Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11138–11142, November 1994
Chemistry

"Libraries from libraries": Chemical transformation of combinatorial libraries to extend the range and repertoire of chemical diversity

(transformation/peralkylated combinatorial libraries/permethylated peptides/peptide library/antimicrobial)

JOHN M. OSTRESH*, GREGORY M. HUSAR*, SYLVIE E. BLONDELLE*, BARBARA DÖRNER*, PATRICIA A. WEBER†, AND RICHARD A. HOUGHTEN*‡

*Torrey Pines Institute for Molecular Studies, San Diego, CA 92121; and †Houghten Pharmaceuticals, San Diego, CA 92121

Communicated by Bruce Merrifield, April 21, 1994 (received for review November 15, 1993)

ABSTRACT **The generation of diverse chemical libraries using a "libraries from libraries" concept is described. The central features of the approaches presented are the use of well-established solid-phase synthesis methods for the generation of combinatorial libraries, combined with the chemical transformation of such libraries while they remain attached to the solid support. The chemical libraries that are generated by this process have very different physical, chemical, and biological properties compared to the libraries from which they were derived. A wide range of chemical transformations are possible for peptide-based or other libraries, and an almost unlimited range of useful chemical diversities can be envisioned. In the example presented, the amide functionalities in an existing combinatorial library made up of peptides were permethylated while the library remained attached to the solid-phase support used in its synthesis. After removal of the permethylated mixtures from their solid support, this library, now lacking the typical -CONH- amide bonds of peptides, can be tested in solution with virtually all existing assay systems to identify individual compounds having specific biological activities of interest. An illustration of the use of such libraries is presented, in which the described permethylated library was used to identify individual permethylated compounds having potent antimicrobial activity against Gram-positive bacteria.**

Recent innovations in peptide chemistry and molecular biology have enabled libraries consisting of tens to hundreds of millions of peptide sequences to be prepared and used to identify highly active, individual sequences. Such libraries can be divided into three broad categories. (*i*) One category of libraries involves the chemical synthesis of soluble nonsupport-bound peptide and peptoid libraries (1–3). (*ii*) A second category involves the chemical synthesis of support-bound peptide libraries composed of L- or D-amino acid sequences presented on solid supports such as plastic pins (4), resin beads (5), or cotton (6). (*iii*) A third category uses molecular biology approaches to prepare peptides or proteins on the surface of filamentous phage particles or plasmids (7). More recently, the production of small collections of nonpeptidic compounds has been described (8–10).

As first presented by this laboratory, soluble, nonsupport-bound peptide libraries [termed synthetic peptide combinatorial libraries (SPCLs)] appear to be usable in virtually all *in vitro* and even *in vivo* assays. Combinatorial libraries of peptides composed of entirely L-amino acids, entirely D-amino acids, or mixtures of L-, D-, and unnatural amino acids have been developed by using this approach. The successful use of these libraries has been reported for the study of antibody/antigen interactions (1, 2) and for the development of receptor-active opioid peptides (11, 12), enzyme inhibitors (6), and antimicrobial agents (1). The recent development of the soluble positional scanning (PS) approach for the production and screening of SPCLs (2, 12) enables individual, biologically active peptides to be identified in a single screening assay.

In a continuing effort to expand the available repertoire of chemical diversities, we present here an example of a soluble chemical library obtained by the chemical transformation of an existing peptide library. This library, prepared in a positional scanning format, is composed of fully permethylated compounds derived from the direct chemical modification of resin-bound peptide libraries. To our knowledge, a study of individual peptides being permethylated while attached to solid-phase synthesis resins has not yet been reported. The use of chemically transformed libraries is illustrated here by the use of this permethylated PS-SPCL for the identification of potent individual compounds selectively active against the Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus sanguis*.

MATERIALS AND METHODS

The benzyl protecting group was used for the side-chain protection of asparagine, glutamate, serine, and threonine; methoxybenzyl for cysteine; dinitrophenyl for histidine; chlorobenzyloxycarbonyl for lysine; sulfoxide for methionine; tosyl for arginine; formyl for tryptophan; and bromobenzyloxycarbonyl for tyrosine. Other reagents and materials used have been described (13).

