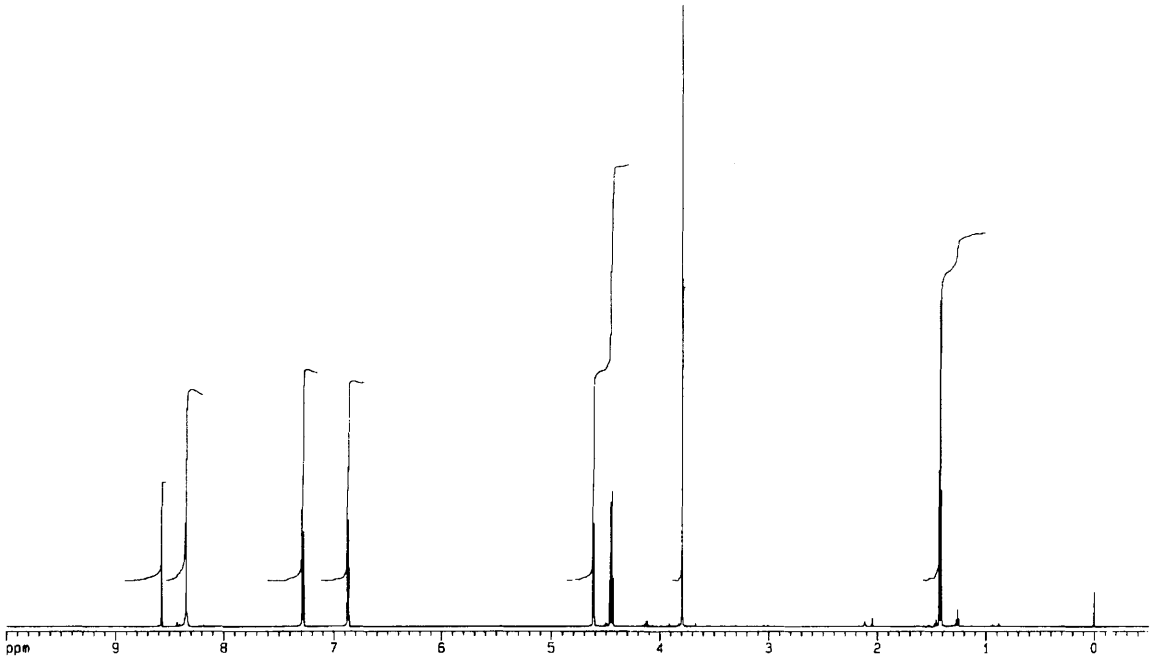
4-Bromo-2-(ethyl ester)-6-(*p*-methoxybenzylamide)-pyridine (20).

A. Isolation of mixed adduct from diacid chloride: To a chilled (0 °C) solution of 4-bromo-2,6-pyridinedicarboxylic acid chloride **11** (3.13 g, 11.07 mmol) in CH_2Cl_2 (160 mL) was slowly added a chilled (0 °C) solution of 4-methoxybenzylamine (1.52 mL, 11.62 mmol), EtOH (0.68 mL, 11.62 mmol) and Et_3N (6 mL) in CH_2Cl_2 (160 mL). After 15 min additional EtOH (2.7 mL, 46.5 mmol) was added. The solution was stirred under nitrogen for 20 h, warming to rt. The solution was diluted to 600 mL with CH_2Cl_2 and was washed with 0.5 M HCl (3 × 300 mL), saturated $NaHCO_3$ (3 × 200 mL) and brine (2 × 200 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated to a yellow oil. The heterosubstituted compound was separated from the diamide and the diester by silica gel column chromatography (8:3 hexane/EtOAc) and **20** (1.45 g, 67%) was isolated as a clear oil that crystallized on standing. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.57 (d, J = 1.8 Hz, 1H), 8.35 (d, J = 2.0 Hz, 1H), 8.35 (br. t, 1H), 7.29 (d, J = 8.7 Hz, 2H), 6.87 (d, J = 8.7 Hz, 2H), 4.62 (d, J = 6.2 Hz, 2H), 4.45 (q, J = 7.1 Hz, 2H), 3.80 (s, 3H), 1.42 (t, J = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$, 151 MHz): δ 163.42, 162.40, 159.23, 151.27, 147.98, 135.58, 130.62, 130.10, 129.37, 128.98, 114.24, 62.61, 55.47, 43.22, 14.40. LRMS (ESI +) m/z calcd for $[C_{17}H_{17}BrN_2O_4 + H]^+$ 393/395 found 393/395.

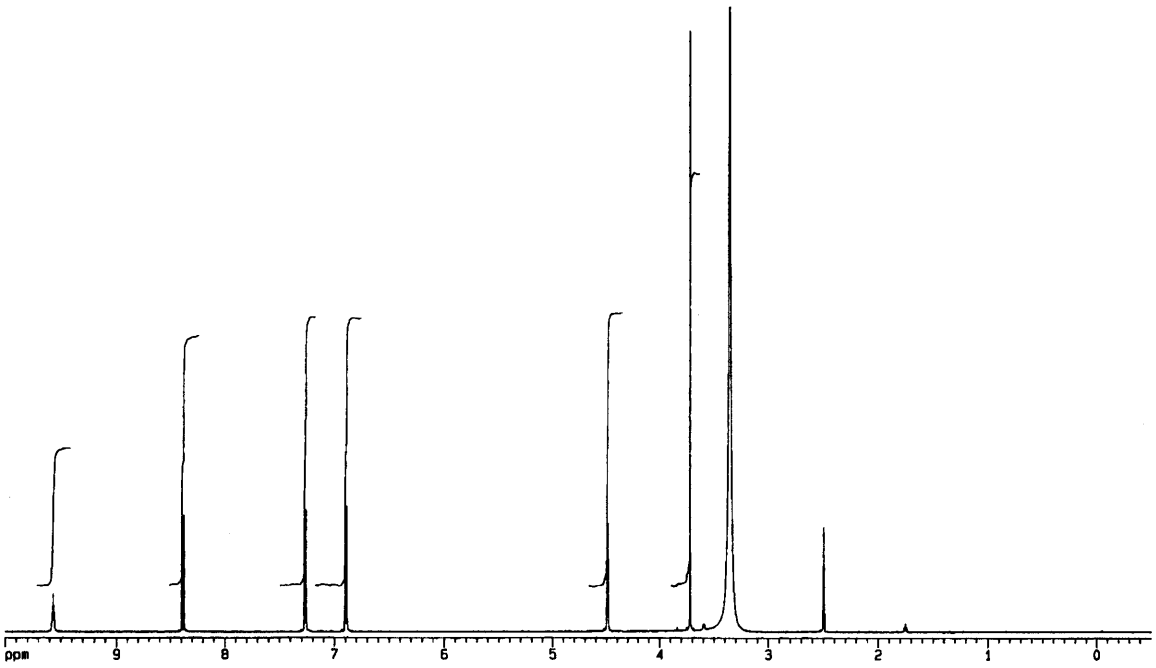
B. PyBOP coupling of ester/acid **23**: Bromopyridine monoacid/monoester **23** (660 mg, 2.40 mmol) and PyBOP (1.38 g, 2.64 mmol) were dissolved in 50 mL DMF. To this stirred solution was added N-methylmorpholine (292 μL, 2.64 mmol) and *p*-methoxybenzylamine (345 μL, 2.64 mmol), and the final solution was stirred under nitrogen for 17 h. The solvent was removed by rotary evaporation and the yellow oil remaining was taken up in CH_2Cl_2 and was washed with HCl, saturated $NaHCO_3$, and brine. The organic phase was dried over magnesium sulfate, was filtered, and was reduced to an oil by rotary evaporation. The oil was

purified by silica gel column chromatography (2:1 hexane/EtOAc), yielding **20** (708 mg, 75%) as a white powder upon sonication of the resulting oil in ether.



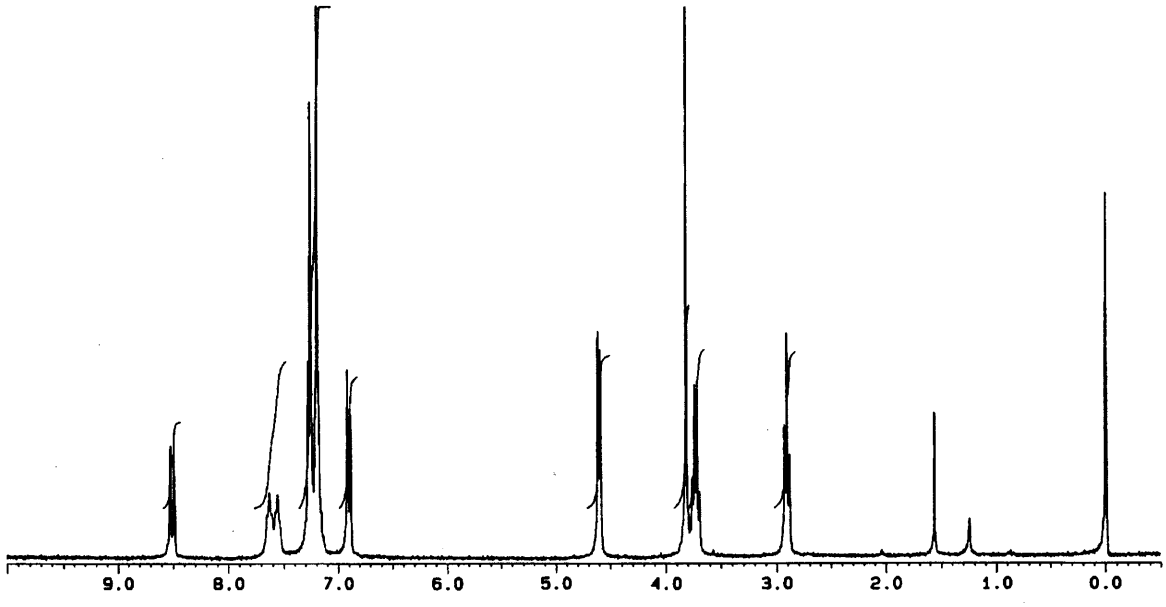
4-Bromo-2-(carboxylic acid)-6-(*p*-methoxybenzylamide)-pyridine (21).

To a solution of **20** (1.19 g, 3.0 mmol) in THF (40 mL) was added a solution of lithium hydroxide monohydrate (250 mg, 6.0 mmol) in water (10 mL). This was stirred vigorously for 1 h, then the solution was acidified by addition of 1 M HCl (230 mL). Solids were formed and the mixture was sonicated, filtered, and dried yielding **21** (0.97 g, 89%) as a white powder. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.58 (t, J = 6.2 Hz, 1H), 8.40 (d, J = 1.7 Hz, 1H), 8.38 (d, J = 1.8 Hz, 1H), 7.27 (d, J = 8.6 Hz, 2H), 6.90 (d, J = 8.6 Hz, 2H), 4.49 (d, J = 6.2 Hz, 2H), 3.72 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 163.9, 161.8, 158.4, 150.6, 147.7, 135.3, 130.9, 129.4, 128.9, 128.2, 113.8, 55.1, 42.0. LRMS (ESI -) calcd for $[C_{15}H_{13}BrN_2O_4 - H]^-$ 363/365 found 363/365.



4-Bromo-2-(phenethylamide)-6-(*p*-methoxybenzylamide)-pyridine (22).

To a solution of **21** (826 mg, 2.26 mmol) in DMF (45 mL) were added N-methylmorpholine (275 μL, 2.5 mmol), PyBOP (1.3 g, 2.5 mmol) and phenethylamine (313 μL, 2.5 mmol). This was stirred under nitrogen for 30 min, then concentrated to a pale yellow oil. The oil was dissolved in CH_2Cl_2 (100 mL) and was extracted with 0.5 M HCl (2 × 100 mL), water (100 mL) and brine (100 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated to a clear oil. Silica gel column chromatography (1:1 hexane/EtOAc) yielded **22** (1.09g, 92%) as a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.52 (d, J = 1.5 Hz, 1H), 8.48 (d, J = 1.5 Hz, 1H), 7.62 (br. t, 1H), 7.55 (br. t, 1H), 7.24–7.18 (m, 7H), 6.89 (d, J = 8.4 Hz, 2H), 4.59 (d, J = 6.1 Hz, 2H), 3.80 (s, 3H), 3.72 (appar. q, J = 6.5 Hz, 2H), 2.90 (t, J = 6.8 Hz, 2H). LRMS (ESI +) m/z calcd for $[C_{23}H_{22}BrN_3O_3 + Na]^+$ 490/492 found 490/492.



Chapter 4. Bipyridines as PAI-1 Antagonists[1]

4.1 Plasminogen activator inhibitor

Tissue-type plasminogen activator (t-PA), a 68-kDa member of the (chymo)trypsin family of serine proteases, catalyzes the rate-limiting step in the endogenous fibrinolytic cascade. This step is activation of the circulating zymogen plasminogen into the active enzyme, plasmin, which then degrades the fibrin meshwork of thrombi (see Figure 1).[2] To prevent systemic activation of plasminogen, blood plasma contains several inhibitors of t-PA, the most important of which is plasminogen activator inhibitor type 1 (PAI-1), a member of the serpin superfamily.[3]

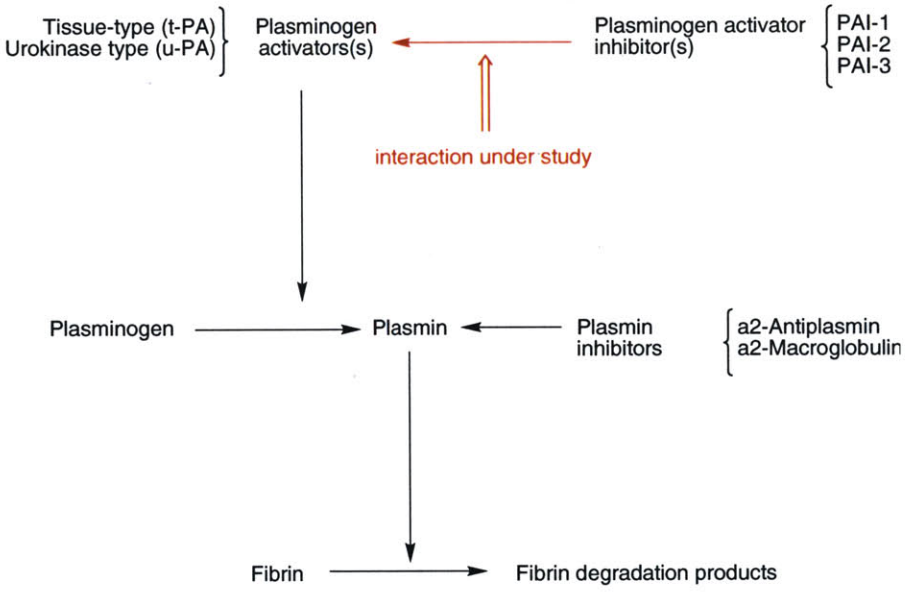


Figure 1. A simplified depiction of the fibrinolytic cascade.

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[1] This work was carried out in collaboration with Edwin L. Madison, Ph. D., Director, Molecular Biology, Corvas International, Inc. and his research group. Technical assistance was provided by Ellen Choi and Christina Cueto.

[2] (a) Bachmann, F.; Kruithof, E. K. O. *Semin. Throm. Haemost.* **1984**, *10*, 6–17. (b) Gerard, R. D.; Chien, K. R.; Meidell, R. S. *Molec. Biol. Med* **1986**, *67*, 1529–1541.

[3] Carrell, R.; Boswell, G. in *Protease Inhibitors* eds. Barrett, A. J.; Salvenson, G. S. 403–425, Elsevier, Amsterdam, 1986.

Recombinant t-PA that is resistant to PAI-1 is used theraputically to induce thrombolysis in a variety of clinical settings, most notably myocardial infarction.[4] A small molecule that inhibits the interaction between t-PA and PAI-1 can be used to elucidate the nature of the interaction, and might also be of great theraputic value. To date, the best reported inhibitors are diketopiperazines with activities in the range of several micromolar (see Figure 2).[5]

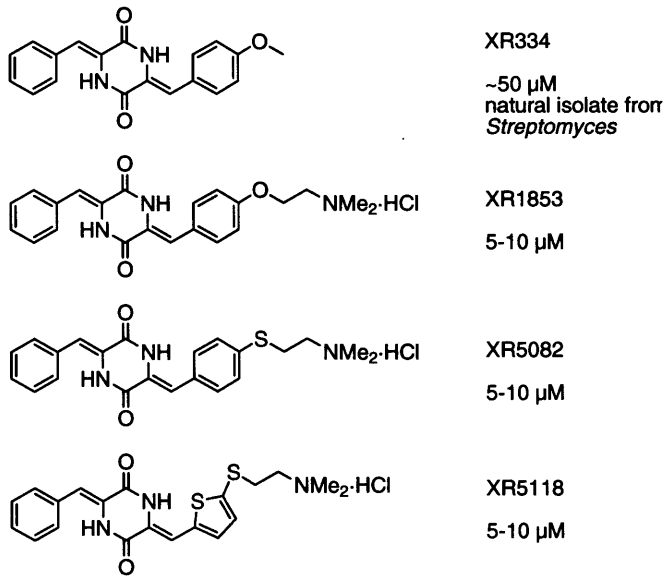

Figure 2. Diketopiperazine inhibitors of PAI-1.

4.2 Initial library synthesis

We prepared a series of twenty libraries, 15 composed of approximately 7000 members and five composed of approximately 25,000 members. Ten libraries were prepared on the xanthene tetraacid chloride core molecule and ten were prepared on the bipyridine

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[4] Bergmann, S. R.; Fox, K. A. A.; Ter-Pogossian, M. M.; and Sobel, B. E. *Science* **1983**, *220*, 1181–1183.

[5] (a) Charlton, P.; Faint, R.; Barnes, C.; Bent, F.; Folkes, A.; Templeton, D.; Mackie, I; Machin, S.; Bevan, P. *Fibrinolysis & Proteolysis* **1997**, *11*, 51–56. (b) Charlton, P. A.; Faint, R. W.; Bent, F.; Bryans, J.; Chicarelli-Robinson, I.; Mackie, I.; Machin, S.; Bevan, P. *Thrombosis & Haemostasis* **1996**, *75*, 808–15.

tetraacid chloride core (Figure 3).[6] The building blocks were chosen randomly from among amino acid derivatives and various other amines with some degree of overlap among them. Initial screening[7] of these libraries identified five libraries active in inhibiting the t-PA/PAI-1 interaction with each core molecule. Synthesis of four sublibraries (two with each core) using building blocks common to several of the active libraries resulted in the identification of an active library of 1225 compounds (seven building blocks) based on the xanthene core. A series of seven deletion libraries identified benzylamine, furfurylamine, and butylamine as particularly important building blocks.

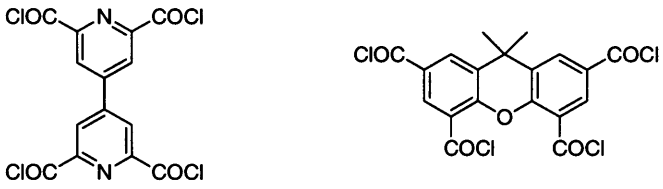

Figure 3. Bipyridine tetraacid chloride and xanthene tetraacid chloride core molecules for initial PAI-1 antagonism libraries.

At this point, we used combinations of these building blocks on a number of different cores to see how important the geometry of the building block presentation was to the activity of the prepared libraries. Surprisingly, the bipyridine libraries produced at this stage exceeded the activity of the original xanthene libraries, with activity levels of ~50 μM. Other cores (see Figure 4), such as the *cis*-glycoluril bis(pentafluorophenyl ester) (to be discussed in Chapter 5), 2,6-pyridine diacid chloride, 4-bromo-2,6-pyridine diacid chloride, and xanthene diacid chloride yielded inactive libraries.

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[6] See experimental section for library details.

[7] Experimental details regarding the screens are reported in the experimental section.

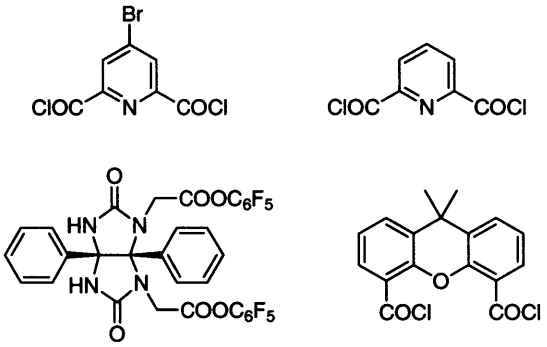


Figure 4. Other core molecules used to examine shape-space dependence of building blocks. No libraries with these cores proved active in PAI-1 antagonism assays.

It was found that the libraries had similar activity in a binding inhibition assay with urokinase-type plasminogen activator (u-PA) and PAI-1. Additionally, a pre-incubation period with PAI-1 increased the activity of the libraries. These two results both imply that the mode of action of the libraries is by binding to PAI-1 in such a way as to disable its ability to bind t-PA and u-PA. Our current hypothesis is that this class of PAI-1 antagonists is causing an allosteric change in the protein upon binding, rather than interfering directly with the protein-protein interface.

4.3 Second round of library synthesis

Since the initial libraries were composed of randomly-chosen building blocks, a second set of libraries was prepared; this time the building blocks were chosen based on their similarity to the building blocks already identified as providing active libraries (see Figure 5).

The three building blocks already identified were included in every library. To these were added four of six other amines, for a total of seven libraries which contained all possible combinations of the nine building blocks in at least one of them. Library S3-3 (Figure 5) was the most active of this set of libraries.

	S3-1	S3-2	S3-3	S3-4	S3-5	S3-6	S3-7
1-(2-aminoethyl)pyrrolidine	x			x	x	x	x
4-(2-aminoethyl)morpholine	x	x			x	x	x
2-(2-aminoethyl)pyridine	x	x	x			x	x
furfurylamine	x	x	x	x	x	x	x
4-methoxybenylamine	x	x	x	x			x
4-(aminomethyl)pyridine	x	x	x	x	x		
benzylamine	x	x	x	x	x	x	x
phenethylamine		x	x	x	x	x	
isobutylamine			x	x	x	x	x
butylamine	x	x	x	x	x	x	x

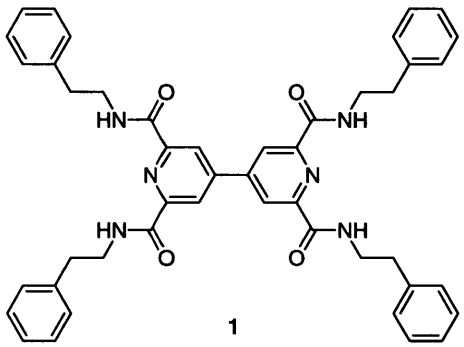
	S4-1	S4-2	S4-3	S4-4	S4-5	S4-6	S4-7	S4-8
2-(2-aminoethyl)pyridine		x	x	x	x	x	x	x
furfurylamine	x		x	x	x	x	x	x
4-methoxybenylamine	x	x		x	x	x	x	x
4-(aminomethyl)pyridine	x	x	x		x	x	x	x
benzylamine	x	x	x	x		x	x	x
phenethylamine	x	x	x	x	x		x	x
isobutylamine	x	x	x	x	x	x		x
butylamine	x	x	x	x	x	x	x	

	1	2	3	4	5	6	7	8	9	10	11
2-(2-aminoethyl)pyridine	x				x	x		x	x		
furfurylamine			x		x		x	x	x		x
4-methoxybenylamine		x				x	x	x	x	x	x
4-(aminomethyl)pyridine											
benzylamine											
phenethylamine				x					x	x	x
isobutylamine											
butylamine											

Figure 5. Directed libraries and sublibraries.

Another set of deletion libraries was prepared (Figure 5), and the four least active of these (implying that the missing building block was important to the activity of the library) were identified. We then prepared a series of individual compounds and small libraries with these four building blocks [2-(2-aminoethyl)pyridine, furfurylamine, 4-methoxybenzylamine, 4-(aminomethyl) pyridine, and phenethylamine]. The most potent single compound prepared

from this series of libraries was the tetra(phenethylamine) adduct of bipyridine tetraacid chloride **1** (library 4 in Figure 5; see Figure 6), which was found to have an activity of 16 μM. However, some of the small libraries were even more active on a mass basis, implying that some of the heterosubstituted compounds very likely had even higher activity.



1

Figure 6. The tetrakis(phenethylamine) adduct of bipyridine tetraacid chloride, the most potent single compound identified in the second round of library screening.

4.4 An unusually active library

Unusual activity was found in one particular library using phenethylamine and *p*-methoxybenzylamine as building blocks. HPLC of this library showed it to be a more complex mixture than the expected six compounds (see Figure 7). Preparative HPLC separation of this mixture allowed the isolation of several individual samples. Unfortunately, the collaboration was interrupted at this point for legal reasons, so it was not known which of the samples would prove active. It was therefore decided to simply go forward with the synthesis of all six compounds that were expected to be found in the library. Two of them (the two tetra-homosubstituted compounds **1** and **2**) had already been prepared. What remained were the four heterosubstituted compounds (Figure 8). These were synthesized according to methods involving a Stille coupling between two individually-prepared half-molecules as described in Chapter 3 (see compounds **7**-**12**, **14**-**16** in Scheme 1 below).

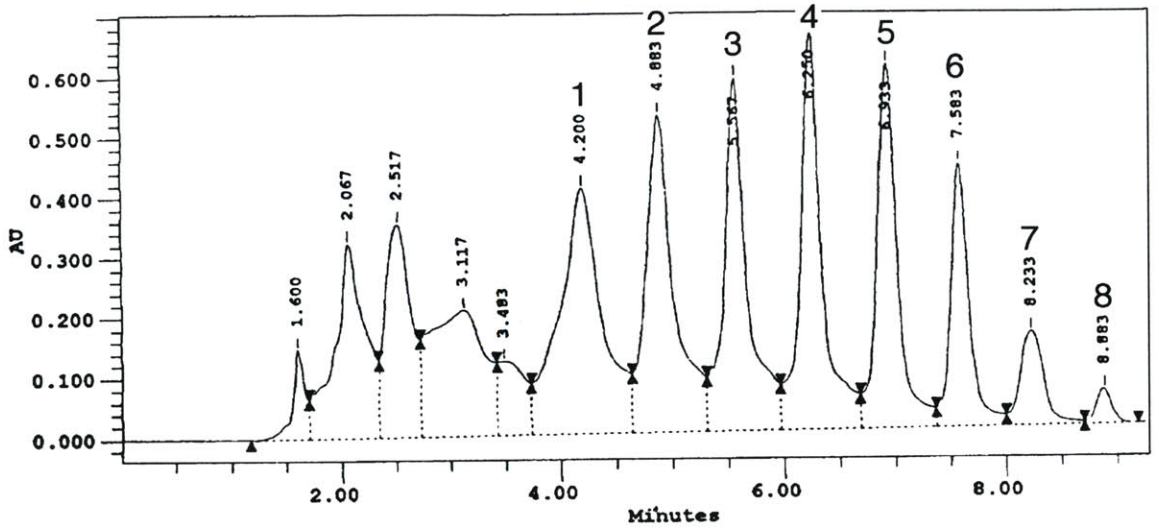


Figure 7. HPLC trace of the most active library—note that the library should theoretically have six components. Fraction 2 exhibited >5 x the activity of the entire library.

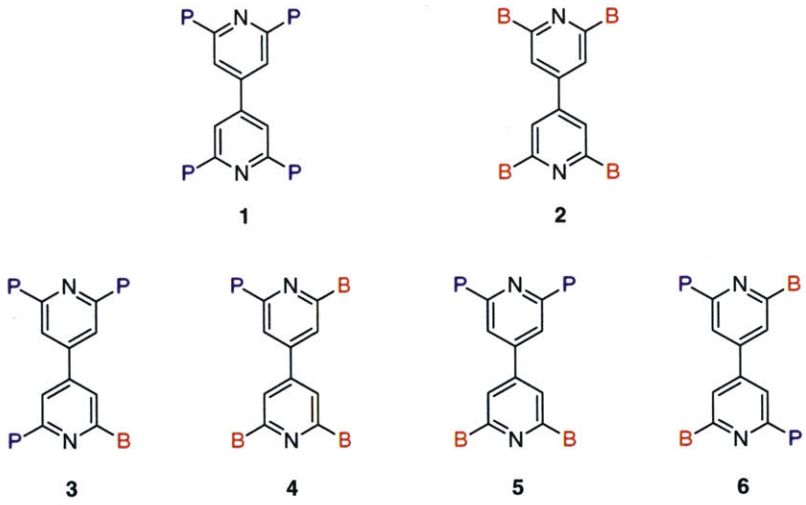


Figure 8. The six compounds expected to be formed from the reaction of the bipyridine tetraacid chloride core molecule with phenethylamine (P) and *p*-methoxybenzylamine (B).

In this particular case, however, the two building blocks were so similar to each other that chromatographic separation of the heterosubstituted pyridine diamide from the two homosubstituted variants was prohibited, and required application of the alternative synthetic routes to heterosubstituted bromopyridines.

After each of the compounds had been synthesized, the screens were performed on the prep-HPLC fractions and the pure synthetic compounds. It was discovered that the six individual compounds were all similar in activity, ranging from about 16–30 μM. However, one fraction from the HPLC samples (fraction 2 in Figure 7) exhibited much greater activity. MS analysis of this compound found a mass consistent with a triamide monoacid bipyridine (see Figure 9).

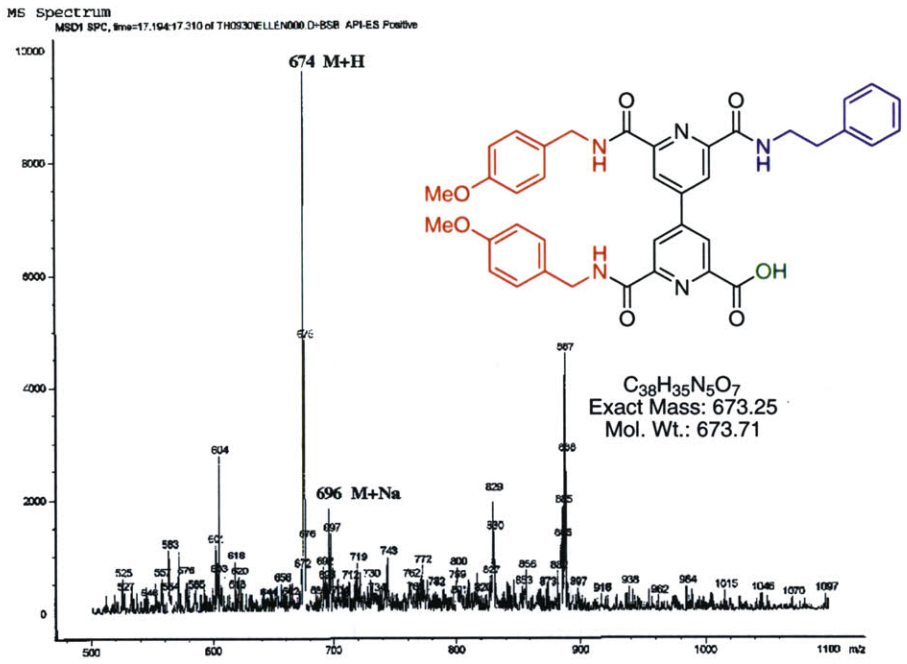
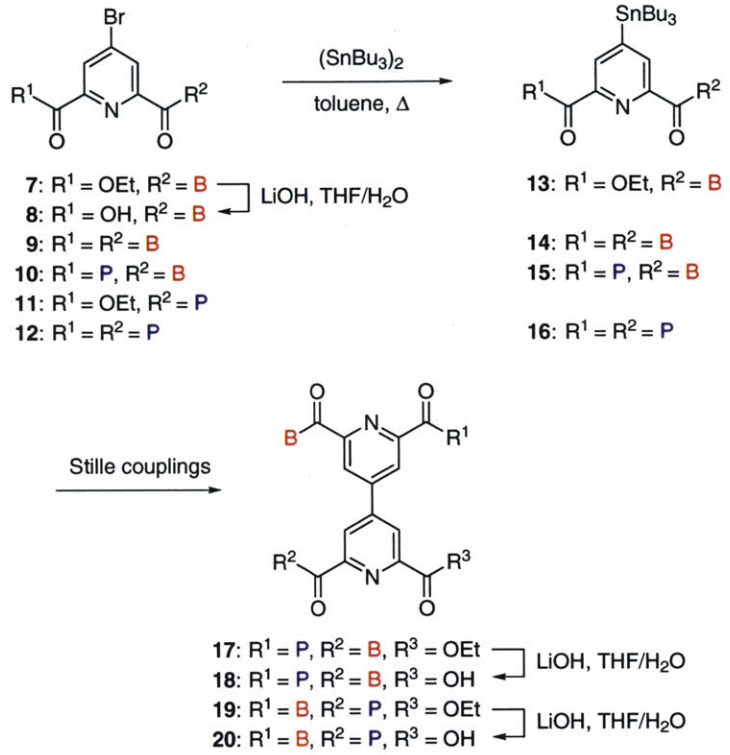


Figure 9. MS spectrum of fraction 2 from the preparative HPLC of the most active library. Fraction 2 was the most active component of the mixture, and the mass is consistent with that of a triamide monoacid.

4.5 Mixed amide/acid compounds

We then synthesized the two isomers **18** and **20** of that mass (Scheme 1). In the penultimate step of the synthesis the corresponding ethyl esters **17** and **19** were isolated, and these were also kept for testing. Additionally, the bromopyridine mono(*p*-methoxy-

benzylamide) monoacid **8** and the bromopyridine monoamide mono(ethyl esters) **7** and **11** were all screened.



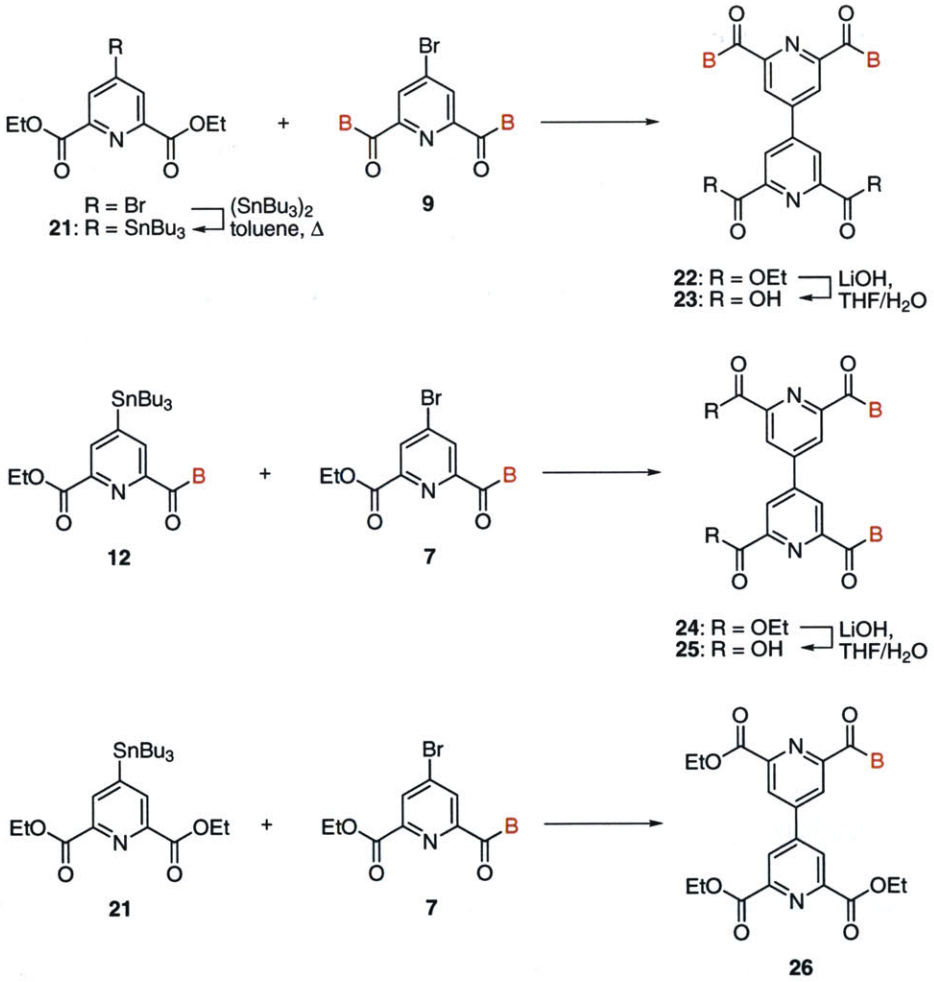
Scheme 1. Synthesis of the two isomers **18** and **20** corresponding to the mass of the most active HPLC fraction. Some intermediates presented here were used in the synthesis of tetraamides **3–6**. P = phenethylamine, B = *p*-methoxybenzylamine.

The screening data demonstrated that both isomers **18** and **20** exhibit significant inhibitory activity in the assay, while both ethyl esters **17** and **19**, as well as the half-molecules containing either esters or free acids, are all essentially inactive. The activity of both triamide isomers is very nearly the same as the strongly active fraction isolated by prep-HPLC above, at about 8–10 μM.

After confirmation of the activity of the triamide monoacid compounds, a series of other mixed amide/acid compounds was prepared. Since the activity of both triamide isomers were very similar, it was decided to use only *p*-methoxybenzylamine in subsequent

compounds to simplify the synthesis; if it were determined that significant activity was found in the new compounds, other building blocks could be used to fine-tune the activity.

Accordingly, diacids **23** and **25** were prepared, as well as triester **26** (Scheme 2). Surprisingly, attempted saponification of **26** using standard conditions (LiOH, THF/H_2O) resulted in failure. It is not known at this time what, precisely, went wrong, but there is little reason to think that the expected triacid could not be synthesized by adjusting the conditions or workup procedure.



Scheme 2. Synthesis of di- and monoamide compounds for screening.

The screening results discouraged further pursuit of the triacid, however. Diacids **23** and **25** both showed activity, but at a much-reduced level (>100 μM). The tetraacid core

molecule itself exhibited no activity, mirroring the results of diesters **22** and **24** and triester **26**.

4.6 Experimental

General

All reagents were purchased from Aldrich Chemical Company and were used without further purification except as noted. Amino acid esters, PyBOP, and EDC•MeI were acquired from Novabiochem (San Diego, CA). Deuterated solvents were obtained from Cambridge Isotopes Laboratories and deuterated chloroform was dried over 4Å molecular sieves. HCl refers to a 1N stock solution. NMR spectra were recorded on either a Bruker AC-250, a Bruker AM-300, or a Bruker DRX-600; TMS was used as a reference in some chloroform-*d* spectra, otherwise residual solvent was used as a reference. Either a Finnegan Mat 8200 (for EI) or a VG ZAB-VSE (for FAB) mass spectrometer was used to ascertain masses. FT-IR spectra were obtained on a Perkin Elmer Paragon 1000 PC FT-IR Spectrometer. Silica gel chromatography was performed with Silica Gel 60 (EM Science or Bodman, 230–400 mesh). TLC analysis was performed using glass-bound Silica Gel 60 (F254) plates. DMAP = *N,N*-dimethyl-4-aminopyridine, DPPA = diphenylphosphoryl azide, EDC•MeI = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide, PyBOP = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate.

General procedure for library synthesis

Note: The solvent system and scale of the synthesis varied slightly in some cases, and several different core molecules were used; the procedure below exemplifies the procedure and can be considered fairly general.

In a 25 mL round bottom flask was stirred 0.072 mmoles of each of 15 amine building blocks (4.4 equivalents total, 1.1 equivalents per core reactive site), added neat or in DMF

solution. DMF was added to bring the total DMF volume to 2 mL. To this solution was added 1 mL triethylamine. A solution of 2,2',6,6'-(4,4'-bipyridine)tetracarboxylic acid chloride (100 mg, 0.246 mmole) in 4 mL warm methylene chloride was added to the chilled (0 °C) amine solution with stirring under nitrogen and the solution was diluted with methylene chloride to a total volume of 10 mL. This solution was left to stir for 1.5 h under nitrogen. The solution was diluted with methylene chloride to ~30 mL and was washed twice with water and once with brine. The organic layer was dried over $MgSO_4$ and was filtered. The solution was concentrated by rotary evaporation to an oil, then was further dried under high vacuum for 3 hours.

This oil was then stirred in 10 mL TFA overnight to effect the deprotection of *tert*-butyl ester functionalities.[8] Removal of TFA by rotary evaporation yielded a yellow oil which was lyophillized to yield a pale yellow powder.

PAI-1 Screen

PAI-1 antagonists were assayed in a standard, indirect chromogenic assay.[9] Briefly, fixed concentrations of active site titrated t-PA and standardized PAI-1 were incubated in the presence or absence of antagonist candidates for 10–15 min at 20 °C. The samples were then diluted and residual enzymatic activity was measured and compared to assays containing t-PA only.

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[8] In cases where no protecting groups were present among the building blocks, the TFA deprotection step was skipped.

[9] Madison, E. L.; Sambrook, J. F. *Methods in Enzymology* **1993**, *223*, 249–271.

Initial PAI-1 bipyridine tetraamide libraries

Fifteen building blocks were used in each library as outlined below, theoretically yielding 7260 compounds.

Building blocks	1	2	3	4	5	6	7	8	9	10
#1. 2-amino-5-diethylaminopentane		x	x		x			x		
#3. 2(2-aminoethyl)-1-methylpyrolidine		x	x		x	x		x	x	
#4. 1-(2-aminoethyl)-pyrrolidine		x		x			x		x	
#5. 4-(2-aminoethyl)-morpholine		x			x	x		x		
#6. 2-(2-aminoethyl)-pyridine		x			x		x		x	
#8. 1-amino-4-methylpiperazine		x		x			x		x	
#9. 4-aminomorpholine		x					x			x
#10. furfurylamine		x		x	x		x		x	x
#11. 4-methoxybenylamine		x		x		x		x		
#12. 1-aminopiperidine		x		x		x			x	x
#13. 4-(aminoethyl)pyridine		x				x		x		x
#14. H-Ala-OMe•HCl		x	x		x		x	x		x
#15. H-Ala-OtBu•HCl	x			x		x			x	
#16. H-Asn-OtBu•HCl	x			x	x		x	x	x	x
#17. H-Asp(OtBu)-OMe•HCl	x	x						x		
#18. H-Asp(OtBu)-OtBu•HCl				x		x				
#19. H-Glu(OtBu)-OtBu•HCl	x		x		x		x	x		x
#20. H-Gly-OMe•HCl	x							x	x	x
#21. H-Ile-OMe•HCl		x	x	x	x		x	x		
#22. H-Ile-OtBu•HCl	x			x		x			x	
#23. H-Leu-OtBu•HCl	x		x		x	x	x			x
#24. H-Lys(BOC)-OMe•HCl	x		x			x				x
#25. H-Lys(BOC)-OtBu•HCl				x	x		x	x		
#26. H-Met-OMe•HCl	x		x	x	x	x	x			x
#27. H-Phe-OtBu•HCl	x								x	
#28. H-Pro-OtBu•HCl	x			x	x		x	x	x	x
#29. H-Ser(tBu)-OtBu•HCl	x		x	x		x	x			
#30. H-Ser-OMe•HCl		x	x		x					x
#31. H-Thr(tBu)-OMe•HCl	x		x	x	x	x				x
#32. H-Tyr-OMe•HCl			x				x			
#33. H-Val-OMe•HCl	x		x			x		x	x	x
#34. H-Val-OtBu•HCl			x						x	
#35. H-Tyr(tBu)-OMe•HCl	x		x			x		x	x	

Initial PAI-1 xanthene tetraamide libraries

Fifteen building blocks were used in libraries 11–15, theoretically yielding 25,425 compounds and eleven building blocks were used in libraries 16–20, theoretically yielding 7381 compounds as outlined below.

Building blocks	11	12	13	14	15	16	17	18	19	20
#1. 2-amino-5-diethylaminopentane		x			x					
#3. 2(2-aminoethyl)-1-methylpyrolidine		x		x			x			
#4. 1-(2-aminoethyl)-pyrrolidine		x	x							
#5. 4-(2-aminoethyl)-morpholine		x		x	x		x			
#6. 2-(2-aminoethyl)-pyridine		x				x		x		
#8. 1-amino-4-methylpiperazine		x	x					x		
#9. 4-aminomorpholine		x							x	
#10. furfurylamine		x	x	x		x			x	
#11. 4-methoxybenylamine		x	x		x					x
#12. 1-aminopiperidine		x	x							x
#13. 4-(aminoethyl)pyridine		x		x						
#14. H-Ala-OMe•HCl	x			x		x			x	
#15. H-Ala-OtBu•HCl			x		x		x			
#16. H-Asn-OtBu•HCl	x		x			x		x		
#17. H-Asp-OMe-OtBu•HCl		x								
#18. H-Asp(OtBu)-OtBu•HCl	x		x		x				x	
#19. H-Glu(OtBu)-OtBu•HCl	x			x	x	x		x	x	
#20. H-Gly-OMe•HCl		x					x			x
#21. H-Ile-OMe•HCl	x		x				x		x	x
#22. H-Ile-OtBu•HCl			x		x					
#23. H-Leu-OtBu•HCl	x			x		x	x	x		
#24. H-Lys(BOC)-OMe•HCl	x	x								
#25. H-Lys(BOC)-OtBu•HCl	x		x	x	x		x		x	x
#26. H-Met-OMe•HCl	x	x	x		x					
#27. H-Phe-OtBu•HCl						x				x
#28. H-Pro-OtBu•HCl	x		x	x		x		x	x	x
#29. H-Ser(tBu)-OtBu•HCl	x		x							
#30. H-Ser-OMe•HCl					x			x		
#31. H-Thr(tBu)-OMe•HCl	x		x	x		x			x	
#32. H-Tyr-OMe•HCl	x							x		
#33. H-Val-OMe•HCl	x						x	x		x
#34. H-Val-OtBu•HCl					x					
#35. H-Tyr(tBu)-OMe•HCl	x				x		x			x
#41. aniline				x						

#42. benzylamine				x		x				
#43. phenethylamine				x			x			
#45. isobutylamine				x			x			
#46. butylamine					x			x		
#47. N,N-diethylethylenediamine						x		x		
#48. 3-(dimethylamino)propylamine					x				x	
#49. aminomethyl cyclopropane									x	
#50. 4-amino-1-benzyl piperidine										x
#51. 4-(3-aminopropyl) morpholine				x						x
#52. 1-(3-aminopropyl)-2-pyrrolidinone										
#53. ethyl 4-amino-1-piperidine carboxylate					x					

Bipyridine tetrakis(phenethylamide) (1).

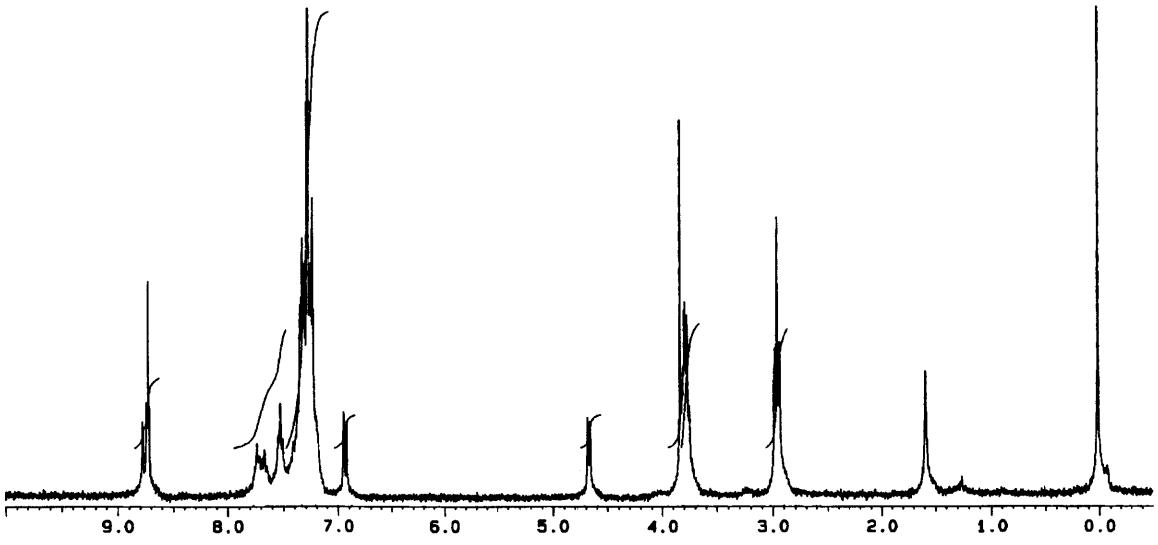
Produced according to general library procedure and screened without characterization.

Bipyridine tetrakis(*p*-methoxybenzylamide) (2).

See Chapter 3, compound **2**.