Permethylation of Protected Peptides. Twenty model peptide resins (110 mg, 0.1 milliequivalent each) with defined sequences (represented as *O*GGFL-resin, "*O*" individually representing each of the 20 naturally occurring amino acids) were prepared on methylbenzhydrylamine resin (mBHA) (0.90 milliequivalent per g) using *t*-butoxycarbonyl (Boc) chemistry combined with simultaneous multiple peptide-synthesis techniques as described (13). After removal of the final *N*-α-Boc protecting group, each of the side-chain-protected resins were then permethylated as described below. The resin-bound PS-SPCL was prepared as described (2, 12).

In a typical example, AGGFL-mBHA resin (90 mg, 0.1 milliequivalent), contained within an individual polypropylene mesh packet (13), was shaken on a reciprocating shaker (Eberbach, Ann Arbor, MI) at 25°C for 16 hr in a 0.25 M

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Abbreviations: PS, positional scanning; RP-HPLC, reversed-phase high-performance liquid chromatography; SPCL, synthetic peptide combinatorial library; MRSA, methicillin-resistant *Staphylococcus aureus*. All amino acids used were of the L configuration unless otherwise noted.

‡To whom reprint requests should be addressed.

solution of NaH/dimethyl sulfoxide (32 ml, 8 milliequivalents). Neat methyl iodide (1.5 ml, 24 milliequivalents) was then added to the reaction mixture, and the methylation reaction allowed to proceed for 15 min at 25°C. After successive washes with dimethylformamide (three times, 5 ml), isopropanol (two times, 5 ml), dichloromethane (three times, 5 ml), and methanol (once, 5 ml), the resin was dried under high vacuum. The resin-bound permethylated peptide was cleaved by using 7.5% (vol/vol) anisole/HF (5 ml) for 1 hr at 0°C, dried under high vacuum, extracted with 10 ml of water, and lyophilized. The permethylated peptide was assayed for purity by reversed-phase high-performance liquid chromatography (RP-HPLC) and identified by laser desorption-mass spectral analysis (Kratos). Individual compounds were purified using preparative RP-HPLC. The PS-SPCL was permethylated, cleaved, and analyzed similarly.

Biological Assays. Individual permethylated compounds and their nonpermethylated counterparts were assayed for their resistance to proteolytic breakdown by trypsin and chymotrypsin. The assays were done in 1 ml of 0.1 M NH_4HCO_3, pH 7.8, at room temperature for 16 hr at a peptide concentration of 1.0 mg/ml. Enzyme-to-peptide concentration was 1:50. The degradation reaction was monitored by RP-HPLC.

The strains *S. aureus* ATCC 29213, methicillin-resistant *S. aureus* (MRSA) ATCC 33591, *S. sanguis* ATCC 10566, *Escherichia coli* ATCC 25922, and *Candida albicans* ATCC 10231 were used in the bioassays. The assays were done in 96-well tissue culture plates (Costar) as described (14).

Hemolytic activity of the individual compounds identified was determined by using a 0.25% suspension of human red blood cells as described (15).

RESULTS

Optimization of Permethylation Conditions. Various methods for permethylation have been described (16, 17). In such permethylations, strongly basic conditions are reported to favor N-methylation over O-methylation. Although unreported in these earlier studies, the synthetic conditions used also permit permethylation of peptides while they remain attached to the solid-phase resins used in their synthesis. The strength of the solid-phase approach (18) is that all excess reagents can be removed by simple wash procedures.

Satisfactory permethylation conditions for resin-bound peptides were studied using AGGFL-NH_2 due to its ease of synthesis, nonreactive side chains, and its availability from other ongoing studies (12). Temperature, reaction time, reagent ratios, and solvents were studied to determine the most effective and mildest conditions for the formation of the amide anions and their subsequent methylation. Temperatures studied ranged from 25°C to 60°C. Reaction times tested for the generation of the amide anions ranged from 20 min to 16 hr. It was found that while complete amide anion formation occurred within 16 hr at 25°C, higher temperatures led to degraded products, as evidenced by mass spectral analysis and RP-HPLC. Methylation reaction times examined ranged from 1 to 30 min. Because permethylation of a free α-amino peptide leads to both methylation of the amide backbone and quaternary salt formation, it was necessary to study the relative rates of these two reactions. It was found that while methylation of the backbone amides was complete within the first minute, longer reaction times were required for the quaternary salt formation. Complete methylation of the backbone amide anions within the first minute was demonstrated with the use of Ac-AGGFL-NH_2. Of the conditions tested, a 16-hr room-temperature treatment of the resin-bound protected peptides, using a 10-fold excess of NaH in dimethyl sulfoxide over the reactive sites of the resin-bound peptide, followed by a 15-min treatment of the resulting amide anions with a 30-fold excess of methyl iodide over the reactive sites, yielded the best results (Fig. 1). Under these conditions AGGFL-NH_2 was obtained in permethylated form in >90% yield and purity.

Permethylation of Model Peptides. Once the permethylation conditions had been selected, 20 model peptide resins (represented as *O*GGFL-NH_2, where "*O*" is one of the 20 naturally occurring L-amino acids) were synthesized. Resin compartmentalization (13) permits the simultaneous permethylation of multiple peptide resins. Mass spectral and RP-HPLC analysis of an HF-cleaved aliquot of the nonpermethylated starting resins indicated that the average crude purity of the nonpermethylated peptides was >95%. The peptide resins were permethylated to determine the stability and susceptibility to modification of the 20 naturally occurring L-amino acids. Mass spectral analyses showed that the nitrogen of each amide bond was methylated, including the C-terminal amide resin linkage. In addition to the quaternization of the α-amino group, small amounts of the mono- and dimethylated α-amino products were also formed, generally to an extent of <15%. While a preliminary exhaustive N-α-methylation of the model peptides before the sodium hydride/methyl iodide treatment eliminated the mono- and dimethylated products, the strongly basic conditions used in the sodium hydride treatment gave partial Hoffman elimination of the quaternary salts formed. In contrast, while exhaustive N-α-methylation after the initial permethylation did not yield the byproducts associated with Hoffman elimination, the quaternization reaction was not driven to completion under the conditions used.

The subsequent cleavage of the permethylated model peptides from the resin yielded the desired products in ≈90% purity for amino acids having nonreactive side chains (Fig. 2). Table 1 summarizes the number of methyl groups incorporated (including side-chain modifications) for each of the other analogs. Similar results were obtained using peptide resins in which the amino acid at the second position was varied, demonstrating the lack of a positional dependence on the modification of the side chains (data not shown).

Although the major product of the permethylation of the model peptide containing cysteine was the methyl thioether,

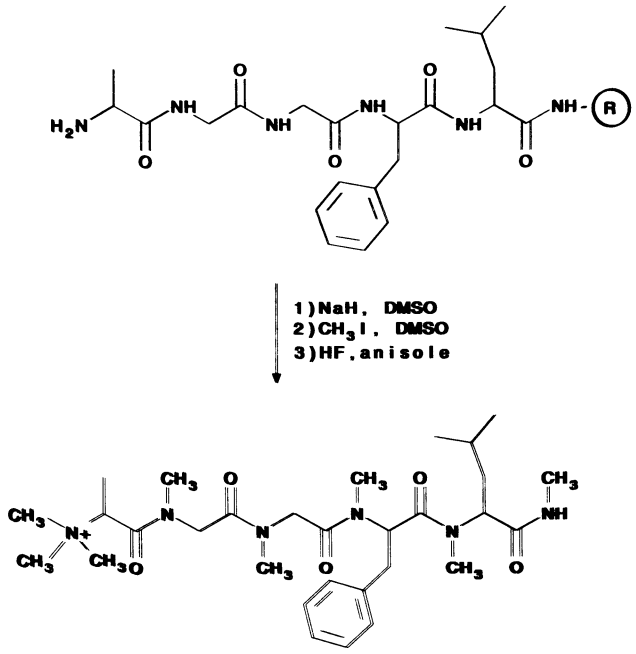

FIG. 1. Illustration of the method used for the permethylation of peptides. DMSO, dimethyl sulfoxide.