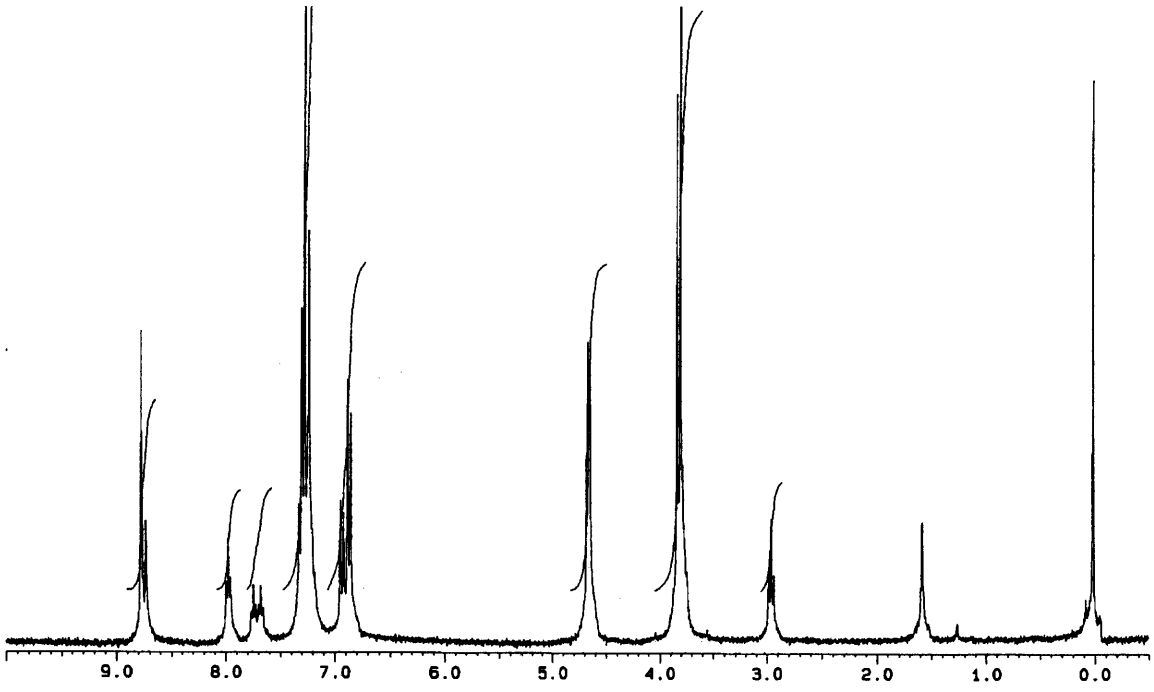
Bipyridine tris(phenethylamide) *p*-methoxybenzylamide (3).

To a stirred solution of bis(phenethylamide)pyridine tributylstannane **16** (201 mg, 0.30 mmol) in 11 mL toluene was added mixed pyridine bromide **10** (141 mg, 0.30 mmol) and $PdCl_2(PPh_3)_2$ (22 mg, 0.031 mmol). The reaction mixture was refluxed under nitrogen for 16 h. The solvent was removed by rotary evaporation and the residue was taken up in chloroform and filtered through Celite. After removing the solvent by rotary evaporation, the residue was triturated with EtOAc/hexane (1:1) and **3** (70 mg, 31%) was isolated by filtration as a pale yellow powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.77–8.71 (m, 4H), 7.73–7.23 (m, 21H), 6.93 (d, J = 8.5 Hz, 2H), 4.66 (d, J = 6.0 Hz, 2H), 3.83 (s, 3H), 3.82–3.74 (m, 6H), 2.96 (t, J = 6.9 Hz, 6H). LRMS (EI +) m/z calcd for $[C_{46}H_{44}N_6O_5 + Na]^+$ 783 found 783.



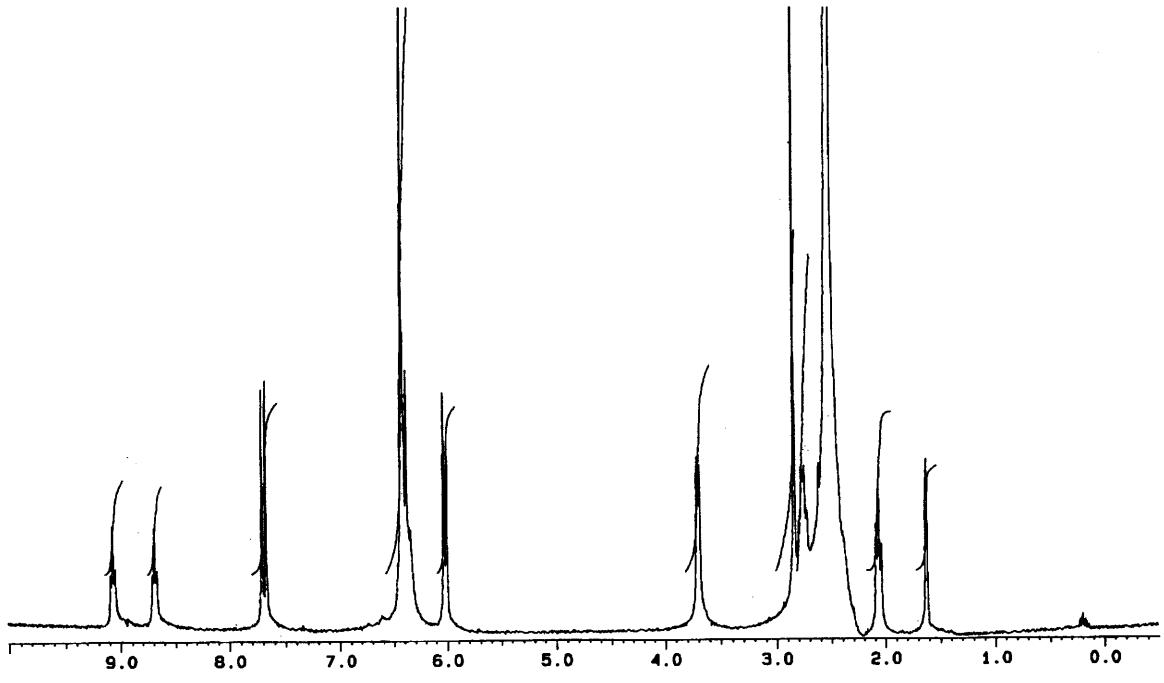
Bipyridine tris(*p*-methoxybenzylamide) phenethylamide (4).

To a stirred solution of bis(*p*-methoxybenzylamide)pyridine tributylstannane **14** (175 mg, 0.252 mmol) in 10 mL toluene was added mixed pyridine bromide **10** (123 mg, 0.263 mmol) and $PdCl_2(PPh_3)_2$ (11 mg, 0.016 mmol). The reaction mixture was refluxed under nitrogen for 16 h. The solvent was removed by rotary evaporation and the residue was taken up in chloroform and filtered through Celite. After removing the solvent by rotary evaporation, the residue was triturated with EtOAc and **4** (152 mg, 76%) was isolated by filtration as a pale yellow powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.79–8.73 (m, 4H), 7.97 (t, J = 6.0 Hz, 2H), 7.75 (t, J = 5.8 Hz, 1H), 7.68 (t, J = 6.0 Hz, 1H), 7.33–7.23 (m, 11H), 6.93 (d, J = 8.6 Hz, 2H), 6.86 (d, J = 8.6 Hz, 4H), 3.83 (s, 3H), 3.80 (s, 6H), 2.96 (t, J = 6.5 Hz, 2H). HRMS (FAB, NBA/CsI) m/z calcd for $[C_{46}H_{44}N_6O_7 + Cs]^+$ 925.2326 found 925.2304.



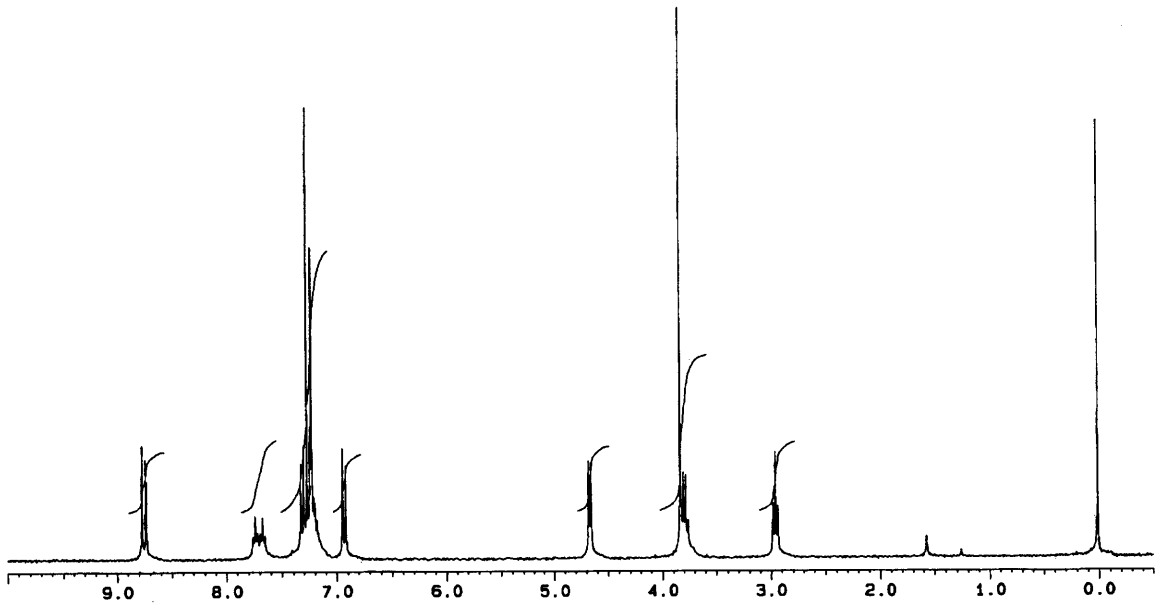
Bipyridine 2,6-bis(*p*-methoxybenzylamide)-2',6'-bis(phenethylamide) (5).

To a stirred solution of bis(phenethylamide)pyridine tributylstannane **16** (138 mg, 0.209 mmol) in 5 mL toluene was added bis(*p*-methoxybenzylamide)pyridine bromide **9** (101 mg, 0.209 mmol) and $PdCl_2(PPh_3)_2$ (16 mg, 0.022 mmol). The reaction mixture was refluxed under nitrogen for 16 h. The solvent was removed by rotary evaporation and the residue was sonicated in EtOAc/hexane (3:2) and the resulting solids were isolated by filtration. The solids were then sonicated in chloroform and filtered. After removing the solvent by rotary evaporation, the residue was triturated with EtOAc and **5** (49 mg, 30%) was isolated by filtration as a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.78 (s, 2H), 8.72 (s, 2H), 7.96 (t, J = 5.9 Hz, 2H), 7.53 (t, J = 5.9 Hz, 2H), 7.37–7.25 (m, 14H), 6.87 (d, J = 8.6 Hz, 4H), 4.66 (d, J = 6.0 Hz, 4H), 3.80 (s, 6H), 3.77 (t, J = 6.7 Hz, 4H), 2.96 (d, 6.9 Hz, 4H). HRMS (FAB, NBA/CsI) m/z calcd for $[C_{46}H_{44}N_6O_6 + Cs]^+$ 909.2377 found 909.2356.



Bipyridine 2,2'-bis(*p*-methoxybenzylamide)-6,6'-bis(phenethylamide) (6).

To a stirred solution of mixed pyridine stannane **15** (270 mg, 0.40 mmol) in 15 mL toluene was added mixed pyridine bromide **10** (101 mg, 0.209 mmol) and $PdCl_2(PPh_3)_2$ (16 mg, 0.022 mmol). The reaction mixture was refluxed under nitrogen for 16 h. The solvent was removed by rotary evaporation and the residue was sonicated in EtOAc/hexane (3:2) and the resulting solids were isolated by filtration. The solids were then sonicated in chloroform and were filtered. After removing the solvent by rotary evaporation, the residue was triturated with EtOAc and **5** (49 mg, 30%) was isolated by filtration as a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.78 (s, 2H), 8.72 (s, 2H), 7.96 (t, J = 5.9 Hz, 2H), 7.53 (t, J = 5.9 Hz, 2H), 7.37–7.25 (m, 14H), 6.87 (d, J = 8.6 Hz, 4H), 4.66 (d, J = 6.0 Hz, 4H), 3.80 (s, 6H), 3.77 (t, J = 6.7 Hz, 4H), 2.96 (d, 6.9 Hz, 4H). HRMS (FAB, NBA/CsI) m/z calcd for $[C_{46}H_{44}N_6O_6 + Cs]^+$ 909.2377 found 909.2356.



4-Bromo-2-(ethyl ester)-6-(*p*-methoxybenzylamide)-pyridine (**7**).

See Chapter 3, compound **20**.

4-Bromo-2-(carboxylic acid)-6-(*p*-methoxybenzylamide)-pyridine (**8**).

See Chapter 3, compound **21**.

4-Bromo-2,6-bis(*p*-methoxybenzylamide)-pyridine (**9**).

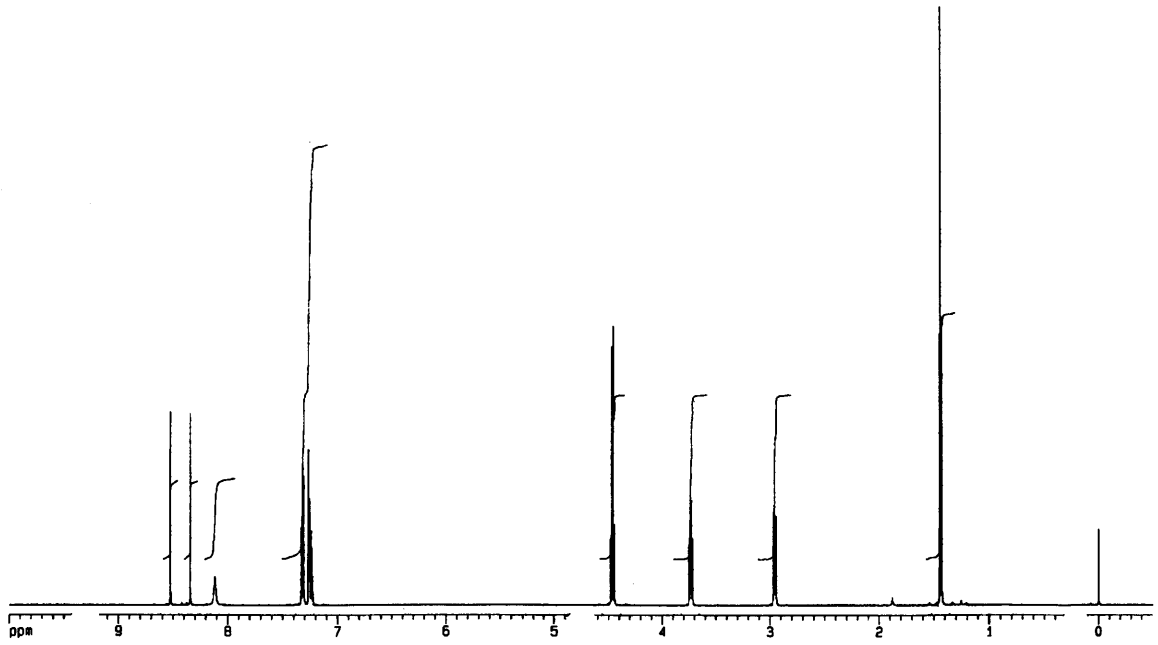
See Chapter 3, compound **12**.

4-Bromo-2-(phenethylamide)-6-(*p*-methoxybenzylamide)-pyridine (**10**).

See Chapter 3, compound **22**.

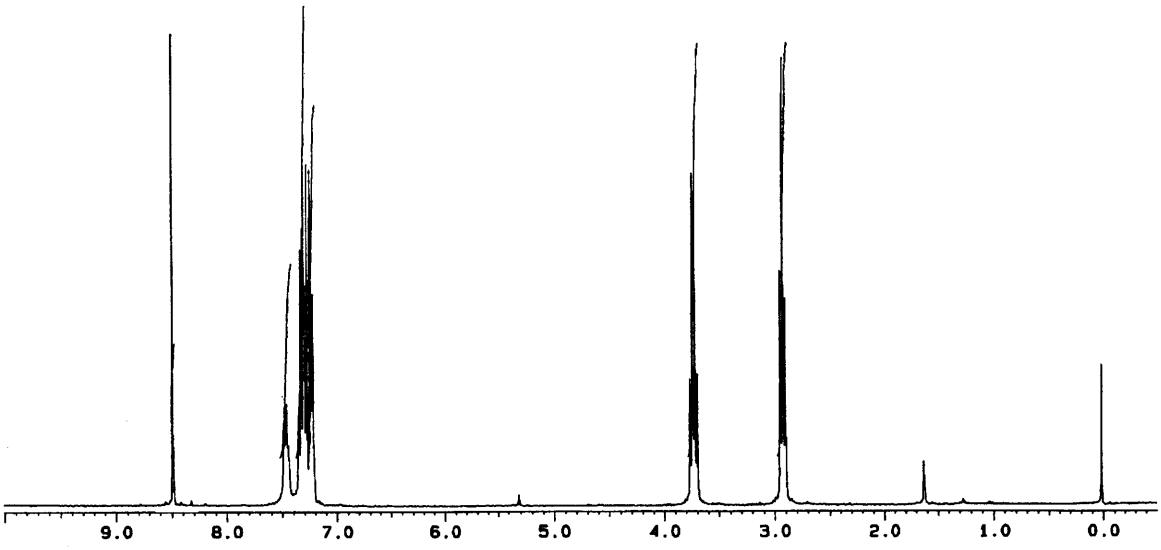
4-Bromo-2-(ethyl ester)-6-(phenethylamide)-pyridine (11).

To a solution of bromopyridine monoacid/monoester (compound **23**, Chapter 3) (330 mg, 1.20 mmol) in DMF (25 mL) were added N-methylmorpholine (150 μL, 1.36 mmol), PyBOP (690 mg, 1.33 mmol), and phenethylamine (170 μL, 1.35 mmol). This was stirred under nitrogen for 20 h, then was concentrated to a yellow oil. The oil was dissolved in CH_2Cl_2 (60 mL) and was extracted with 0.5 M HCl (2 × 100 mL), water (60 mL) and brine (60 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated to a clear oil. Silica gel column chromatography (1:1 hexane/ EtOAc) yielded **11** (430 mg, 95%) as a clear oil that crystallized upon standing. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.53 (d, J = 1.8 Hz, 1H), 8.34 (d, J = 1.8 Hz, 1H), 8.12 (br. t, 1H), 7.34–7.23 (m, 5H), 4.47 (q, J = 7.1 Hz, 2H), 3.75–3.72 (m, 2H), 2.96 (t, J = 7.3 Hz, 2H), 1.44 (t, J = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 163.49, 162.49, 151.25, 147.98, 138.87, 135.61, 130.60, 128.98, 128.85, 128.80, 126.76, 62.60, 41.18, 36.01, 14.46. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{17}H_{17}BrN_2O_3$ + H]$^+$ 377.0501/379 found 377.0506/379.



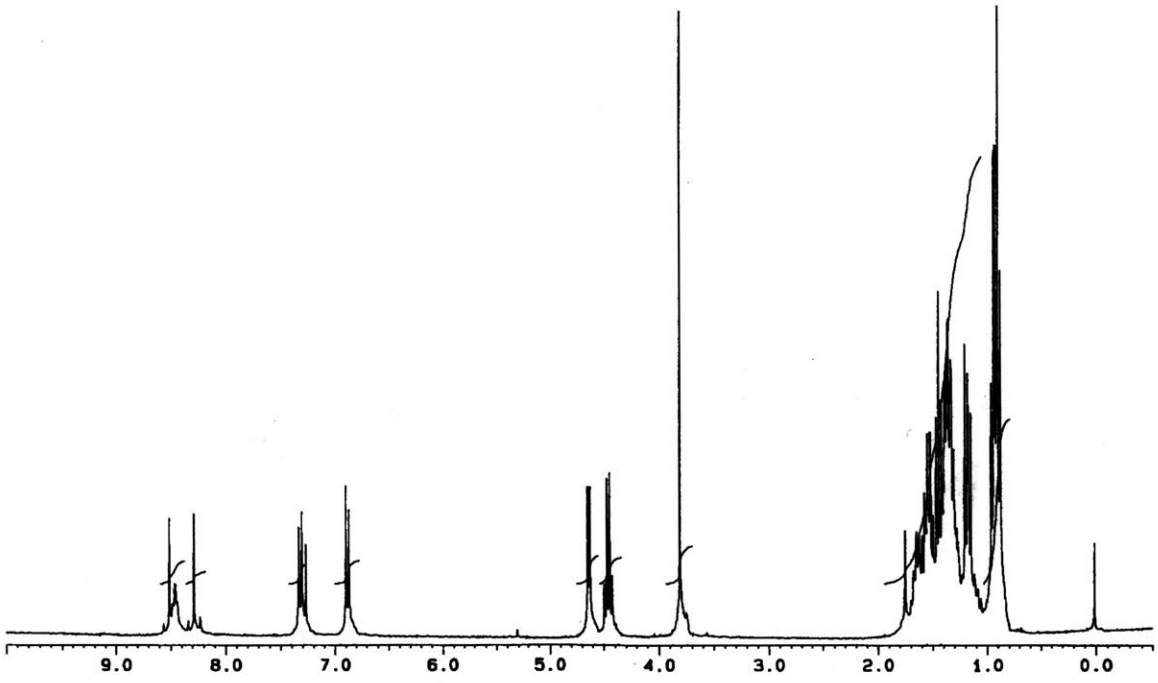
4-Bromo-2,6-bis(phenethylamide)-pyridine (12).

To 175 mL methylene chloride chilled in an ice bath was added bromopyridine diacid chloride (compound **11**, Chapter 3) (922 mg, 3.26 mmol). While stirring, phenethylamine (0.95 mL, 7.57 mmol) and triethylamine (1.5 mL) were added. The ice bath was removed and the mixture was allowed to warm to room temperature with stirring. After 2 h, the solution was washed 3 x with 1 M citric acid, 2 x with saturated sodium bicarbonate, and once with brine. The solution was then dried over magnesium sulfate and filtered. An oil was left by the removal of solvent by rotary evaporation; upon exposure of this oil to high vacuum **12** (1.20 g, 82% yield) was isolated as a foam. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.49 (s, 2H), 7.46 (br. t, 2H), 7.34–7.21 (m, 10H), 3.72 (appar. q, J = 6.7 Hz, 4H), 2.92 (t, J = 7.0 Hz). HRMS (FAB, NBA/NaI) m/z calcd for $[C_{23}H_{22}BrN_3O_2 + H]^+$ 452.0974/454 found 452.0959/454.



4-(Tributyl tin)-2-(ethyl ester)-6-(*p*-methoxybenzylamide)-pyridine (**13**).

A solution of bromopyridine ester/amide **7** (630 mg, 1.60 mmol), bis(tributyltin) (2.69 mL, 5.13 mmol) and dichlorobis(triphenylphosphine) palladium (II) (17 mg, 0.024 mmol) in toluene (15 mL) was stirred and heated to 80 °C. A brown color developed as the reaction progressed. The solution was heated for 20 h, then after cooling it was concentrated to a dark brown oil. Silica gel column chromatography (8:1→3:1 hexane/EtOAc), after elution of excess bis(tributyltin), yielded **13** (277 mg, 28%) as a clear oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.50 (s, 1H), 8.44 (t, J = 6.1 Hz, 1H), 8.28 (s 1H), 7.31 (d, J = 8.5 Hz, 2H), 6.87 (d, J = 8.6 Hz, 2H), 4.63 (d, J = 6.2 Hz, 2H), 4.45 (q, J = 7.1 Hz, 2H), 1.56–1.51 (m, 6H), 1.43 (t, J = 7.1 Hz, 3H), 1.38–1.30 (m, 6H), 1.21–1.12 (m, 6H), 0.89 (t, J = 7.2 Hz, 9H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 165.5, 164.4, 159.2, 157.7, 147.08, 145.0, 135.1, 133.4, 130.6, 129.4, 114.2, 62.1, 55.5, 43.1, 29.1, 27.4, 14.5, 13.8, 10.0. LRMS (ESI +) m/z calcd for $[C_{29}H_{45}N_2O_4Sn + H]^+$ 603/605, found 603/605.

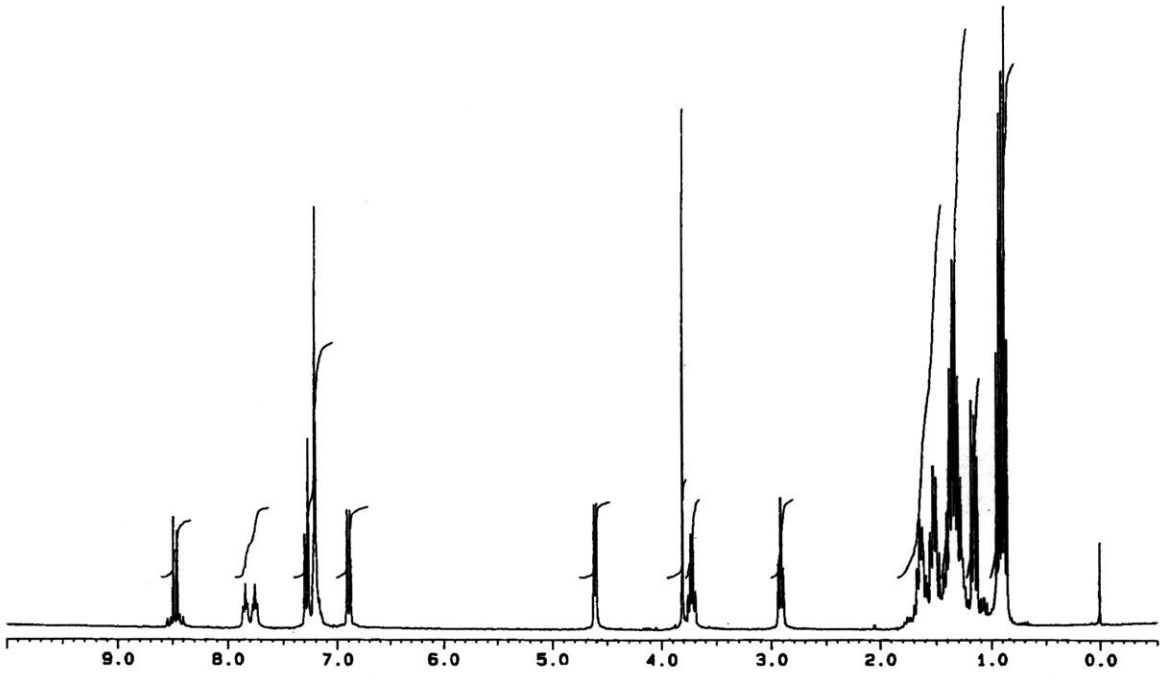


4-(Tributyl tin)-2,6-bis(*p*-methoxybenzylamide)-pyridine (14).

See Chapter 3, compound **13**.

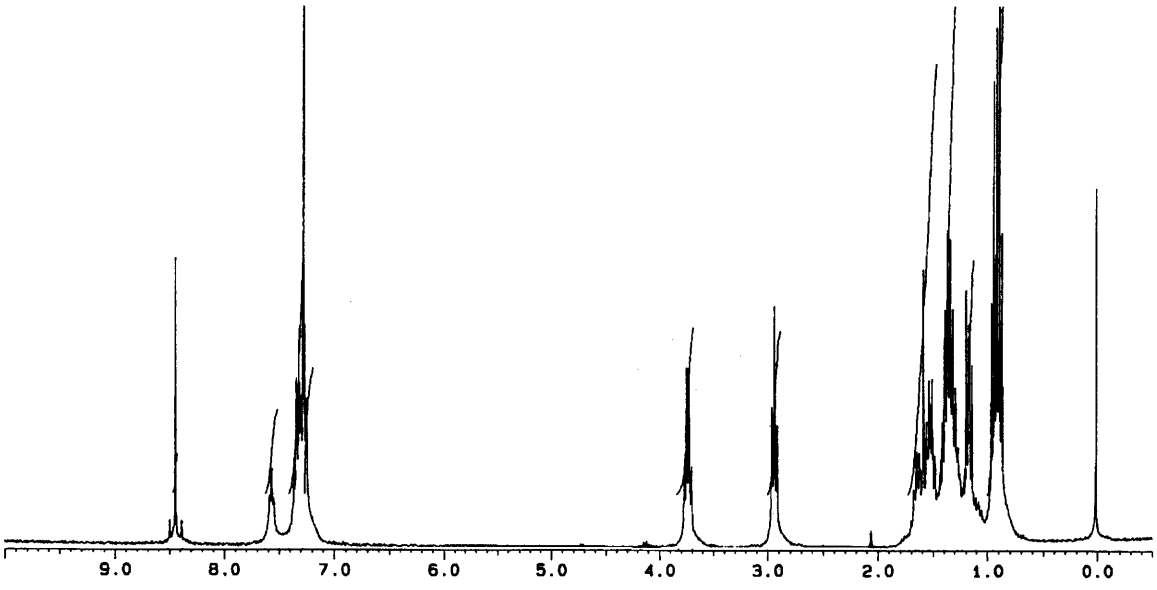
4-(Tributyl tin)-2-(phenethylamide)-6-(*p*-methoxybenzylamide)-pyridine (15).

A solution of bromopyridine heterodiamide **10** (220 mg, 0.47 mmol), bis(tributyltin) (0.78 mL, 1.54 mmol) and dichlorobis(triphenylphosphine) palladium (II) (20 mg, 0.029 mmol) in toluene (6 mL) was stirred and heated to 80 °C. A brown color developed as the reaction progressed. The solution was heated for 5 h, then after cooling it was filtered through Celite. After removal of solvent by rotary evaporation, silica gel column chromatography (8:1→5:1→3:1→1:1 hexane/EtOAc), after elution of excess bis(tributyltin), yielded **15** (270 mg, 85%) as a clear oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.49 (s, 1H), 8.46 (s, 1H), 7.84 (t, J = 6.1 Hz, 1H), 7.76 (t, J = 5.9 Hz, 1H), 7.29 (d, J = 8.6 Hz, 2H), 7.22–7.17 (m, 5H), 6.90 (d, J = 8.6 Hz, 2H), 4.61 (d, J = 6.2 Hz, 2H), 3.81 (s, 3H), 3.72 (appar q., J = 6.6 Hz, 2H), 2.91 (t, J = 6.9 Hz, 2H), 1.64–0.86 (m, 27H). LRMS (ESI +) m/z calcd for $[C_{35}H_{49}N_3O_3Sn + H]^+$ 676/678/680, found 676/678/680.



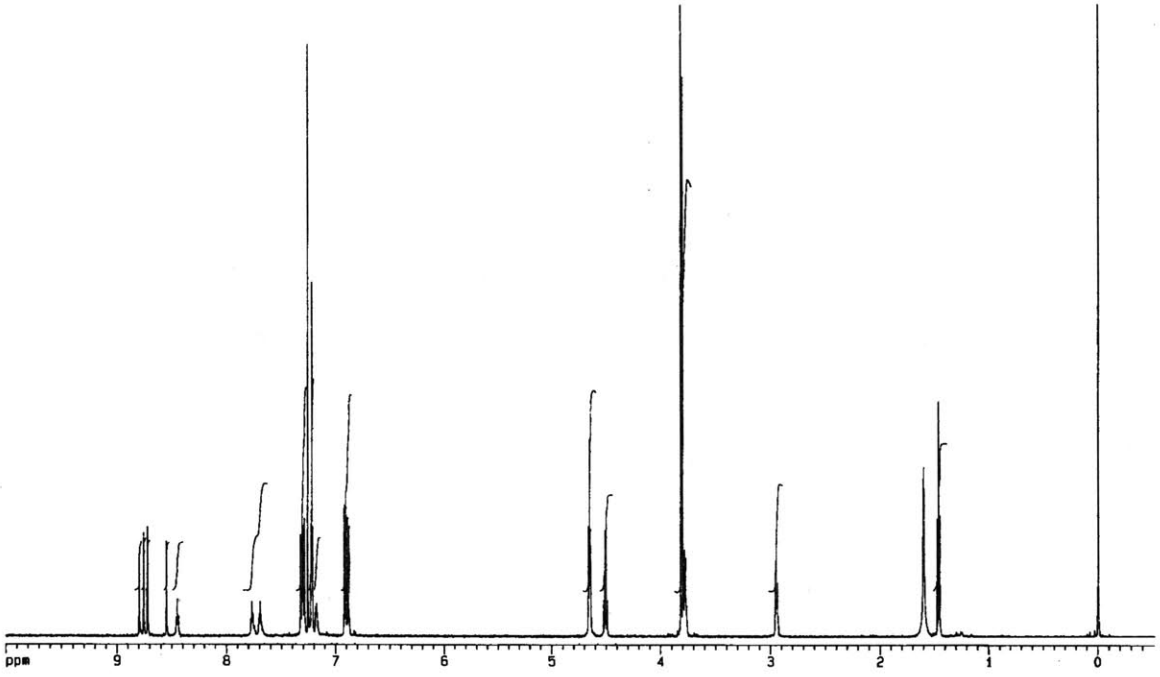
4-(Tributyltin)-2,6-bis(phenethylamide)-pyridine (**16**).

A solution of bromopyridine diamide **12** (977 mg, 2.16 mmol), bis(tributyltin) (3.4 mL, 6.7 mmol) and dichlorobis(triphenylphosphine) palladium (II) (17 mg, 0.024 mmol) in toluene (12 mL) was stirred and heated to 80 °C. A brown color developed as the reaction progressed. The solution was heated for 2 d, then after cooling the solvent was removed by rotary evaporation, and silica gel column chromatography (8:1→2:1→1:2 hexane/EtOAc), after elution of excess bis(tributyltin), yielded **16** (1.22 g, 85%) as a brown oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.44 (s, 2H), 7.57 (t, J = 6.0 Hz, 2H), 7.35–7.23 (m, 10H), 3.74 (appar q., J = 6.8 Hz, 4H), 2.94 (t, J = 7.1 Hz, 4H), 1.65–0.88 (m, 27H). HRMS (FAB, NBA/CsI) m/z calcd for $[C_{35}H_{49}N_3O_2Sn + Cs]^+$ 796.1901 found 796.1929.



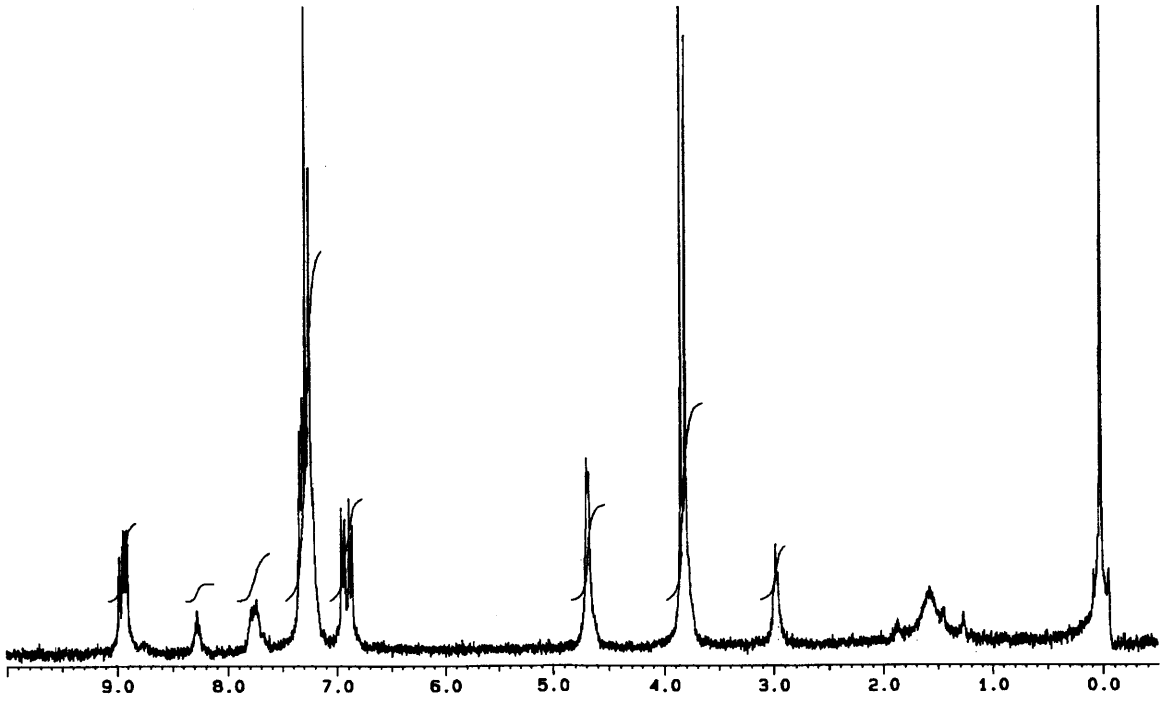
2,2' - Bis(*p*-methoxybenzylamide) - 6 - phenethylamide - 6' - (ethyl ester) - 4,4'-bipyridine (17).

A solution of stannane **13** (275 mg, 0.46 mmol), bromodiamide **10** (213 mg, 0.46 mmol) and dichlorobis(triphenylphosphine) palladium (II) (20 mg, 0.027 mmol) in toluene (15 mL) was refluxed for 24 h, turning from yellow to dark brown in color over this period. The solution was concentrated to brown solids, which were sonicated in $CHCl_3$ and filtered through Celite. The filtrate was concentrated to a brown oil. Silica gel column chromatography (1:1→3:1 EtOAc/hexane) yielded a pale yellow powder. Recrystallization from EtOAc/Hexane yielded **17** (112 mg, 35%) as a white powder. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.80 (d, J = 1.5 Hz, 1H), 8.76 (d, J = 1.7 Hz, 1H), 8.72 (d, J = 1.6 Hz, 1H), 8.55 (d, J = 1.7 Hz, 1H), 8.45 (t, J = 6.1 Hz, 1H), 7.77 (t, J = 6.2 Hz, 1H), 7.70 (t, J = 5.9 Hz, 1H), 7.32 (d, J = 8.6 Hz, 2H), 7.30 (d, J = 8.5 Hz, 2H), 7.23-7.18 (m, 5H), 6.92 (d, J = 8.6 Hz, 2H), 6.89 (d, J = 8.6 Hz, 2H), 4.66 (d, J = 5.9 Hz, 2H), 4.65 (d, J = 5.7 Hz, 2H), 4.51 (q, J = 7.1 Hz, 2H), 3.82 (s, 3H), 3.80 (s, 3H), 3.78 (q, J = 6.4 Hz, 2H), 2.95 (t, J = 6.7 Hz, 2H), 1.46 (t, J = 7.2 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 163.0, 159.5, 151.8, 150.5, 150.4, 148.4, 147.7, 147.3, 130.3, 130.2, 129.5, 129.3, 129.1, 128.9, 126.9, 125.0, 123.4, 123.2, 123.0, 114.4, 114.3, 62.7, 55.6, 55.5, 43.3, 40.8, 35.9, 14.6. LRMS (ESI +) m/z calcd for $[C_{40}H_{39}N_5O_7 + H]^+$ 702 found 702.


ppm
9
8
7
6
5
4
3
2
1
0

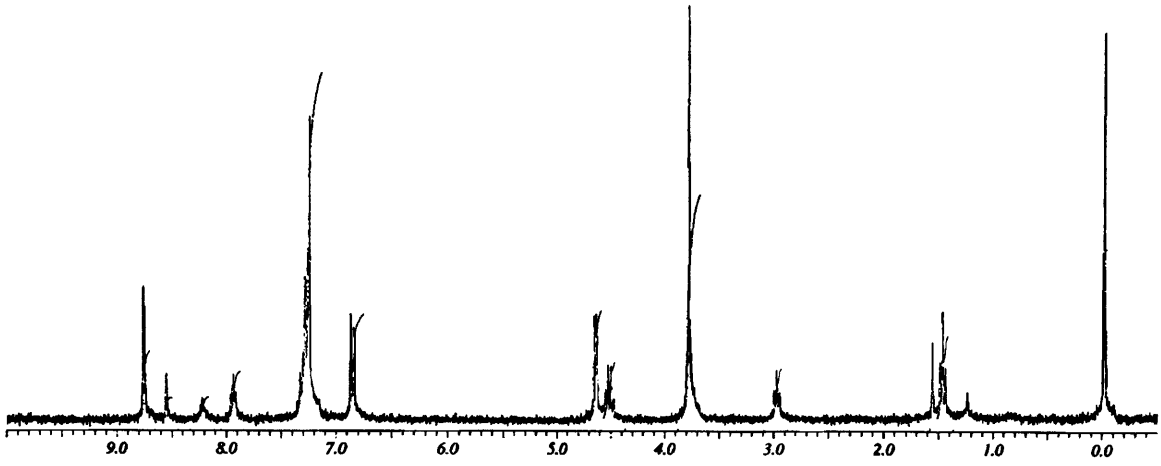
2,2' - Bis(*p*-methoxybenzylamide) - 6 - phenethylamide - 6' - (carboxylic acid) - 4,4'-bipyridine (18).

To a solution of ethyl ester **17** (66 mg, 0.094 mmol) in THF (4 mL) was added a solution of lithium hydroxide monohydrate (8 mg, 0.19 mmol) in water (1 mL). This was stirred vigorously for 30 min, then the solution was acidified by the addition of 1 M HCl (25 mL). Solids were formed and isolated by filtration; drying yielded **18** (45 mg, 72%) as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.97 (s, 1H), 8.93 (s, 1H), 8.92 (s, 1H), 8.90 (s, 1H), 8.27 (br. t, 1H), 7.77–7.70 (m, 2H), 7.33–7.21 (m, 9H), 6.93 (d, J = 8.5 Hz, 2H), 6.87 (d, J = 8.6 Hz, 2H), 4.67 (d, 5.4 Hz, 4H), 3.83 (s, 3H), 3.79 (s, 3H), 2.97 (m, 2H), 1.86 (m, 2H). LRMS (ESI +) m/z calcd for $[C_{38}H_{35}N_5O_7 + H]^+$ 674 found 674.



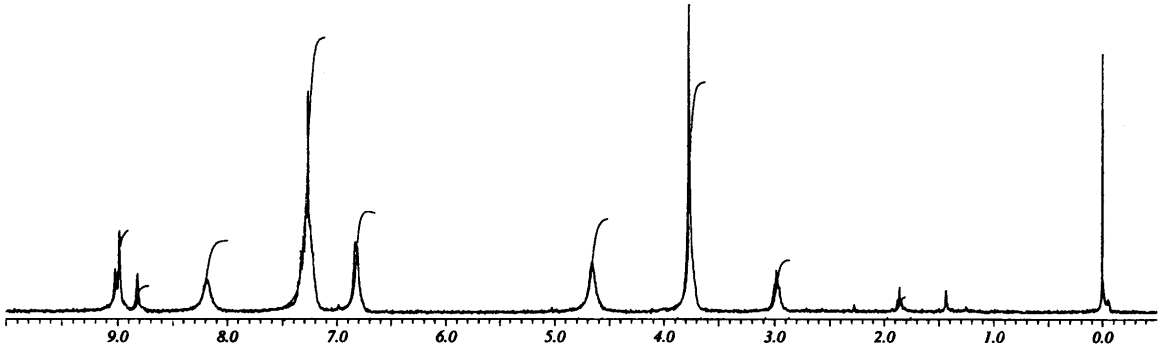
2,6 - Bis(*p*-methoxybenzylamide) - 2' - phenethylamide - 6' - (ethyl ester) - 4,4'-bipyridine (19).

A solution of stannane **14** (500 mg, 0.72 mmol), bromopyridine **11** (271 mg, 0.72 mmol) and dichlorobis(triphenylphosphine) palladium (II) (31 mg, 0.045 mmol) in toluene (25 mL) was refluxed for 24 h, turning from yellow to dark brown in color over this period. The solution was concentrated to brown solids which were sonicated in $CHCl_3$ and filtered through Celite. The filtrate was concentrated to a yellow oil. Silica gel column chromatography (1:1→3:1 EtOAc/hexane) yielded **19** (280 mg, 55%) as a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.76–8.74 (m, 3H), 8.55 (s, 1H), 8.22 (br. t, 1H), 7.92 (t, J = 5.7 Hz, 2H), 7.33–7.25 (m, 9H), 6.85 (d, J = 8.8 Hz, 4H), 4.64 (d, J = 6.1 Hz, 4H), 4.51 (q, J = 7.2 Hz, 2H), 3.79 (s, 6H), 3.79–3.76 (m, 2H), 2.98 (t, J = 7.7 Hz, 2H), 1.47 (t, J = 7.0 Hz, 3H). LRMS (ESI +) m/z calcd for $[C_{40}H_{39}N_5O_7 + H]^+$ 702 found 702.



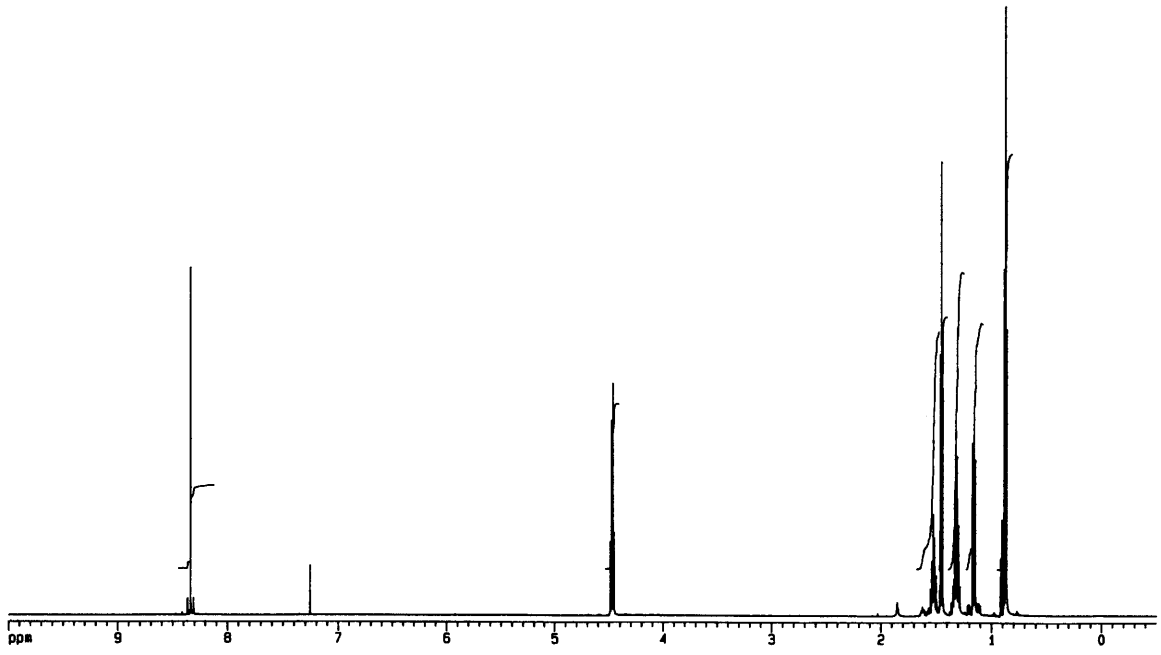
2,6 - Bis(*p*-methoxybenzylamide) - 2' - phenethylamide - 6' - (carboxylic acid) - 4,4'-bipyridine (20).

To a solution of ethyl ester **19** (123 mg, 0.175 mmol) in THF (8 mL) was added a solution of lithium hydroxide monohydrate (17 mg, 0.41 mmol) in water (2 mL). This was stirred vigorously for 45 min, then the solution was acidified by the addition of 1 M HCl (40 mL). Solids were formed and were isolated by filtration; drying yielded **18** (59 mg, 50%) as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.97–8.83 (m, 4H), 8.07 (m, 3H), 7.26 (m, 9H), 6.85 (m, 4H), 4.68 (m, 4H), 3.79 (s, 6H), 3.00 (m, 2H), 1.86 (br. t, 2H). LRMS (ESI +) *m/z* calcd for $[C_{38}H_{35}N_5O_7 + H]^+$ 674 found 674.



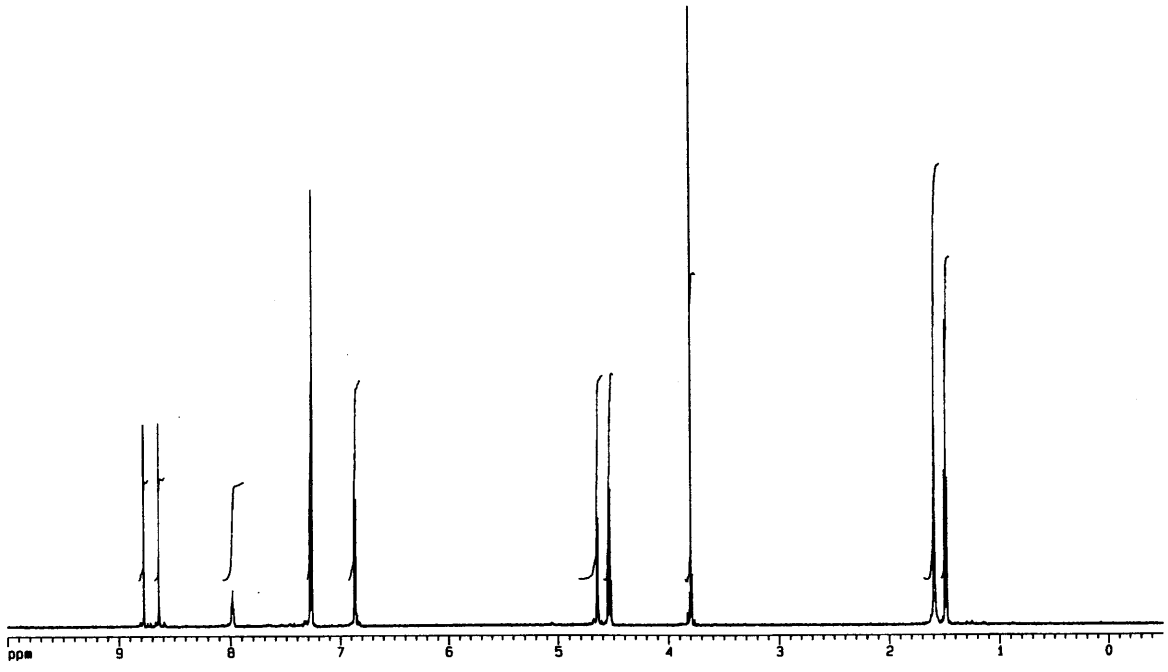
4-(Tributyl tin)-2,6-bis(ethyl ester)-pyridine (21).