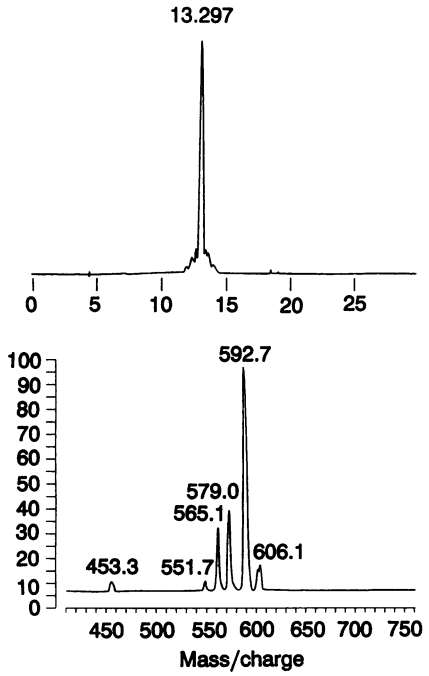

FIG. 2. RP-HPLC and mass spectral analysis of the permethylated form of SGGFL-NH_2.

the presence of unmodified side chain suggests incomplete removal of the methoxybenzyl protecting group. It was found that the benzyl ester side-chain, protecting groups of aspartate and glutamate were removed during the sodium hydride treatment. The lower purity found suggests that internal cyclization of these compounds occurred (19). The dinitrophenyl protecting group of histidine was found to be removed by the sodium hydride treatment. Methylation of the histidine side chain gave two major products of identical molecular weight, which are assumed to be the τ- and π-imidazole side-chain methylation products. The low purity of KGGFL-NH_2, along with the presence of low-molecular-weight species as evidenced by mass spectral analysis, indicates additional byproducts are due to internal cleavage by nucleophilic attack of the ε-amino group on the peptide backbone.

The sulfoxide of methionine, which is stable to the reaction conditions, also serves to prevent the internal cleavage of the peptides via methyl sulfonium salts of methionine (20). Unexpectedly, some free methionine product was found resulting from reduction of the sulfoxide. The stability of the tosyl group of arginine to the permethylation reaction resulted in three methyl groups being incorporated onto the guanidinium group of the arginine-containing peptide. The tosyl group was then removed during the standard high HF cleavage procedure (21). Methylation of the rings of aromatic amino acids (22) was not seen.

Treatment of the permethylated peptide resins by using standard low HF conditions (23) was found to result in partial cleavage of permethylated peptides from the resin. The lability of secondary amide bonds relative to primary amide bonds under acidic conditions is well documented (24, 26). In addition, the peptide remaining on the resin after low HF treatment was found to be substantially degraded as determined by RP-HPLC and mass spectral analysis after HF cleavage.

Racemization Study. From the literature, it is anticipated that the conditions used here for permethylation would result in minimal racemization or C_α-methylation (17). Because the increased acidity of the C_α-hydrogen of aromatic amino acids such as phenylalanine (17) makes them more prone to racemization and/or C_α-methylation, a test series was devised in which the four possible stereoisomers of GGFL-NH_2 were synthesized and analyzed by RP-HPLC. An aliquot of the GGFL-NH_2 resin was treated with NaH/dimethyl sulfoxide to form the amide anions and then quenched by washing with 1% water/dimethyl sulfoxide. After amino acid cleavage from the resin, the maximum percentage of the D/L, L/D enantiomeric pair, as seen by RP-HPLC, was <0.75%, establishing that the extent of racemization, and therefore potential C_α-methylation, was <1% (Fig. 3).

Enzymatic Susceptibility. The stability of N-permethylated compounds to proteolysis was examined for two permethylated sequences, AGGFL-NH_2 and RGGFL-NH_2; their nonpermethylated equivalents were used as controls. Treatment of these four compounds by trypsin and chymotrypsin was monitored by RP-HPLC and mass spectral analysis. Rapid cleavage of AGGFL-NH_2 by chymotrypsin and RGGFL-NH_2 by trypsin was observed (<1 hr), whereas <1% cleavage of the equivalent permethylated peptides was seen after overnight enzyme exposure (3).