A solution of 4-bromo-2,6-bis(ethyl ester) pyridine (compound **9**, Chapter 3) (1.61 g, 5.32 mmol), bis(tributyltin) (8.6 mL, 17.02 mmol) and dichlorobis(triphenylphosphine) palladium (II) (101 mg, 0.14 mmol) in toluene (50 mL) was stirred and heated to 80 °C. A brown color developed as the reaction progressed. The solution was heated for 20 h, then after cooling it was concentrated to a dark brown oil. Silica gel column chromatography (8:1→5:1 hexane/EtOAc), after elution of excess bis(tributyltin), yielded **21** (1.00 g, 37%) as a clear oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.34 (s, 2H), 4.48 (q, J = 7.2 Hz, 4H), 1.54–1.51 (m, 6H), 1.45 (t, J = 7.2 Hz, 6H), 1.34–1.30 (m, 6H), 1.18–1.15 (m, 6H), 0.88 (t, J = 7.3 Hz, 9H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 165.68, 157.47, 146.63, 135.79, 62.43, 29.08, 27.43, 14.43, 13.81, 10.09. LRMS (ESI +) m/z calcd for $[C_{23}H_{39}NO_4Sn + H]^+$ 512/514 found 512/514.



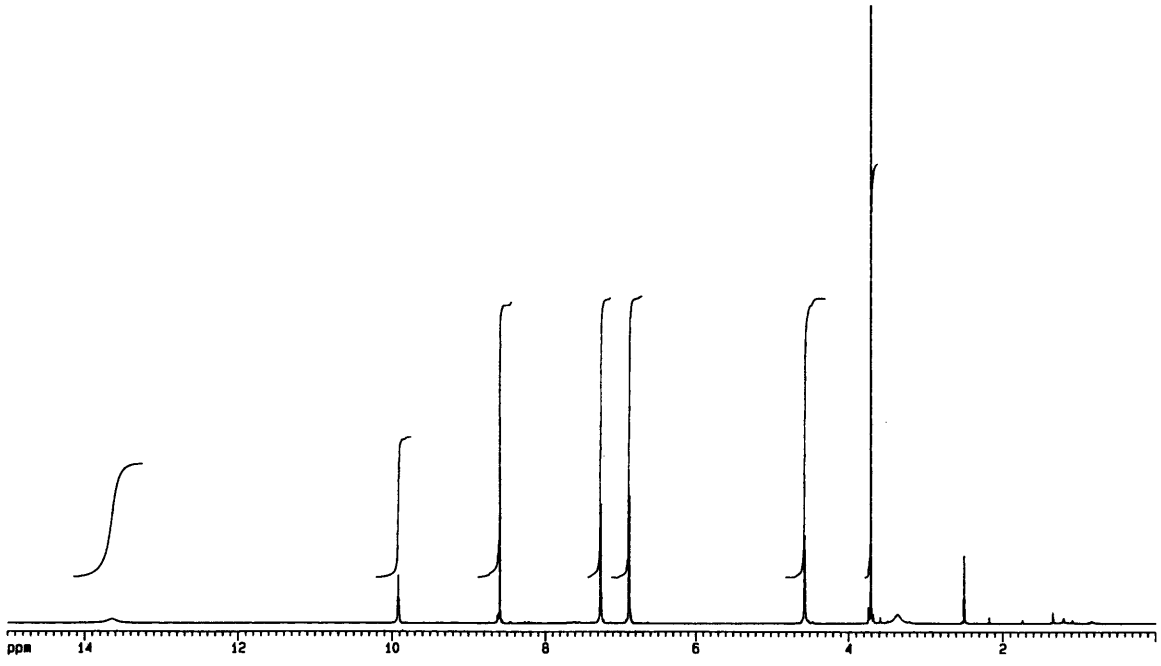
2,6-Bis(*p*-methoxybenzylamide)-2',6'-bis(ethyl ester)-4,4'-bipyridine (22).

A solution of bromopyridine **9** (475 mg, 0.98 mmol), stannane **21** (500 mg, 0.98 mmol) and dichlorobis(triphenylphosphine) palladium (II) (43 mg, 0.06 mmol) in toluene (25 mL) was refluxed for 24 h, turning from yellow to dark brown in color over this period. The solution was concentrated to brown solids which were sonicated in $CHCl_3$ and filtered through Celite. The filtrate was concentrated to a brown oil. Silica gel column chromatography (2:1 EtOAc/hexane) yielded **22** (325 mg, 53%) as pale yellow solids. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.77 (s, 2H), 8.64 (s, 2H), 7.98 (t, J = 6.0 Hz, 2H), 7.28 (d, J = 8.6 Hz, 4H), 6.86 (d, J = 8.6 Hz, 4H), 4.65 (d, J = 6.1 Hz, 4H), 4.54 (q, J = 7.1 Hz, 4H), 3.80 (s, 6H), 1.49 (t, J = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 164.3, 163.0, 159.4, 150.6, 150.2, 147.4, 147.0, 130.0, 129.4, 125.6, 123.2, 114.4, 62.9, 55.5, 43.4, 14.5. LRMS (ESI +) m/z calcd for $[C_{34}H_{34}N_4O_8 + Na]^+$ 649 found 649. LRMS (ESI -) m/z calcd for $[C_{34}H_{34}N_4O_8 - H]^-$ 625 found 625.



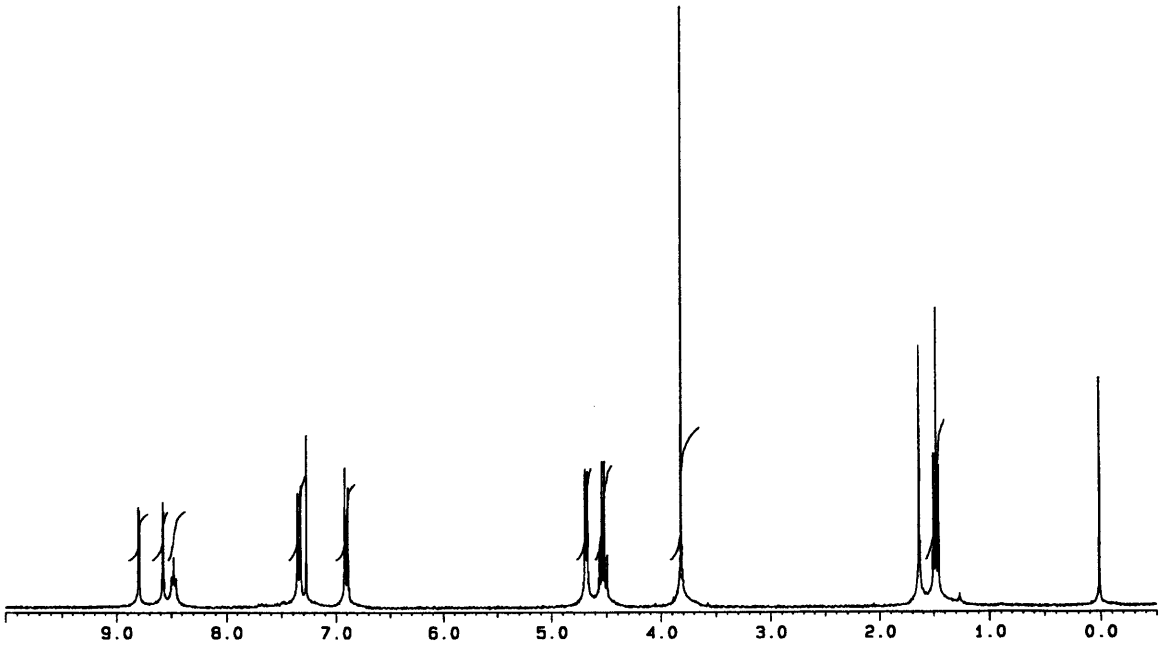
2,6-Bis(*p*-methoxybenzylamide)-2',6'-bis(carboxylic acid)-4,4'-bipyridine (**23**).

To a solution of diethyl ester **22** (160 mg, 0.255 mmol) in THF (10 mL) was added a solution of lithium hydroxide monohydrate (54 mg, 1.277 mmol) in water (7 mL). This was stirred vigorously for 45 min, then the solution was acidified by the addition of 1 M HCl (40 mL). Solids were formed and were isolated by filtration, yielding **23** (105 mg, 72%) as pale yellow solids. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.65 (br. s, 2H), 9.92 (t, J = 6.3 Hz, 2H), 8.60 (s, 4H), 7.27 (d, J = 8.6 Hz, 4H), 6.89 (d, J = 8.6 Hz, 4H), 4.57 (d, J = 6.2 Hz, 4H), 3.71 (s, 6H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 165.4, 162.9, 158.3, 150.0, 149.6, 146.7, 146.6, 131.1, 128.3, 125.2, 122.3, 113.8, 55.1, 41.7. LRMS (ESI +) m/z calcd for $[C_{30}H_{26}N_4O_8$ + Na$]^+$ 571 found 571.



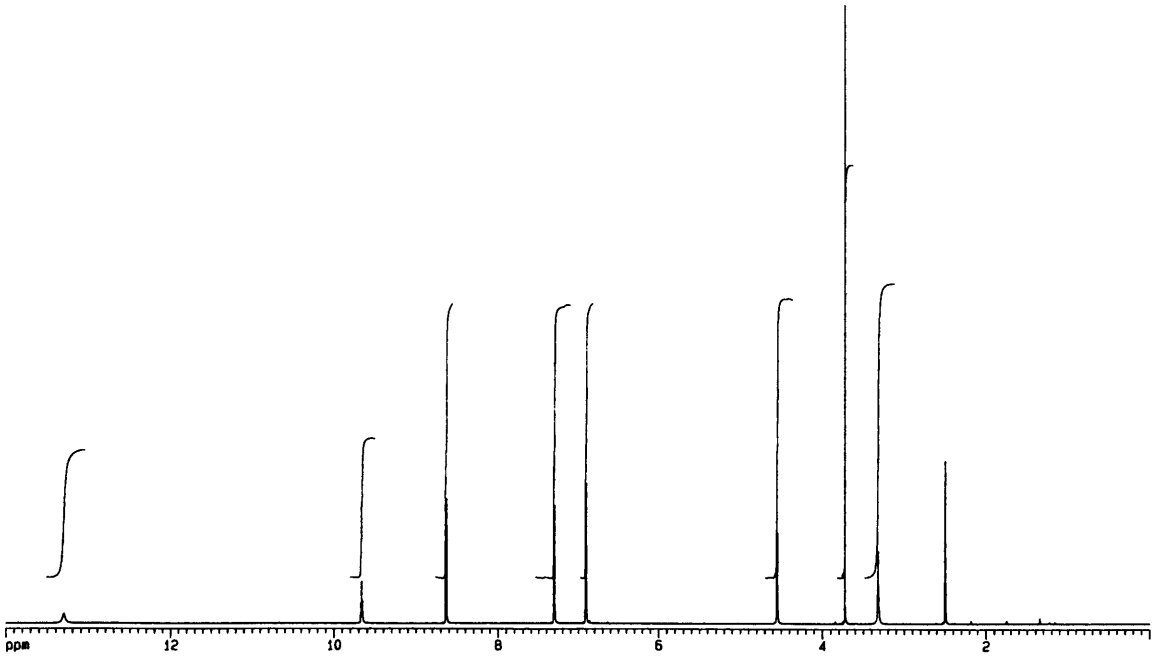
2,2'-Bis(*p*-methoxybenzylamide)-6,6'-bis(ethyl ester)-4,4'-bipyridine (24).

A solution of bromopyridine **7** (252 mg, 0.64 mmol), stannane **12** (380 mg, 0.63 mmol) and dichlorobis(triphenylphosphine) palladium (II) (28 mg, 0.04 mmol) in toluene (20 mL) was refluxed for 24 h, turning from yellow to dark brown in color over this period. The solution was concentrated to brown solids. Silica gel column chromatography (1:1→2:1 EtOAc/hexane) yielded **24** (230 mg, 58%) as white solids. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.80 (d, J = 1.6 Hz, 2H), 8.57 (d, J = 1.6 Hz, 2H), 8.48 (t, J =5.9 Hz, 2H), 7.34 (d, J = 8.6 Hz, 4H), 6.90 (d, J = 8.6 Hz, 4H), 4.68 (d, J = 6.1 Hz, 4H), 4.53 (q, J = 7.1 Hz, 4H), 3.82 (s, 6H), 1.48 (t, J = 7.1 Hz, 6H). LRMS (ESI +) m/z calcd for $[C_{34}H_{34}N_4O_8 + Na]^+$ 649 found 649.



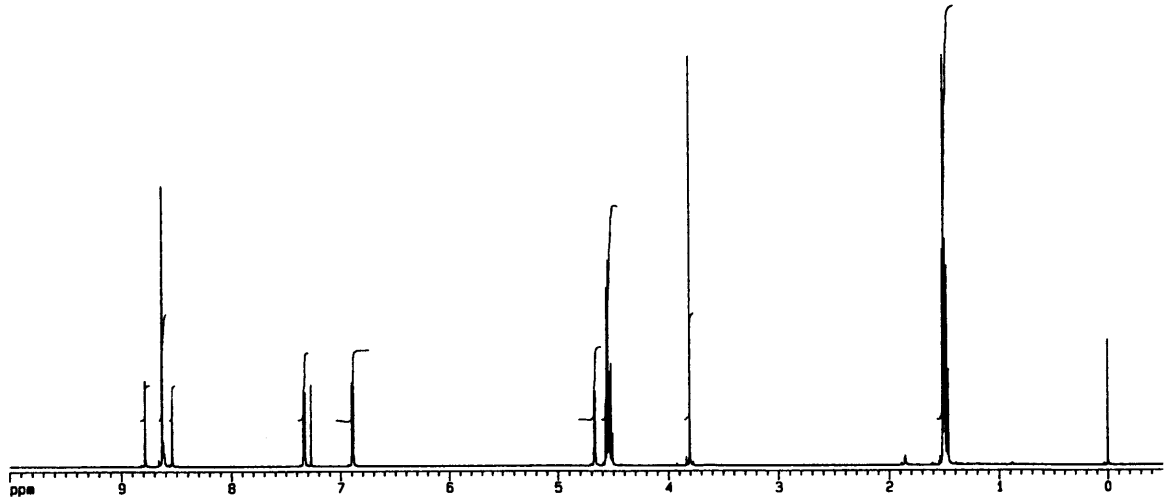
2,2'-Bis(*p*-methoxybenzylamide)-6,6'-bis(carboxylic acid)-4,4'-bipyridine (25).

To a solution of diethyl ester **24** (41 mg, 0.065 mmol) in THF (3.5 mL) was added a solution of lithium hydroxide monohydrate (14 mg, 0.33 mmol) in water (2 mL). This was stirred vigorously for 1 h 15 min, then the solution was acidified by the addition of 1 M HCl (10 mL). Solids were formed and were isolated by filtration, yielding **25** (25 mg, 67%) as white solids. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.31 (br. s, 2H), 9.67 (t, J = 6.2 Hz, 2H), 8.65 (d, J = 1.7 Hz, 2H), 8.63 (d, J = 1.6 Hz, 2H), 7.31 (d, J = 8.6 Hz, 4H), 6.91 (d, J = 8.6 Hz, 4H), 4.55 (d, J = 6.2 Hz, 4H), 3.73 (s, 6H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 164.61, 162.57, 158.38, 150.54, 147.55, 146.99, 131.02, 128.83, 124.55, 123.28, 113.82, 55.08, 41.94. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{30}H_{26}N_4O_8 + Cs]^+$ 703.0805 found 703.0788.



2-(*p*-Methoxybenzylamide)-2',6,6'-tris(ethyl ester)-4,4'-bipyridine (26).

A solution of bromopyridine **7** (385 mg, 0.98 mmol), stannane **21** (500 mg, 0.98 mmol) and dichlorobis(triphenylphosphine) palladium (II) (42 mg, 0.06 mmol) in toluene (25 mL) was refluxed for 24 h, turning from yellow to dark brown in color over this period. The solution was concentrated to brown solids which were sonicated in $CHCl_3$ and filtered through Celite. The filtrate was concentrated to a brown oil. Silica gel column chromatography (1:1→2:1 EtOAc/hexane) yielded a clear oil. Crystallization from ethanol/hexane yielded **26** (326 mg, 62%) as a white powder. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.79 (d, J = 1.7 Hz, 1H), 8.63 (s, 2H), 8.54 (d, J = 1.8 Hz, 1H), 7.34 (d, J = 8.6 Hz, 2H), 6.89 (d, J = 8.7 Hz, 2H), 4.67 (d, J = 6.2 Hz, 2H), 4.56 (q, J = 7.1 Hz, 4H), 4.53 (q, J = 7.1 Hz, 2H), 3.81 (s, 3H), 1.50 (t, J = 7.2 Hz, 6H), 1.47 (t, J = 7.2 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 164.4, 164.2, 163.1, 159.3, 151.9, 150.2, 148.5, 147.0, 147.0, 130.2, 129.5, 125.6, 124.9, 123.4, 114.3, 63.0, 62.8, 55.5, 43.3, 14.5, 14.5. LRMS (ESI +) m/z calcd for $[C_{28}H_{29}N_3O_8 + Na]^+$ 558 found 558. LRMS (ESI -) m/z calcd for $[M - H]^-$ 534, found 534.



Chapter 5. Glycoluril Core Molecules[1]

5.1 Introduction

Glycolurils have found application in a number of settings, including light stabilization,[2] polymer cross-linking,[3] explosives,[4] and molecular recognition.[5] Previous work in this laboratory in the last of these applications[6] and in solution-phase combinatorial chemistry[7] led to consideration of the glycoluril framework for use as a core molecule for the synthesis of combinatorial libraries.

We have developed a series of glycoluril derivatives which allow functionalization at various positions about the core structure via amide linkages. Using either a one-pot reaction or a stepwise reaction sequence, either mixtures or individual compounds can be synthesized. The core molecules we envisioned were variously substituted glycoluril polyacids or polyacid derivatives, and three examples of such are shown in Figure 1. In each case, the bicyclic glycoluril centerpiece was formed by the condensation of two equivalents of an appropriately-substituted urea with benzil or a substituted benzil derivative.

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[1] Portions of this chapter have been incorporated into a manuscript in press and are being used with permission from The American Chemical Society. Pryor, K. E.; Rebek, J., Jr. *Org. Lett.* **1999**, in press.

[2] Krause, A.; Aumueller, A.; Korona, E.; Trauth, H. U.S. Patent 5,670,613, 1997.

[3] Wang, A.; Bassett, D. U.S. Patent 4,310,450, 1982.

[4] (a) Fang, Y.; Wu, G. *Hanneng Cailiao* **1997**, *5*, 9. (b) Boileau, J.; Carail, M.; Wimmer, E.; Gallo, R.; Pierrot, M. *Propellants, Explos., Pyrotech.* **1985**, *10*, 118.

[5] (a) Dave, P. R.; Forohar, F.; Kaselj, M.; Gilardi, R.; Trivedi, N. *Tetrahedron Lett.* **1999**, *40*, 447. (b) Elemans, J. A. A. W.; de Gelder, R.; Rowan, A. E.; Nolte, R. J. M. *Chem. Commun.* **1998**, *15*, 1553. (c) Reek, J. N. H.; Wlwmans, J. A. A. W.; Nolte, R. J. M. *J. Org. Chem.* **1997**, *62*, 2234. (d) Murray, B. A.; Whelan, G. S. *Pure Appl. Chem.* **1996**, *68*, 1561. (e) Coolen, H. K. A. C.; van Leeuwen, P. W. N. M.; Nolte, R. J. M. *J. Org. Chem.* **1996**, *61*, 4739. (f) Sijbesma, R. P.; Nolte, R. J. M. *Top. Curr. Chem.* **1995**, *175*, 25.

[6] (a) Meissner, R.; Rebek, J., Jr., de Mendoza, J. *Science* **1995**, *270*, 1485. (b) Valdez, C.; Spitz, U. P.; Toledo, L. M.; Kubik, S. W.; Rebek, J. Jr. *J. Am. Chem. Soc.* **1995**, *117*, 12733.

[7] Pryor, K. E.; Shipps, G. W., Jr.; Skyler, D. A.; Rebek, J., Jr. *Tetrahedron*, **1998**, *54*, 4107 and references therein.

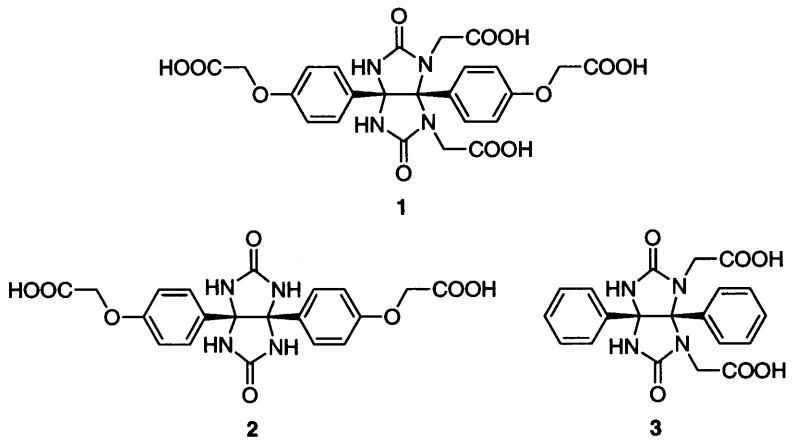

Figure 1. Three glycoluril polyacid core molecules.

5.2 The glycoluril tetraacid core molecule

The first member of this class, tetraacid **1**, can be synthesized by saponification of the corresponding tetraethyl ester **8** (Scheme 1). Following a published procedure, commercially available anisil **4** was demethylated with pyridine•HCl.[8] The resulting 4,4'-dihydroxybenzil **5** was then alkylated with ethyl bromoacetate to provide the requisite diketone **6** for condensation. The other partner in the condensation, ethyl hydantoate **7**, is commercially available, but can also be conveniently prepared on large scale from glycine ethyl ester by conversion of the amino functionality into a urea upon reaction with KOCN in $EtOH/H_2O$.

The TFA-catalyzed condensation was performed with azeotropic removal of water to afford tetraester **8**. Upon saponification to the tetraester, however, the resulting tetraacid **1** was so highly water soluble that separation of the compound from the salt by-products of the reaction was impossible. By replacing the ethyl esters with benzyl esters (compound **11**) and performing a hydrogenolysis instead of a saponification, tetraacid **1** could be accessed cleanly and in high yield. This optimized procedure is shown in Scheme 2.

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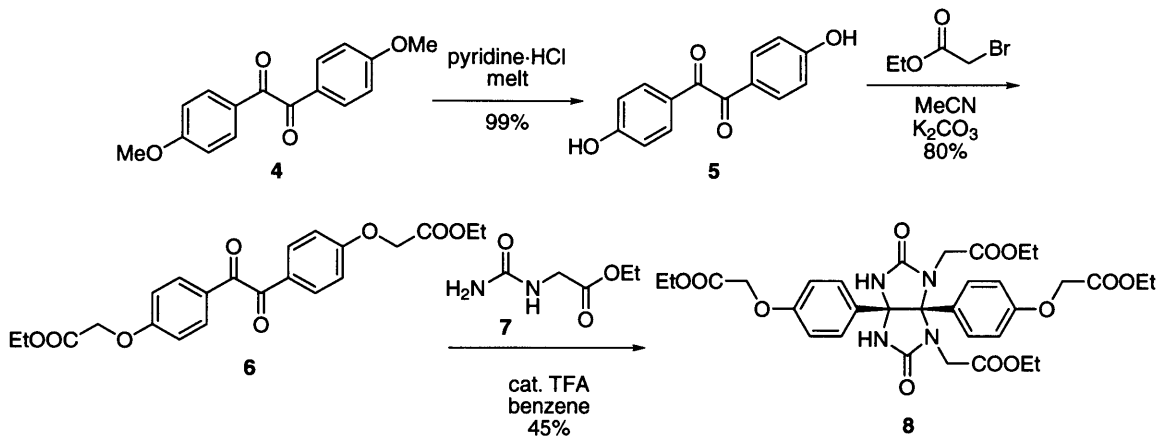
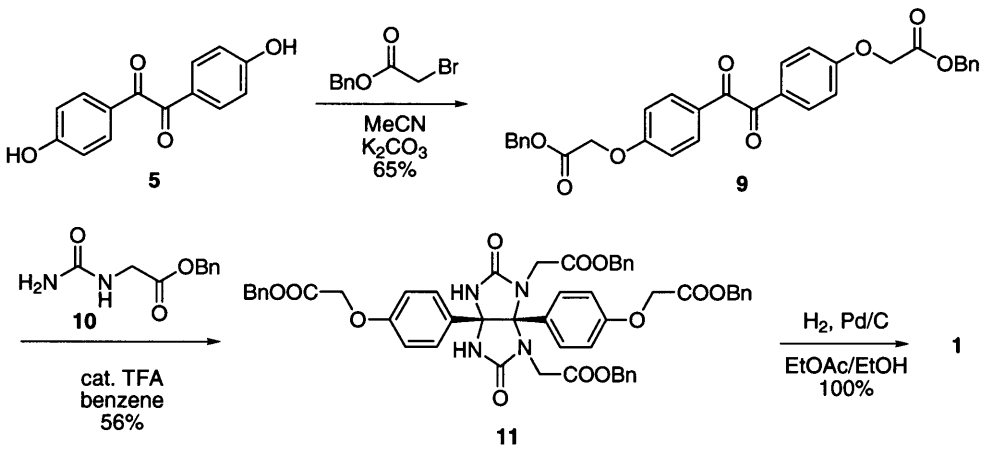
[8] Somin, I. N.; Kuznetsov, S. G. *Khim. Nauka I Prom.* **1959**, *4*, 801.



Scheme 1. Synthesis of glycoluril tetrakis(ethyl ester) **8**.

Dihydroxybenzil **5** is now simply alkylated with benzyl bromoacetate to provide the bis(benzyl ester) diketone **9**. Benzyl hydantoate **10**[9] is prepared in two steps from glycine by initial esterification with benzyl alcohol followed by similar treatment with KOCN in EtOH/H_2O. Tetraester **11** is converted in quantitative yield to tetraacid **1** by catalytic hydrogenolysis.



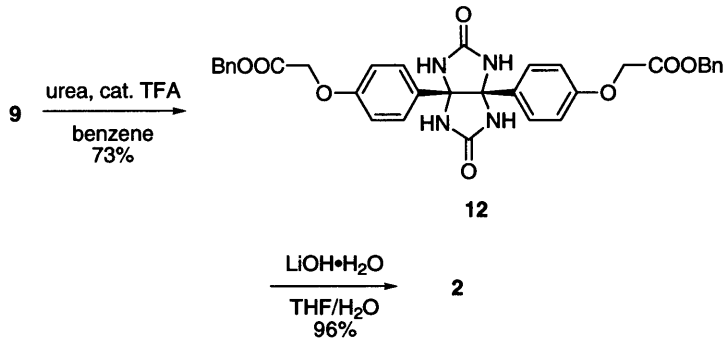
Scheme 2. Synthesis of glycoluril tetraacid **1**.

[9] Previously reported in Kotani, T.; Ishii, A.; Nhagaki, Y.; Toyomaki, Y.; Yago, H.; Suehiro, S.; Okukado, N.; Okamoto, K. *Chem. Pharm. Bull.* **1997**, *45*, 297.

5.3 Glycoluril diacid core molecules

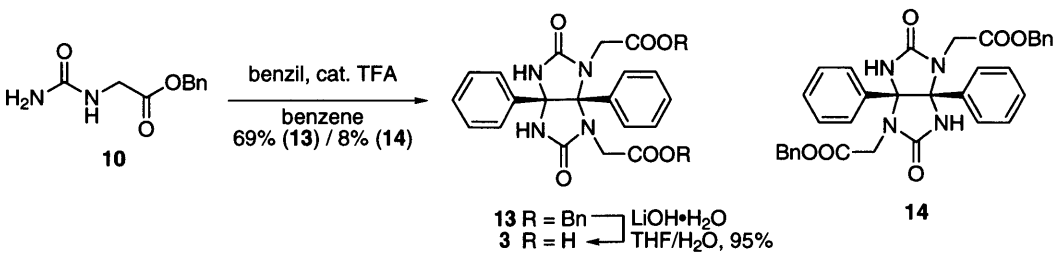
Mindful of the remarkable water solublity of **1**, we based our initial syntheses of other analogs with fewer acid substituents on the hydrogenolysis of the corresponding benzyl esters, as well. However, issues of solubility and product recovery limited yields for this step in both cases to approximately 35–55%.

Fortunately, subsequent trials proved that the diacids were also less soluble in water than the tetraacid, so saponification of either the ethyl or benzyl esters was a viable option; yields of the saponification reactions are routinely greater than 90%. Condensation of substituted benzil **9** with urea yields glycoluril diester **12** (Scheme 3). Saponification of the diester with LiOH yields diacid **2**, which precipitates immediately upon pouring the reaction mixture into 1M HCl.



Scheme 3. Synthesis of glycoluril diacid **2**.

Similarly, benzyl hydantoate **10** can be condensed with benzil to yield diester **13** (Scheme 4). Diacid **3** crystallizes upon chilling after pouring the reaction mixture into 1M HCl after saponification of diester **13** with LiOH.



Scheme 4. Synthesis of *cis*- and *trans*-glycoluril diesters **13** and **14** and *cis*-glycoluril diacid **3**.

The *cis*-substituted product **13** is isolated in 63% yield, while the corresponding *trans*-substituted product **14** is produced in only 8% yield. This is thought to be the result of the production of intermediate **15** (Figure 2), which, particularly under acidic conditions, reacts with a second equivalent of benzyl hydantoate **10** to preferentially form the *cis*-isomer. The two isomers can be isolated from the crude reaction mixture (after aqueous extraction) by selective crystallization.

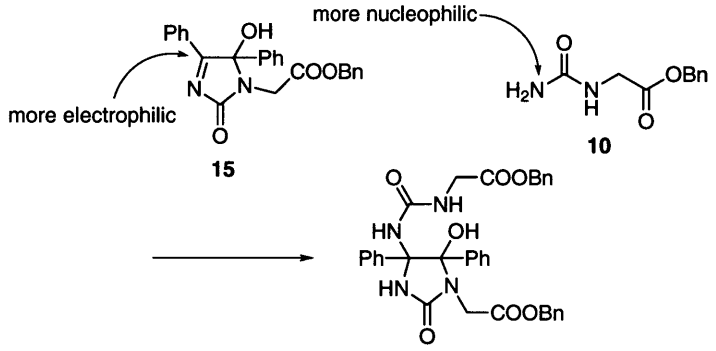
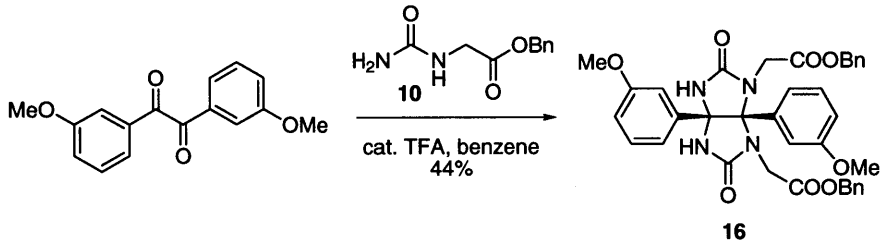


Figure 2. *Cis*-substituted derivatives are favored 8:1 over the *trans*-substituted derivatives.

In order to investigate how the incorporation of *meta*-methoxyphenyl groups would affect the core molecules, the synthesis of bis(benzyl ester) **16** was undertaken (Scheme 5). A straightforward condensation between benzyl hydantoate **10** and commercially-available 3,3'-dimethoxybenzil provided **16** in 44% yield.



Scheme 5. Synthesis of *meta*-methoxy-substiuted glycoluril diester **16**.

It is clear from the broad and poorly-defined ^{1}H NMR spectrum that the ring substituents cause hindered rotation of the aryl groups. A comparison between the spectra of unsubstitued diester **13** and compound **16** illustrates this point and is shown below in Figure 3.

5.4 Activation of the glycoluril core molecules

Once the synthesis of the polyacids was accomplished, it became necessary to develop methodology to couple building blocks to them in a clean, efficient manner. Historically,[7] activation of core acids as acid chlorides has been a simple, effective way to accomplish this goal. In the case of the glycoluril core molecules, however, the presence of the urea functionality precludes activation as acid chlorides.

Alternatives to acid chloride activation are, happily, numerous. One option is to make use of a coupling reagent to directly couple the amine building blocks to the polyacid core. Another option is to activate the acids on the core as some functional group other than as acid chlorides and isolate the activated core for use in a subsequent amidation step.

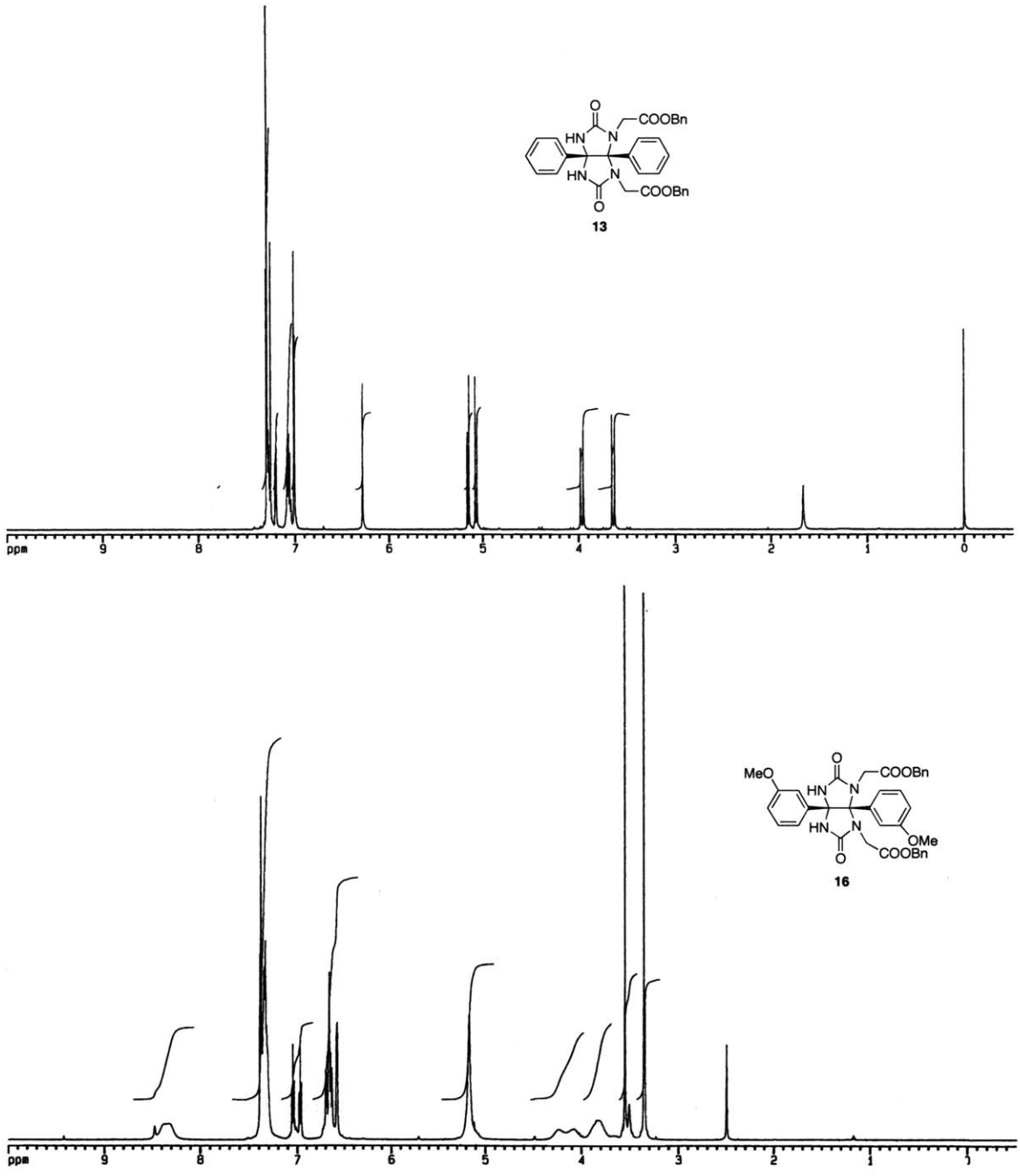
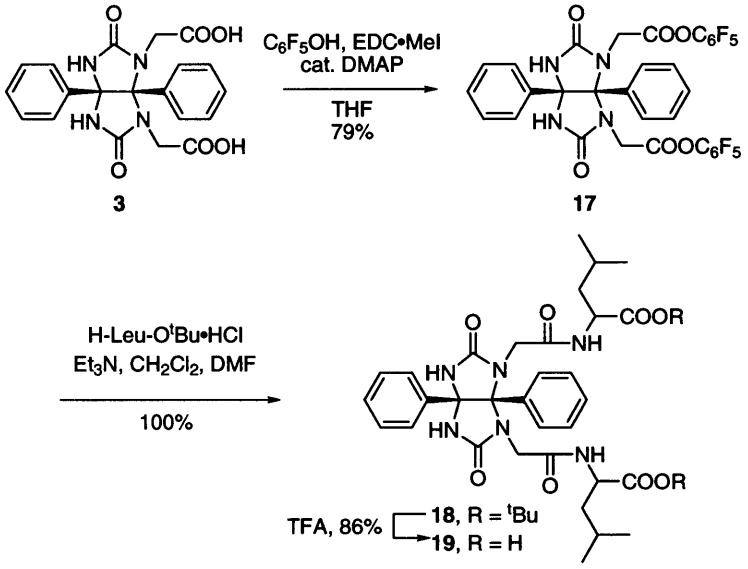


Figure 3. ^{1}H NMR spectra of **13** (top) and **16** (bottom). Note the broadness of the lower spectrum.

Pentafluorophenyl esters

Exemplifying the latter route, we activated diacid **3** as the bis(pentafluorophenyl ester) **17** by coupling the diacid and pentafluorophenol with EDC•MeI and catalytic DMAP in THF (see Scheme 6). The resulting active ester reacts readily with a variety of amines, including relatively unreactive anilines.[10] Additionally, both unreacted excess amines and pentafluorophenol can be removed by aqueous extraction during work-up. The glycoluril core is unaffected by the reaction conditions used for deprotection of *t*-butyl ester derivatives of amino acids used as building blocks (neat TFA, 12 h), as demonstrated by the synthesis of bis(leucine *tert*-butyl ester) derivative **18** and its subsequent TFA deprotection to yield diacid **19**.

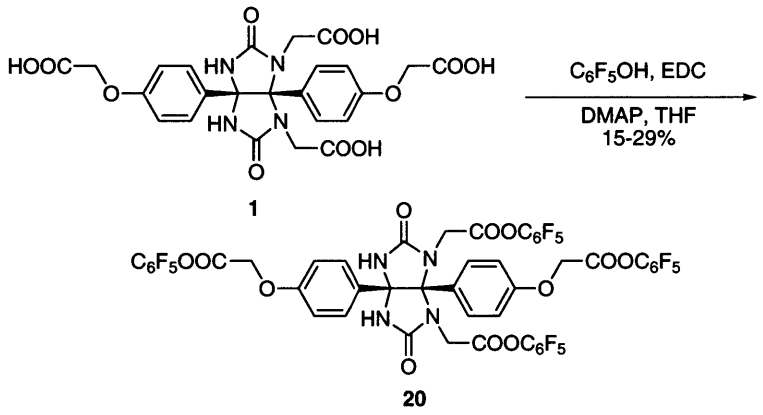


Scheme 6. Activation of glycoluril diacid **3** and reaction of the resulting activated ester **17**.

[10] Mass spectrometry of libraries formed by the reaction of the glycoluril core with aniline and several other single amines showed that all three masses expected for the two homo- and the heterosubstituted products were present.

A number of small libraries based on this core have been synthesized. HPLC analysis of small libraries indicates clean reactions with approximately statistical product distributions.

Unfortunately, attempts to activate diacid **2** or tetraacid **1** as pentafluorophenyl esters led to poor yields. Tetrakis(pentafluorophenyl ester) **20** was obtained in 15-29% yield by reaction with EDC•MeI or PyBOP (see Scheme 7). Fortunately, the entire reaction pathway is easily scaleable, and gram quantities of tetrakis(pentafluorophenyl ester) **20** can be obtained despite the less-than-optimal yields of the final step. The bis(pentafluorophenyl ester) corresponding to diacid **2** appeared to be isolated in approximately 60% yield, but mass spectrometry of the isolate indicated that it consisted of some compound of 110 higher mass units, and the compound failed to react in the expected manner in a test reaction.

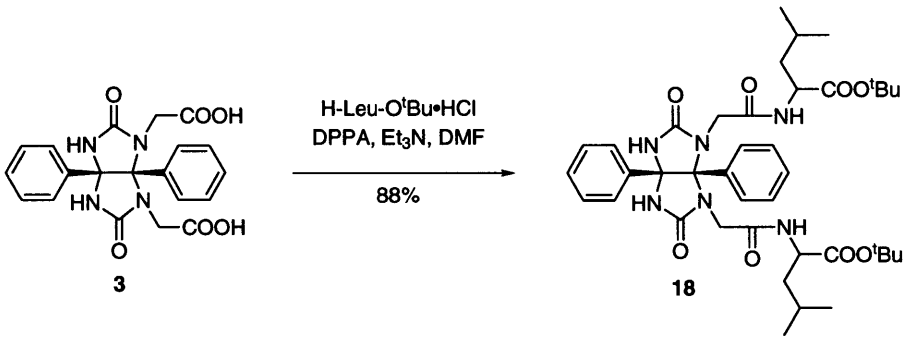


Scheme 7. Synthesis of glycoluril tetrakis(pentafluorophenyl ester) **20**.

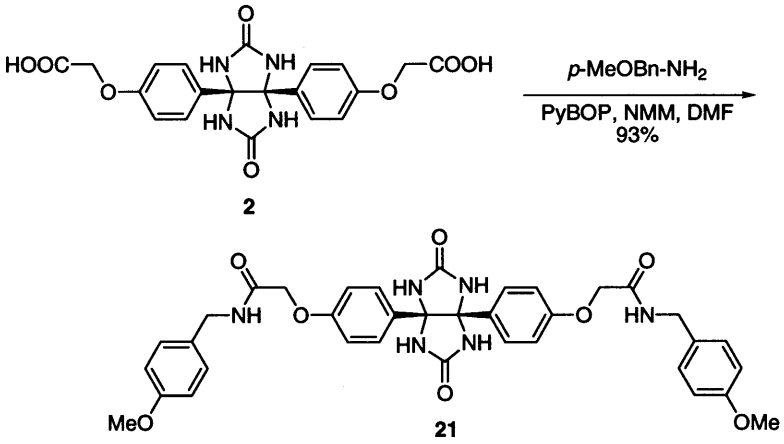
Use of coupling reagents

Attempts to synthesize libraries by the direct reaction between the polyacid cores and amine building blocks using coupling reagents have been stymied in many cases by an inability to clean the library of byproducts sufficiently. A number of coupling reagents have been used to test their efficacy, including DCC, EDC•MeI, PyBOP, PyBrOP, and DPPA. Many of the reactions worked fairly well, but it was impossible to purify the products

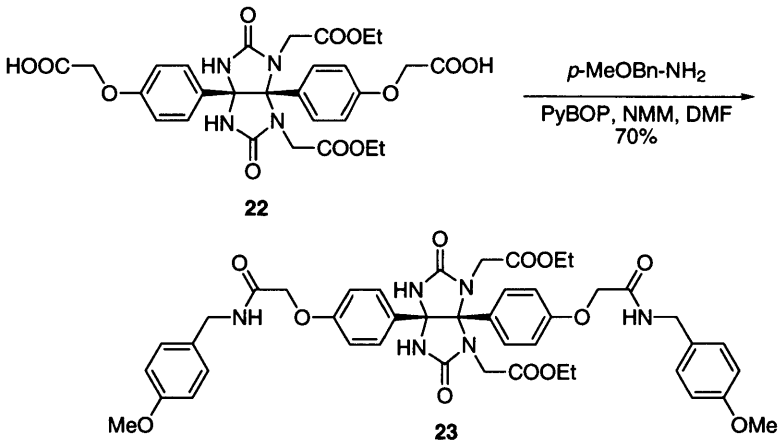
completely without resorting to chromatography. Two exceptions have been the use of DPPA with glycoluril diacid **3**, and PyBOP with diacids **2** and **22** (see section 5.5 below), examples of which are shown below in Schemes 8–10. This, fortunately, provides a way to access cleanly compounds based on derivatives of core **2**.



Scheme 8. Use of DPPA directly with diacid **3** to give diamide **18**.



Scheme 9. Use of PyBOP to install amides on diacid **2**.

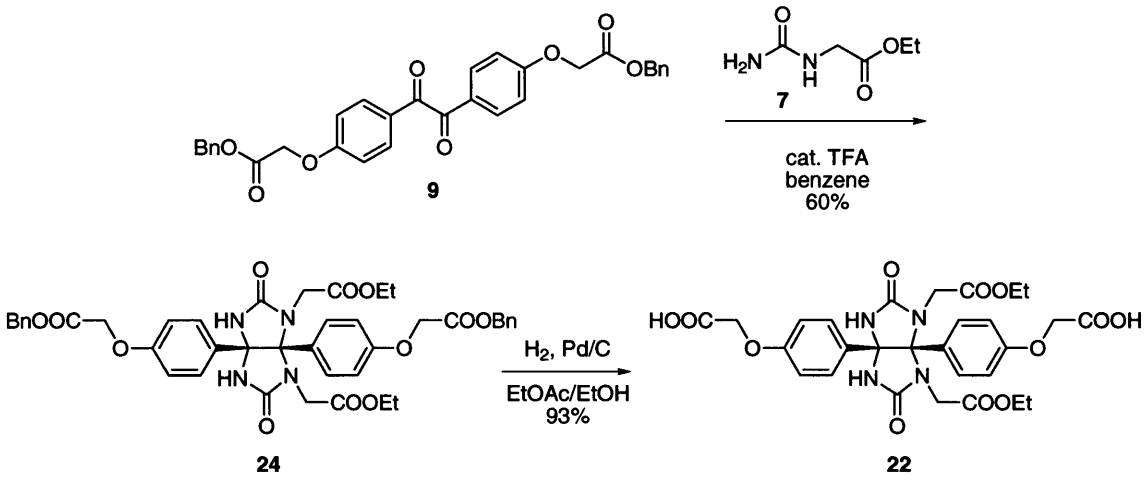


Scheme 10. Installation of building blocks with PyBOP on diacid **22**, one of the compounds developed for deconvolution of the glycoluril tetrasubstituted core molecule (see section 5.5 below).

5.5 Site differentiation on the tetrasubstituted glycoluril core

Bis(ethyl ester)/bis(benzyl ester)

In order to synthesize smaller libraries and individual compounds with the tetrasubstituted core molecule **1**, it is necessary to incorporate different protecting groups into the molecule that would allow orthogonal deprotection. One such compound is bis(ethyl ester)/bis(benzyl ester) **24**. Conveniently synthesized by the condensation of ethyl hydantoate **7** and diketone **9**, tetraester **24** can be selectively deprotected by cleavage of the benzyl esters by catalytic hydrogenolysis to afford diester/diacid **22** (Scheme 11). As discussed above in Section 5.4, this diacid can be functionalized by using PyBOP-mediated coupling reactions. Additionally, although the reaction was not attempted, the success of the reaction of diester/diacid **29** to give bis(pentafluorophenyl ester) **30** (Scheme 12) implies that the analogous activation of diacid **22** as the bis(pentafluorophenyl ester) should be possible.



Scheme 11. Synthesis of bis(ethyl ester)/bis(benzyl ester) **24** and its selective deprotection.

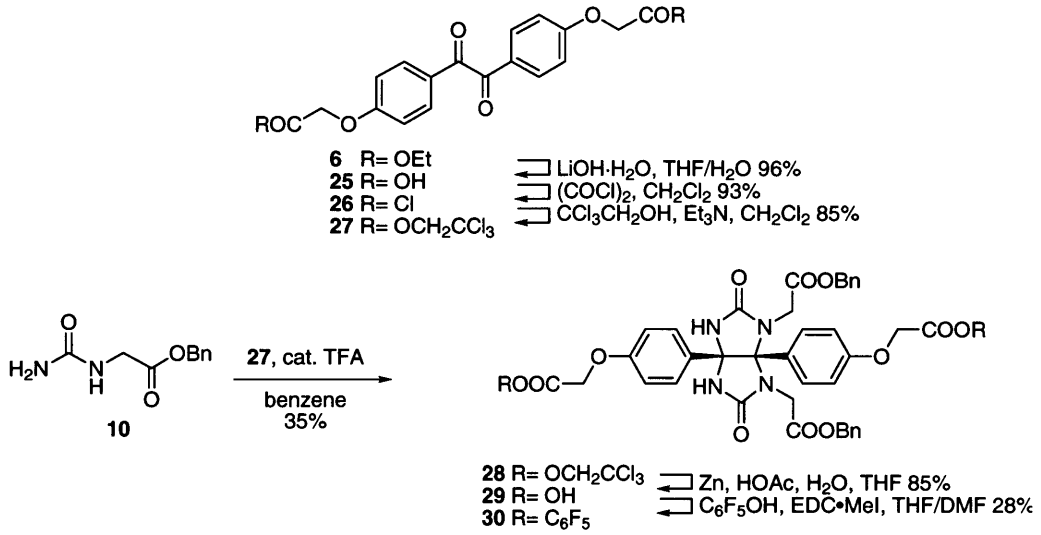
Bis(benzyl ester)/bis(2,2,2-trichloroethyl ester)

A further refinement of the deconvolution conditions was made to allow deprotection in the second step of deconvolution under conditions that would not epimerize amino acid substituents. Unlike bis(ethyl ester) **24** above, the bis(2,2,2-trichloroethyl ester)/bis(benzyl ester) **28** provides us with this ability. Deprotection of the trichloroethyl esters yields the diacid/diester **29**; subsequent derivatization at the acid positions [*via* bis(pentafluorophenyl ester) **30**], followed by mild hydrogenolysis, yields a diacid for further reaction without resort to basic conditions.

A synthetic scheme for diester/diacid **29** is shown in Scheme 12. The ethyl ester substituted benzil **6** was saponified to **25** in almost quantitative yield with LiOH in aqueous THF. Conversion of this diacid to the diacid chloride **26** was accomplished using oxalyl chloride in good yield. Esterification with 2,2,2-trichloroethanol provided the protected dione **27**.

This dione was condensed with benzyl hydantoate **10** to yield the differentially-protected glycoluril **28**. Deprotection of the trichloroethyl esters was accomplished by

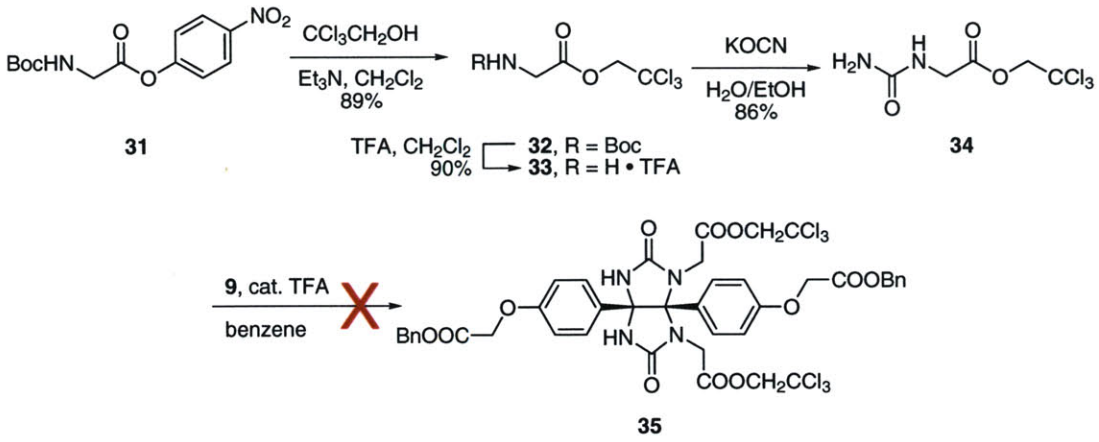
treatment with zinc and acetic acid to give diacid **29**, which was activated as bis(pentafluorophenyl ester) **30**.



Scheme 12. Site differentiation *via* mixed trichloroethyl and benzyl esters.

Attempts to condense the free diacid **25** in a glycoluril-forming reaction only met with success when unsubstituted urea was used, yielding glycoluril diacid **2**. In several attempts with benzyl hydantoate, the acid-catalyzed cyclization of the hydantoate to give hydantoin proceeded to the exclusion of the desired condensation; it is thought that the poor solubility of diacid **25** in the reaction medium is to blame.

A synthesis of the "reverse" glycoluril **35** (Scheme 13), in which the benzyl and trichloroethyl esters are switched relative to compound **28**, also met with failure. The trichloroethyl hydantoate **34** (available in three steps from *N*-α-Boc-L-glycine-4-nitrophenyl ester **31**) is more labile to hydantoin formation than the benzyl analog, and glycoluril formation was not competetive in this case, despite the favorable solubility properties of the reactants.



Scheme 13. An attempted synthesis of a "reverse" differentially-protected glycoluril.

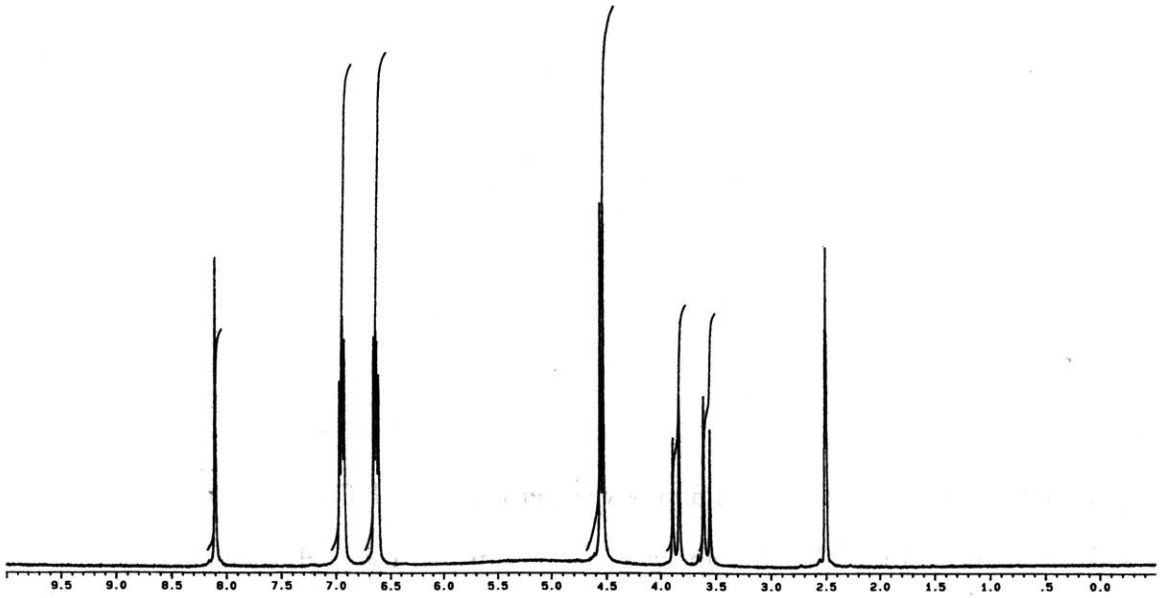
5.6 Experimental

General

All reagents were purchased from Aldrich Chemical Company and were used without further purification except as noted. Amino acid esters, PyBOP, and EDC•MeI were acquired from Novabiochem (San Diego, CA). Deuterated solvents were obtained from Cambridge Isotopes Laboratories and deuterated chloroform was dried over 4Å molecular sieves. HCl refers to a 1N stock solution. NMR spectra were recorded on either a Bruker AC-250, a Bruker AM-300, or a Bruker DRX-600; TMS was used as a reference in some chloroform-*d* spectra, otherwise residual solvent was used as a reference. Either a Finnegan Mat 8200 (for EI) or a VG ZAB-VSE (for FAB) mass spectrometer was used to ascertain masses. FT-IR spectra were obtained on a Perkin Elmer Paragon 1000 PC FT-IR Spectrometer. Silica gel chromatography was performed with Silica Gel 60 (EM Science or Bodman, 230–400 mesh). TLC analysis was performed using glass-bound Silica Gel 60 (F254) plates. DMAP = *N*,*N*-dimethyl-4-aminopyridine, DPPA = diphenylphosphoryl azide, EDC•MeI = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide, PyBOP = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate.

Glycoluril tetraacid (1).

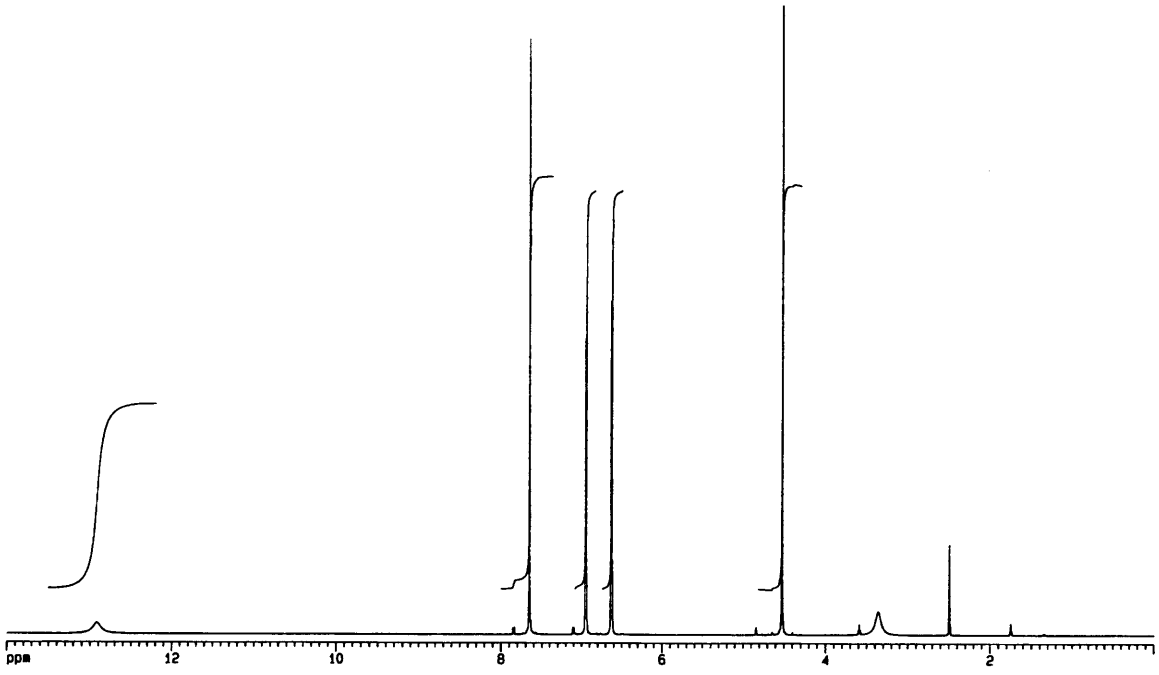
Glycoluril tetrakis(benzyl ester) **11** (4.00 g, 4.35 mmol) was dissolved in 400 mL 1:1 EtOAc/MeOH and 10% palladium on activated carbon (412 mg, cat.) was added. The slurry was stirred under an atmosphere of hydrogen for 15 hours, and was then filtered through Celite. The filtrate was placed on a rotary evaporator and the solvent was removed, leaving a white foam. Compound **1** (2.53g, quant.) was isolated after further drying the foam under high vacuum. m.p. 126 °C (dec.) ^{1}H NMR (600 MHz, DMSO-d_6): δ 12.65 (br. s, 4H), 8.09 (s, 2H), 6.96–6.93 (m, 4H), 6.65–6.62 m, 4H), 4.55 (s, 2H), 4.52 (s, 2H), 3.86 (d, J = 17.6 Hz, 2H), 3.59 (d, J = 17.6 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 170.78, 170.01, 169.97, 159.14, 157.82, 157.52, 130.13, 129.31, 128.49, 126.09, 114.13, 113.40, 88.26, 79.66, 64.53, 64.47, 42.93. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{24}H_{22}N_4O_{12} + Cs]^+$ 691.0289 found 691.0313. FT-IR (neat) 3375 (br., strong), 1720, 1513, 1479, 1305, 1228, 1075, 965, 838, 536 cm^{-1}.



Glycoluril diacid (2).

A. Saponification of bis(benzyl ester) **12.** To a stirred suspension of glycoluril bis(benzyl ester) **12** (1.00 g, 1.61 mmol) in 17.5 mL THF was added a solution of $LiOH•H_2O$ (0.16 g, 3.9 mmol) in 3.5 mL water. The resulting mixture was vigorously stirred at RT for 17 h. The mixture was poured into 90 mL HCl. The resulting white solids were isolated by filtration and were dried at elevated temperature, yielding the diacid **2** (0.68 g, 96%). m.p. 287 °C (dec.) ^{1}H NMR (600 MHz, DMSO-d_6): δ 12.92, (br. s, 2H), 7.66 (s, 4H), 6.95 (d, J = 8.8 Hz, 4H), 6.63 (d, J = 8.7 Hz, 4H), 4.53 (s, 4H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 170.00, 160.64, 157.23, 130.90, 128.28, 113.36, 81.59, 64.38. HRMS (FAB, NBA/NaI) m/z calcd for $[C_{20}H_{18}N_4O_8 + H]^+$ 443.1203 found 443.1233. FT-IR (neat) 3381, 3221 (br., v.s.), 2361, 1726, 1684, 1610, 1513, 1476, 1445, 1419, 1302, 1235, 1185, 1110, 1074, 954, 839, 780, 737, 634 cm^{-1}.

B. Condensation of 4,4'-bis(methoxycarboxylic acid) benzil **25** *with urea.* Benzil **25** (3.00 g, 8.37 mmol), urea (1.52 g, 25.3 mmol), and trifluoroacetic acid (2 mL) was dissolved in 40 mL benzene. The solution was refluxed under a Dean-Stark trap in a nitrogen atmosphere for 40 hours. Ethanol was added to the cooled solution and the undissolved white solids were removed by filtration. Drying at elevated temperatures overnight yielded **2** (3.42 g, 92%) as white solids.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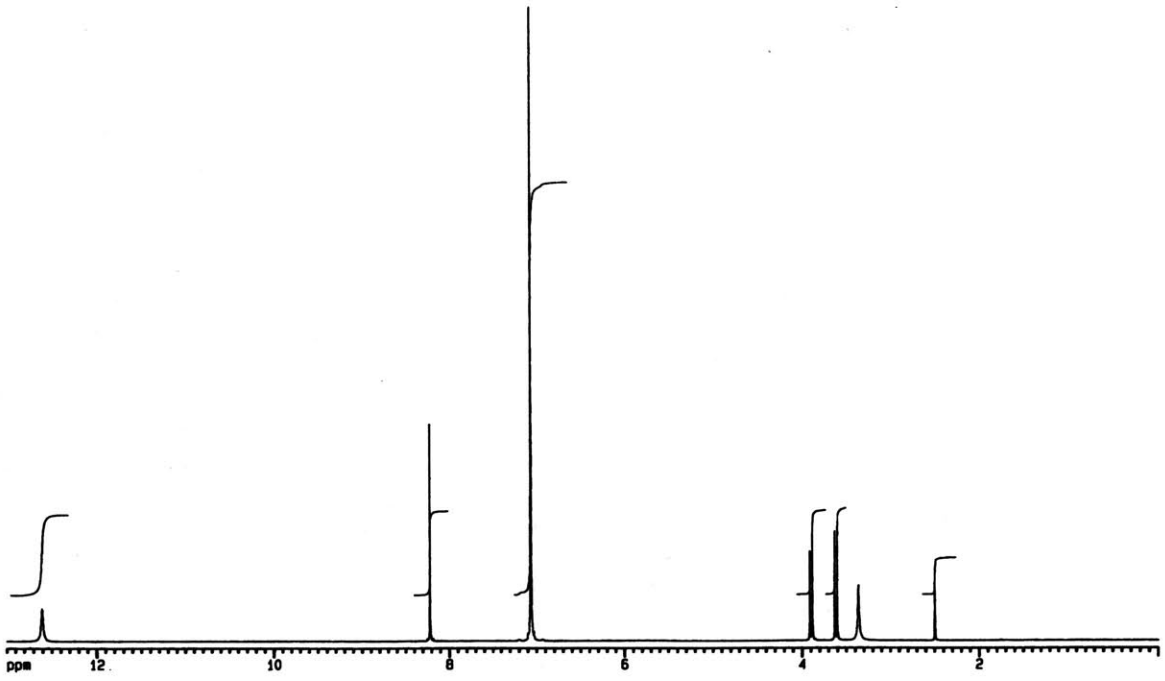
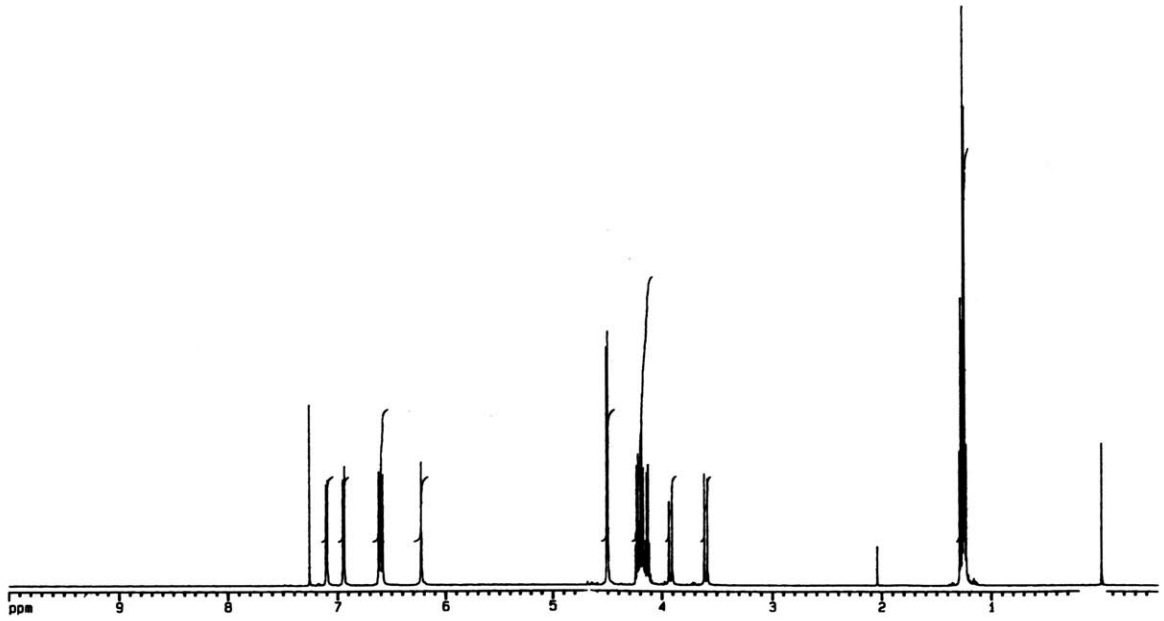
Glycoluril diacid (3).

To a stirred suspension of glycoluril bis(benzyl ester) **13** (0.590 g, 1.00 mmol) in 10 mL THF was added a solution of $LiOH•H_2O$ (0.101 g, 2.40 mmol) in 2 mL H_2O. The mixture was stirred at RT for 15 h. The mixture was then poured into 50 mL HCl. The resulting solution was chilled at 5 °C for 24 hours, whereupon colorless crystals developed. The crystals were isolated by filtration and were dried at elevated temperature, yielding diacid **3** (0.388 g, 95%). m.p. 292–294 °C (dec.) ^{1}H NMR (600 MHz, DMSO-d_6): δ 12.62 (br. s, 2H), 8.21 (s, 2H), 7.07 (s, 10H), 3.90 (d, J = 17.6 Hz, 2H), 3.62 (d, J = 17.6 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 171.06, 159.54, 137.87, 133.96, 128.79, 128.38, 128.35, 128.19, 127.62, 127.51, 88.64, 79.90, 43.08. HRMS (FAB, NBA/NaI) m/z calcd for $[C_{20}H_{18}N_4O_6 + H]^+$ 411.1305 found 411.1317. FT-IR (neat) 3456.6, 3228.0 (br.), 1734.8, 1700.2, 1474.6, 1449.8, 1399.8, 1340.7, 1225.6, 1147.2, 985.8, 963.9, 944.5, 780.7, 703.7, 667.9 cm^{-1}.



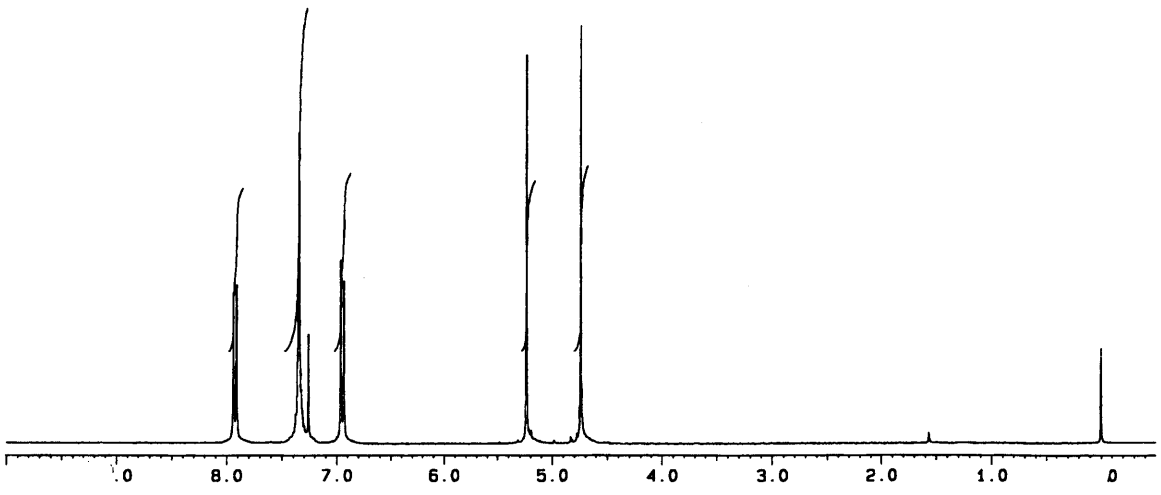
Glycoluril tetrakis(ethyl ester) (**8**).

To 200 mL benzene was added diethyl 4,4'-bis(methoxycarboxylate)-benzil **18** (20.00 g, 48.30 mmol) and ethyl hydantoate (20.0 g, 137 mmol). Trifluoroacetic acid (10 mL) was added and the solution was refluxed with a Dean-Stark trap for 48 hours. The solvent was removed by rotary evaporation, then the residue was taken up in a minimum of chloroform and was washed with water (3x) and brine (1x). The organic phase was dried over $MgSO_4$, filtered, and the solvent was removed by rotary evaporation. The resulting orange oil was crystallized from hot EtOH/EtOAc (2:1) to yield **8** (14.51 g, 45%) as colorless microcrystals in two crops. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.11 (d, J = 9.0 Hz, 2H), 6.95 (d, J = 8.9 Hz, 2H), 6.62 (d, J = 8.9 Hz, 2H), 6.60 (d, J = 9.0 Hz, 2H), 6.35 (s, 2H), 4.52 (s, 2H), 4.50 (s, 2H), 4.25–4.12 (m, 8H), 3.92 (d, J = 17.5 Hz, 2H), 3.60 (d, J = 17.5 Hz, 2H), 1.30–1.23 (m, 12H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 169.11, 168.88, 168.59, 159.36, 158.49, 158.12, 129.74, 129.69, 128.91, 125.14, 114.82, 114.16, 89.14, 80.76, 65.19, 64.96, 62.02, 61.61, 43.16, 14.33, 14.30, 14.26. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{32}H_{38}N_4O_{12} + Cs]^+$ 803.1541 found 803.1521. FT-IR (neat) 3351.7, 2983.0, 2252.4, 1731.8, 1609.9, 1512.7, 1462.8, 1305.6, 1204.3, 1182.9, 1082.4, 1027.6, 835.9, 730.5 cm^{-1}.



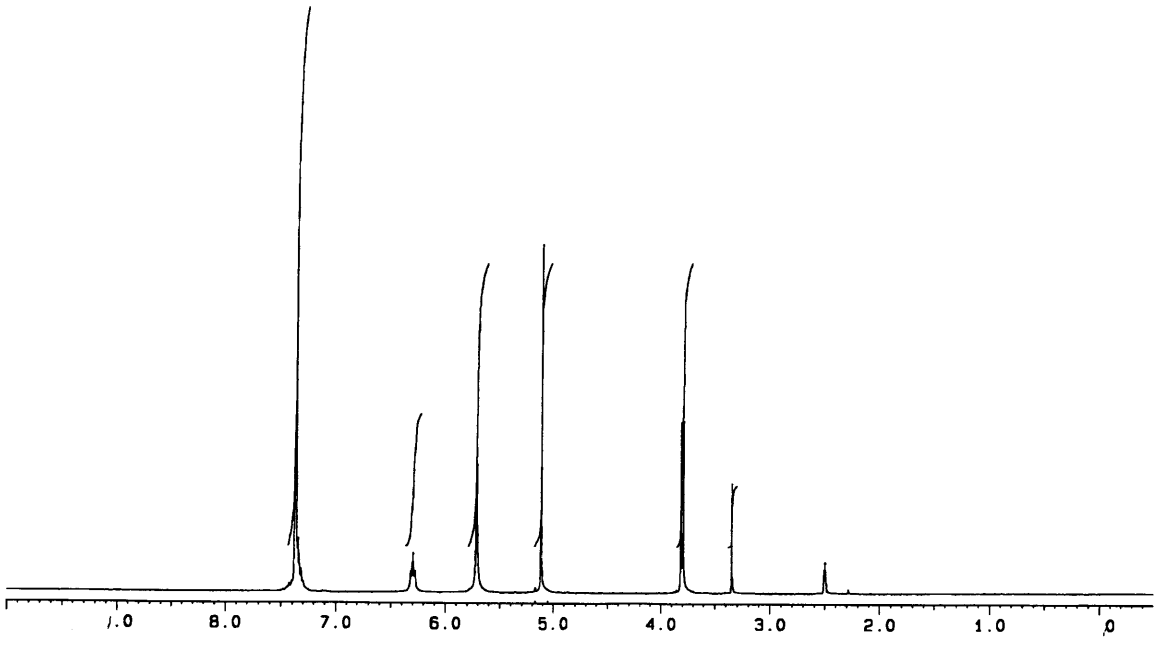
Dibenzyl 4,4'-bis(methoxycarboxylate)-benzil (9).

To a flame-dried 1000 mL round-bottom flask was added 4,4'-dihydroxybenzil (30.00 g, 123.8 mmol), benzyl bromoacetate (102.2 g, 446 mmol), and 500 mL dry acetonitrile. This solution was stirred vigorously and potassium carbonate (84.8 g, 614 mmol) was added. The resulting suspension was stirred and refluxed under nitrogen for 24 hours. The insoluble salts were removed from the solution by filtration, then the acetonitrile solution was poured into ~1900 mL stirring water. The brown precipitate was removed by filtration and was washed thoroughly with diethyl ether. The solids were partially taken up in methylene chloride and then were precipitated with ether, yielding **9** (43.22 g, 65%) as a bright white powder. m.p. 121 °C (dec.) ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.74 (s, 4H), 5.24 (s, 4H), 6.95 (d, J = 8.9 Hz, 4H), 7.30–7.35 (m, 10H), 7.92 (d, J = 8.9 Hz, 4H). ^{13}C NMR (76 MHz, $CDCl_3$): δ 65.28, 67.50, 115.04, 127.20, 128.76, 128.88, 132.57, 135.00, 162.96, 168.01, 193.31. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{32}H_{26}O_8 + Cs]^+$ 671.0682 found 671.0664. FT-IR (neat) 1755, 1665, 1597, 1508, 1422, 1161, 1075, 884, 834, 753, 698 cm^{-1}.



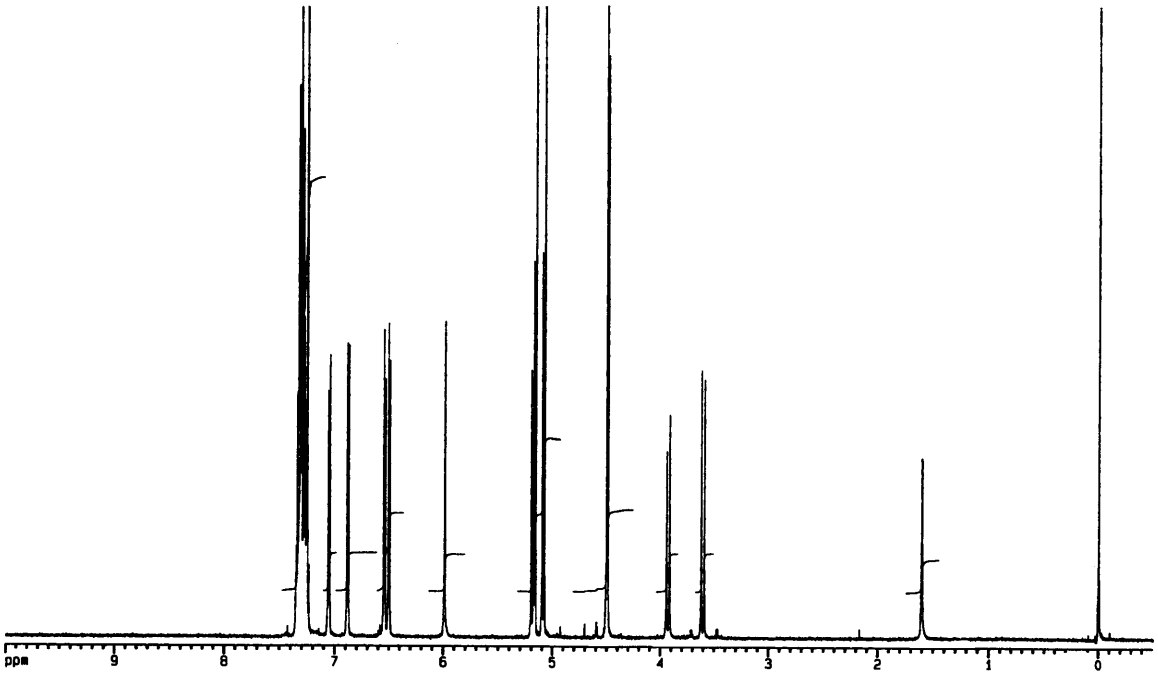
Benzyl hydantoate (10).

Glycine benzyl ester tosylate (60.1 g, 178 mmol) was dissolved in 250 mL hot ethanol. To this solution was added a solution of potassium cyanate (14.50 g, 179 mmol) in 250 mL water. The resulting clear solution was stirred at room temperature for 15 hours, then ~3/5 of the solution was removed by rotary evaporation and the white product **10** (34.33 g, 93%) crystallized from the remaining solution upon cooling. m.p. 119–121 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.37–7.31 (m, 5H), 6.29 (t, J = 5.7 Hz, 1H), 5.71 (s, 2H), 5.11 (s, 2H), 3.80 (d, J = 6.1 Hz, 2H).



Glycoluril tetrakis(benzyl ester) (11).

Dibenzyl 4,4'-bis(methoxycarboxylate)-benzil **9** (10.78 g, 20.0 mmol), benzyl hydantoate **10** (10.42 g, 50.0 mmol), and trifluoroacetic acid (5 mL) were dissolved in 100 mL benzene. The solution was raised to reflux under a Dean-Stark trap in a nitrogen atmosphere, and was held there for 28 h. The solvent was removed by rotary evaporation and the residue was dissolved in chloroform and was washed with water until achieving neutral pH in the aqueous phase (washing with base caused a difficult emulsion) then was washed with brine. The organic phase was dried over magnesium sulfate and filtered, and the solvent was reduced by rotary evaporation. Ethanol was added to the mixture and the resulting solids were isolated by filtration. Recrystallization from ethanol yielded **11** (6.73 g, 56%) as brilliant white crystals. m.p. 103–106 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.33–7.24 (m, 20H), 7.06 (d, J = 8.8 Hz, 2H), 6.88 (d, J = 8.8 Hz, 2H), 6.54 (d, J = 9.0 Hz, 2H), 6.50 (d, J = 9.0 Hz, 2H), 6.38 (s, 2H), 5.17–5.15 (m, 4H), 5.07–5.05 (m, 4H), 4.50 (s, 2H), 4.46 (s, 2H), 3.91 (d, J = 17.5 Hz, 2H), 3.59 (d, J = 17.5 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 135.41, 135.34, 135.31, 129.79, 129.00, 128.94, 128.91, 128.88, 128.78, 128.76, 128.73, 125.19, 114.91, 114.31, 89.20, 80.77, 67.64, 67.20, 67.14, 65.12, 64.87, 43.11. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{52}H_{46}N_4O_{12} + Cs]^+$ 1051.2167 found 1051.2123. FT-IR (neat) 3364, 3033, 1752, 1609, 1586, 1512, 1456, 1388, 1306, 1178, 1118, 1081, 1006, 962, 881, 837, 753, 699, 666 cm^{-1}.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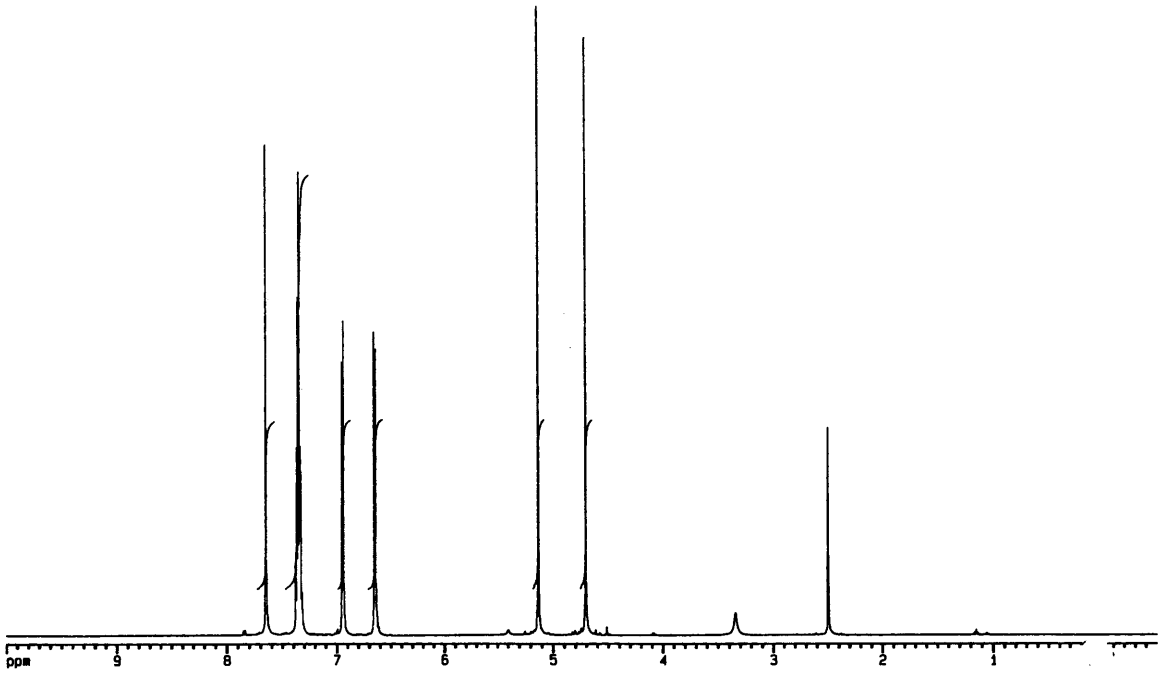
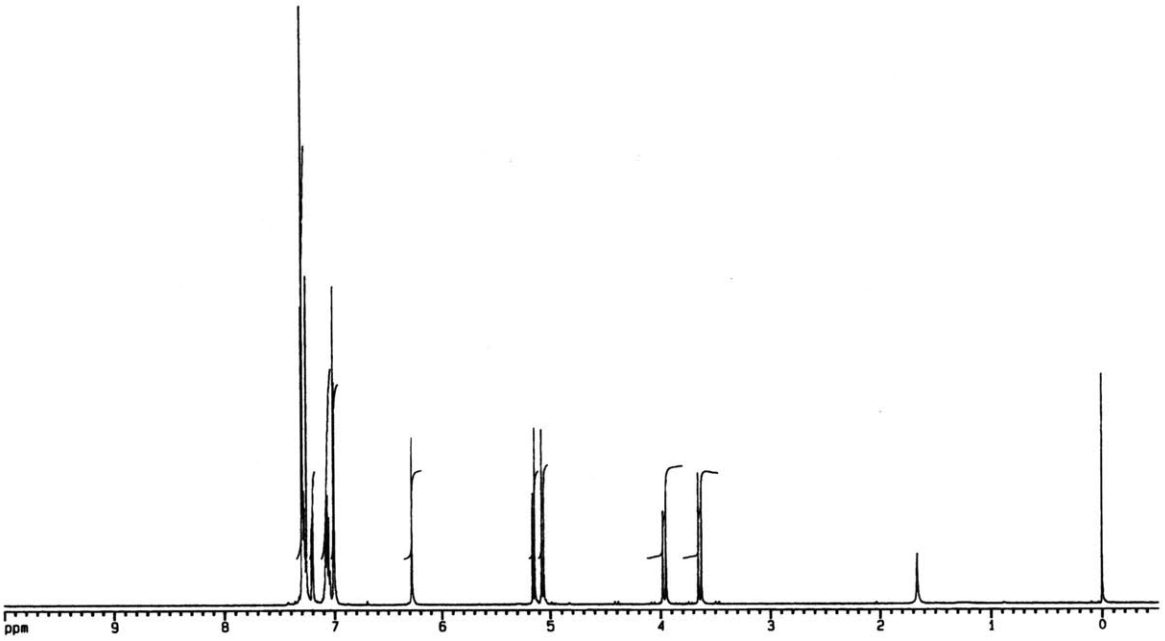
Glycoluril bis(benzyl ester) (12).

Dibenzyl 4,4'-bis(carboxymethoxy)benzil (43.82 g, 81.4 mmol) and urea (12.22 g, 203.5 mmol) were dissolved in 400 mL benzene. TFA (20 mL) was added and the solution was refluxed under a Dean-Stark trap in a nitrogen atmosphere for 16 h. EtOH was added to the cooled solution and the solid precipitate was isolated by filtration. The solids were purified by stirring overnight in boiling EtOH. The resulting solids were isolated by filtration and dried *in vacuo*, yielding **12** (36.97 g, 73%) as a white powder. m.p. 213–217 °C (dec.) ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.65 (s, 4H), 7.33–7.38 (m, 10H), 6.94 (d, J = 8.8 Hz, 4H), 6.65 (d, J = 8.8 Hz, 4H), 5.13 (s, 4H), 4.71 (s, 4H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 168.66, 160.79, 157.24, 135.82, 131.30, 128.60, 128.42, 128.34, 128.20, 113.55, 81.60, 65.98, 64.56. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{34}H_{30}N_4O_8 + Cs]^+$ 755.1118 found 755.1148. FT-IR (neat) 3229.6, 1760.0, 1721.7, 1682.8, 1667.3, 1610.2, 1494.5, 1454.6, 1416.5, 1175.5, 1140.7, 1110.5, 1082.9, 1024.5, 955.0, 836.7, 736.1, 696.6 cm^{-1}.

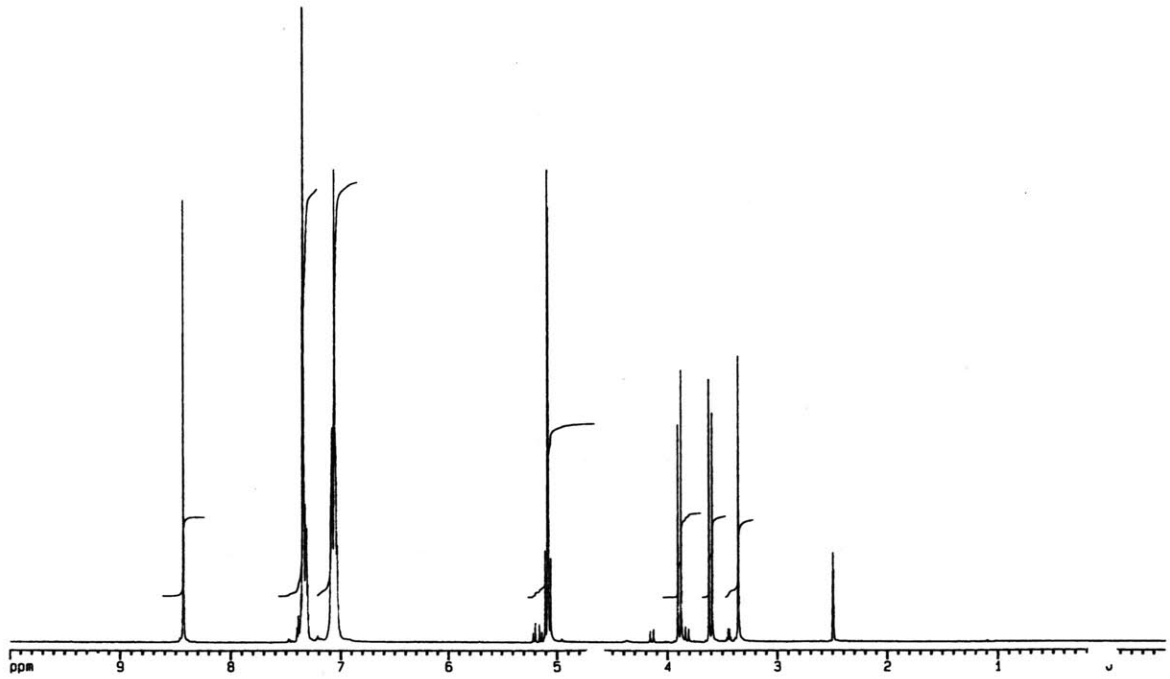


Cis- and *trans*-glycoluril bis(benzyl esters) (13 and 14).

Benzyl hydantoate **10** (18.0 g, 86.4 mmol), benzil (6.00 g, 28.5 mmol), and trifluoroacetic acid (7.5 mL) were dissolved in 150 mL benzene. The solution was refluxed under a Dean-Stark trap in a nitrogen atmosphere for 20 hours. A precipitate was removed by filtration, then the filtrate was concentrated to a yellow oil by rotary evaporation, was taken up in chloroform, and was washed with brine until the aqueous phase was neutral. The organic layer was dried over magnesium sulfate, was filtered, and was concentrated again to a yellow oil. Sonication with methylene chloride/ether produced a yellow solution with white solids. The solids were isolated by filtration and were combined with the solids removed directly from the reaction solution and were recrystallized from hot ethanol. Upon cooling to room temperature, **13** (11.56 g, 69%) was isolated by filtration as white solids. Concentration of the mother liquor caused the precipitation of **14** (1.27 g, 8%). *Compound* **13**: m.p. 213–218 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.31–7.27 (m, 10H), 7.20 (d, J = 6.8 Hz, 2H), 7.10–7.05 (m, 4H), 7.01 (d, J = 4.2 Hz, 4H), 6.28 (s, 2H), 5.15 (d, J = 12.0 Hz, 2H), 5.07 (d, J = 12.0 Hz, 2H), 3.97 (d, J = 17.5 Hz, 2H), 3.64 (d, J = 17.5 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 136.34, 135.38, 132.15, 129.66, 129.26, 128.99, 128.91 (large), 128.78, 128.78, 128.53, 128.30, 127.56, 89.62, 81.04, 67.64, 43.30. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{34}H_{30}N_4O_6 + Cs]^+$ 723.1220 found 723.1245. FT-IR (neat) 3252, 3064, 2248, 1707, 1450, 1388, 1307, 1191, 1144, 1012, 960, 940, 910, 877, 778, 734, 698 cm^{-1}.

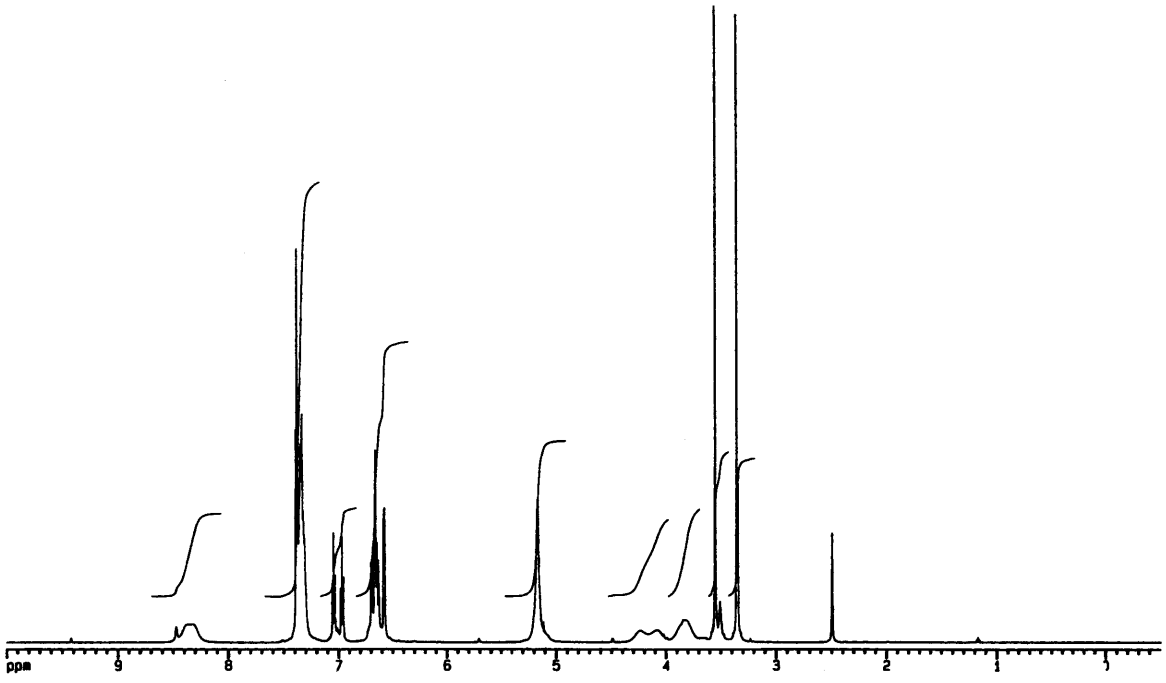


Compound **14***:* ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.43 (s, 2H), 7.36–7.32 (m, 10H), 7.08–7.04 (m, 10H), 5.10 (d, J = 12.6 Hz, 2H), 3.90 (d, J = 17.8 Hz, 2H), 3.61 (d, J = 17.8 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 168.82, 159.05, 136.07, 135.50, 128.62, 128.53, 128.15, 128.04, 127.87, 127.81, 83.92, 65.92, 42.24. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{34}H_{30}N_4O_6 + Cs]^+$ 723.1220 found 723.1202.



Bis(3-methoxyphenyl)/bis(benzyl ester) glycoluril (**16**).

Benzyl hydantoate **10** (9.22 g, 44.3 mmol) and 3,3'-dimethoxybenzil (4.00 g, 14.8 mmol) were refluxed in a solution of 120 mL benzene and 6 mL trifluoroacetic acid under a Dean-Stark trap for 24 h. After cooling, the solution was filtered to remove hydantoin byproduct and the solvent was removed by rotary evaporation. The residue was taken up in methylene chloride and the solution was washed thrice with water and once with satd. NaCl soln. The organic layer was dried over magnesium sulfate, filtered, and the solvent was removed by rotary evaporation. Sonication of this yellow oil in ethyl acetate caused the release of a white solid. Isolation by filtration followed by drying at elevated temperature yielded **16** (4.25 g, 44%) as a white powder. m.p. 177–181 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.37, (br. s, 2H), 7.39–7.31 (m, 10H), 7.05 (t, J = 7.9 Hz, 1H), 6.97 (t, J = 8.0 Hz, 1H), 6.73–6.58 (m, 6H), 5.17 (br. s, 4H), 4.16 (b., 2H), 3.84 (b., 2H), 3.55 (s, 3H), 3.36 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 169.69, 159.31, 158.67, 139.12, 136.04, 135.28, 129.30, 128.57, 128.54, 128.18, 128.01, 119.92, 119.77, 115.77, 114.26, 112.99, 112.64, 88.38, 80.06, 66.08, 54.87, 54.62, 43.00. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{36}H_{35}N_4O_8 + Cs]^+$ 651.2455 found 651.2478. FT-IR (neat) 3245.4, 1706.2, 1602.8, 1465.5, 1265.5, 1187.6, 946.7, 734.3 cm^{-1}.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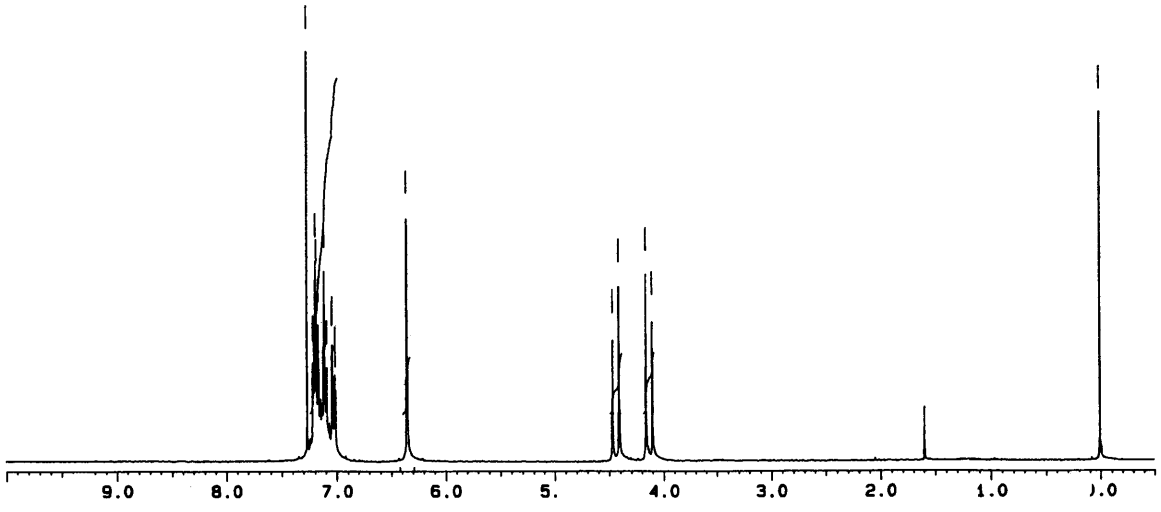
Glycoluril bis(pentafluorophenyl ester) (17).