Preparation of the Permethylated Library. The six separate positional sublibraries of a resin-bound PS-SPCL (2), each consisting of 20 separate peptide mixtures, were permethylated and cleaved as described. The resulting library consisted of 120 hexamer mixtures, in which one position was defined by each of the 20 permethylated amino acids (represented as *O*), with the remaining five positions made up of mixtures of 18 permethylated amino acids (represented as *X*;

Table 1. Side-chain modifications of amino acids in permethylated peptides

Parent sequence	Parent M_r	Methylated M_r found	Number methyls	Side-chain modification	Nonmethylated purity,† %	Methylated* purity,† %
CGGFL-NH_2	494.8	620.8	9	Methyl thioether	86	50
DGGFL-NH_2	506.7	633.6	9	Methyl ester	97	60
EGGFL-NH_2	520.7	647.8	9	Methyl ester	90	75
HGGFL-NH_2	528.8	655.8	9	Methyl imidazole	98	40
KGGFL-NH_2	519.8	673.0	11	Quaternary salt	99	30
LGGFL-NH_2	504.8	617.2	8	Unmodified	99	81
M[O]GGFL-NH_2	538.8	651.8	8	Unmodified	98	70
NGGFL-NH_2	505.7	646.9	10	Dimethyl amide	85	86
QGGFL-NH_2	519.7	660.0	10	Dimethyl amide	85	80
RGGFL-NH_2	703.3	547.8	11	Trimethyl guanidine	88	75
WGGFL-NH_2	577.8	704.8	9	Methyl indole	98	70
YGGFL-NH_2	554.8	681.5	9	Methyl ether	99	81

*Sum of the mono-, di-, and trimethylated α-amine products.

†Purity of the crude compounds as determined by analytical RP-HPLC. Repetition of these experiments showed a maximum variation of 20% from the values listed. Purities of the unlisted nonpermethylated model peptides were >99%; purities of the unlisted permethylated compounds were >90%. Side chains of the unlisted compounds were unmodified by permethylation treatment.

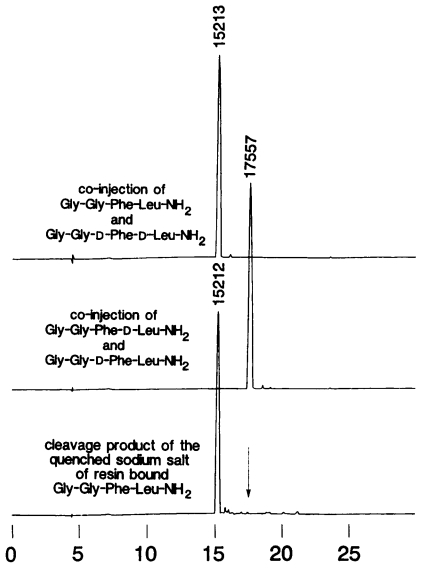

FIG. 3. RP-HPLC analysis of the four stereoisomers of GGFL-NH_2 after the formation and quenching of their amide anions by NaH. Arrow represents the retention time of the D/L and L/D stereoisomers.

cysteine and tryptophan were excluded). Each of the six permethylated sublibraries (represented as pm[*OXXXXX*], pm[*XOXXXX*], pm[*XXOXXX*], pm[*XXXOXX*], pm[*XXXXOX*], and pm[*XXXXXO*]) contained 37,791,360 (20×18^5) permethylated compounds in approximately equimolar amounts.