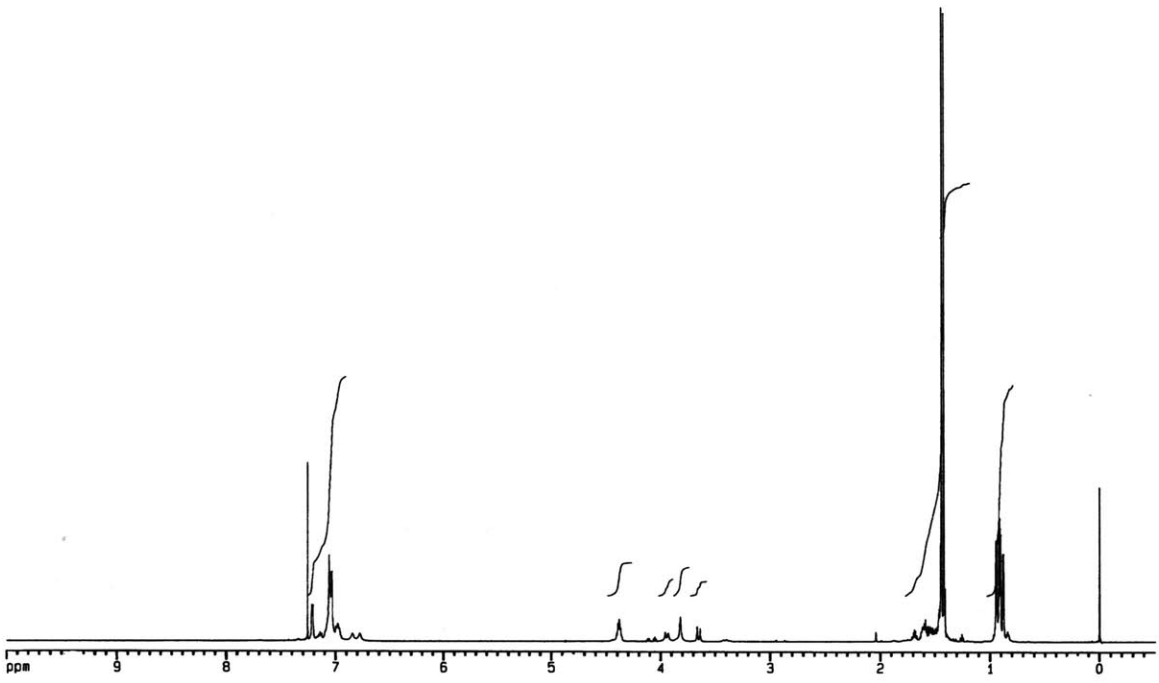
To a stirred suspension of glycoluril diacid **3** (1.00 g, 2.44 mmol) in 100 mL THF was added pentafluorophenol (1.80 g, 9.76 mmol), EDC•MeI (3.12 g, 10.5 mmol) and catalytic DMAP. The mixture was stirred at RT for 14 h. The solvent was removed by rotary evaporation and the resulting paste was sonicated in EtOAc and filtered to remove insoluble material. The filtrate was run through a plug of silica gel with EtOAc. The filtrate was concentrated and sonicated in Et_2O to remove pentafluorophenol. The white powder was isolated by filtration, rinsed with Et_2O and dried *in vacuo*, yielding the diester **17** (1.42 g, 79%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.58 (s, 2H), 7.15–7.03 (m, 10H), 4.77 (d, J = 18.4 Hz, 2H), 4.38 (d, J = 18.4 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 166.77, 159.03, 141.52 (m), 139.83 (m), 138.54 (m), 137.06, 136.89 (m), 133.31, 129.08, 128.55, 127.79, 127.69, 127.31, 88.34, 80.48, 42.39. ^{19}F-NMR (565 MHz, DMSO-d_6): δ -152.61 (d, J = 24 Hz), -157.44 (t, J = 24 Hz), -162.16 (t, J = 24 Hz). HRMS (FAB, NBA/CsI) m/z calcd for $[C_{32}H_{16}F_{10}N_4O_6 + Cs]^+$ 874.9964 found 875.0004. FT-IR (neat) 1788.7, 1714.4, 1521.7, 1450.0, 1103.7, 998.1 cm^{-1}.



Glycoluril bis(leucine t-butyl ester) adduct (18).

A. Reaction of bis(pentafluorophenyl ester) **17.** To a solution of leucine *t*-butyl ester hydrochloride (34.8 mg, 0.155 mmol) in 1 mL DMF and 0.5 mL Et_3N chilled in an ice bath was added a partial solution/suspension of glycoluril bis(pentafluorophenyl ester) **17** (50.4 mg, 0.0683 mmol) in 3.5 mL CH_2Cl_2. The solution warmed over 2.5 h, then was diluted with CH_2Cl_2 and was washed with water (3x) and brine (3x). The organic phase was dried over $MgSO_4$, was filtered, and was concentrated by rotary evaporation to pale yellow solids which were dried *in vacuo*, yielding **18** (53 mg, quant.). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.24–7.21 (m, 2H), 7.16–6.97 (m, 10H), 6.85 (br. s, 1H), 6.78 (br. s, 1H), 4.41–4.37 (m, 2H), 3.95 (d, J = 16.3 Hz, 1H), 3.82 (s, 2H), 3.65 (d, J = 16.5 Hz, 1H), 1.70–1.46 (m, 6H), 1.44 (s, 9H), 1.42 (s, 9H), 0.95 (d, J = 6.6 Hz, 3H), 0.93 (d, J = 6.5 Hz, 3H), 0.93 (d, J = 6.4 Hz, 3H), 0.88 (d, J = 6.4 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 172.57, 172.41, 168.57, 168.52, 160.98, 160.59, 136.57, 131.90, 129.51, 129.38, 129.00, 128.82, 1283.63, 128.27, 127.57, 123.48, 120.50, 120.47, 90.79, 82.10, 82.02, 80.69, 51.93, 51.75, 45.96, 45.79, 41.46, 41.33, 28.08, 28.06, 25.00, 24.98, 22.83, 22.74, 22.33, 22.21. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{40}H_{56}N_6O_8 + Cs]^+$ 881.3214 found 881.3236. FT-IR (neat) 3242.3, 2958.7, 1701.9, 1458.4, 1150.0 cm^{-1}.

B. Reaction of diacid **3** *with DPPA.* A stirred solution of glycoluril diacid **3** (200 mg, 0.487 mmol) and leucine *t*-butyl ester hydrochloride (240 mg, 1.07 mmol) in 2 mL DMF was chilled in a salt/ice bath. To this solution was added diphenylphosphoryl azide (231 μL, 294 mg, 1.07 mmol) and Et_3N, (298 μL, 217 mg, 2.14 mmol) and the solution was stirred 17 h, warming to RT. The solvent was removed by rotary evaporation and the residue was taken up in EtOAc and was washed with water (2x) and brine (3x). The organic phase was dried over $MgSO_4$, was filtered, and was concentrated by rotary evaporation to an off-white foam **18** (323 mg, 88%).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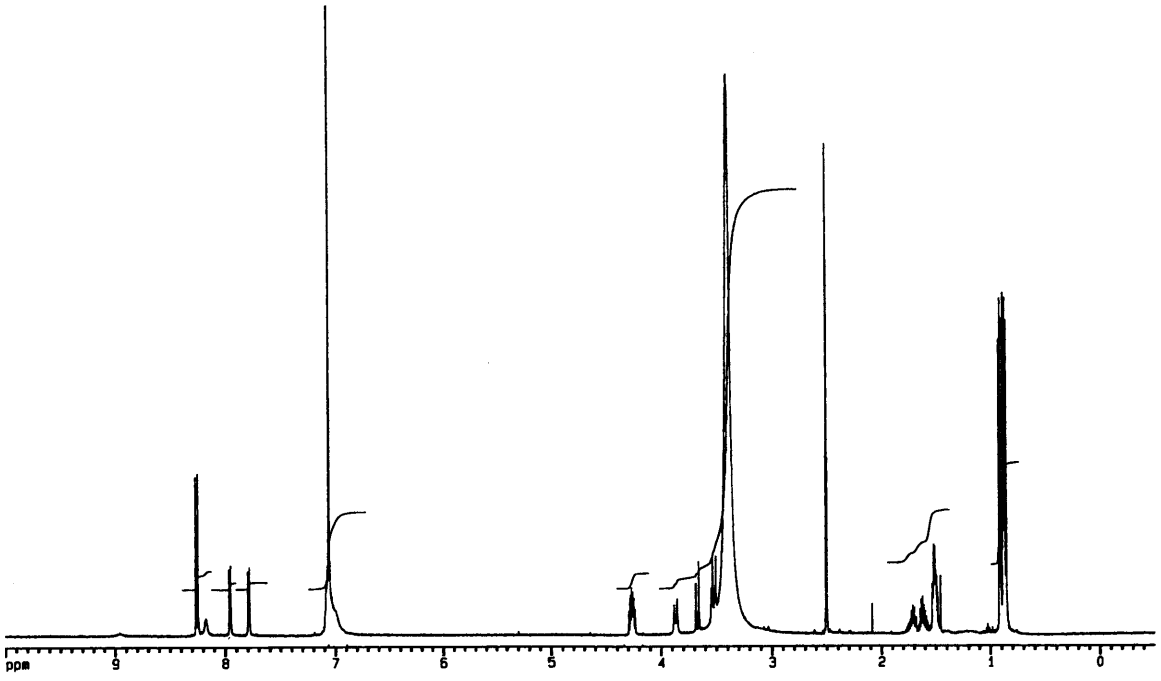
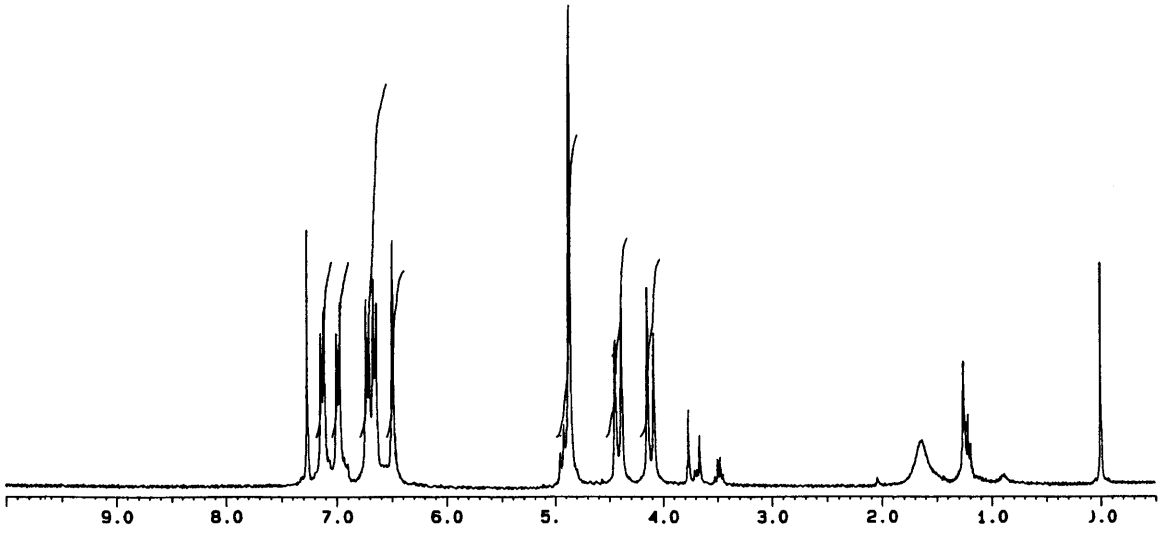
Glycoluril bis(leucine) adduct (19).

Glycoluril bis(leucine *t*-butyl ester adduct) **18** (45 mg, 0.060 mmol) was stirred in 5 mL TFA for 15 h. The TFA was removed by rotary evaporation and the resulting oil was sonicated in 1:1 Et_2O/hexane. The resulting precipitate was isolated by filtration and was rinsed with Et_2O, yielding **19** (34 mg, 86%) as a white powder. ^{1}H NMR (600 MHz, DMSO–d_6): δ 8.27 (s, 1H), 8.25 (s, 1H), 7.96 (d, J = 7.9 Hz, 1H), 7.79 (d, J = 7.8 Hz, 1H), 7.09–7.02 (m, 10H), 4.28–4.26 (m, 2H), 3.85 (d, J = 16.6 Hz, 1H), 3.66 (d, J = 16.8 Hz, 1H), 3.53 (d, J = 16.7 Hz, 1H), 3.52 (d, J = 16.8 Hz, 1H), 1.79–1.70 (m, 1H), 1.70–1.62 (m, 1H), 1.55–1.48 (m, 4H), 0.92–0.85 (m, 12H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 174.42, 174.19, 168.47, 168.39, 159.73, 159.69, 137.62, 133.37, 128.72, 128.26, 127.49, 127.36, 89.12, 79.61, 50.30, 45.77, 44.22, 44.05, 40.28, 24.21, 22.85, 22.79, 21.41, 8.58. LRMS (FAB, NBA/CsI) m/z calcd for $[C_{32}H_{40}N_6O_8 + Cs]^+$ 769 found 769.



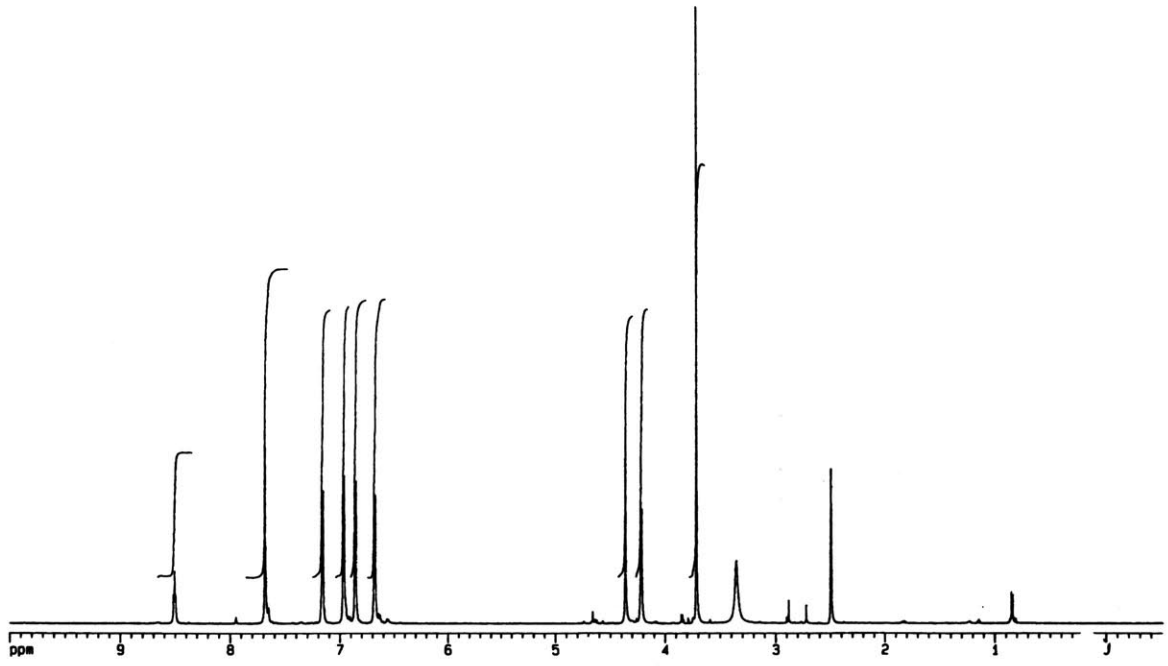
Glycoluril tetrakis(pentafluorophenyl ester) (20).

A solution of glycoluril tetraacid **1** (200 mg, 0.358 mmol), pentafluorophenol (527 mg, 2.86 mmol), EDC•MeI (847 mg, 2.85 mmol), and catalytic DMAP in 20 mL THF was stirred at RT for 8 h. The solvent was removed by rotary evaporation and the residue was taken up in EtOAc and shaken. Insoluble material was removed by filtration and the filtrate was concentrated and purified by silica gel chromatography (100% EtOAc). Product-containing fractions were combined and concentrated by rotary evaporation. Excess pentafluorophenol co-eluted with the product and was removed by sonicating the resulting oil in Et_2O. White solids were isolated by filtration and were dried *in vacuo* giving **20** (62 mg, 15%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.53 (s, 2H), 6.99 (d, J = 8.8 Hz, 2H), 6.98 (d, J = 8.7 Hz, 2H), 6.76 (d, J = 8.3 Hz, 4H), 5.22 (s, 2H), 5.21 (s, 2H), 4.77 (d, J = 18.3 Hz, 2H), 4.37 (d, J = 18.3 Hz, 2H). HRMS (FAB, NBA/CsI) m/z calcd for $[C_{48}H_{18}F_{20}N_4O_{12} + H]^+$ 1223.0680 found 1223.0609.



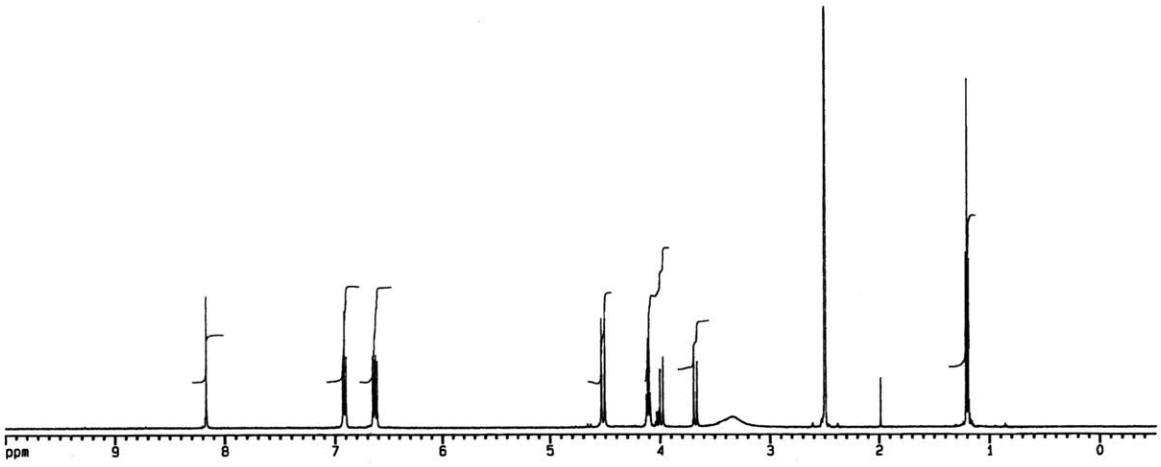
Glycoluril bis(*p*-methoxybenzyl amide) (21).

To a suspension of glycoluril diacid **2** (0.200 g, 0.452 mmol) in 10 mL DMF was added N-methylmorpholine (104 μL, 0.096 g, 0.97 mmol), PyBOP (0.470 g, 0.903 mmol), and 4-methoxybenzylamine (124 μL, 0.130 g, 0.949 mmol). The resulting solution was stirred under nitrogen for 15 h. The solvent was removed by rotary evaporation and the residue was taken up in methylene chloride and insoluble material was isolated by filtration, yielding **21** (0.286 g, 93%) as white solids. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.51 (t, J = 5.9 Hz, 2H), 7.69 (s, 4H), 7.16 (d, J = 8.6 Hz, 4H), 6.97 (d, J = 8.8 Hz, 4H), 6.86 (d, J = 8.6 Hz, 4H), 6.68 (d, J = 8.9 Hz, 4H), 4.67 (s, 4H), 4.22 (d, J = 5.9 Hz, 4H), 3.72 (s, 6H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 167.29, 160.63, 158.23, 157.12, 131.25, 131.12, 128.70, 128.29, 113.65, 81.56, 66.87, 55.08, 41.24. HRMS (FAB in NBA/CsI) m/z calcd for $[C_{36}H_{36}N_6O_8$ + Cs$]^+$ 813.1649 found 813.1628. FT-IR (neat) 3234.2, 1722.5, 1671.8, 1611.1, 1512.9, 1302.6, 1248.7, 1179.3, 110.6, 837.4, 778.4, 526.8 cm^{-1}.



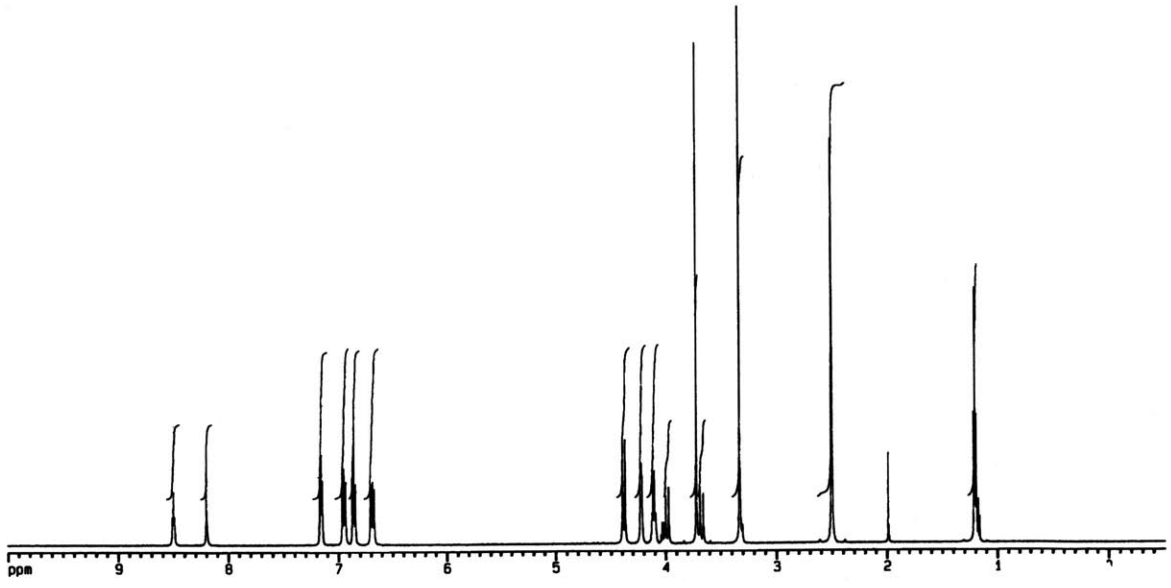
Glycoluril bis(ethyl ester) diacid (22).

To a solution of **24** (1.00 g, 1.26 mmol) in 80 mL ethyl acetate and 20 mL ethanol was added 10% palladium on carbon (0.125 g). The flask was evacuated and backfilled with hydrogen three times, then the suspension was stirred under hydrogen overnight. The solution was filtered through Celite, then the solvent was removed by rotary evaporation. The resulting clear foamy oil was redissolved in ethyl acetate, and precipitation with hexane yielded **22** (0.72 g, 93%) as white powder. ^{1}H NMR (600 MHz, DMSO-d_6): δ 12.95 (br. s, 2H), 8.18 (s, 2H), 6.94–6.91 (m, 4H), 6.65 (d, J = 9.0 Hz, 2H), 6.63 (d, J = 9.0 Hz, 2H), 4.56 (s, 2H), 4.53 (s, 2H), 4.13–4.09 (m, 4H), 4.39 (d, J = 17.6 Hz, 2H), 3.68 (d, J = 17.6 Hz, 2H), 1.20 (t, J = 7.1 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 169.96, 169.93, 169.41, 159.01, 157.85, 157.60, 129.84, 129.18, 128.45, 125.74, 114.13, 113.41, 88.08, 79.97, 64.45, 60.64, 42.90, 14.03. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{28}H_{30}N_4O_{12} + Cs]^+$ 747.0915 found 747.0935. FT-IR (neat) 3588.2, 3204.0, 1718.7, 1609.9, 1512.6, 1309.2, 1231.8, 1069.7, 1025.1, 885.7, 835.3, 774.5 cm^{-1}.



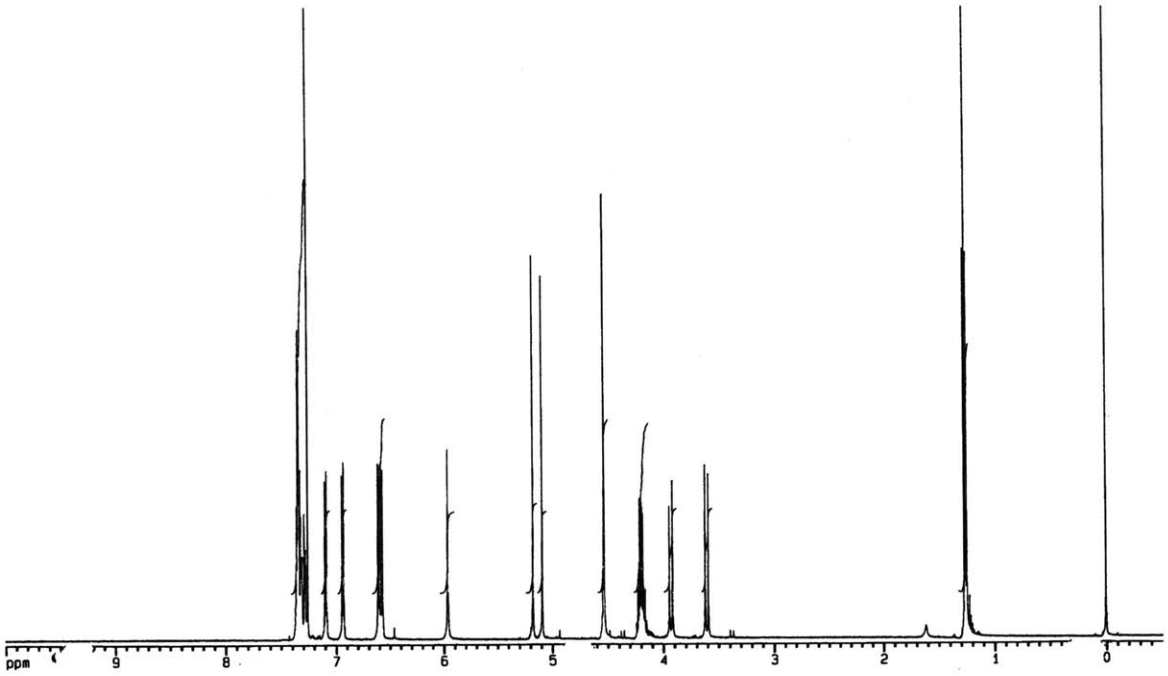
Glycoluril bis(ethyl ester) bis(*p*-methoxybenzyl amide) (23).

To a solution of glycoluril diacid **22** (0.100 g, 0.163 mmol), in 5 mL DMF was added PyBOP (0.170 g, 0.327 mmol), N-methylmorpholine (37 μL, 34 mg, 0.336 mmol), and *p*-methoxybenzylamine (43 μL, 45 mg, 0.329 mmol). The resulting clear solution was stirred at RT under nitrogen for 6 h. The solution was then poured into HCl and a white precipitate formed. The suspension was extracted with three portions of EtOAc, then the organic was washed with 1M NaOH (3x) and brine (3x). Solids insoluble in either layer after the final wash were removed by filtration, yielding **23** (0.096 g, 70%) as a white powder. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.50 (t, J = 6.0 Hz, 2H), 8.20 (s, 2H), 7.15 (dd, J = 8.3, 6.6 Hz, 4H), 6.95 (dd, J = 11.0, 8.6 Hz, 4H), 6.87–6.84 (m, 4H), 6.69 (dd, J = 13.9 Hz, 8.7 Hz, 4H), 4.39 (br. s, 2H), 4.37 (br. s, 2H), 4.23–4.22 (m, 4H), 4.13–4.09 (m, 4H), 3.99 (d, J = 17.6 Hz, 2H), 3.72 (s, 6H), 3.67 (d, J = 17.6 Hz, 2H), 1.21–1.16 (m, 6H). LRMS (FAB, NBA/CsI) m/z calcd for $[C_{44}H_{48}N_6O_{12} + Cs]^+$ 985 found 985.



Glycoluril bis(ethyl ester) bis(benzyl ester) (24).

Dibenzyl 4,4'-bis(methoxycarboxylate)-benzil **5** (5.00 g, 9.29 mmol), ethyl hydantoate (4.06 g, 27.8 mmol), and trifloroacetic acid (2.5 mL) were dissolved in 50 mL benzene. The solution was refluxed under a Dean-Stark trap in a nitrogen atmosphere for 14 hours. The solution was diluted with 200 mL chloroform and was extracted with water, saturated sodium bicarbonate solution (caused a bad emulsion), and brine. The organic layer was dried over magnesium sulfate and was filtered. The solvent was removed by rotary evaporation and the residue was crystallized from 2:1 EtOH/EtOAc, yielding **24** (4.46 g, 60%) as white microcrystals. m.p. 146–149 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.36–7.31 (m, 10H), 7.09 (d, J = 9.0 Hz, 2H), 6.93 (d, J = 9.0 Hz, 2H), 6.61 (d, J = 9.0 Hz, 2H), 6.58 (s, J = 9.0 Hz, 2H), 6.39 (s, 2H), 5.17 (s, 2H), 5.07 (s, 2H), 4.53 (s, 2H), 4.52 (s, 2H), 4.20–4.14 (m, 4H), 3.91 (d, J = 17.5 Hz, 2H), 3.59 (d, J = 17.5 Hz, 2H), 1.23 (t, J = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 169.10, 168.70, 168.45, 159.39, 158.42, 158.06, 135.21, 135.19, 129.72, 129.70, 128.90, 128.83, 128.78, 128.68, 128.63, 125.15, 114.84, 114.22, 89.15, 80.75, 67.22, 67.17, 65.12, 64.92, 62.04, 43.15, 14.25. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{42}H_{42}N_2O_{12} + Cs]^+$ 927.1854 found 927.1819. FT-IR (neat) 3356.8, 2982.16, 2252.3, 1731.9, 1609.9, 1512.6, 1455.9, 1306.3, 1180.0, 1081.9, 1028.0, 912.5, 835.7, 733.6, 698.8 cm^{-1}.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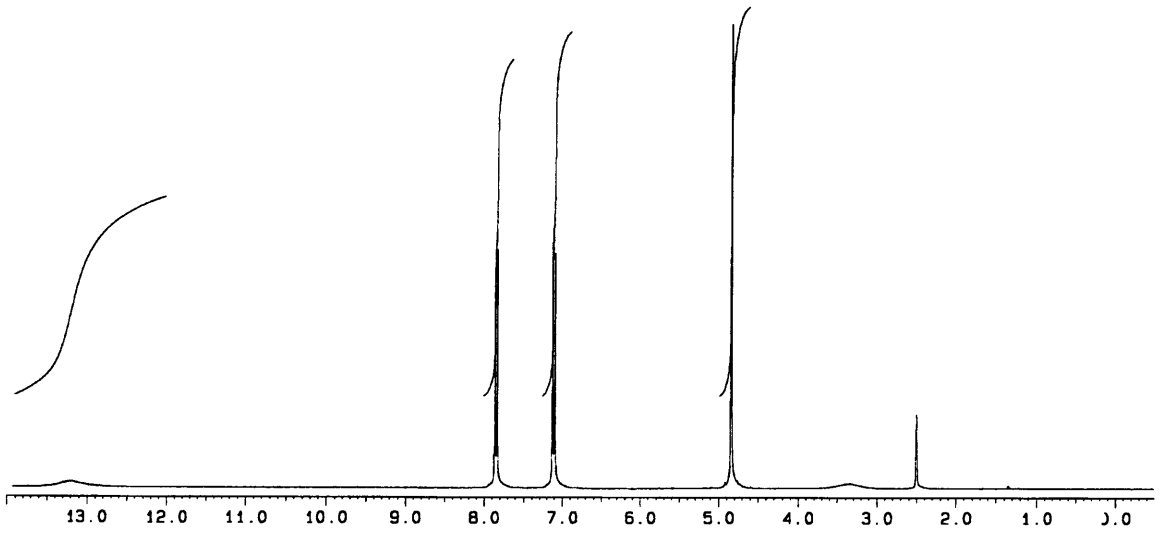
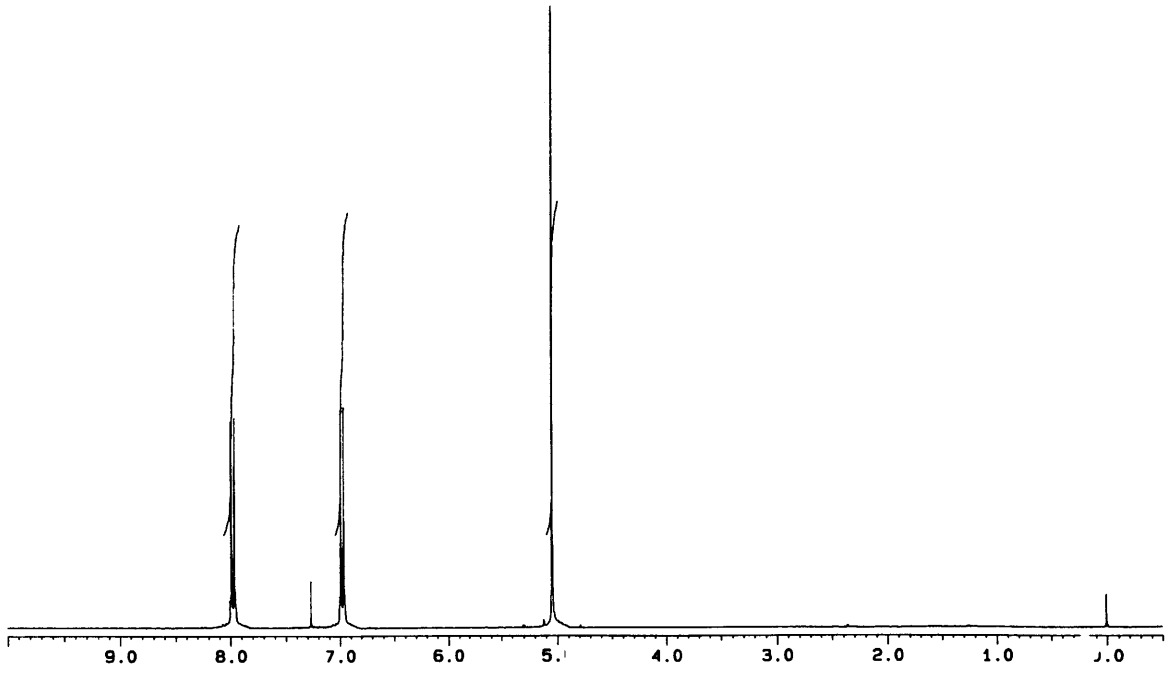
4,4'-Bis(methoxycarboxylic acid) benzil (25).

Diester **18** (12.0 g, 29.0 mmol) was dissolved in 250 mL THF. To this solution was added a solution of lithium hydroxide monohydrate (2.92 g, 70.0 mmol) in 50 mL water. The resulting biphasic solution was vigorously stirred for 15 h. After this period, solids had developed. The slurry was poured into 1400 mL 1M HCl and bright yellow solids formed. This material was removed by filtration and dried at 50 °C overnight, yielding **25** as a yellow powder (9.99 g, 96%). m.p. 225–227 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.22 (br. s, 2H), 7.86 (d, J = 9.0 Hz, 4H), 7.11 (d, J = 9.0 Hz, 4H), 4.85 (s, 4H). ^{13}C NMR (151 MHz, DMSO–d_6): δ 193.85, 169.83, 163.56, 132.22, 125.98, 115.53, 64.76. FT-IR (neat) 2913.5, 1748.0, 1709.9, 1663.0, 1601.5, 1577.1, 1507.4, 1419.7, 1312.9, 1252.6, 1171.7, 882.9, 846.4 cm^{-1}. HRMS (FAB in NBA/NaI) m/z calcd for $[C_{18}H_{15}O_8 + H]^+$ 359.0767 found 359.0756.



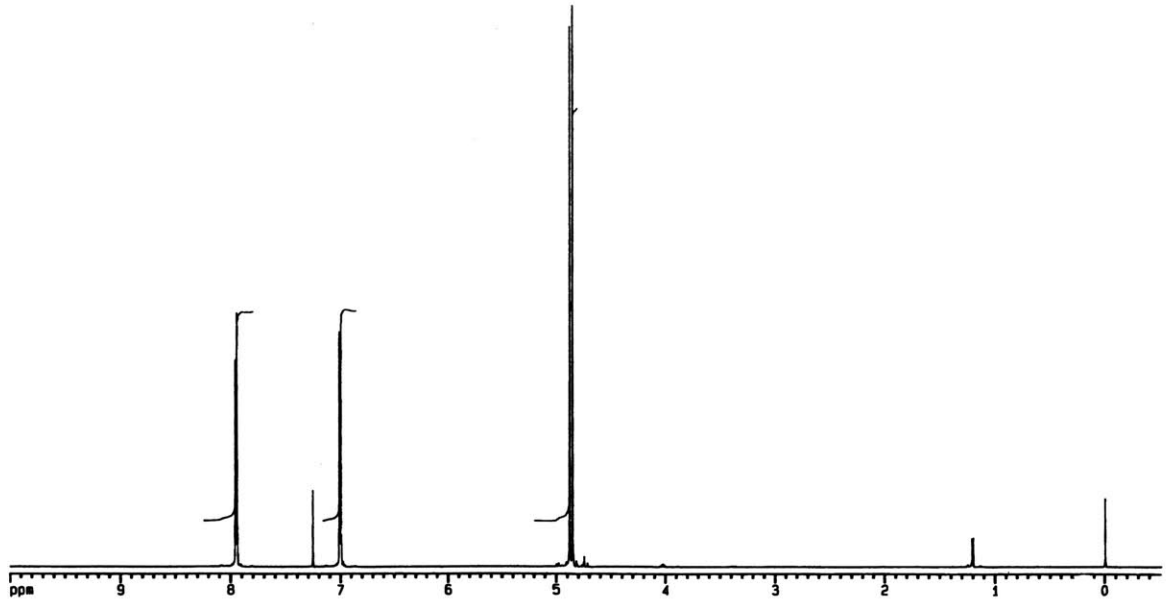
4,4'-Bis(methoxy carboxylic acid chloride) benzil (26).

Diacid **25** (6.00 g, 16.7 mmol) and oxalyl chloride (5.8 mL, 8.4 g, 66 mmol) were stirred in 240 mL methylene chloride. To the stirred suspension was added 8 drops of a 4% solution of DMF in methylene chloride. The mixture was refluxed for 9 h. The resulting solution was filtered through Celite and the solvent was removed by rotary evaportation. The residue was taken up in toluene and the solvent was again removed. Drying under high vacuum for 2 h yielded **26** as a yellow powder (6.18 g, 93%). m.p. 99–102 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.99 (d, J = 8.8 Hz, 4H), 6.99 (d, J = 8.7 Hz, 4H), 5.05 (s, 4H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 193.06, 169.68, 162.01, 132.84, 128.02, 115.18, 72.08. FT-IR (neat) 2908.6, 2359.2, 1804.6, 1667.4, 1597.6, 1578.5, 1508.6, 1418.0, 1309.7, 1269.6, 1222.6, 1164.6, 945.8, 884.7, 840.8, 766.9, 736.8 cm^{-1}. Sample too labile for mass spectral analysis.



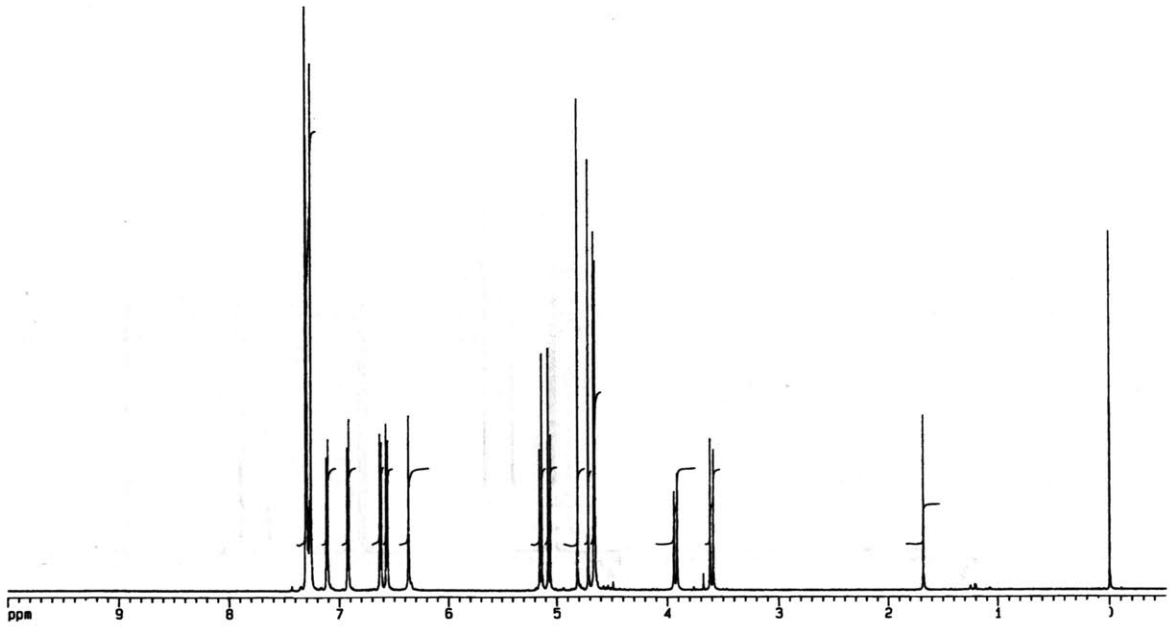
4,4'-Bis[(2,2,2-trichloroethyl)methoxycarboxylate] benzil (27).

To a chilled (0 °C) solution of diacid chloride **26** (5.00 g, 12.7 mmol) in 150 mL methylene chloride was added a solution of 2,2,2-trichloroethanol (2.7 mL, 4.2 g, 28 mmol) and triethylamine (7.1 mL, 5.2 g, 51 mmol) in 100 mL methylene chloride. The resulting solution was stirred under nitrogen, slowly warming to room temperature over the course of 14 h. The solution was then diluted with more methylene chloride and was washed thrice with HCl and once with brine. The organic phase was dried over magnesium sulfate, filtered, and the solvent was removed by rotary evaporation. Sonication of the resulting amber oil in 1:1 ether/hexane yielded, upon filtration, **27** (6.67 g, 85%) as a pale yellow powder. m.p. 101–103 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.95 (d, J = 8.5 Hz, 4H), 7.01 (d, J = 8.8 Hz, 4H), 4.89 (s, 4H), 4.86 (s, 4H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 193.33, 166.94, 162.85, 132.71, 127.55, 115.16, 94.44, 74.36, 64.79. HRMS (FAB in NBA/CsI) m/z calcd for $[C_{22}H_{16}Cl_6O_8 + Cs]^+$ 750.8031, found 750.8001. FT-IR (neat) 2958.7, 1775.1, 1770.8, 1667.7, 1598.6, 1509.1, 1276.0, 1224.2, 1159.5, 1071.1, 728.7 cm^{-1}.



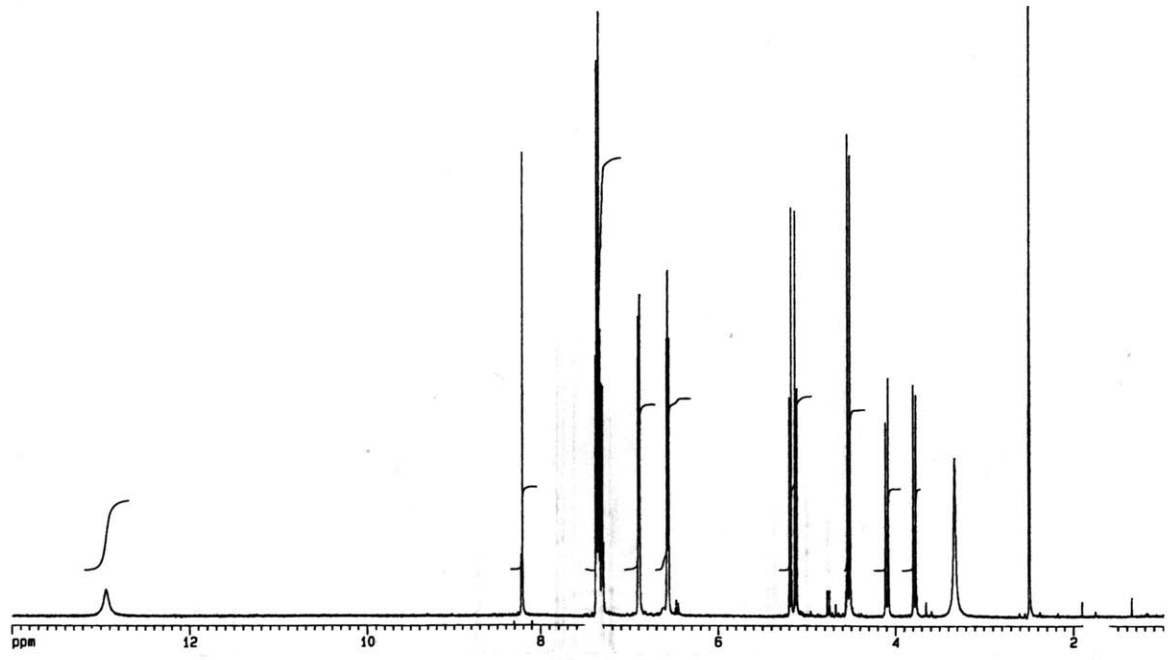
Bis(trichloroethyl ester)/bis(benzyl ester) glycoluril (**28**).

Diketone **27** (7.00 g, 11.3 mmol) and benzyl hydantoate **10** (7.03 g, 33.8 mmol) were stirred in 120 mL benzene. The addition of 6 mL trifluoroacetic acid caused all solids to dissolve. The solution was refluxed under a Dean-Stark trap for 46 h. The hydantoin byproduct was removed from the raw reaction mixture by filtration and the solvent and trifluoroacetic acid was removed by rotary evaporation. Purification of the yellow oil by silica gel chromatography (40:1 methylene chloride/methanol) yielded **28** as a white powder (3.98 g, 35%). m.p. 141–144 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.35–7.29 (m, 10H), 7.13 (d, J = 8.8 Hz, 2H), 6.95 (d, J = 8.9 Hz, 2H), 6.65 (d, J = 9.0 Hz, 2H), 6.59 (d, J = 8.9 Hz, 2H), 6.09 (s, 2H), 5.19 (d, J = 12.0 Hz, 2H), 5.11 (d, J = 12.1 Hz, 2H), 4.84 (s, 2H), 4.76 (s, 2H), 4.69 (s, 4H), 3.96 (d, J = 17.5 Hz, 2H), 3.64 (d, J = 17.5 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 169.11, 167.64, 167.37, 159.55, 158.37, 158.02, 135.37, 130.00, 129.89, 129.07, 128.91 (2 peaks), 128.78, 125.51, 115.02, 114.43, 94.63, 94.59, 89.21, 80.84, 74.22, 74.16, 67.67 (2 peaks), 64.70, 64.46, 43.12. HRMS (FAB , NBA/CsI) m/z calcd for $[C_{42}H_{36}Cl_6N_4O_{12}$ + Cs]$^+$ 1132.9486 found 1132.9428. FT-IR (neat) 3366.4, 3255.2, 1735.0, 1609.9, 1512.4, 1457.4, 1175.9, 729.3 cm^{-1}.



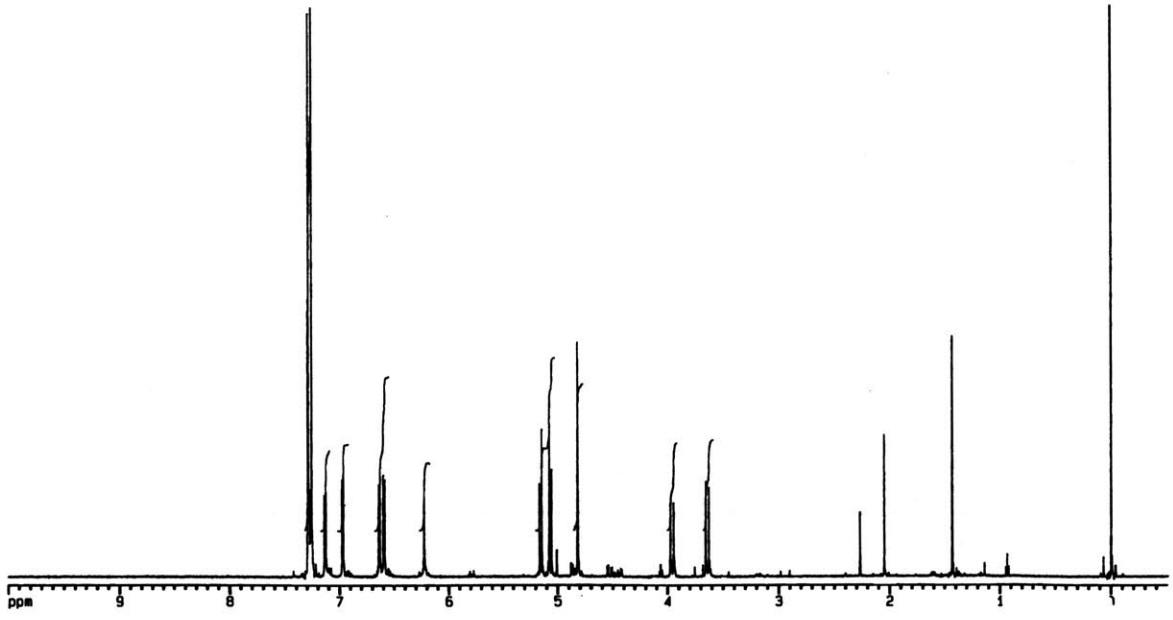
Bis(benzyl ester) diacid glycoluril (29).

Glycoluril **28** (1.33 g, 1.33 mmol) was dissolved in 40 mL acetic acid, 9 mL THF, and 4.5 mL water. As the solution was stirred in an ice bath at 0 °C, zinc dust (0.67 g, 10.2 mmol) was added. The suspension was stirred at 0 °C for 2.5 h, then the zinc was removed by filtration. The filtrate was poured into 300 mL water. A white precipitate developed and was isolated by filtration after the mixture was chilled in a refrigerator overnight. The filter cake was dried under high vacuum, yielding **29** as a white powder (0.83 g, 85%). m.p. 235–237 °C (dec). ^{1}H NMR (600 MHz, DMSO-d_6): δ 12.95 (br. s, 2H), 8.23 (s, 2H), 7.40–7.31 (m, 10H), 6.91 (d, J = 9.0 Hz, 2H), 6.90 (d, J = 9.0 Hz, 2H), 6.59 (d, J = 8.8 Hz, 2H), 6.58 (d, J = 8.8 Hz, 2H), 5.20 (d, J = 12.6 Hz, 2H), 5.13 (d, J = 12.6 Hz, 2H), 4.55 (s, 2H), 4.52 (s, 2H), 4.11 (d, J = 17.7 Hz, 2H), 3.79 (d, J = 17.7 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 170.20, 170.17, 169.65, 159.28, 158.04, 157.75, 136.08, 129.98, 129.36, 128.60 (overlapping peaks), 128.20, 128.04 (two peaks), 127.53, 125.93, 114.22, 113.49, 88.24, 80.02, 66.04, 64.45, 42.95. HRMS (FAB in NBA/CsI) m/z calcd for $[C_{38}H_{34}N_4O_{12} + Cs]^+$ 871.1228 found 871.1265. FT-IR (neat) 1717.2, 1511.4, 1472.5, 1213.5, 1181.2, 1071.7, 836.1, 699.4 cm^{-1}.



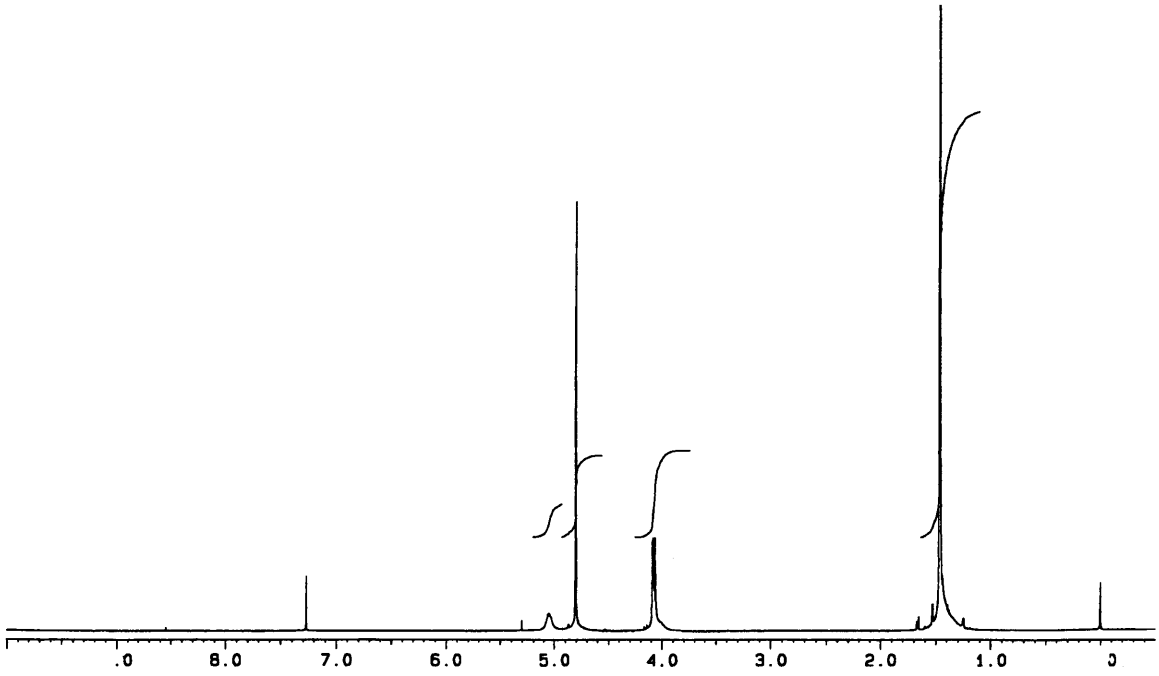
Glycoluril bis(benzyl ester)/bis(pentafluorophenyl ester) (30).

Glycoluril diacid **29** (148 mg, 0.200 mmol) and pentafluorophenol (155 mg, 0.842 mmol) were dissolved in 18 mL THF and 2 mL DMF. To this stirred solution were added EDC•MeI (256 mg, 0.861 mmol) and DMAP (cat.). After stirring under nitrogen at RT for 17 h the solvent was removed by rotary evaporation. The resulting oil was taken up in EtOAc and was washed with water (2x) and brine (1x). The organic phase was dried over $MgSO_4$, filtered, and concentrated to a pale yellow oil *in vacuo*. The oil was purified by silica gel column chromatography (1:1 hexane/EtOAc). A clear oil crystallized upon sitting to give **30** (59 mg, 28%) as colorless crystals. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.30–7.27 (m, 10H), 7.13 (d, J = 8.9 Hz, 2H), 6.97 (d, J = 8.9 Hz, 2H), 6.64 (d, J = 8.9 Hz, 2H), 6.59 (d, J = 8.9 Hz, 2H), 6.23 (s, 2H), 5.15 (d, J = 12.0 Hz, 2H), 5.07 (d, J = 12.0 Hz, 2H), 4.83 (s, 2H), 4.82 (s, 2H), 3.96 (d, J = 17.5 Hz, 2H), 3.64 (d, J = 17.6 Hz, 2H). HRMS (FAB, NBA/CsI) m/z calcd for $[C_{50}H_{32}F_{10}N_4O_{12} + Cs]^+$ 1203.0911 found 1203.0961.



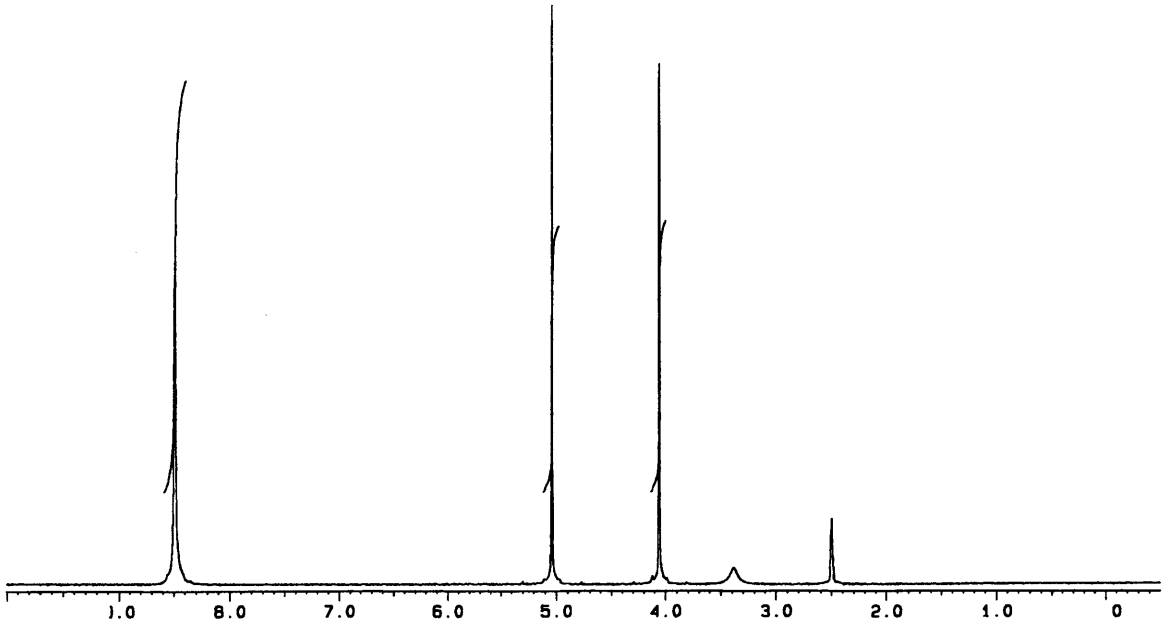
N-α-Boc-L-glycine-α-2,2,2-trichloroethyl ester (32).

N-Boc-glycine-*p*-nitrophenyl ester (15.39 g, 51.9 mmol) (NovaBiochem) and 2,2,2-trichloroethanol (7.5 mL, 11.7 g, 77.9 mmol) were dissolved in 200 mL dichloromethane. Upon the addition of triethylamine (11 mL, 8.0 g, 79 mmol) a deep yellow color formed, and the solution was stirred for 15 hours. The solution was then extracted with saturated sodium bicarbonate solution until the yellow color disappeared. The organic phase was dried over magnesium sulfate and filtered, then the solvent was removed by rotary evaporation. Upon drying the resulting oil by exposure to high vacuum, **32** (14.14 g, 89%) was isolated as an off white solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.11 (br. t, 1H), 4.80 (s, 2H), 4.07 (d, J = 5.8 Hz, 2H), 1.46 (s, 9H). LRMS (FAB, NBA/NaI) m/z calcd for $[C_9H_{14}Cl_3NO_4 + H]^+$ 306/308, $[C_9H_{14}Cl_3NO_4 + Na]^+$ 328/330 found 306/308, 328/330.



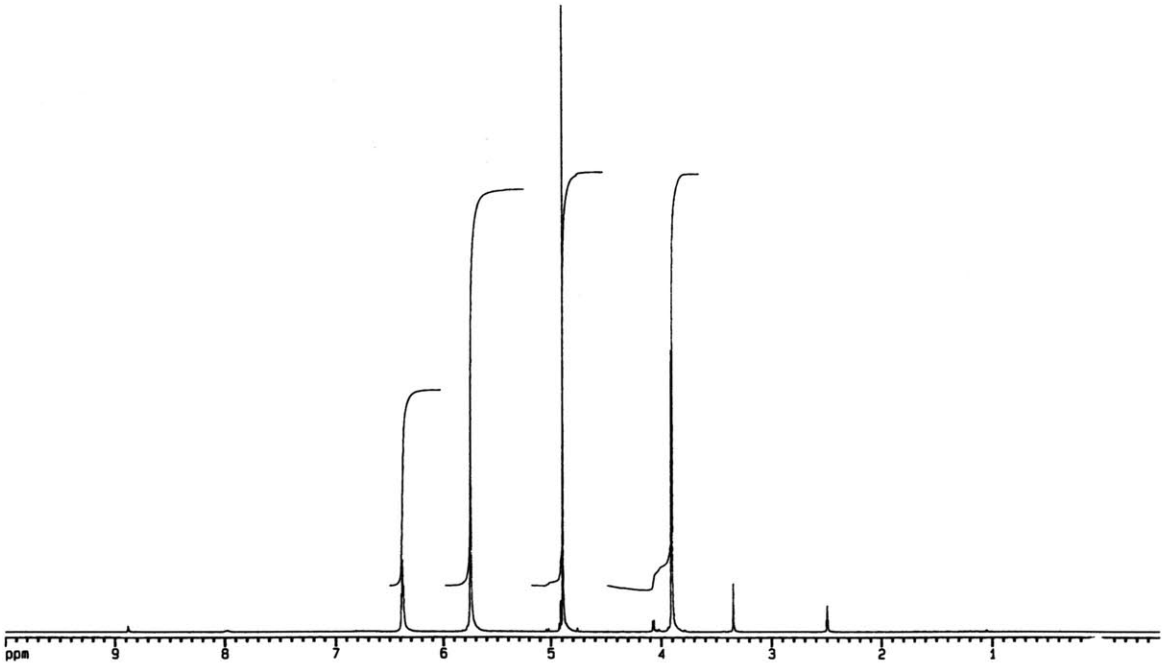
Glycine-α-2,2,2-trichloroethyl ester, trifluoroacetic acid adduct (**33**).

Compound **32** (14.14 g, 46.13 mmol) was dissolved in a solution of 50 mL trifluoroacetic acid and 50 mL dichloromethane and the solution was stirred for 40 minutes. The solvent was removed by rotary evaporation and the resulting oil was crystallized from cold ether/hexane, yielding **33** (13.36 g, 90%) as colorless crystals. m.p. 85–86 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.50 (br. s, 3H), 4.83 (s, 2H), 4.04 (s, 2H).



2,2,2-Trichloroethyl-glycylurea (**34**).

A solution of **33** (10.00 g, 31.2 mmol) in 25 mL ethanol was added to a solution of potassium cyanate (2.61 g, 32.2 mmol) in 50 mL water. The solution was stirred at room temperature for 16 hours. White solids formed after 2 hours. Most of the ethanol was removed by rotary evaporation; at the elevated temperatures of the water bath the solids redissolved. Upon cooling in a refrigerator the compound again crystallized. Isolation of this material by filtration followed by rinsing with water and drying at elevated temperatures yielded **34** (6.68 g, 86%) as white, mica-like crystals. m.p. 91–93 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 6.40 (t, J = 5.8 Hz, 1H), 5.76 (s, 2H), 4.89 (s, 2H), 3.91 (d, J = 6.2 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 170.41, 158.83, 95.33, 73.36, 41.22. HRMS (FAB, NBA/NaI) m/z calcd for $[C_5H_7Cl_3N_2O_3 + H]^+$ 250.9517 found 250.9579. FT-IR (neat) 3445.4, 3397.1, 3222.5, 2360.2, 2341.5, 1756.9, 1654.7, 1618.2, 1526.9, 1380.4, 1341.3, 1270.1, 1167.1, 877.2, 800.8, 720.3, 668.0 cm^{-1}.



Chapter 6. Reduced Amide Linkers[1]

6.1 Introduction

Since secondary amides are notorious for poor bioavailability, the conversion of the multiple amides found in, for instance, the tetra-substituted bipyridine libraries into simple methylamino groups should make any active compounds identified from such libraries more attractive as potential starting points for medicinal chemistry. Accordingly, we undertook a project to develop methods to use reduced amides as linkers between the core molecule and the building blocks.

Broadly, there are two methods for producing the linkers under study: starting from a core substituted with methyl alcohols, the Mitsunobu reaction[2] can be used, and with a polyaldehyde core reductive amination reactions[3] can be used (see Figure 1).

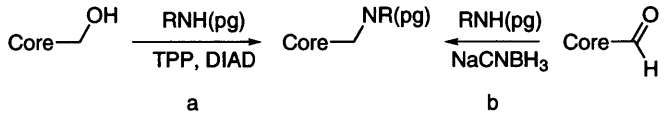


Figure 1. Two routes to reduced amides: a. Mitsunobu reaction, b. reductive amination.

Since partial reductions of carboxylic acids and acid derivatives to aldehydes tend to be rather sensitive and prone to overreduction, and given that our core molecules all contain multiple acids, we decided that the greatest likelihood for success in isolating polyaldehydes lay instead in the full reduction of the acids to alcohols followed by partial oxidation back up to the aldehydes. Given that, we decided to focus initial efforts on the Mitsunobu reaction since the requisite polyalcohols would be isolated as intermediates along the path to the polyaldehydes anyway.

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[1] Technical assistance was provided by Ellen Choi and Christina Cueto.

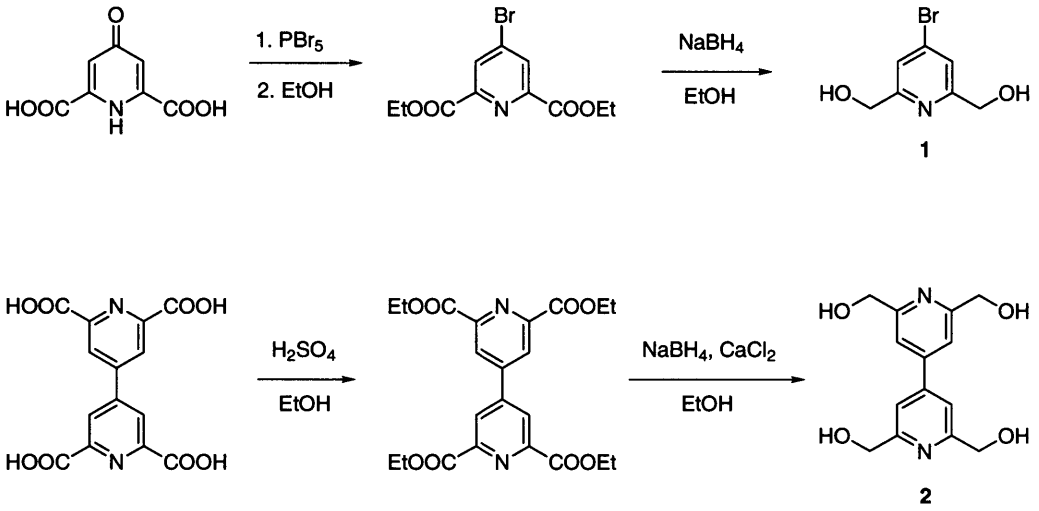
[2] (a) Mitsunobu, O.; Wada, M.; Sano, T. *J. Am. Chem. Soc.* **1972**, *94*, 679. (b) Mitsunobu, O. *Synthesis* **1981**, 1.

[3] (a) Borch, R. F. *Org. Synth.* **1972**, *52*, 124. (b) Mattson, R. J.; Pham, K. M.; Leuck, D. J.; Cowen, K. A. *J. Org. Chem.* **1990**, *55*, 2552.

The bipyridine core molecule was chosen as the initial target for development due to the interest expressed by Novartis AG in it. Concurrently with the full core molecule, the 4-bromopyridine-2,6-disubstituted half-core for deconvolution was investigated, as well.

6.2 Synthesis of core polyalcohols

Reduction of core polyesters[4] to polyalcohols was effected by either sodium borohydride or a combination of sodium borohydride and calcium chloride, which is thought to form the more strongly-reducing species calcium borohydride *in situ*.[5] Subsequent treatment with aqueous base followed by solid-phase extraction provided the core polyalcohols (Scheme 1).



Scheme 1. Reduction of polyesters yields polyalcohols **1**[6] and **2** for use as core molecules in Mitsunobu amination reactions.

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[4] For the bromopyridine diester, see Chapter 3, compound **9**. The bipyridine tetraester was synthesized according to Hünig, S.; Wehner, I. *Synthesis* **1989**, 552–554.

[5] (a) Narasimhan, S.; Prasad, K.; Ganeshwar; Madhavan, S. *Synth. Commun.* **1995**, *25*, 1689–1697. (b) Brown, H. C.; Narasimhan, S.; Choi, Y. M. *J. Org. Chem.* **1982**, *47*, 4702–4708. (c) Xinyan, W.; Yongbin, H.; Chengtai, W. *Wuhan Univ. J. Nat. Sci.* **1996**, *1*, 105–106.

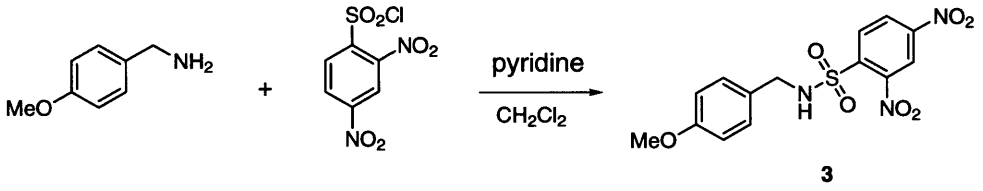
[6] Takalo, H.; Pasanen, P.; Kankare, J. *Acta Chem. Scand., Ser. B* **1988**, *42*, 373–377.

The yields of the reductions were fair to poor. The bromopyridine diester **1** was reduced in 68% yield, consistent with literature precedent for that reaction.[6] A test reaction on the unsubstituted pyridine diethyl ester using the more powerful conditions incorporating calcium chloride proceeded in 75% yield, providing hope that the bipyridine tetraester would react similarly. However, the yield of tetraalcohol **2** was disappointingly (and consistently) low (21–22%) in initial attempts. It is hoped that this yield can be improved by further refinement of the reaction and workup conditions.

6.3 Mitsunobu reactions with 2,4-dinitrobenzenesulfonamides

Synthesis of 2,4-dinitrobenzenesulfonamides

A method for protecting the amine building blocks reported by Fukuyama, *et al.*, as 2,4-dinitrobenzenesulfonamides[7] was successfully implemented to serve a two-fold purpose: to increase the acidity of the building block by conversion from an amine to a sulfonamide, thereby making it a better partner in the Mitsunobu reaction, and to remove the possibility that a second reaction might occur after the primary amine was alkylated to a secondary amine. The protecting group contains two nitro groups which help the sulfonamide products to crystallize, aiding workup. This method is outlined in Scheme 2.



Scheme 2. Amines are protected as 2,4-dinitrobenzenesulfonamides for the Mitsunobu reaction.

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[7] Fukuyama, T.; Cheung, M.; Jow, C.-K.; Hidai, Y.; Kan, T. *Tetrahedron Lett.* **1997**, *38*, 5831–5834.

In order to test the generality of this protection scheme, several other dinitrobenznesulfonamides were also synthesized. These are shown below in Figure 2. The syntheses, in most cases, were essentially the same as that for sulfonamide **3**. In some cases where there was a basic nitrogen elsewhere in the compound, however, a slightly modified workup was applied. This consisted of isolating the corresponding ammonium salt, followed by freebasing the amine in a second step.

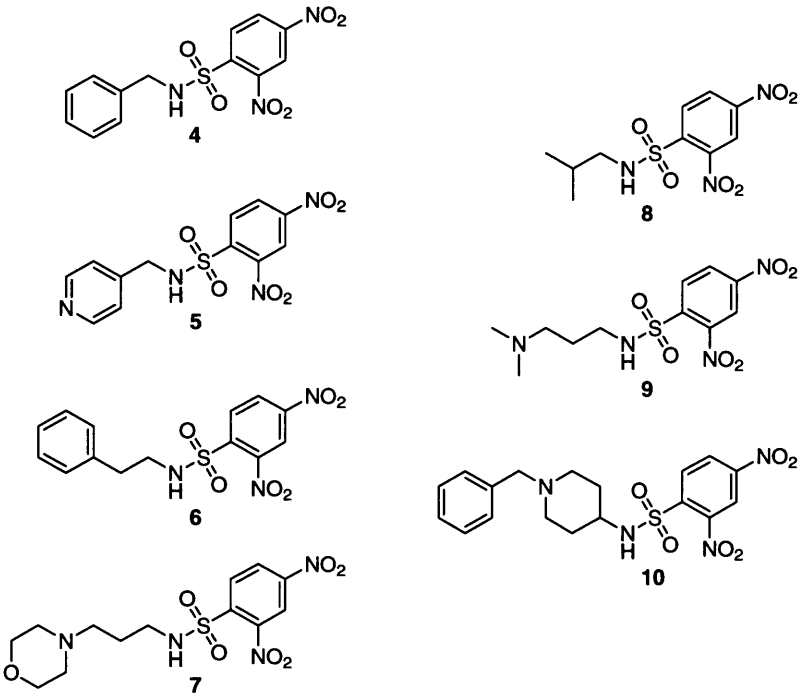
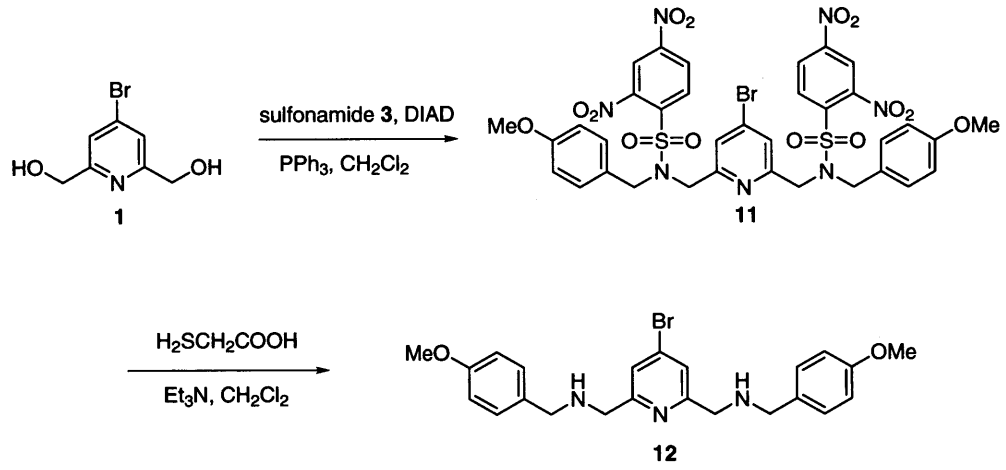


Figure 2. A series of 2,4-dinitrobenznesulfonamides.

The Mitsunobu reaction

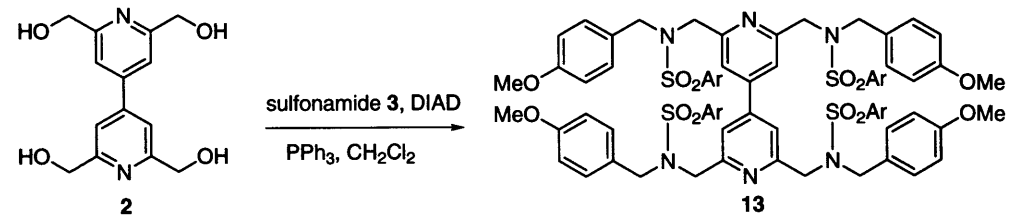
The Mitsunobu reaction itself proceeded very cleanly and in high yields under standard conditions with the bromo-substituted dialcohol **1** and (*p*-methoxybenzyl)-2,4-dinitrobenzenesulfonamide **3**. After removal of solvent from the crude reaction mixture, the residue was sonicated briefly in diethyl ether and the pure product **11** precipitated in 87% isolated yield. Deprotection of the two sulfonamides with the water soluble nucleophile

mercaptoacetic acid proceeded in approximately quantitative yield and the final diamine **12** was purified by extraction of the organic reaction mixture with aqueous base. (See Scheme 3).



Scheme 3. Test Mitsunobu reaction and deprotection with mercaptoacetic acid.

The Mitsunobu reaction with bipyridine tetraalcohol **2** (Scheme 4) was somewhat disappointing when compared to the case of the bromodialcohol. The reaction proceeded in 42% yield and was not entirely pure upon precipitation. It is likely that the poor solubility of the tetraalcohol in methylene chloride is to blame—the bromodialcohol reaction became optically clear very rapidly upon addition of DIAD to the reaction mixture, while the tetraalcohol reaction remained very hazy even after 24 hours. The insoluble material removed at the end of the reaction constituted a significant fraction of the original starting material.

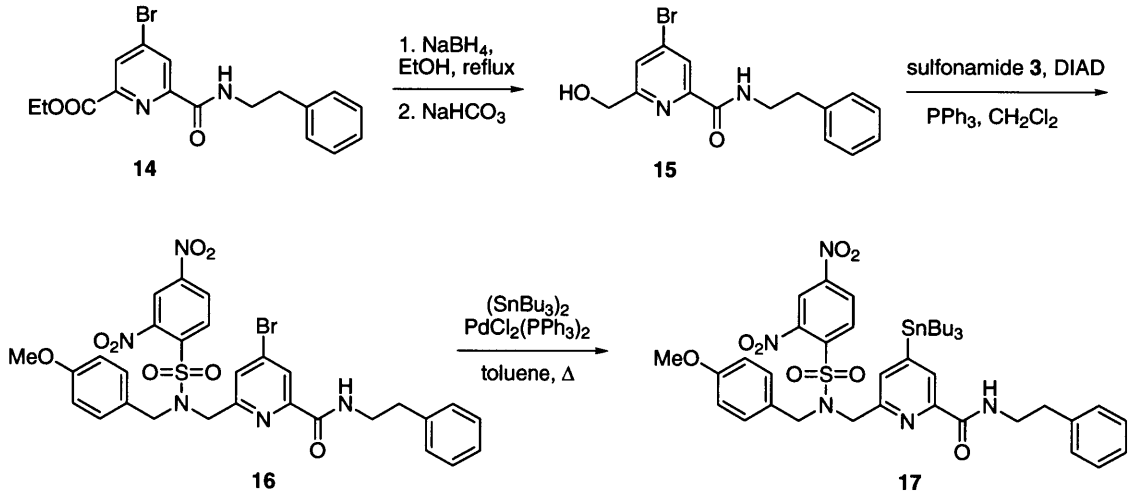


Scheme 4. Test Mitsunobu reaction with the bipyridine tetraalcohol core.

6.4 Mixed amide/amine linkers

Synthesis of a mixed amide/amine stannane

In order to expand the types of compounds that can be produced on the bipyridine core, several test reactions were run to install both amides and amines into a single bipyridine compound. The first step was to demonstrate the feasibility of synthesizing a stannylpyridine incorporating one amide and one protected amine. The synthesis of mixed compound **17** is shown below in Scheme 5.



Scheme 5. Synthesis of a mixed amide/protected amine stannylpyridine.

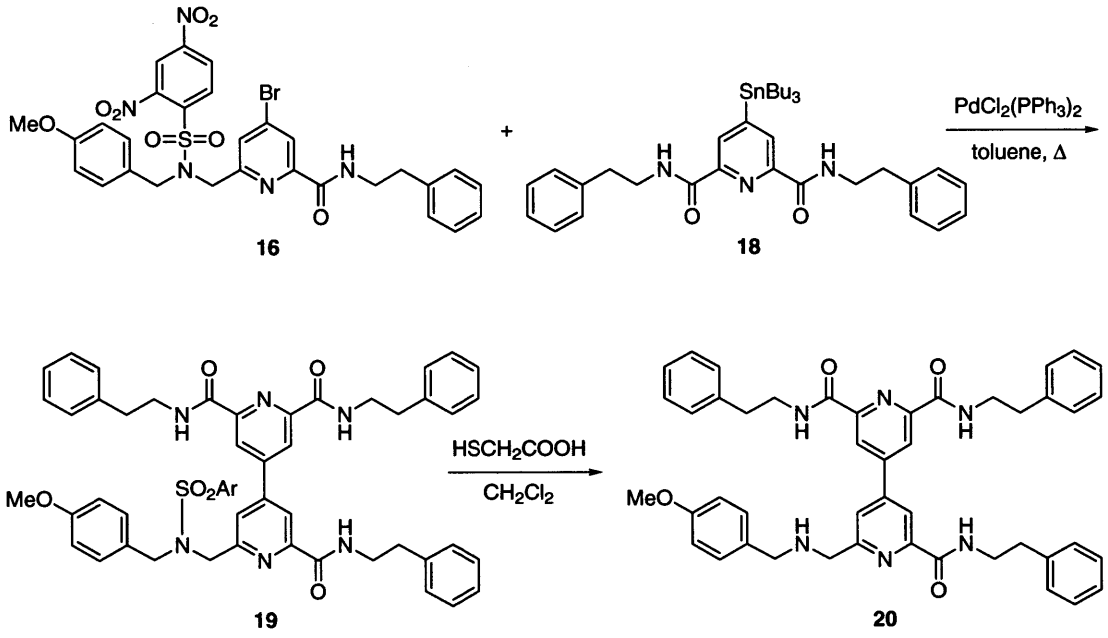
Starting from mixed ester/amide **14** (see Chapter 4), selective reduction of the ester was accomplished with $NaBH_4$ in 62% yield. The resulting monoalcohol **15** was subjected to a Mitsunobu reaction with sulfonamide **3**, affording bromopyridine **16** in 46% yield. This, in turn, was converted into the stannylpyridine **17** with bis(tributyl tin) and catalytic palladium in hot toluene.

Surprisingly, the final step of the reaction sequence went in unusually low yield—10%. A large amount of starting material (37%) was recovered from the reaction

mixture after workup, implying that perhaps higher temperatures might be used to accelerate the reaction and more efficiently accomplish the transformation.

Synthesis of a mixed amide/amine bipyridine

Our next step was to synthesize a complete bipyridine compound containing both amides and amines. Since so little of stannane **17** was isolated, bromopyridine **16** was reacted with stannylpyridine diamide **18** in a first attempt to provide bipyridine **19** (Scheme 6). The adduct was then deprotected with mercaptoacetic acid to provide the final triamide/monoamine **20**, which, unfortunately, was not obtained in high purity.

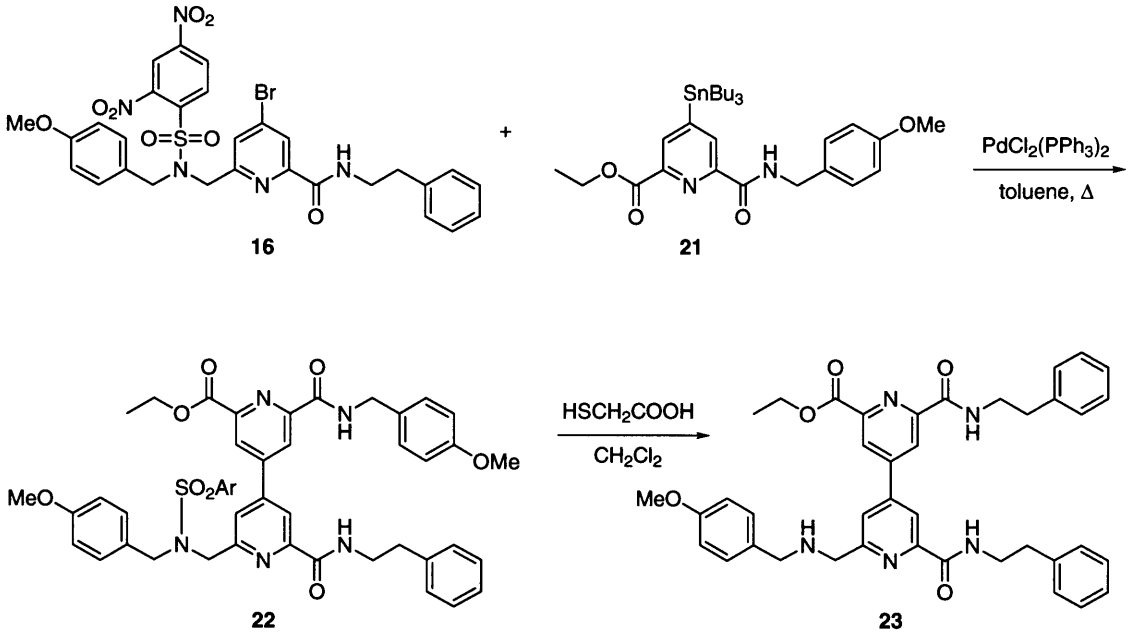


Scheme 6. Synthesis of mixed amide/amine bipyridine **20**.

Synthesis of a bipyridine with three types of substituents

In a further extension of chemistry utilizing the bipyridine core, we undertook the synthesis of the mixed amide/amine/ester **23** (Scheme 7). Compounds in this class would be interesting to screen for PAI-1 antagonism due to their similarity to the triamide/monoacid antagonists already identified (see Chapter 4). Bromopyridine **16** was used again, this time

being coupled to monoester/monoamide stannane **21**, although in vanishingly low yield. The small portion of adduct **22** was deprotected with mercaptoacetic acid as usual to yield the final deprotected amine **23**. Unfortunately, initial attempts to purify the final product by preparative TLC were insufficient to clean the final product completely, but we are confident that other means could be found to do so in future syntheses.



Scheme 7. Synthesis of mixed amide/amine/ester bipyridine **23**.

6.5 Experimental

General

All reagents were purchased from Aldrich Chemical Company and were used without further purification except as noted. Amino acid esters, PyBOP, and EDC•MeI were acquired from Novabiochem (San Diego, CA). Deuterated solvents were obtained from Cambridge Isotopes Laboratories and deuterated chloroform was dried over 4Å molecular sieves. HCl refers to a 1N stock solution. NMR spectra were recorded on either a Bruker AC-250, a Bruker AM-300, or a Bruker DRX-600; TMS was used as a reference in some

chloroform-*d* spectra, otherwise residual solvent was used as a reference. Either a Finnegan Mat 8200 (for EI) or a VG ZAB-VSE (for FAB) mass spectrometer was used to ascertain masses. FT-IR spectra were obtained on a Perkin Elmer Paragon 1000 PC FT-IR Spectrometer. Silica gel chromatography was performed with Silica Gel 60 (EM Science or Bodman, 230–400 mesh). TLC analysis was performed using glass-bound Silica Gel 60 (F254) plates. DMAP = *N,N*-dimethyl-4-aminopyridine, DIAD = diisopropyl azodicarboxylate, DPPA = diphenylphosphoryl azide, EDC•MeI = 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide methiodide, PyBOP = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate, TPP = triphenylphosphine.

General preparation of 2,4-dinitrobenenesulfonamides

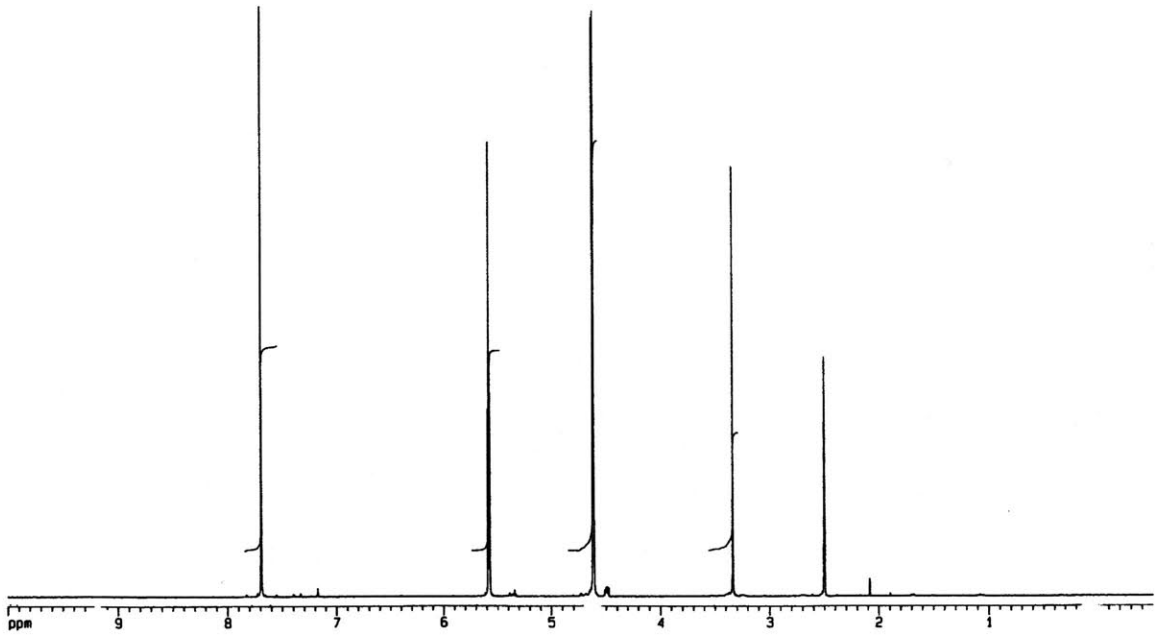
To a 0.2 M methylene chloride solution of 2,4-dinitrobenzenesulfonyl chloride (1 eq) was added amine (1.1 eq) and pyridine (1.2 eq). The mixture was stirred under nitrogen for 2 h. The solution was washed with 0.5 M HCl (3 x), saturated $NaHCO_3$ solution (3 x), and brine (1 x). The organic layer was dried over magnesium sulfate and was filtered. In some cases the product was crystallized by cooling or by the addition of cosolvents such as hexane or ether. In other cases, silica gel column chromatography or prep-TLC was used to purify the final product.

4-Bromo-2,6-pyridinedimethanol (1).

Synthesized according to ref. 6., Takalo, H.; Pasanen, P.; Kankare, J. *Acta Chem. Scand., Ser. B* **1988**, *42*, 373–377.

2,2',6,6'-(4,4'-Bipyridine)tetramethanol (2).

To a stirred solution of 2,2',6,6'-(4,4'-bipyridine)tetrakis(ethyl ester) (1.03 g, 2.32 mmol) in 30 mL ethanol was added $NaBH_4$ (210 mg, 5.56 mmol) in several portions over 2 min. A deep blue color developed. Next was added powdered $CaCl_2$ (617 mg, 5.56 mmol) portionwise over 2 min, and the color changed from blue to violet. The mixture was stirred at RT for 18 h. The solvent was removed by rotary evaporation and the violet residue was taken up in saturated K_2CO_3 solution and was briefly boiled. The violet color changed to a pale yellow. The sample was freeze-dried and the resulting foam was pulverized and continuously extracted with acetone for 48 h. Upon cooling of the acetone extract, tetraalcohol **2** (139 mg, 22%) precipitated as an off-white powder. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.69 (s, 4H), 5.58 (d, J = 5.8 Hz, 4H), 4.62 (d, J = 5.8 Hz, 8H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 162.43, 146.17, 115.36, 64.11. HRMS (FAB, NBA/CsI) m/z calcd for $[C_{14}H_{16}N_2O_4 + H]^+$ 277.1188 found 277.1186.

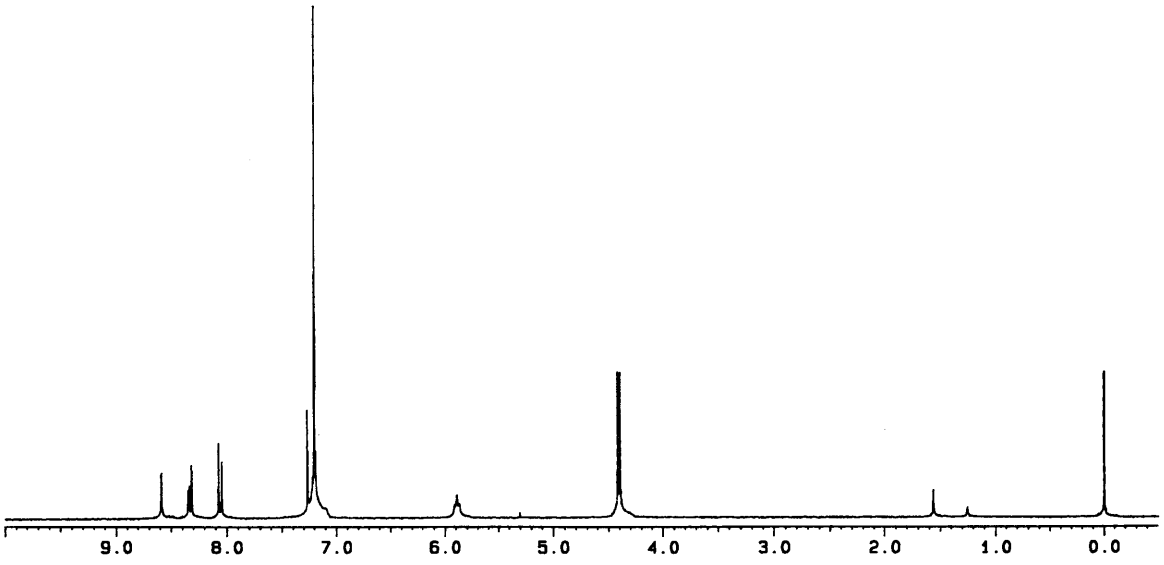


(*p*-Methoxybenzyl)-2,4-dinitrobenznesulfonamide (3).

Synthesized according to ref. 7, Fukuyama, T.; Cheung, M.; Jow, C.-K.; Hidai, Y.; Kan, T. *Tetrahedron Lett.* **1997**, *38*, 5831–5834.

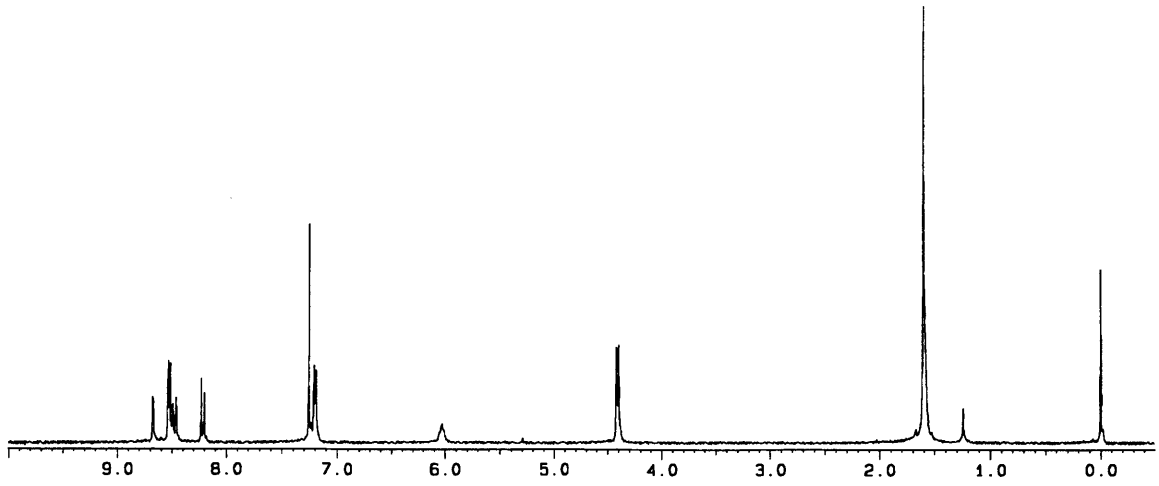
Benzyl-2,4-dinitrobenznesulfonamide (4).

Prepared according to general procedure, 52%.^{1}H NMR (300 MHz, $CDCl_3$): δ 8.59 (d, J = 2.2 Hz, 1H), 8.33 (dd, J = 8.5, 2.2 Hz, 1H), 8.06 (d, J = 8.5 Hz, 1H), 7.20 (br. s, 5H), 5.89 (t, J = 6.1 Hz, 1H), 4.40 (d, J = 6.4 Hz, 2H). LRMS (ESI +) m/z calcd for $[C_{13}H_{11}N_3O_6S + Na]^+$ 360 found 360.



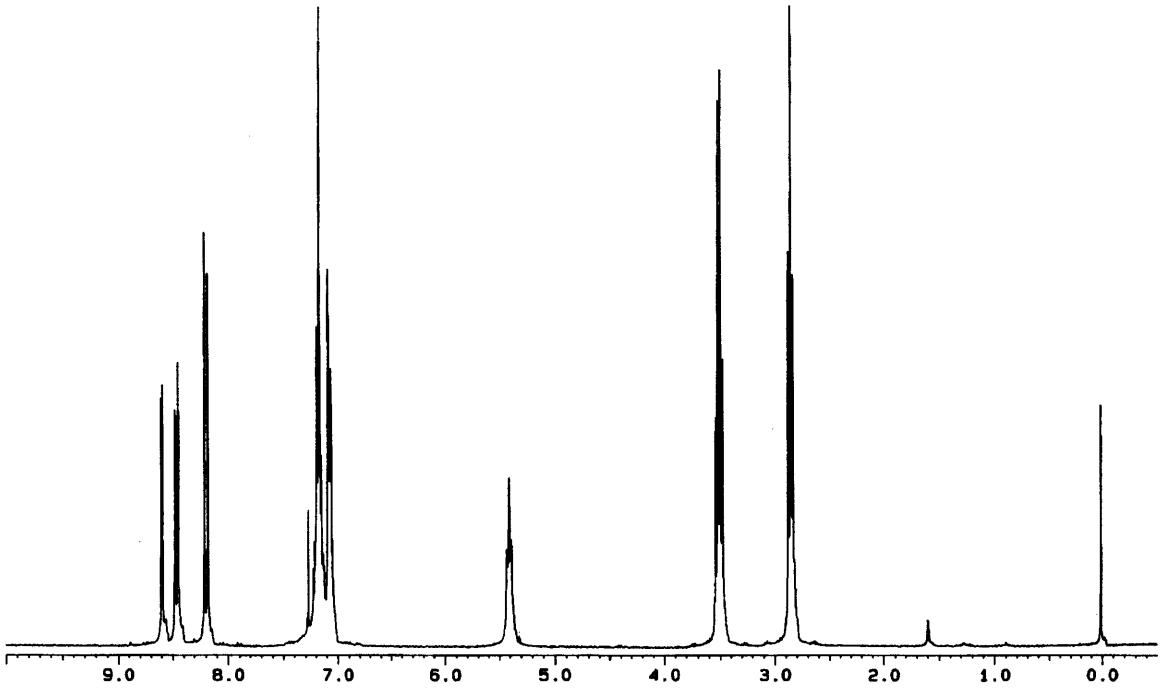
(4-Pyridylmethyl)-2,4-dinitrobenznesulfonamide (5).