Antimicrobial Activity. Each of the 120 permethylated mixtures was assayed at an initial concentration of 2.5 mg/ml for its ability to inhibit the growth of *S. aureus*. A number of permethylated mixtures from each of the six libraries inhibited *S. aureus* growth (Fig. 4). None of the mixtures showed significant hemolytic activity or *in vitro* toxicity as determined by an MTT assay using McCoy cells ATCC 1696-CRL [MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (25). The amino acids chosen at each of the defined positions from the most active permethylated mixtures were used to generate a series of individual permethylated compounds. Peptides were thus synthesized and permethylated representing all combinations of the amino acids chosen for the first position (W, F, Y, H, I, L), the second position (F), the third position (W, I, F), the fourth position (W, F), the fifth position (F, H), and the sixth position (F, H). The resultant 144 individual, crude permethylated compounds were screened for their antimicrobial activity. Forty-one compounds had IC_{50} values <50 μg/ml, whereas 51 compounds had IC_{50} values ≥250 μg/ml against *S. aureus*. Although the permethylated forms of FFIFFF-NH_2, FFFFFF-NH_2, and LFIFFF-NH_2 exhibited the greatest activity of the series (Table 2), the quaternary trimethyl ammonium salt of nonpermethylated FFFFFF-NH_2 showed no activity (IC_{50} >250 μg/ml). Similar activities were found against MRSA and *S. sanguis*, whereas none of the permethylated compounds or mixtures exhibited activity against *E. coli* or *C. albicans* (IC_{50} values >600 μg/ml) or showed significant toxicity as evidenced by lysis of red blood cells (<2% hemolysis at 100 μg/ml). These compounds showed activities similar to a range of previously described peptides made up of L-amino acids (1). However, in contrast to the L-amino acid peptides, these compounds appear completely stable to proteolytic enzymes. Mass spectral and RP-HPLC analysis showed that, in some cases, incomplete methylation had occurred. For the permethylated form of FFFFFF-NH_2, similar antimicrobial activity was found after purification, suggesting that incompletely methylated byproducts also had antimicrobial activity.

The potent antimicrobial activity of the permethylated hexaphenylalanine prompted a study to determine the length at which a permethylated polyphenylalanine has the greatest antimicrobial activity. The set of compounds prepared varied in length from one to eight residues. The antimicrobial activities of these compounds against the two Gram-positive bacteria, *S. aureus* and *S. sanguis*, are shown in Table 2. Significant activities were found for those permethylated compounds having a length of at least five residues, with a optimal length of seven residues. In addition, the seven- and eight-residue permethylated polyphenylalanine compounds exhibited similar activities against MRSA.