Prepared according to general procedure, except that the corresponding ammonium ion was isolated by filtration from the biphasic solution following the acid wash. In a second step, the ammonium ion was shaken with chloroform and saturated $NaHCO_3$ solution and the final product was isolated after removal of solvent by rotary evaporation, 10%. Even after this treatment, a considerable amount of product material remained out of the chloroform solution, and the crude residue constituted another 41% of theoretical. ^1H NMR (300 MHz, $CDCl_3$): δ 8.67 (d, J = 2.2 Hz, 1H) 8.52 (d, J = 5.5 Hz, 2H), 8.47 (dd, J = 8.5, 2.2 Hz, 1H), 8.21 (d, J = 8.5 Hz, 1H), 7.20 (d, J = 5.6 Hz, 2H), 6.03 (br. t, 1H), 4.41 (d, J = 6.3 Hz, 2H). LRMS (ESI +) m/z calcd for $[C_{12}H_{10}N_4O_6S + H]^+$ 339 found 339.



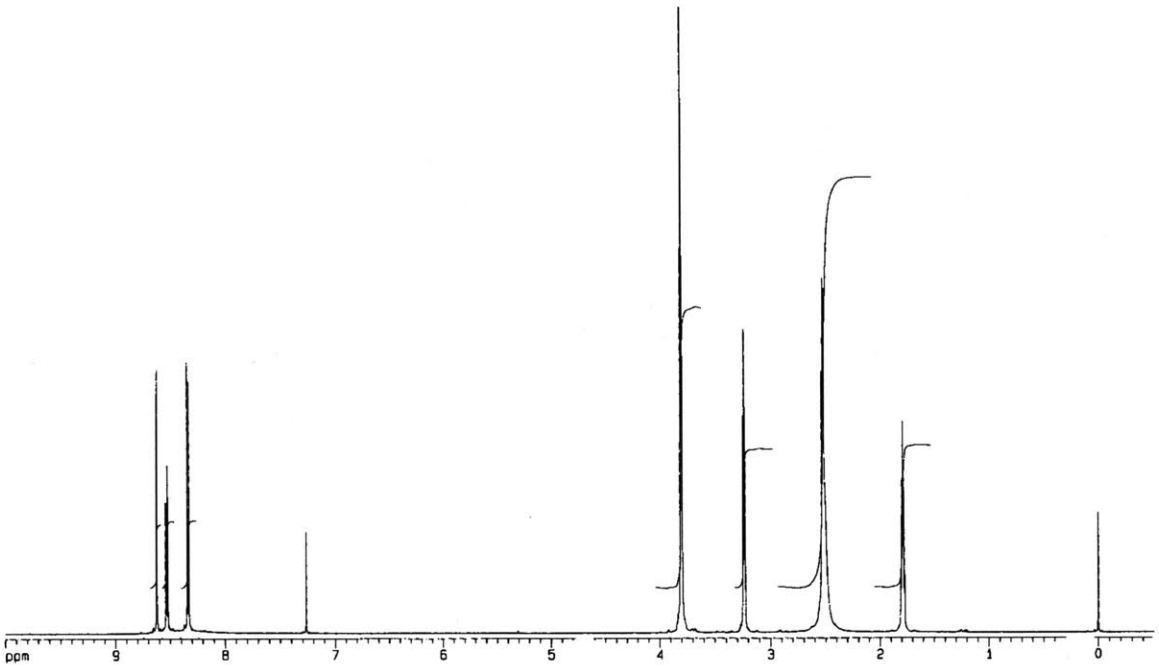
Phenethyl-2,4-dinitrobenznesulfonamide (6).

Prepared according to general procedure, 44%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.59 (d, J = 2.2 Hz, 1H), 8.45 (dd, J = 8.4, 2.2 Hz, 1H), 8.19 (d, J = 8.6 Hz, 1H), 7.22–7.06 (m, 5H), 5.40 (t, J = 5.7 Hz, 1H), 3.50 (appar. q, J = 6.5 Hz, 2H), 2.85 (t, J = 6.7 Hz, 2H). LRMS (ESI +) m/z calcd for $[C_{14}H_{13}N_3O_6S + Na]^+$ 374 found 374.



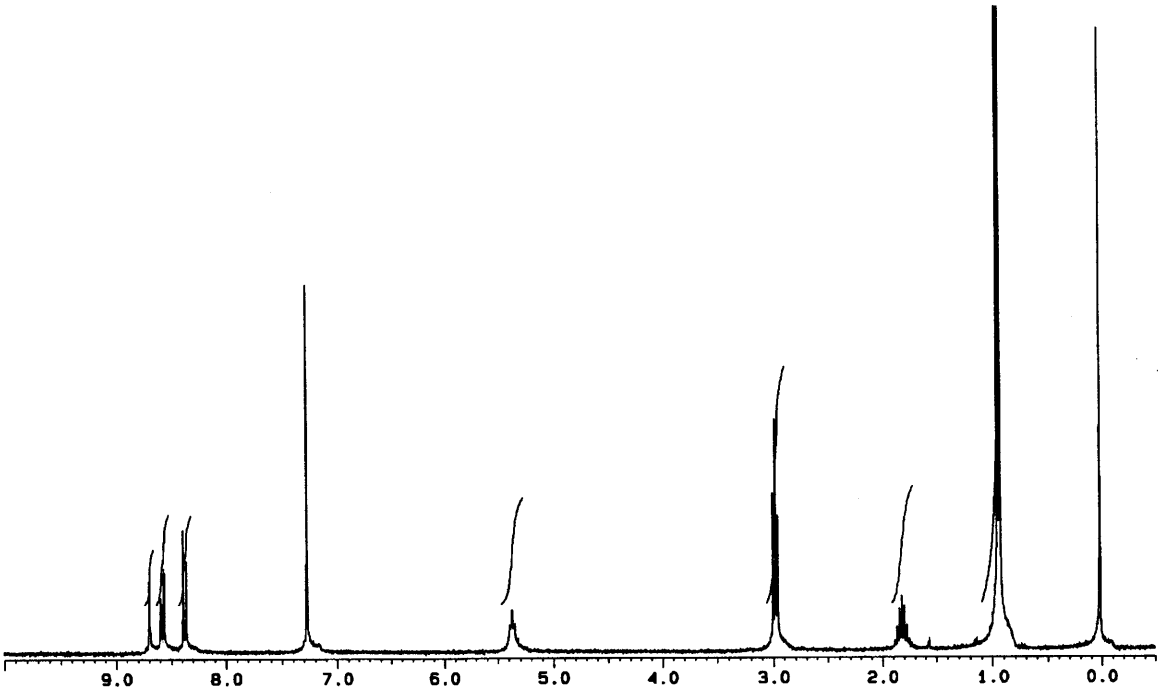
3-(N-Morpholino)propyl-2,4-dinitrobenznesulfonamide (7).

Prepared according to general procedure, except that the corresponding ammonium ion was isolated by filtration from the biphasic solution following the acid wash. In a second step, the ammonium ion was shaken with chloroform and saturated $NaHCO_3$ solution and the final product was isolated after removal of solvent by rotary evaporation, 28%. ^1H NMR (600 MHz, $CDCl_3$): δ 8.62 (d, J = 2.2 Hz, 1H), 8.53 (dd, J = 8.6, 2.2 Hz, 1H), 8.34 (d, J = 8.6 Hz, 1H), 3.80 (t, J = 4.6 Hz, 4H), 3.24 (t, J = 5.7 Hz, 2H), 2.53–2.50 (m, 6H), 1.79 (quint, J = 5.6 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 149.81, 148.48, 139.58, 132.94, 126.91, 120.52, 66.75, 58.85, 53.86, 44.91, 23.67. HRMS (FAB, NBA/NaI) m/z calcd for $[C_{13}H_{18}N_4O_7S + H]^+$ 375.0974 found 375.0977.



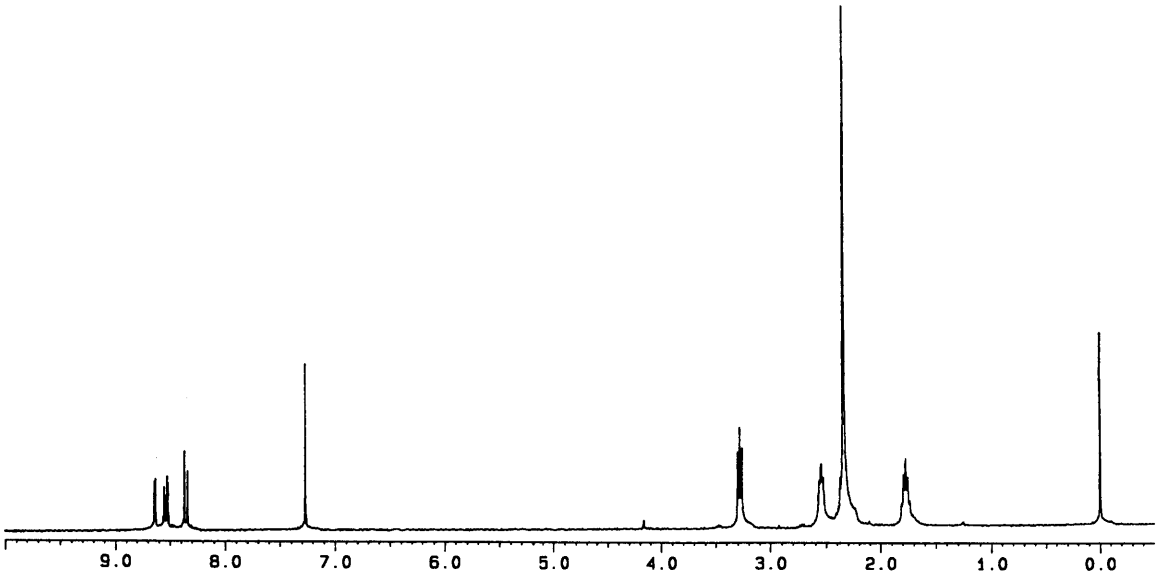
Isobutyl-2,4-dinitrobenznesulfonamide (8).

Prepared according to general procedure, 42%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.69 (d, J = 2.2 Hz, 1H), 8.57 (dd, J = 8.6, 2.2 Hz, 1H), 8.37 (d, J = 8.6 Hz, 1H), 5.35 (t, J = 6.4 Hz, 1H), 2.97 (appar. t, J = 6.5 Hz, 2H), 1.81 (m, 1H), 0.93 (d, J = 6.8 Hz, 6H). LRMS (ESI –) m/z calcd for $[C_{10}H_{13}N_3O_6S - H]^-$ 302 found 302.



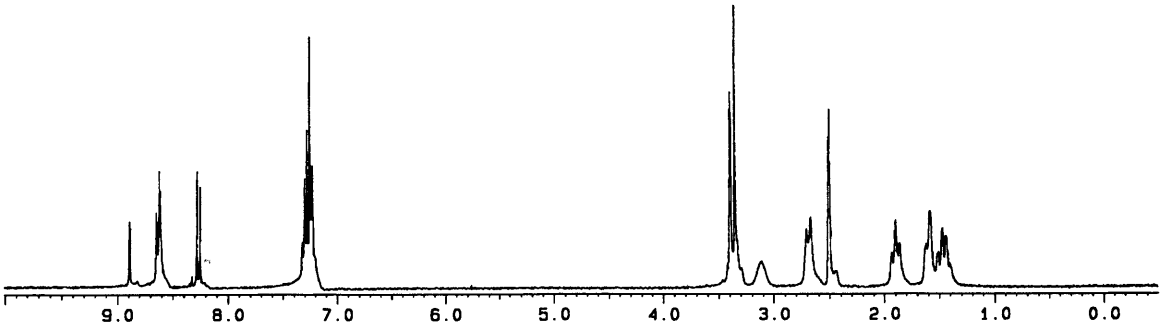
3-(Dimethylamino)propyl-2,4-dinitrobenznesulfonamide (9).

Prepared according to general procedure, 39%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.63 (d, J = 2.2 Hz, 1H), 8.53 (dd, J = 8.5, 2.2 Hz, 1H), 8.35 (d, J = 8.6 Hz, 1H), 3.28 (t, J = 5.7 Hz, 1H), 2.54 (m, 2H), 2.37 (m, 8H), 1.77 (m, 2H). LRMS (ESI +) m/z calcd for $[C_{11}H_{16}N_4O_6S + H]^+$ 333 found 333.



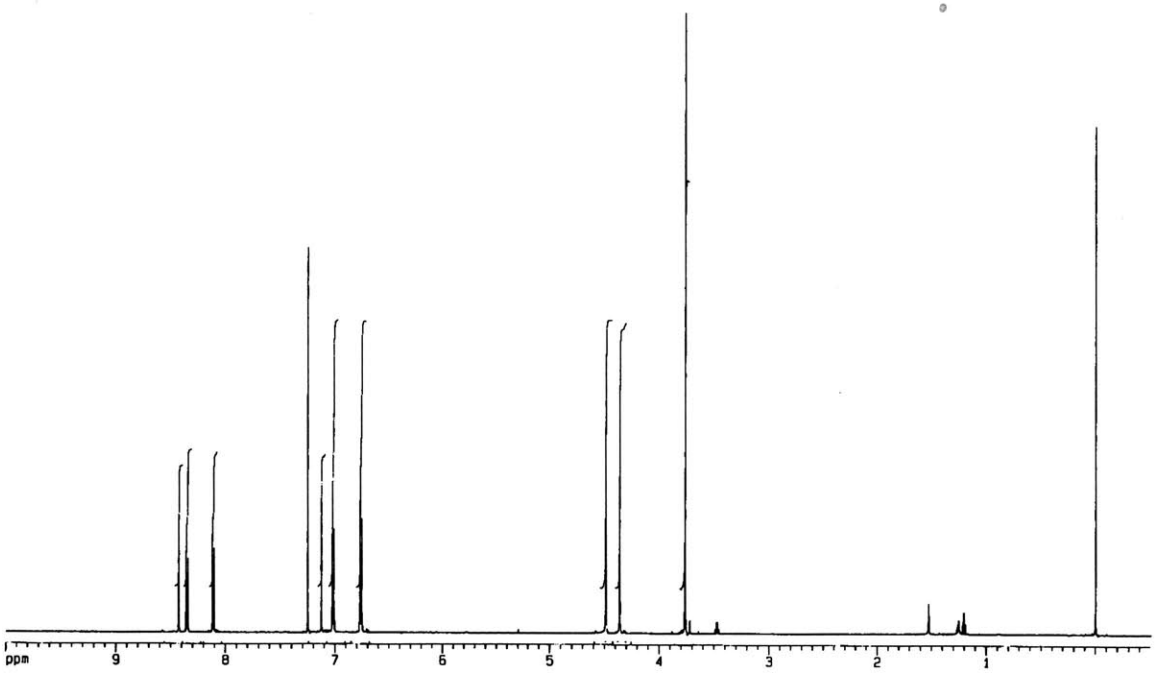
N-(2,4-Dinitrobenznesulfonamide)-1-benzylpiperidine (10).

Prepared according to general procedure, except that the corresponding ammonium ion was isolated by filtration from the biphasic solution following the acid wash. In a second step, the ammonium ion was shaken with chloroform and saturated $NaHCO_3$ solution and the final product was isolated after removal of solvent by rotary evaporation, 38%. ^1H NMR (300 MHz, DMSO-d_6): δ 8.88 (d, J = 2.2 Hz, 1H), 8.64–8.60 (m, 2H), 8.26 (d, J = 8.6 Hz, 1H), 7.32–7.22 (m, 5H), 3.47 (s, 2H), 3.11 (br. s, 1H), 2.70–2.66 (m, 2H), 1.93–1.86 (m, 2H), 1.62–1.40 (m, 4H). LRMS (ESI +) m/z calcd for $[C_{18}H_{20}N_4O_6S + H]^+$ 421 found 421.



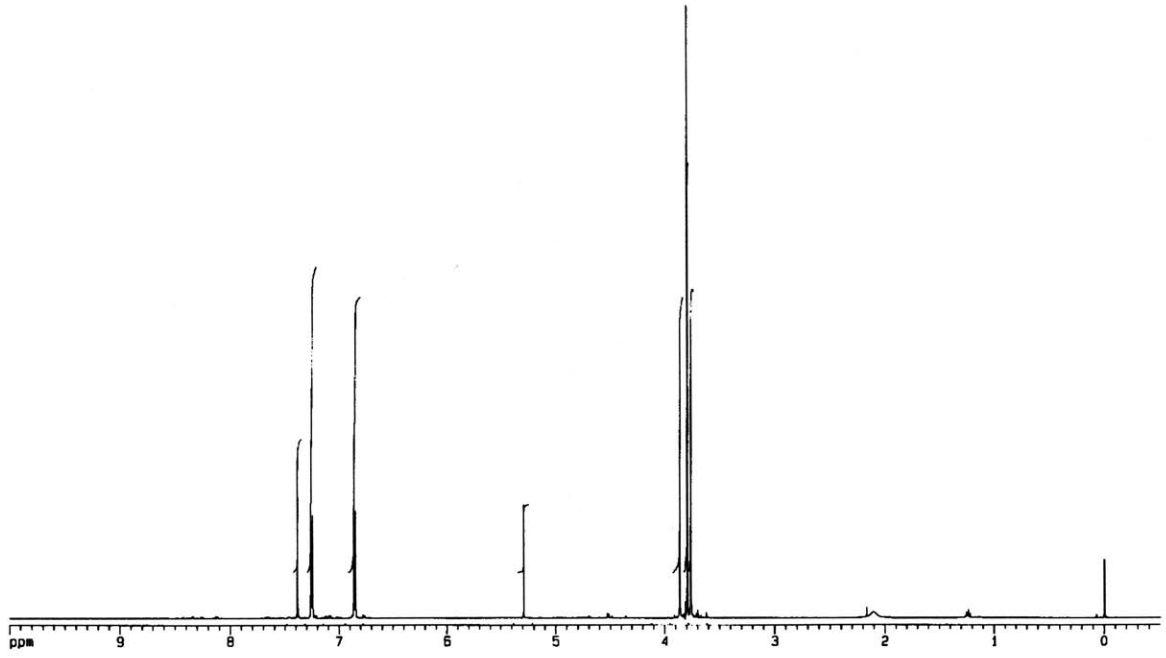
4-Bromo-2,6-bis[(*p*-methoxybenzyl)-2,4-dinitrobenznesulfonamide]pyridine (11).

To a stirred suspension of 4-bromo-2,6,-pyridinedimethanol **1** (151 mg, 0.500 mmol), TPP (328 mg, 1.25 mmol), and (*p*-methoxybenzyl)-2,4-dinitrobenzene-sulfonamide **3** (459 mg, 1.25 mmol) in 15 mL methylene chloride was added DIAD (246 μL, 253 mg, 1.25 mmol) and all solids dissolved rapidly. The orange solution was stirred at RT for 22 h and the solvent was removed by rotary evaporation. The resulting oil was sonicated in Et_2O and a precipitate formed. Compound **11** (389 mg, 87%) was isolated by filtration as an orange powder. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.43 (d, J = 2.2 Hz, 2H), 8.36 (dd, J = 8.7, 2.2 Hz, 2H), 8.12 (d, J = 8.6 Hz, 2H), 7.13 (s, 2H), 7.02 (d, J = 8.6 Hz, 4H), 6.77 (d, J = 8.6 Hz, 4H), 4.49 (s, 4H), 4.37 (s, 4H), 3.77 (s, 6H). HRMS (FAB, NBA/CsI) m/z calcd for $[C_{35}H_{30}BrN_7O_{14}S_2 + Cs]^+$ 1047.9530/1050 found 1047.9574/1050.



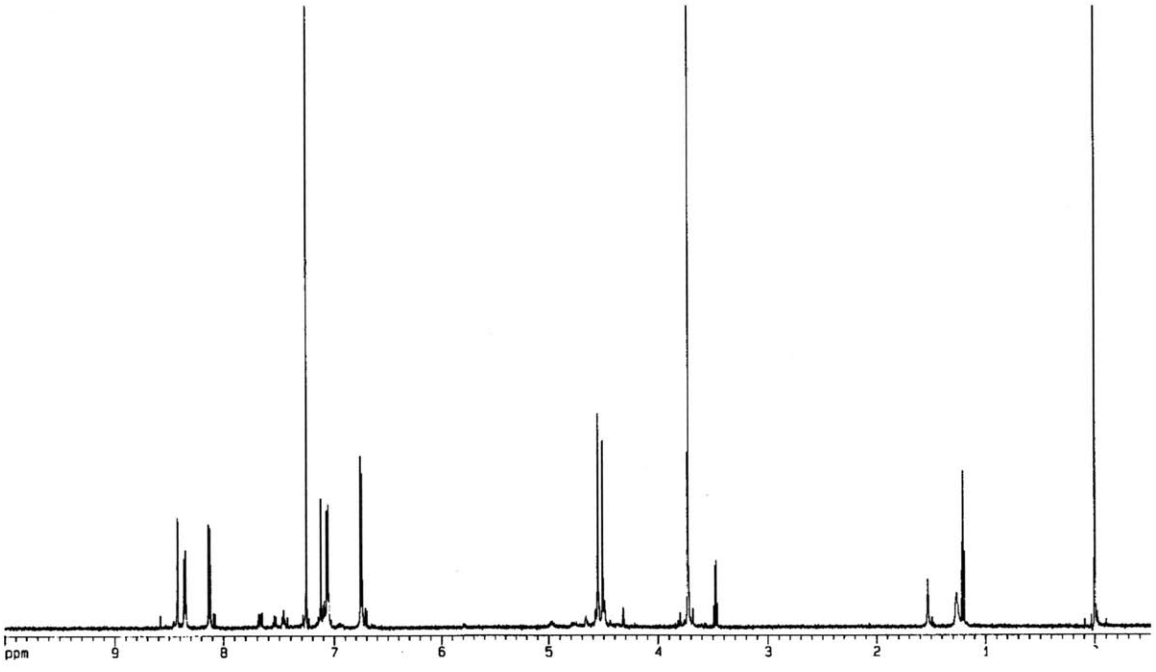
4-Bromo-2,6-bis(*p*-methoxybenzylamino)pyridine (12).

To a solution of bis(sulfonamide) **11** (50 mg, 0.11 mmol) and Et_3N (4 drops) in 4 mL methylene chloride was added mercaptoacetic acid (3 drops). The solution was stirred at RT for 10 min, then was washed with saturated $NaHCO_3$ solution several times until the yellow color disappeared from the organic phase. The organic phase was dried over magnesium sulfate and was filtered. The solvent was removed by rotary evaporation, yielding **12** (24 mg, 96%) as a pale straw oil. ^1H NMR (600 MHz, $CDCl_3$): 7.39 (s, 2H), 7.26 (d, J = 8.6 Hz, 4H), 6.86 (d, J = 8.6 Hz, 4H), 3.87 (s, 4H), 3.80 (s, 6H), 3.77 (s, 4H). LRMS (ESI +) m/z calcd for $[C_{23}H_{26}BrN_3O_2 + H]^+$ 456/458 found 456/458.



2,2',6,6'-Tetrakis[(*p*-methoxybenzyl)-2,4-dinitrobenznesulfonamide]-4,4'-bipyridine (13).

A suspension of bipyridinetetramethanol **2** (69 mg, 0.25 mmol), (*p*-methoxybenzyl)-2,4-dinitrobenzenesulfonamide **3** (459 mg, 1.25 mmol), TPP (328 mg, 1.25 mmol), and DIAD (246 μL, 253 mg, 1.25 mmol) in 15 mL methylene chloride was stirred at RT for 24 h. Insoluble material was removed by filtration and the filtrate was concentrated by rotary evaporation. Ether was added and the mixture was sonicated. The resulting precipitate was isolated by filtration and dried at elevated temperature, yielding **13** (174 mg, 42%) as pale yellow powder. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.42 (d, J = 2.2 Hz, 4H), 8.36 (dd, J = 8.7, 2.2 Hz, 4H), 8.13 (d, J = 8.6 Hz, 4H), 7.12 (s, 4H), 7.06 (d, J = 8.6 Hz, 8H), 6.75 (d, J = 8.6 Hz, 8H), 4.55 (s, 8H), 4.51 (s, 8H), 3.72 (s, 12H). LRMS (ESI +) m/z calcd for $[C_{70}H_{60}N_{14}O_{28}S_4 + H]^+$ 1673 found 1673.

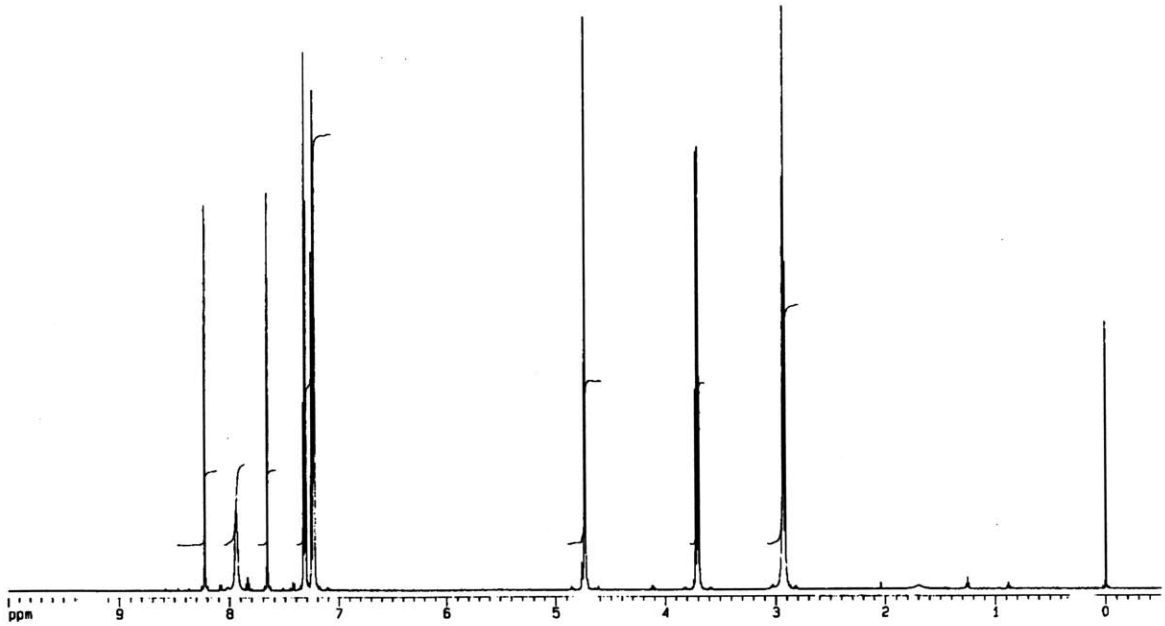


4-Bromo-2-(phenethylamide)-6-(ethyl ester) pyridine (14).

See Chapter 4, compound **11**.

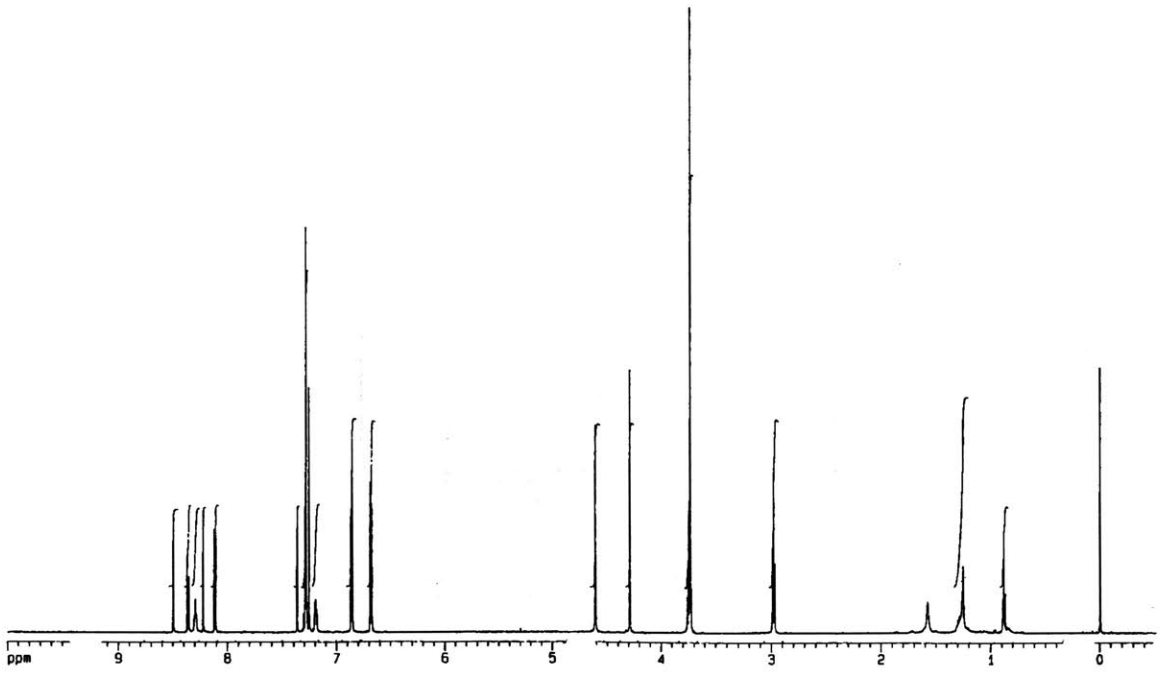
4-Bromo-2-(phenethylamide)-6-methanolpyridine (15).

To a solution of ethyl ester **14** (1.51 g, 4.00 mmol) in 50 mL EtOH was added $NaBH_4$ (0.225 g, 5.95 mmol). The resulting wheat-colored solution was gently refluxed for 4 h. The solvent was removed by rotary evaporation and saturated $NaHCO_3$ solution was added. This mixture was boiled briefly and the sample was freeze-dried. The residue was shaken in $CHCl_3$ and the insoluble material was removed by filtration. The solvent from the filtrate was removed by rotary evaporation, and crystallization of the residue from hexane/EtOAc yielded **15** (835 mg, 62%) as colorless crystals. ^1H NMR (600 MHz, $CDCl_3$): δ 8.23 (d, J = 1.6 Hz, 1H), 7.94 (br. t, 1H), 7.67 (d, J = 1.6 Hz, 1H), 7.34–7.23 (m, 5H), 4.74 (s, 2H), 3.71 (appar. q, J = 7.0 Hz, 2H), 2.95–2.92 (m, 3H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 163.04, 160.35, 150.21, 138.86, 135.41, 129.00, 128.90, 126.88, 126.42, 124.73, 64.44, 40.95, 35.94. HRMS (FAB , NBA/NaI) m/z calcd for $[C_{15}H_{15}BrN_2O_2 + H]^+$ 335.0395/337 found 335.0405/337. FT-IR (neat) 3375.7, 2930.1, 1662.5, 1573.9, 1530.7, 1454.2, 1298.0, 1074.7, 699.8 cm^{-1}.



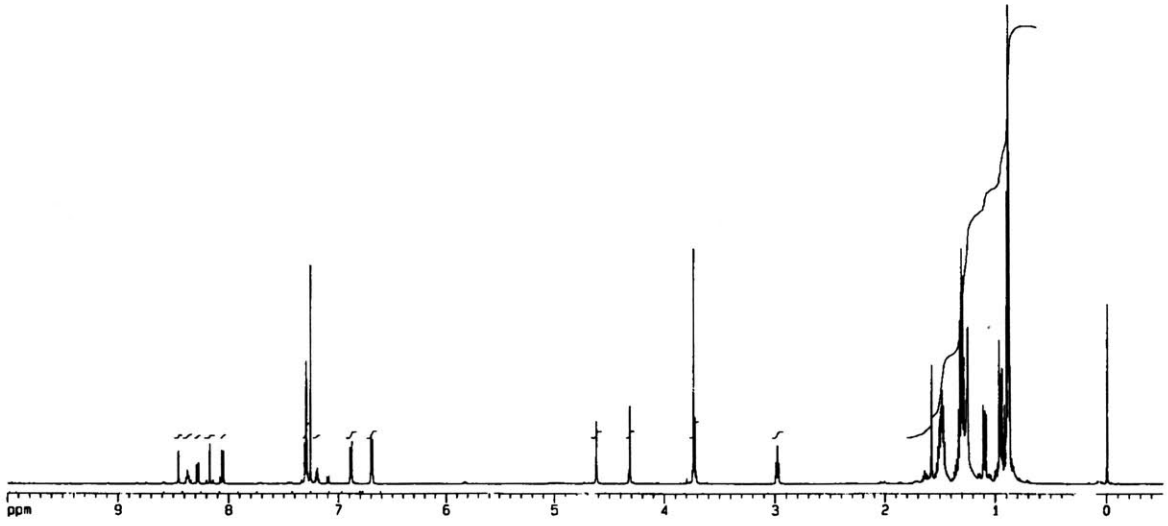
4-Bromo-2-(phenethylamide)-6-[(*p*-methoxybenzyl)-2,4-dinitrobenzne-sulfonamide]-pyridine (16).

A solution of pyridinemethanol **15** (503 mg, 1.50 mmol), (*p*-methoxybenzyl)-2,4-dinitrobenzenesulfonamide **3** (661 mg, 1.8 mmol), TPP (484 mg, 1.8 mmol), and DIAD (354 μL, 364 mg, 1.8 mmol) in 45 mL methylene chloride was stirred at RT for 17 h. The crude reaction mixture was purified by silica gel column chromatography (100% CH_2Cl_2), yielding **16** (472 mg, 42%) as a yellow powder. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.50 (d, J = 2.2 Hz, 1H), 8.37 (dd, J = 8.6, 2.2 Hz, 1H), 8.30 (t, J = 5.9 Hz, 1 H) 8.23 (d, J = 1.4 Hz, 1H), 8.12 (d, J = 8.6 Hz, 1H), 7.37 (d, J = 1.2 Hz, 1H), 7.32–7.28 (m, 4H), 7.21–7.19 (m, 1H), 6.87 (d, J = 8.6 Hz, 2H), 6.69 (d, J = 8.6 Hz, 1H), 4.62 (s, 2H), 4.30 (s, 2H), 3.77–3.73 (m, 5H), 2.98 (t, J = 7.3 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 162.97, 159.99, 155.30, 150.83, 149.83, 147.86, 139.27, 139.19, 135.43, 133.19, 130.15, 129.07, 128.76, 127.28, 126.65, 126.15, 125.64, 124.92, 120.08, 114.37, 55.52, 52.24, 51.98, 41.08, 35.82. HRMS (FAB , NBA/CsI) m/z calcd for $[C_{29}H_{26}BrN_5O_8S + Cs]^+$ 815.9740/818 found 815.9722/818. FT-IR (neat) 3406.0 (br.), 1672.5, 1537.5, 1513.8, 1349.6, 1250.7, 1162.0, 736.5 cm^{-1}.



4-(Tributyl tin)-2-(phenethylamide)-6-[(*p*-methoxybenzyl)-2,4-dinitrobenzne-sulfonamide]-pyridine (17).

To a solution of bromopyridine **16** (400 mg, 0.584 mmol) and $PdCl_2(PPh_3)_2$ (7.0 mg, 0.0121 mmol) in 8 mL toluene was added bis(tributyltin) (0.94 mL, 1.08 g, 1.87 mmol), and the solution turned brown. The solution was stirred at 60 °C for 18 h, then the solvent was removed by rotary evaporation and the residue was purified by silica gel column chromatography (4:1→2:1 hexane/EtOAc). Starting material **16** (297 mg) was recovered, and product **17** (50 mg, 10% [37% based on recovered starting material]) was isolated as a white powder. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.46 (d, J = 2.2 Hz, 1H), 8.38 (t, J = 5.9 Hz, 1H), 8.28 (dd, J = 8.7, 2.2, 1H), 8.17 (s, 1H), 8.05 (d, J = 8.6 Hz, 1H), 7.33–7.31 (m, 5H), 7.23–7.20 (m, 1H), 6.90 (d, J = 8.6 Hz, 2H), 6.71 (d, J = 8.6 Hz, 2H), 4.64 (s, 2H), 4.33 (s, 2H), 3.76–3.73 (m, 5H), 3.00 (t, J = 7.3 Hz, 2H), 1.60–0.88 (m, 27H). LRMS (ESI -) m/z calcd for $[C_{41}H_{53}N_5O_8SSn + Cl]^-$ 928/930 found 928/930.

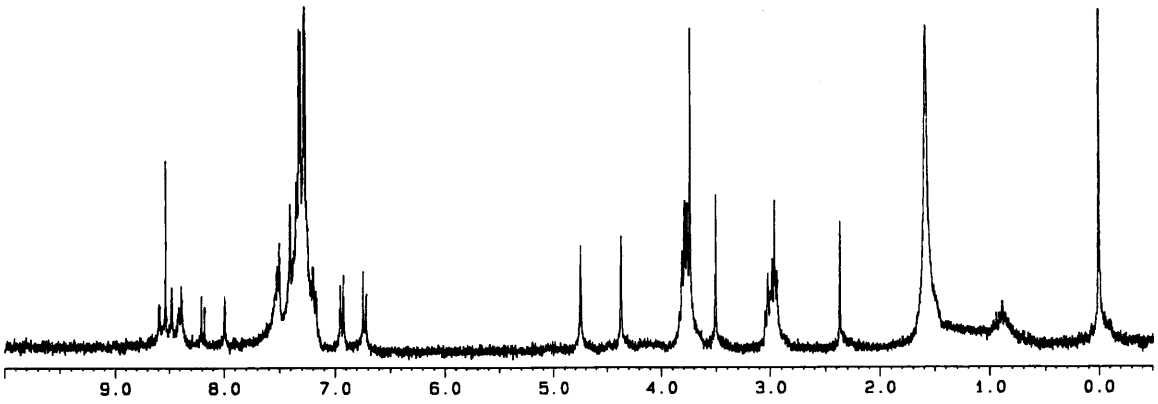


4-(Tributyl tin)-2,6-bis(phenethylamide)-pyridine (18).

See Chapter 4, compound **16**.

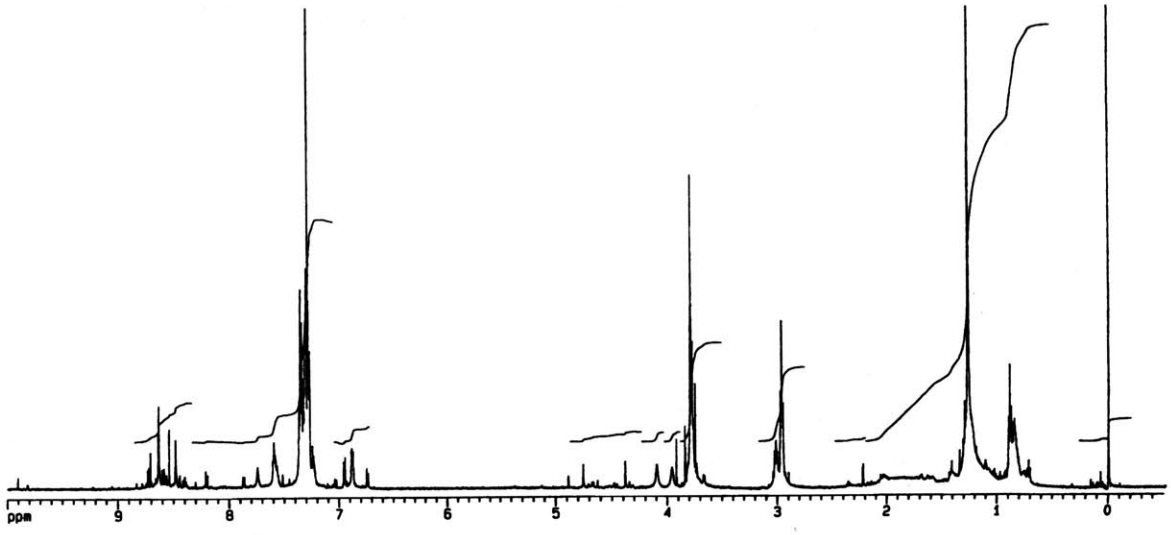
2,2',6-Tris(phenethylamide)-6'-[(*p*-methoxybenzyl)-2,4-dinitrobenzne-sulfonamide]-4,4'-bipyridine (19).

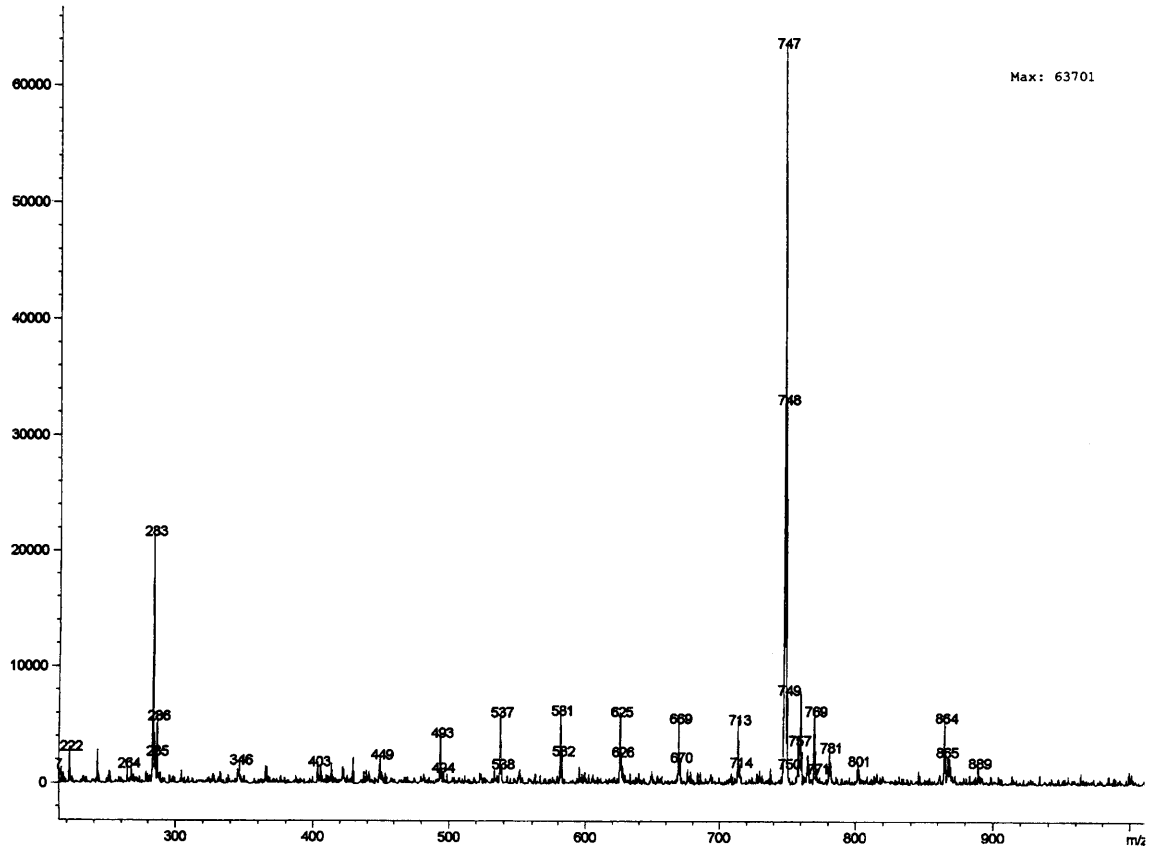
A solution of stannane **18** (206 mg, 0.312 mmol), bromopyridine **16** (224 mg, 0.327 mmol) and dichlorobis(triphenylphosphine) palladium (II) (11 mg, 0.016 mmol) in toluene (3 mL) was stirred at 110 °C for 24 h, turning from yellow to dark brown in color over this period. The solution was concentrated to brown solids. The residue was taken up in $CHCl_3$ and the crude product (33%) was precipitated with 2:3 hexane/EtOAc. Preparative TLC of this compound (1:2 hexane/EtOAc) yielded **19** (21 mg, 7%) as an off-white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.58 (d, J = 2.2 Hz, 1H), 8.52 (s, 2H), 8.47 (s, 1H), 8.39 (dd, J = 2.2, 8.6 Hz, 1H), 8.18 (d, J = 8.6 Hz, 1H), 7.99 (s, 1H), 7.52–7.17 (m, 19H), 6.93 (d, J = 8.6 Hz, 2H), 6.72 (d, J = 8.6 Hz, 2H), 4.74 (s, 2H), 4.36 (s, 2H), 3.80–3.73 (m, 7H), 3.01–2.93 (m, 6H). LRMS (ESI +) m/z calcd for $[C_{52}H_{48}N_8O_{10}S + Na]^+$ 999 found 999.



2,2',6-Tris(phenethylamide)-6'-(*p*-methoxybenzylamine)-4,4'-bipyridine, *crude* (20).

To a solution of sulfonamide **19** (21 mg, 0.0215 mmol) and Et_3N (4 drops) in 4 mL methylene chloride was added mercaptoacetic acid (3 drops). The solution was stirred at RT for 50 min, then was washed with saturated $NaHCO_3$ solution several times until the yellow color disappeared from the organic phase. The organic phase was dried over magnesium sulfate and was filtered. The solvent was removed by rotary evaporation. The residue was partially purified by preparative thin layer chromatography, yielding crude **20** (2.4 mg, 15%) as a pale yellow oil. ^1H NMR (600 MHz, $CDCl_3$): crude product. HRMS (FAB, NBA/CsI) *m/z* calcd for $[C_{46}H_{46}N_6O_4 + H]^+$ 747.3658found 747.365.

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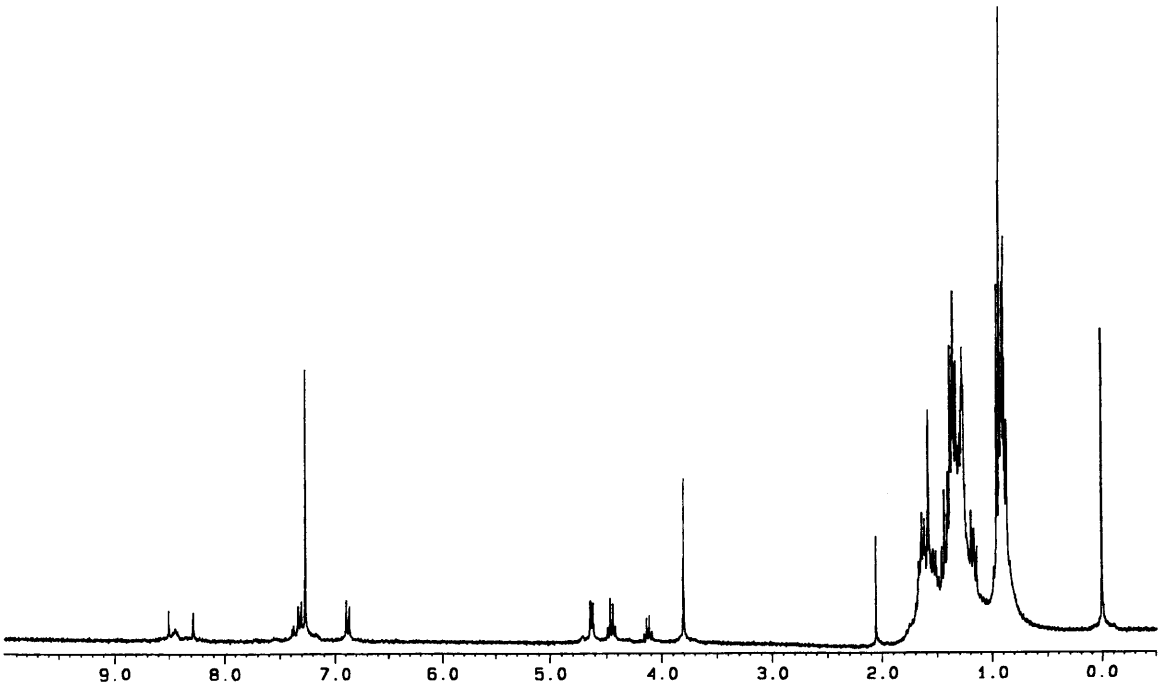
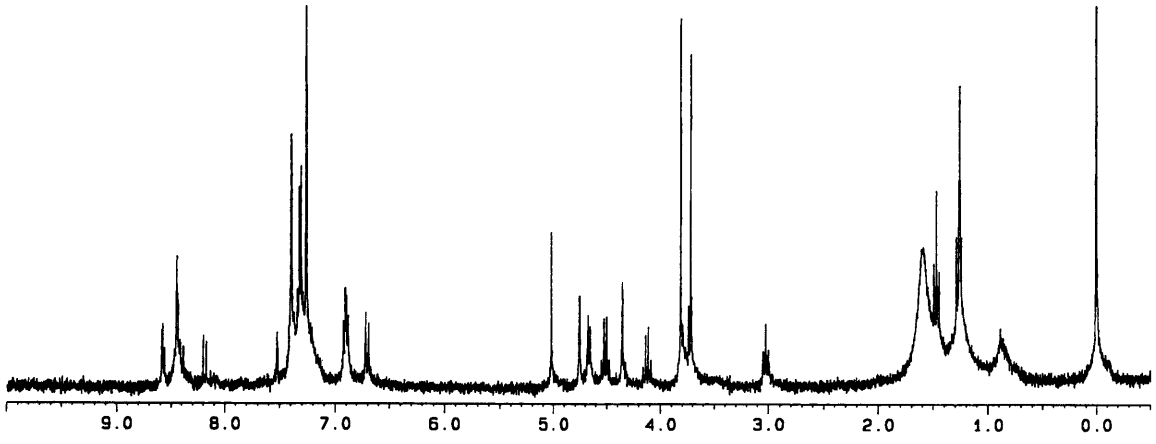
4-(Tributyl tin)-2-(ethyl ester)-6-(*p*-methoxybenzylamide)-pyridine (21).