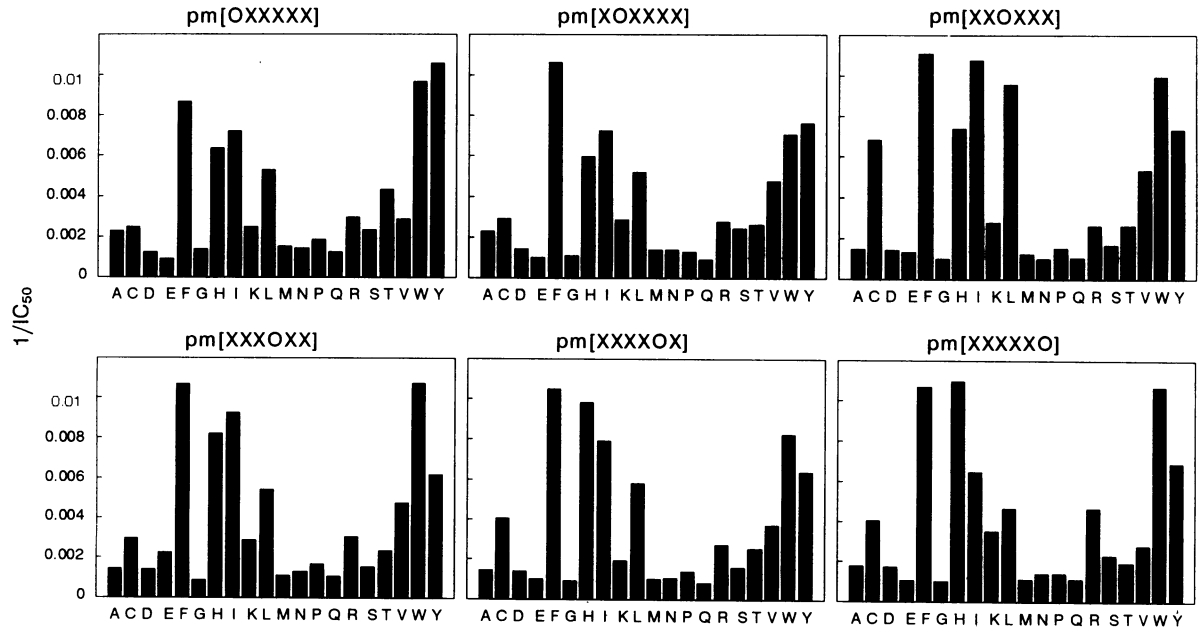

FIG. 4. Antimicrobial activity of the permethylated (pm-) PS-SPCL against *S. aureus*. Each individual bar represents the inverse of the IC_{50} of a permethylated peptide mixture defined in the "*O*" position with one of the 20 naturally occurring amino acids.

Table 2. Antimicrobial and hemolytic activities of permethylated peptides derived from a permethylated SPCL

Parent sequence	*S. aureus*		*S. sanguis*	
	IC_{50}, μg/ml	MIC, μg/ml	IC_{50}, μg/ml	MIC, μg/ml
LFIFFF-NH_2	6	11–15	1.7	3–5
FFIFFF-NH_2	6	11–15	14	20–40
FFFFFF-NH_2	7	11–15	9	15–20
LFFFFF-NH_2	10	21–31	14	20–40
F-NH_2	>500	>500	>500	>500
FF-NH_2	>500	>500	>500	>500
FFF-NH_2	288	>500	446	>500
FFFF-NH_2	116	250–500	85	125–250
FFFFF-NH_2	19	21–31	20	25–30
FFFFFF-NH_2	7	11–15	5	7–8
FFFFFFF-NH_2	2.5	3–4	2.3	3–4
FFFFFFFF-NH_2	5	6–8	7	8–9

MIC, lowest concentration at which no growth is detected after 21-hr incubation.

DISCUSSION

Biologically active peptides can now be readily identified through the use of SPCLs and PS-SPCLs (1, 2, 11, 12). Peptides, however, have limitations as potential therapeutics; this is due to their lack of oral activity, rapid breakdown by proteolytic enzymes, rapid clearance from circulation, and typical inability to pass through the blood–brain barrier to effect central nervous system activity. The generation of libraries consisting of nonpeptidic compounds can be expected to circumvent a number of these limitations. We are pursuing a library approach in which existing and/or readily accessible peptide or other chemical diversities are chemically transformed to yield libraries (termed here chemically transformed libraries) having more desirable physical and chemical properties.

For the chemical transformation of peptide libraries to be of practical use, there are two requirements. (*i*) One must begin with a well-defined peptide library, and (*ii*) one must have access to chemical reagents that can effectively alter chemical moieties in a known manner, while leaving either all of the compound mixture on the resin or alternatively removing all of the mixture from the resin. The solid–phase synthesis of individual peptides (13, 18) and peptide libraries (1, 2) satisfies the first requirement because the preparation of libraries on solid supports can be done with a high degree of confidence and exactitude by recently described approaches (1, 2, 5, 12). Such libraries can then be chemically altered before their resin cleavage. The integrity of the peptide library used in the present study has been well demonstrated in earlier work (2, 12), and this library can be used for chemical transformation with confidence. The second requirement is satisfied here by the successful generation of chemically transformed peptide libraries. Initial studies with individual peptides indicate that peptides can be fully permethylated while bound to the resin support used in their synthesis. The average yield was >85% and was achieved independently of the sequence being permethylated.

Peptoid libraries (3), which are derived from the step-wise synthesis of amide-functionalized polyglycines, consist of compounds having a number of physical–chemical properties similar to the permethylated peptides described here (resistance to enzymes, favorable aqueous/lipid partitioning, etc.). We believe the use and transformation of existing resin-bound peptide libraries offer significant advantages, due to their general availability and to the familiarity of the procedures for the synthesis of peptides (13, 18) and peptide libraries (1, 2, 5, 12).

Thus, the transformation described here is an example of a more general approach to produce large diversities from readily accessible existing peptide libraries. We believe that the chemical transformation of a wide range of existing and future combinatorial libraries, as exemplified by the permethylated library presented here, offers a straightforward and rapid means to increase the available chemical diversity for use in basic research and drug discovery.

We thank Ema Takahashi for her expert technical assistance, Eileen Silva for help in document preparation. This work was funded by Houghten Pharmaceuticals, Inc., San Diego.

1. Houghten, R. A., Pinilla, C., Blondelle, S. E., Appel, J. R., Dooley, C. T. & Cuervo, J. H. (1991) *Nature (London)* **354**, 84–86.
2. Pinilla, C., Appel, J. R., Blanc, P. & Houghten, R. A. (1992) *Biotechniques* **13**, 901–905.
3. Simon, R. J., Kania, R. S., Zuckermann, R. N., Huebner, V. D., Jewell, D. A., Banville, S., Ng, S., Wang, L., Rosenberg, S., Marlowe, C. K., Spellmeyer, D. C., Tan, R., Frankel, A. D., Santi, D. V., Cohen, F. E. & Bartlett, P. A. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 9367–9371.
4. Geysen, H. M., Rodda, S. J. & Mason, T. J. (1986) *Mol. Immunol.* **23**, 709–715.
5. Lam, K. S., Salmon, S. E., Hersh, E. M., Hruby, V. J., Kazmierski, W. M. & Knapp, R. J. (1991) *Nature (London)* **354**, 82–84.
6. Eichler, J. & Houghten, R. A. (1993) *Biochemistry* **32**, 11035–11041.
7. Scott, J. K. & Craig, L. (1994) *Curr. Opin. Biotechnol.* **5**, 40–48.
8. DeWitt, S. H., Kiely, J. S., Stankovic, C. J., Schroeder, M. C., Cody, D. M. R. & Pavia, M. R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6909–6913.
9. Chen, C., Ahlberg-Randall, L. A., Miller, R. B., Jones, A. D. & Kurth, M. J. (1994) *J. Am. Chem. Soc.* **116**, 2661–2662.
10. Bunin, B. A., Plunkett, M. J. & Ellman, J. A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 4708–4712.
11. Dooley, C. T., Chung, N. N., Schiller, P. W. & Houghten, R. A. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 10811–10815.
12. Dooley, C. T. & Houghten, R. A. (1993) *Life Sci.* **52**, 1509–1517.
13. Houghten, R. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5131–5135.
14. Blondelle, S. E. & Houghten, R. A. (1992) *Biochemistry* **31**, 12688–12694.
15. Blondelle, S. E., Simpkins, L. R., Pérez-Payá, E. & Houghten, R. A. (1993) *Biochim. Biophys. Acta* **1202**, 331–336.
16. Hakomori, S. I. (1964) *J. Biochem. (Tokyo)* **55**, 205.
17. Challis, B. C. & Challis, J. A. (1970) in *The Chemistry of Amides*, ed. Zabicky, J. (Wiley, New York), pp. 731–857.
18. Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85**, 2149–2154.
19. Ibrahim, H. H. & Lubell, W. D. (1993) *J. Org. Chem.* **58**, 6438–6441.
20. Lawson, W. B., Gross, E., Foltz, C. M. & Witkz, B. (1962) *J. Am. Chem. Soc.* **84**, 1715–1718.
21. Houghten, R. A., Bray, M. K., De Graw, S. T. & Kirby, C. J. (1986) *Int. J. Pept. Protein Res.* **27**, 673–678.
22. Russell, G. A. & Weiner, S. A. (1966) *J. Am. Chem. Soc.* **31**, 248–251.
23. Tam, J. P., Heath, W. F. & Merrifield, R. B. (1983) *J. Am. Chem. Soc.* **105**, 6442–6455.
24. Piszkiewicz, D., Landon, M. & Smith, E. L. (1970) *Biochem. Biophys. Res. Commun.* **40**, 1173–1177.
25. Mosmann, T. (1983) *J. Immunol. Methods* **65**, 55–63.
26. Kornreich, W., Anderson, H., Porter, J., Vale, W. & Rivier, J. (1985) *Int. J. Pept. Protein Res.* **25**, 414–420.