A solution of 4-bromo-2-(ethyl ester)-6-(*p*-methoxybenzylamide)-pyridine (see Chapter 3, compound **20**) (225 mg, 0.573 mmol), bis(tributyl tin) (1.06 g, 1.83 mmol), and $PdCl_2(PPh_3)_2$ (8 mg, 0.012 mmol) in 6 mL toluene was refluxed for 23 h. A brown color developed. The solvent was removed by rotary evaporation and the residue was purified by silica gel column chromatography (3:1 hexane/EtOAc), yielding **21** (45 mg, 13%) as a clear oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.50 (s, 1H), 8.44 (br. t, 1H), 8.28 (s, 1H) 7.31 (d, J = 8.6 Hz, 2H), 6.88 (d, J = 8.6 Hz, 2H), 4.63 (d, J = 6.2 Hz, 2H), 4.45 (q, J = 7.1 Hz, 2H), 3.80 (s, 3H), 1.65–0.86 (m, 30H). LRMS (ESI +) m/z calcd for $[C_{29}H_{44}N_2O_4Sn + Na]^+$ 627 found 627.



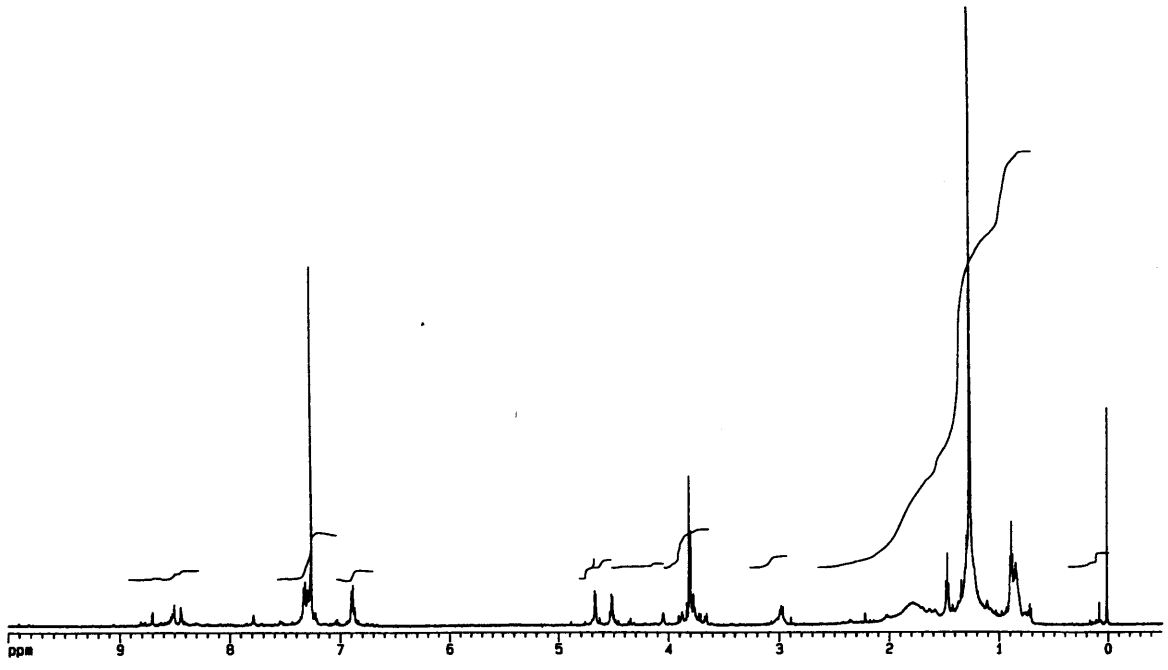
2-(Phenethylamide)-2'-(*p*-methoxybenzylamide)-6-[(*p*-methoxybenzyl)-2,4-dinitrobenznesulfonamide]-6'-(ethyl ester)-4,4'-bipyridine (22).

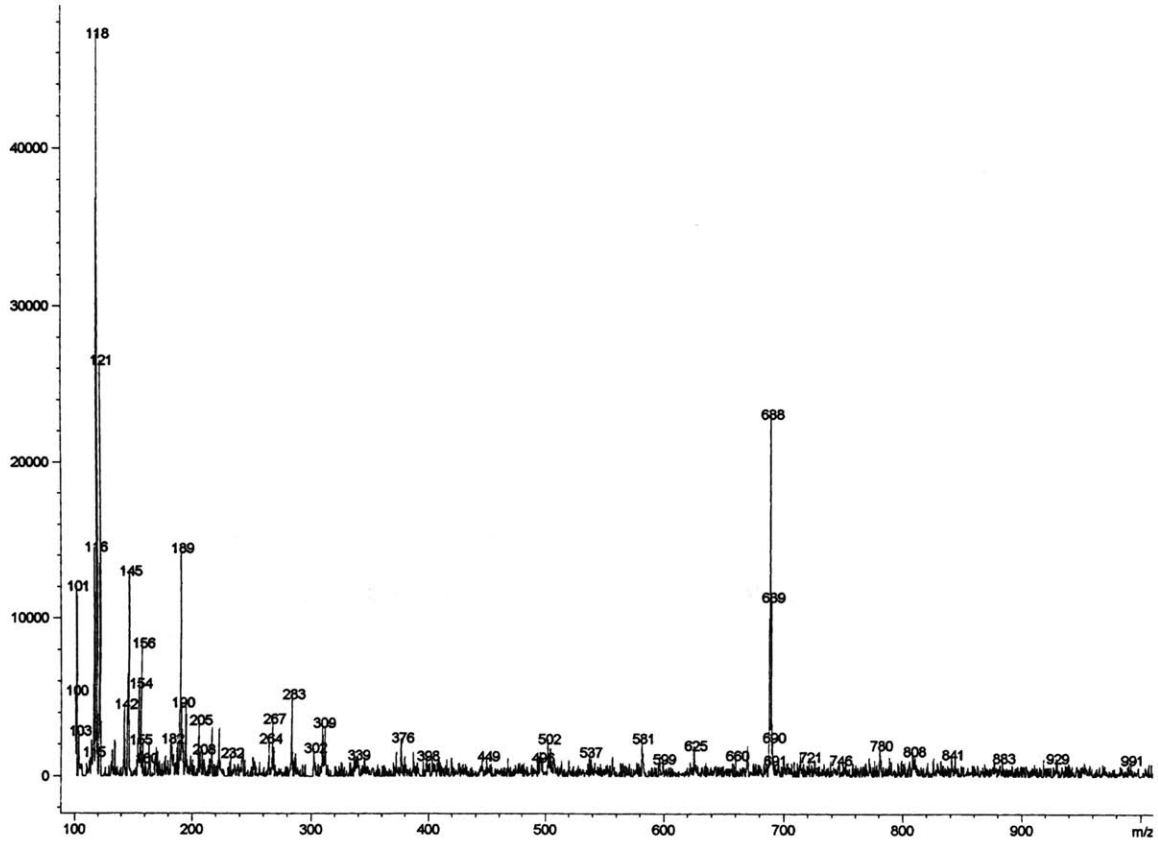
A solution of stannane **21** (40 mg, 0.066 mmol), bromopyridine **16** (48 mg, 0.070 mmol) and dichlorobis(triphenylphosphine) palladium (II) (2.3 mg, 0.0033 mmol) in toluene (5 mL) was stirred at 80 °C for 24 h, turning from yellow to dark brown in color over this period. The solution was concentrated to brown solids. Silica gel column chromatography ($CHCl_3$) yielded an impure brown solid. Preparative TLC of this compound (1:2 hexane/EtOAc) yielded **22** (3 mg, 5%) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.15–8.38 (m, 5H), 8.19 (d, J = 1.7 Hz, 1H), 7.52–7.21 (m, 13H), 6.93–6.88 (m, 2H), 6.71 (d, J = 8.6 Hz, 2H), 4.76 (s, 2H), 4.66 (d, J = 5.7 Hz, 2H), 4.51 (q, J = 7.1 Hz, 2H), 4.35 (s, 2H), 4.12 (q, J = 7.1 Hz, 2H), 3.79 (s, 3H), 3.72 (s, 3H), 3.02 (t, J = 6.7 Hz, 2H), 1.47 (t, J = 7.2 Hz, 3H). LRMS (ESI +) m/z calcd for $[C_{46}H_{43}N_7O_{12}S + Na]^+$ 940 found 940.



2-(Phenethylamide)-2'-(*p*-methoxybenzylamide)-6-(*p*-methoxybenzylamine)-6'-(ethyl ester)-4,4'-bipyridine, *crude* (23).

To a solution of sulfonamide **22** (3 mg, 0.0033 mmol) and Et_3N (5 drops) in 4 mL methylene chloride was added mercaptoacetic acid (4 drops). The solution was stirred at RT for 30 min, then was washed with saturated $NaHCO_3$ solution several times until the yellow color disappeared from the organic phase. The organic phase was dried over magnesium sulfate and was filtered. The solvent was removed by rotary evaporation. The residue was partially purified by preparative thin layer chromatography, yielding crude **23** (1.6 mg, 71%) as a pale yellow oil. ^1H NMR (600 MHz, $CDCl_3$): crude product. HRMS (FAB, NBA/CsI) *m/z* calcd for $[C_{40}H_{41}N_5O_6 + H]^+$ 688.3135 found 688.3145.

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502
581
625
780
808
841
883
929
991
40000
30000
20000
10000
0
100
200
300
400
500
600
700
800
900
m/z

Chapter 7. A Rapid Method to Identify Protease Inhibitors[1]

7.1 Introduction

Small molecule protease inhibitors have become an integral facet of pharmaceutical development.[2] Current advances arising from arrayed and combinatorial methods pose an increasing demand for tools which can efficiently differentiate large numbers of candidates.[3] We have developed an approach which rapidly addresses *exo*-protease inhibition through the implementation of efficient combinatorial and assay technologies.

Proteolytic screening is currently monitored using a combination of substrates which change color, electrochemistry or fluorescence upon reaction.[4] Several of these materials, such as bis-amides of rhodamine 110,[5] can be screened at high-throughput by combining visual analysis with photographic digitization. Unfortunately, this approach currently requires at least μM product formation.[6]

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[1] This work was carried out in collaboration with James J. LaClair, Ph. D., Assistant Professor, Department of Molecular Biology, The Scripps Research Institute. Technical assistance was provided by Ellen Choi. Portions of this chapter have been incorporated into a manuscript in press and are being used with permission from Elsevier Science. Pryor, K. E.; LaClair, J. J. *Bioorg. Med. Chem. Lett.* **1999**, in press.

[2] (a) Whittaker, M. *Curr. Opin. Chem. Biol.* **1998**, *2*, 386. (b) Dolle, R. E. *Mol. Diversity.* **1997**, *2*, 223. (c) Kennedy, A. R. *Pharmacol. Theraputics* **1998**, *78*, 167. (d) Amzel, L. M. *Curr. Opin. Biotech.* **1998**, *9*, 366.

[3] (a) Cargill, J. F.; Lebl, M. *Curr. Opin. Chem. Biol.* **1997**, *1*, 67. (b) Czarnik, A. W. *Curr. Opin. Chem. Biol.* **1997**, *1*, 60. (c) Weber, L. *Curr. Opin. Chem. Biol.* **1998**, *2*, 381. (c) Amzel, L. M. *Curr. Opin. Biotech.* **1998**, *9*, 336. (d) Veber, D. F.; Drake, F. H.; Gowen, M. *Curr. Opin. Chem. Biol.* **1997**, *1*, 151. (e) Houston, J. G.; Banks, M. *Curr. Opin. Biotech.* **1997**, *8*, 734. (f) Brown, D. *Mol. Diversity.* **1997**, *2*, 217.

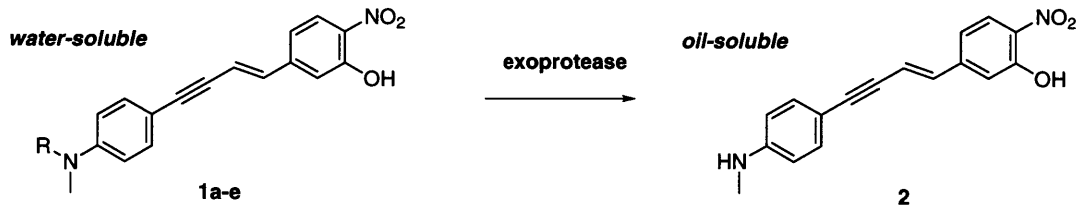
[4] (a) In *Topics in Fluorescence Spectroscopy: Probe Design and Chemical Sensing*; Lakowicz, J. R., Ed. Plenum: New York, 1994, vol 4. (b) Mason, W. T. *Fluorescent and Luminescent Probes for Biological Activity*; Academic, San Diego, 1993.

[5] (a) Leytus, S. P.; Toledo, D. L.; Mangel, W. F. *Biochim. Biophys. Acta.* **1984**, *788*, 74. (b) Leytus, S. P.; Patterson, W. L.; Mangel, W. F. *Biochem. J.* **1983**, *215*, 253. (c) Leytus, S. P.; Melhado, L. L.; Mangel, W. F. *Biochem. J.* **1983**, *203*, 299.

[6] Even the most robust chromaphores (i.e., $\varepsilon \geq 90{,}000\ cm^{-1}M^{-1}$) require a concentration greater than 1 μM to appear colored.

7.2 Assay development

We reduced this limit to the nanomolar level by developing a new class of substrates whose solubility and spectroscopic properties invert with proteolysis. A system was designed such that substrates, water-soluble and non-fluorescent, release an oil soluble fluorophore upon hydrolysis.[7] Using this approach, we could assay trypsin, kallikrein, cathepsin B and elastase activity at enzyme levels reduced by more than 30-fold and in a fraction of the time required using the conventional substrates. Alkenyne (**2**) is sparingly soluble and non-fluorescent, $\Phi_f \sim 10^{-7}$, in aqueous media, and highly soluble and fluorescent in mineral oil, λ_F = 511 nm with $\Phi_f \sim 0.11$ (Figure 1).[8]



enzyme	peptide (R)	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m ($10^5\ M^{-1}s^{-1}$)	visual limit (μU)[9]
trypsin	Z-L-Ile-L-Pro-L-Arg -	97.0	72.5	7.48	22±9
kallikrein	Z-L-Pro-L-Phe-L-Arg -	92.2	65.5	7.13	49±9
cathepsin B	Z-L-Arg-L-Arg -	129.0	121.0	9.37	37±9
elastase	$CH_3O(CH_2CH_2O)_4CO$-L-Ala-L-Ala -	325.0	145.0	4.46	29±6
HLE	L-Tyr-L-Leu-L-Val-	16.1	3.6	2.25	15+3

Figure 1. Application of two-phase colorimetry to monitor *exo*-proteases. Hydrolysis of the water-soluble peptides **1a-e** releases **2**, which becomes fluorescent as it is extracted into a trace amount of mineral oil. The visual limit indicates the lowest level of enzyme required for detection within 1 h. Under the conditions used for each assay, derivatives of rhodamine 110 required ≥ 1 mU.[9]

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[7] La Clair, J. J. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 325.

[8] La Clair, J. J. *J. Am. Chem. Soc.* **1997**, *119*, 7676.

[9] A unit (U) is defined throughout as the quantity of enzyme required to produce 1 μmol of product per minute.

The origin of this enhancement arises from the solvent-induced stabilization of twisted-intramolecular charge transfer (TICT) states. When excited to the singlet state, materials presenting charge transfer undergo facile relaxation from planar-conjugated to orthogonally-twisted or TICT states. Due to their perpendicular orbital alignment, TICT states present greater dipole character than their planar counterparts and no longer relax to the ground state through fluorescent mechanisms. Therefore, factors that increase their expression, such as solvent polarity, also present a net loss in fluorescence.

When functionalized with water soluble peptides, conjugates **1a-e** of **2** become insoluble in mineral oil and rapidly partition into aqueous media, thereby destroying their fluorescence. The extent of this regulation is apparent by the fact that fluorescence was not detected when aqueous solutions of these conjugates were placed under a conventional handheld UV lamp. When mixed with the appropriate enzyme, hydrolysis of the C-terminal amide releases **2**, which is subsequently extracted into mineral oil. Like rhodamine 110, the concentration of indicator **2** must be greater than ~1 μM. However, **2** is now concentrated by the use of two phases. Using 1 μL of mineral oil per 200 μL of aqueous buffer, the analyte is concentrated ~200 fold, thereby reducing the detection limit to require 5 nM of product formation.

The detection limit was defined as the concentration of enzyme required to produce approximately 1 μL of 0.2 μM mineral oil solution of **2**, which is equal to the cleavage and transfer 0.2 pmol of product. Theoretically, this test could detect 3.3 nU in 1 h of an enzyme whose unity is defined as 1 μmol/min. Rhodamine- and fluorescein-based substrates require two equivalents of hydrolysis per substrate. One μU of enzyme would theoretically produce enough product for visual detection in 1 h using these substrates, as indicated by the need to generate 200 μL of a 0.3 μm solution or 120 pmol of hydrolysis products. Solutions of *p*-nitrophenol appear yellow above 5 μM in basic media. The visual detection of its production in this assay would require ~ 17 μU of enzyme, based on the need to produce 1 nmol or 200

μL of a 5 μM solution. The fact that these reagents were not sufficient was clearly seen by the fact that replacement of the corresponding substrates with rhodamine 110 derivatives failed to produce color. While other derivatives such as coumarins, resorufin, 7-hydroxy-9*H*-(1,3-dichoro-9,9-dimethylacridin-2-one, ELF or indoyl substrates also possess only one hydrolytic unit and favorable kinetics, these materials lack the solubility and solvatochromic properties inherent to **2**.

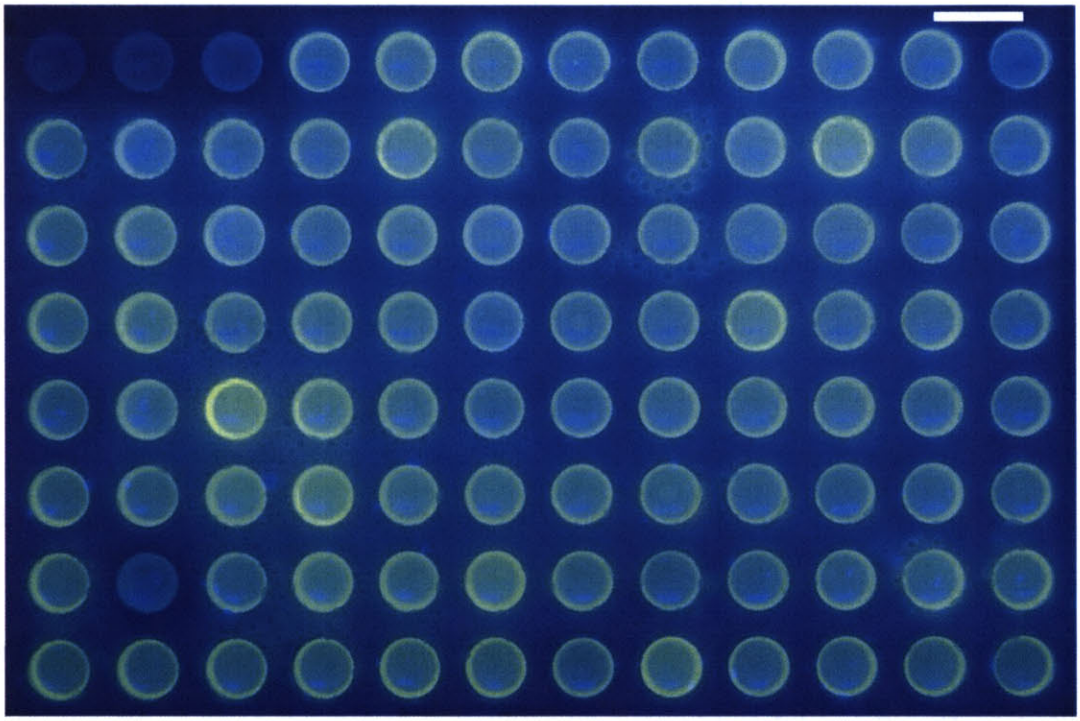

Figure 2.[10] Application of two-phase colorimetry to screen for trypsin inhibition. 92 shelf chemicals were screened by comparison with background (well at bottom right corner) and three known inhibitors (first three trials on top row).[11] Inhibition is indicated by the lack of emitted visible light. Fluorescence was obtained by excitation at 366 nm with a hand-held UV lamp. White bar in upper left indicates 1 cm.

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[10] Photography was provided by Alan McPhee.

[11] Known trypsin inhibitors were displayed along the top row (left to right): 4-aminobenzamidine, phenylmethylsulfonylfluoride and N-tosyl-L-phenylalanine chloromethyl-ketone.

In all cases, catalysis was comparable to that observed using known substrates (see Figure 1), indicating that the phase transfer was not rate limiting. The strength of the assay is demonstrated in Figure 2. Here, a selection of unknowns was assayed for trypsin inhibition. The first three wells (top left) were loaded with known inhibitors for comparison purposes.[11] As indicated, only one additional well lacked fluorescence, thereby indicating that its contents, N-phenylmaleimide, was a weak inhibitor ($K_i \sim 0.8$ mM).[12]

7.3 Xanthene-based libraries targeting HLE

With an efficient screen in hand, we then devised a series of small libraries against human leukocyte elastase (HLE). The libraries were designed to resemble a peptide-sequence recognized by HLE, Ala-Ala-Pro-Val.[13] Accordingly, 9,9-dimethylxanthene-4,5-dicarboxylic acid chloride **3** was reacted with left- and right- termini of Ala-Ala-Pro-Val, with the intention replacing internal amino acid(s) with the core. A hit was found in a series of four libraries produced by incorporating two building blocks per core (Scheme 1). The only library of this group exhibiting micromolar inhibition was that which contained Ala-Ala and Val, library A. Notably, library B was inactive, indicating the importance of the two terminal alanines. Subsequent resynthesis of the individual components **4**–**6** showed that, indeed, the mixed Ala-Ala/Val adduct was responsible for the greatest activity (K_i = 79 μM), exceeding the activity of the bis(Ala-Ala) and bis(Val) adducts by factors of 2 and 6.5, respectively.

Insertion of the core altered both the position of the two amino acid side chains as well as inverting the head-to-tail orientation of the Ala-Ala residue. Interestingly, this

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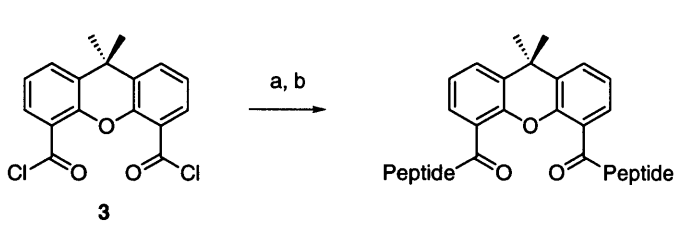
[12] Hall, P. L.; Anderson, C. D. *Biochem.* **1997**, *13*, 2082.

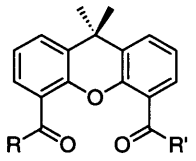
[13] (a) Gutschow, M.; Neumann, U. *J. Med. Chem.* **1998**, *41*, 1729. (b) Buynak, J. D.; Rao, A. S.; Ford, G. P.; Carver, C.; Adam, G.; Geng, B.; Bachmann, B.; Shobassy, S.; Lackey, S. *J. Med. Chem.* **1997**, *40*, 3423. (c) Regan, J.; McGarry, D.; Bruno, J.; Green, D.; Newman, J.; Hsu, C. Y.; Kline, J.; Barton, J.; Travis, J.; Choi, Y. M.; Volz, F.; Pauls, H.; Harrison, R.; Zilberstein, A.; Ben-Sasson, S. A.; Chang. M. *J. Med. Chem.* **1997**, *40*, 3408.

modification induced only a modest loss in inhibition. As apparent upon overlaying **6** into a crystal structure of HLE complexed with MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl,[14] the enzymatic pocket not only allows the critical encapsulation of the terminal Val residue but also readily accommodates the bulky dimethylxanthene.



Libraries	Building Blocks: Peptide	Peptide
A	L-Ala-L-Ala	L-Val
B	L-Ala	L-Val
C	L-Ala	L-Pro-L-Val
D	L-Ala	L-Pro

screen library against HLE



Compounds	R, R'	K_i with Tyr-Leu-Val-1 (μM)	K_i with p-nitroanilide (nM)
4	L-Ala-L-Ala	248	
5	L-Val	515	
6	L-Ala-L-Ala, L-Val	86	128

Scheme 1. Dimethylxanthene core-based libraries targeting HLE (EC 3.4.21.37). Each library was prepared by coupling a mixture of two peptides onto **3**. Each library consists of a mixture of bis(peptide$_1$), bis(peptide$_2$) and peptide$_1$-peptide$_2$. Screening using the method described in Figure 1 indicated that only library A presented μM inhibition. Using this information, the activity of each component was determined independently. Conditions: (a) t-butyl ester of peptide$_1$ (1.1 eq), t-butyl ester of peptide$_2$ (1.1 eq), Et_3N (3 eq), CH_2Cl_2, 1.5 h. (b) neat TFA, 3 h.

Application of two-color colorimetry to solution-based core molecule display has rapidly identified an inhibitor of HLE. While applied here for a specific core and protease, these methods should be directly applicable to a variety of medicinally-significant *exo*-proteases.

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[14] Modeling was performed in conjunction with Mike Pique. Our approach was modeled after this particular inhibitor because a crystal structure of its complex to HLE (pdb file = 1ppg) has been determined. Wei, Z.; Mayr, I.; Bode, W. *FEBS Lett.* **1988**, *234*, 376.

7.4 Experimental

Enzymes

All enzymes were purchased from Sigma [product number] and standardized as follows (enzyme): (trypsin) One unit or 16.6 μg of Type IX from porcine pancreas [T 0134] converted 1 μmol / min of BAEE[15] to *N*-α-benzoylarginine at pH 7.6 at 23 °C. (Kallikrein) One unit or 25.6 μg of kallikrein from porcine pancreas [K 3627] converted 1 μmol / min of BAEE at pH 8.7 at 25 °C. (Cathepsin B) One unit or 30.3 μg of Cathepsin B from bovine spleen [C 6286] hydrolyzed 1 μmol / min of *N*-α-CBZ-lysine-*p*-nitrophenylester at pH 5.0 at 25 °C. (HLE) One unit or 1.2 μg of elastase from human leukocytes [E 8140] released 1 μmol / min of *p*-nitrophenol from N-*t*-BOC-L-alanine-*p*-nitrophenyl ester at pH 6.5 at 37 °C.

Dyes and Substrates

Compound **2** was prepared using a procedure described in La Clair, J. J. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 325. Attachment of **2** to the various peptides was accomplished using standard methods as described in La Clair, J. J. *J. Am. Chem. Soc.* **1997**, *119*, 7676 and Cotenescu, M. G.; La Clair, J. J. *J. Biotech.* **1999**, *in review.*

Apparatus

A 300 μL 96-welled PTFE Teflon plate (Berghof/America) was covered with 1-2 mm thick plate of PTFE Teflon and sealed by wrapping with packing tape.

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[15] For kinetic data on these and other substrates see (a) Schomburg, D.; Salzmann, M. *Enzyme Handbook*; Springer-Verlag: Berlin, 1991. (b) Hofmann, J.; Sernetz, M. *Anal. Biochem.* **1983**, *131*, 180. (c) Assfalg-Machleidt, I.; Rothe, G.; Klingel, S.; Banati, R.; Mangle, W. F.; Valet, G.; Machleidt, W. *Biol. Chem. Hoppe-Seyler* **1992**, *373*, 433-440.

Lamp

Excitation was provided at 366 nm with a common hand-held UV lamp (Mineralight Model UVGL-58). As visual detection is subjective, the detection limit was defined as producing a least 0.2 μM mineral oil solution of **2**. Short-wave lamps with excitation at 254 nm can also be used, albeit with ~ 30% efficiency.

Partitioning

The partitioning of each material was verified by examining the absorption and fluorescence in both layers of mixtures containing 50 μL of 0.1 μM mineral oil solution of **2** and 1 mL of the given buffers or 1 mL of 2 μM solutions of peptide conjugates in appropriate buffers with 50 μL of mineral oil, which were shaken at 255 rpm for 3 hr at 37 °C in 10 ml PTFE Teflon Oak Ridge Centrifuge Tubes (Fisher). For all materials, the absorption or fluorescence in the opposing layer was indistinguishable from background.

Spectroscopic properties

Absorption and fluorescence spectra were collected using solutions which ranged from 0.1–10 μM, 6 repeats were collected and averaged. Absorption spectra were collected on an HP8452 UV/Vis diode ray spectrometer and the fluorescence data on an SLM SPF-500. All quantum yields (Φ_f) were standardized to 0.70 for rhodamine B in ethanol.

General test procedure

A 11.1 μM stock solution of each substrate was prepared by diluting 222 μL of a 50 mM solution of each substrate in spectral grade DMF (Aldrich Chemical Co.) to a total volume of 1000 mL with the appropriate buffer. This solution was readily stored for up to 1 month at -20 °C. A stock of each enzyme was prepared at 10-fold greater concentration than that chosen to examine. One μL of mineral oil [8012-95-1] (Aldrich Chemical Co.) was added to each well using a 10 μL gas tight syringe. Subsequently, each well was charged with

180 μL of the substrate stock in the appropriate buffer, providing a final substrate concentration of 10 μM. To each respective well, 20 μL of the 10-fold concentrated enzyme stock was added to the corresponding wells. Each plate was covered with a thin plate of Teflon and then sealed with tape. Each assembly was positioned vertically and shaken at 37 °C at 256 rpm. Upon completion, the plate was opened and placed under a small UV-lamp.

Spectroscopic analysis was performed by adding 50 μL of spectroscopic grade *n*-heptane followed by immediate freezing at -20 °C. The organic layer was then readily pipetted away from the frozen aqueous layer. Each well was transferred to a quartz 96-well plate warmed to room temperature and read in a plate reader (Molecular Devices Spectra Max 250 monitored on-line by attachment to Power Macintosh 7200/110). Eight runs were collected and their average was used. Each enzyme was examined under the following conditions (enzyme): (trypsin) 10 mM Tris-HCL pH 7.8 at 25 °C; (kallikrein) 50 mM Tris at pH 9.0 at 37 °C; (cathepsin B) 100 mM potassium phosphate, pH 6.0 at 25 °C; (HLE) 10 mM Tris-HCl, pH 7.5 at 25 °C.

Kinetic measurements

K_m was detetermined for each enzyme-substrate pair by running a series of 24 substrate concentrations ranging from 0.1 nM to 100 μM and comparing this data with 10 mU, 1 mU, 100 μU and 10 μU units of enzyme. This was most efficiently achieved on a single 96-well plate. Eight runs were collected for each enzyme. Quantification of each reactions was determined by adding 50 μL of spectral grade *n*-heptane, shaking for 2 minutes, freezing the aqueous layer, transferring the organic layer to a quartz 96-well plate and examining its absorption on a plate reader. The data were tabulated and the K_m values determined using Eadie-Hofstee, Hanes-Woolf, and Johansen-Lumry plots using Enzyme Kinetics v1.1. Data presented were obtained using the Eadie-Hofstee method. K_{cat} was

determined at 10 μM substrate and was examined over a series of 10 enzyme concentrations, ranging from 1 μU to 1 mU.

Screening for trypsin inhibition

An 80 μM stock solution of Z-Ile-Pro-Arg-**2** (**1a**) was prepared by diluting 10 μL of an 8 mM solution of **1a** in spectral grade DMSO (Aldrich Chemical Co.) in a total volume of 1000 mL with 10 mM Tris-HCl at pH 7.8. This solution was readily stored for up to 4 months at -20 °C. A 10 mM stock solution of each candidate (see Table 1 below) was prepared in 10 mM Tris-HCl at pH 7.8. A fresh 0.1 mU stock solution of trypsin was prepared just prior to use in 10 mM Tris-HCl at pH 7.8 and was standardized as described above. 100 μL of both the candidate inhibitor and trypsin stock solution were loaded into a 96-well Teflon plate. After 30 min incubated at 37 °C, 10 μL of mineral oil was added *via* syringe. Next, each well was charged with 180 μL of the substrate stock in the appropriate buffer, providing a final substrate concentration of 10 μM. To each respective well, 20 μL of the substrate stock solution was added. The plate was covered with a thin teflon plate, sealed, and shaken vigorously for 5 minutes. Upon completion, the plate was opened and placed under a small UV-lamp.

Spectroscopic analysis of the four samples exhibiting inhibition (see Figure 2) were compared to two controls by adding 50 μL of spectroscopic grade *n*-heptane followed by immediate freezing at -20 °C. The organic layer was then readily pipetted away from the frozen aqueous layer. Each well was transferred to a quartz 96-well plate warmed to room temperature and read in a plate reader (Molecular Devices Spectra Max 250 monitored on-line by attachment to Power Macintosh 7200/110). Eight runs were collected and averaged.

1,1	**4-aminobenzamidine**	5,1	4-methylaminophenol
1,2	**phenylmethylsulfonylfluoride**	5,2	2-methyl-3-nitroanisole
1,3	**N-tosyl-L-phenylalanine chloromethylketone**	5,3	1-methylpyrrolidine
1,4	acetophenone	5,4	1-methylpiperidine
1,5	acetovanillone	5,5	8-methylquinoline
1,6	adenosine	5,6	methyl-S-lactate
1,7	adenine	5,7	2-methylcyclohexanone
1,8	3-aminobenzoic acid	5,8	1-napthol
1,9	5-aminoindane	5,9	2-napthol
1,10	2-aminophenol	5,10	4-nitrobenzaldoxime
1,11	3-aminophenylacetic acid	5,11	3-nitrobenzoic acid
1,12	arabanose	5,12	4-nitrobenzoic acid
2,1	anisole	6,1	nicotinic acid
2,2	benzoin	6,2	4-nitrocatechol
2,3	4-benzylpyridine	6,3	4-nitrocinnamic acid
2,4	benzylisobutylketone	6,4	3-nitrophenol
2,5	*t*-butylacetate	6,5	4-nitrophenylacetic acid
2,6	caffeine	6,6	3-nitrosalicylic acid
2,7	ε-caprolactam	6,7	5-nitrosalicylic acid
2,8	camphor	6,8	7-nitrotetralone
2,9	chrysene	6,9	1,8-octanediol
2,10	*t*-cinnamic acid	6,10	oxalic acid
2,11	citric acid	6,11	1,5-pentanediol
2,12	cinnamonitrile	6,12	4-pentenoic acid
3,1	*p*-cresol	7,1	phenol
3,2	4-cyanophenylacetonitrile	7,2	**N-phenylmalemide**
3,3	cytidine	7,3	piperazine
3,4	dansylglycine	7,4	2-picoline
3,5	9-decene-1-ol	7,5	phthalic acid
3,6	diacetone glucose	7,6	3-piperidine methanol
3,7	dibenzylketone	7,7	propionitrile
3,8	diethyl-L-tartrate	7,8	2-pyrrolidinone
3,9	3.4-dimethyoxyacetophenone	7,9	4-pyrrolidinopyridine
3,10	2,4-dinitroaniline	7,10	resorcinol
3,11	diphenylacetic acid	7,11	reserpine
3,12	2,2'-dipyridyl	7,12	succinic acid
4,1	2-dodecanol	8,1	solketal
4,2	ethylcyanoacetate	8,2	spermidine
4,3	9-fluorenone	8,3	tartaric acid
4,4	glycidol	8,4	2-*p*-toluenesulfonylethanol
4,5	1-indanone	8,5	m-toluic acid
4,6	limonene	8,6	tetracycline
4,7	maleic acid	8,7	vanillin
4,8	4-methoxybenzylamine	8,8	uridine
4,9	methyl-α-D-glucopyranoside	8,9	δ-valerolactone
4,10	methyl-β-D-arabinopyranoside	8,10	veratrylamine
4,11	7-methylcoumarin	8,11	xanthone
4,12	1-methylcyclohexanol	8,12	*blank*

Table 1. A listing of compounds tested for trypsin inhibition (see Figure 2). Inhibitors are shown in **bold**. Three known inhibitors were included in the first three positions, and no inhibitor candidate was included in the last position as a control. Position is given by (row, column).

Screening for HLE inhibition

The procedures used for the trypsin inhibition were applied with the following modifications. A 40 μM stock solution of L-Tyr-Leu-Val-**2** (**1e**) was prepared by diluting 10 μL of a 4 mM solution of **1e** in spectral grade DMSO (Aldrich Chemical Co.) in total volume of 1000 mL with 10 mM Tris-HCl at pH 7.5. A 10 mM–10 μM stock solution of each candidate was prepared in 10 mM Tris-HCl at pH 7.5. A fresh 50 μU stock of HLE was prepared just prior to use in 10 mM Tris-HCl at pH 7.5 and was standardized as described previously.

Kinetic determination of HLE inhibition

K_i values were collected by screening the previously-described HLE assay with an array of 8 substrate concentrations ranging from 0.1 nM to 100 μM and 5 inhibitor concentrations ranging from 50 μM to 5 mM. Three runs were collected for each inhibitor and their average was calculated. Data was interpreted with Eadie-Hofstee plots using Enzyme Kinetics v1.1. This inhibition was compared to a previously reported procedure[16] which monitored the inhibition of HLE hydrolysis of MeO-Suc-Ala-Ala-Pro-Ala-*p*-nitroanilide with ICI 200880. Inhibition with this substrate required prolonged development (>5 h) and an increased amount of HLE, thereby providing a partial explanation for the difference in the kintetics of inhibition given by the two methods (86 μM for **1e** vs. 128 nM for *p*-nitroanilide, see Scheme 1).

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[16] Regan, J.; McGarry, D.; Bruno, J.; Green, D.; Newman, J.; Hsu, C. Y.; Kline, J.; Barton, J.; Travis, J.; Choi, Y. M.; Volz, F.; Pauls, H.; Harrison, R.; Zilberstein, A.; Ben-Sasson, S. A.; Chang. M. *J. Med. Chem.* **1997**, *40*, 3408.

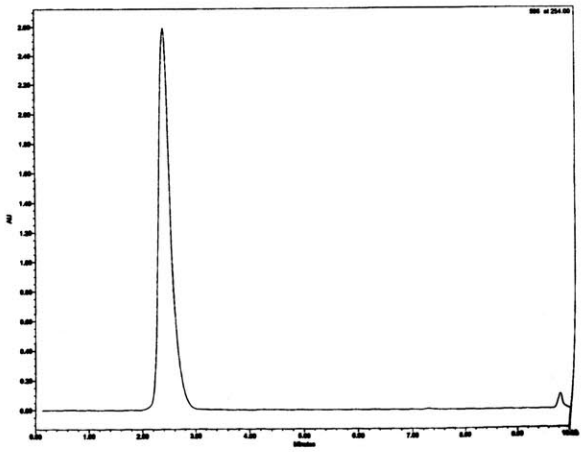
HLE library synthesis

All libraries were prepared similarly. To a solution of two building blocks (0.099 mmol each) in 3 mL CH_2Cl_2 was added triethylamine (0.3 mL) and a solution of xanthene diacidchloride **3** (30 mg, 0.090 mmol) in 1 mL CH_2Cl_2. The solution was stirred for 1.5 h, then was diluted with CH_2Cl_2 and was washed with water (3 x). The organic phase was dried over magnesium sulfate and was filtered. The solvent was removed by rotary evaporation and the residue was deprotected with 10 mL neat TFA for 3 h. The TFA was removed by rotary evaporation and the library was freeze-dried, yielding the library as a pale tan foam.

Single compounds **4–6** were isolated from resynthesized library **A** by preparative HPLC. Each compound was identified by LRMS (ESI +) and their purity was shown to be >95% by analytical HPLC.

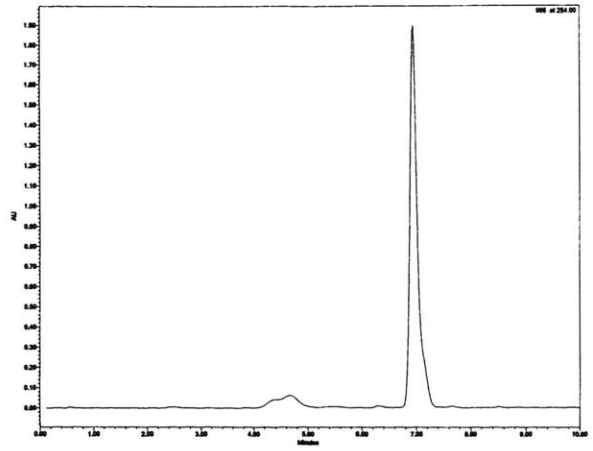
Ala-Ala/Ala-Ala adduct of xanthene diacid chloride (4).

LRMS (ESI +) m/z calcd for $[C_{29}H_{34}N_4O_9 + H]^+$ 583 found 583.



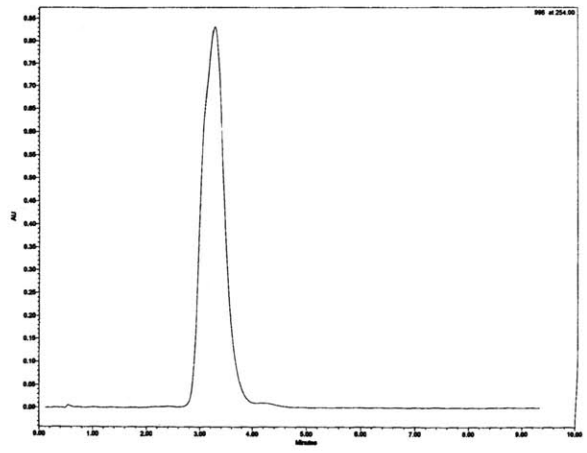
Val/Val adduct of xanthene diacid chloride (**5**).

LRMS (ESI +) m/z calcd for $[C_{27}H_{32}N_2O_7 + H]^+$ 497 found 497.



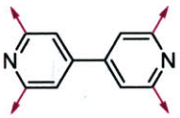
Ala-Ala/Val adduct of xanthene diacid chloride (**6**).

LRMS (ESI +) m/z calcd for $[C_{28}H_{33}N_3O_8 + H]^+$ 540 found 540.



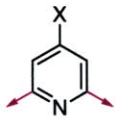
Appendix: Diversity Calculations

A.1 Bipyridine tetra-substituted core



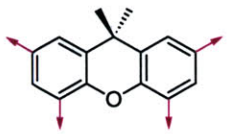
Combination Symm. Mult Combin Rule m	AAAA 1 m!/1!(m-1)!	AAAB 4 m!/2!(m-2)!	AABC 6 m!/3!(m-3)!	ABCD 3 m!/4!(m-4)!	Total
1	1	-	-	-	1
2	2	4	-	-	6
3	3	12	6	-	21
4	4	24	24	3	55
5	5	40	60	15	120
6	6	60	120	45	231
7	7	84	210	105	406
8	8	112	336	210	666
9	9	144	504	378	1035
10	10	180	720	630	1540
11	11	220	990	990	2211
12	12	264	1320	1485	3081
13	13	312	1716	2145	4186
14	14	364	2184	3003	5565
15	15	420	2730	4095	7260
16	16	480	3360	5460	9316
17	17	544	4080	7140	11781
18	18	612	4896	9180	14706
19	19	684	5814	11628	18145
20	20	760	6840	14535	22155
21	21	840	7980	17955	26796
22	22	924	9240	21945	32131
23	23	1012	10626	26565	38226
24	24	1104	12144	31878	45150
25	25	1200	13800	37950	52975

A.2 Pyridine di-substituted core



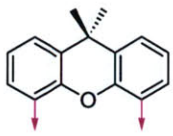
Combination Symm. Mult Combin Rule m	AA 1 m!/1!(m-1)!	AB 1 m!/2!(m-2)!	Total
1	1	-	1
2	2	1	3
3	3	3	6
4	4	6	10
5	5	10	15
6	6	15	21
7	7	21	28
8	8	28	36
9	9	36	45
10	10	45	55
11	11	55	66
12	12	66	78
13	13	78	91
14	14	91	105
15	15	105	120
16	16	120	136
17	17	136	153
18	18	153	171
19	19	171	190
20	20	190	210
21	21	210	231
22	22	231	253
23	23	253	276
24	24	276	300
25	25	300	325

A.3 Xanthene tetra-substituted core



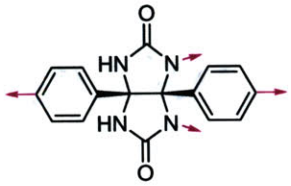
Combination Symm. Mult Combin Rule m	AAAA 1 m!/1!(m-1)!	AAAB 8 m!/2!(m-2)!	AABC 18 m!/3!(m-3)!	ABCD 12 m!/4!(m-4)!	Total
1	1	-	-	-	1
2	2	8	-	-	10
3	3	24	18	-	45
4	4	48	72	12	136
5	5	80	180	60	325
6	6	120	360	180	666
7	7	168	630	420	1225
8	8	224	1008	840	2080
9	9	288	1512	1512	3321
10	10	360	2160	2520	5050
11	11	440	2970	3960	7381
12	12	528	3960	5940	10440
13	13	624	5148	8580	14365
14	14	728	6552	12012	19306
15	15	840	8190	16380	25425
16	16	960	10080	21840	32896
17	17	1088	12240	28560	41905
18	18	1224	14688	36720	52650
19	19	1368	17442	46512	65341
20	20	1520	20520	58140	80200
21	21	1680	23940	71820	97461
22	22	1848	27720	87780	117370
23	23	2024	31878	106260	140185
24	24	2208	36432	127512	166176
25	25	2400	41400	151800	195625

A.4 Xanthene di-substituted core



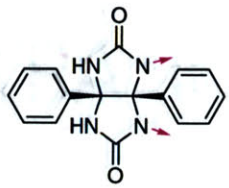
Combination Symm. Mult Combin Rule m	AA 1 m!/1!(m-1)!	AB 1 m!/2!(m-2)!	Total
1	1	-	1
2	2	1	3
3	3	3	6
4	4	6	10
5	5	10	15
6	6	15	21
7	7	21	28
8	8	28	36
9	9	36	45
10	10	45	55
11	11	55	66
12	12	66	78
13	13	78	91
14	14	91	105
15	15	105	120
16	16	120	136
17	17	136	153
18	18	153	171
19	19	171	190
20	20	190	210
21	21	210	231
22	22	231	253
23	23	253	276
24	24	276	300
25	25	300	325

A.5 Glycoluril tetra-substituted core



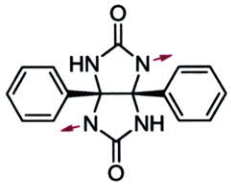
Combination Symm. Mult Combin Rule m	AAAA 1 m!/1!(m-1)!	AAAB 14 m!/2!(m-2)!	AABC 30 m!/3!(m-3)!	ABCD 24 m!/4!(m-4)!	Total
1	1	-	-	-	1
2	2	14	-	-	16
3	3	42	30	-	75
4	4	84	120	24	232
5	5	140	300	120	565
6	6	210	600	360	1176
7	7	294	1050	840	2191
8	8	392	1680	1680	3760
9	9	504	2520	3024	6057
10	10	630	3600	5040	9280
11	11	770	4950	7920	13651
12	12	924	6600	11880	19416
13	13	1092	8580	17160	26845
14	14	1274	10920	24024	36232
15	15	1470	13650	32760	47895
16	16	1680	16800	43680	62176
17	17	1904	20400	57120	79441
18	18	2142	24480	73440	100080
19	19	2394	29070	93024	124507
20	20	2660	34200	116280	153160
21	21	2940	39900	143640	186501
22	22	3234	46200	175560	225016
23	23	3542	53130	212520	269215
24	24	3864	60720	255024	319632
25	25	4200	69000	303600	376825

A.6 Glycoluril *cis*-di-substituted core



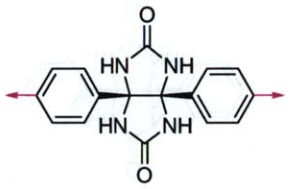
Combination Symm. Mult Combin Rule m	AA 1 m!/1!(m-1)!	AB 2 m!/2!(m-2)!	Total
1	1	-	1
2	2	2	4
3	3	6	9
4	4	12	16
5	5	20	25
6	6	30	36
7	7	42	49
8	8	56	64
9	9	72	81
10	10	90	100
11	11	110	121
12	12	132	144
13	13	156	169
14	14	182	196
15	15	210	225
16	16	240	256
17	17	272	289
18	18	306	324
19	19	342	361
20	20	380	400
21	21	420	441
22	22	462	484
23	23	506	529
24	24	552	576
25	25	600	625

A.7 Glycoluril *trans*-di-substituted core



Combination Symm. Mult Combin Rule m	AA 1 m!/1!(m-1)!	AB 1 m!/2!(m-2)!	Total
1	1	-	1
2	2	1	3
3	3	3	6
4	4	6	10
5	5	10	15
6	6	15	21
7	7	21	28
8	8	28	36
9	9	36	45
10	10	45	55
11	11	55	66
12	12	66	78
13	13	78	91
14	14	91	105
15	15	105	120
16	16	120	136
17	17	136	153
18	18	153	171
19	19	171	190
20	20	190	210
21	21	210	231
22	22	231	253
23	23	253	276
24	24	276	300
25	25	300	325

A.8 Glycoluril di-(phenyl-subsituted) core



Combination Symm. Mult Combin Rule m	AA 1 m!/1!(m-1)!	AB 1 m!/2!(m-2)!	Total
1	1	-	1
2	2	1	3
3	3	3	6
4	4	6	10
5	5	10	15
6	6	15	21
7	7	21	28
8	8	28	36
9	9	36	45
10	10	45	55
11	11	55	66
12	12	66	78
13	13	78	91
14	14	91	105
15	15	105	120
16	16	120	136
17	17	136	153
18	18	153	171
19	19	171	190
20	20	190	210
21	21	210	231
22	22	231	253
23	23	253	276
24	24	276	300
25	25	300	325