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Research Article

Application of One-Bead One-Structure Approach to Identification of Nonpeptidic Ligands

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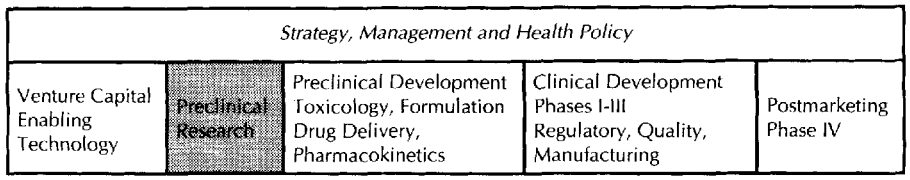

ABSTRACT A synthetic chemical library comprised of alkylated and acylated amino acids was synthesized and screened to determine structures that bind to a model target, streptavidin. The library was prepared using "split synthesis" and screened in a solid phase binding assay. The structure of positively reacting compounds was determined using mass spectroscopy. Positive compounds, together with various structural analogs were synthesized and their binding confirmed. Structures containing both an imidazole moiety and a substituted aromatic residue demonstrated binding.

Key Words: streptavidin, split synthesis, synthetic chemical library

INTRODUCTION

Combinatorial peptide libraries have proven to be a powerful technique to identify lead compounds with desired binding or biological properties. In order to expand the utility of this technique, efforts have been made to synthesize non-peptidic libraries. Large libraries can be prepared by biological methods (filamentous phage [Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990; Felici et al., 1991] and plasmid [Cull et al., 1992] libraries) and by synthetic methods which include (1) the iterative approach [Geysen et al., 1986, 1987; Houghten et al., 1991; Owens et al., 1991; Blake and Litzi-Davis, 1992] and (2) the one-bead one-compound approach [Lam et al., 1991; Lam and Lebl, 1992; Salmon et al., 1993]. Only synthetic techniques are capable of adaptation to a broader array of compounds. Initial efforts in this area have been demonstrated in libraries containing structures with reduced peptide bonds and non-peptide moieties [Bunin and Ellman, 1992; Simon et al., 1992; Cho et al., 1993; Nikolaiev et al., 1993; Chen et al., 1994; Lebl et al., 1994a,b].

The "one-bead one-structure" approach employs split synthesis [Furka et al., 1991; Houghten et al., 1991; Lam et al., 1991], to ensure that only one chemical entity is being synthesized on each bead. The entire bead-bound library can then be screened to determine binding activity against a soluble acceptor molecule for binding [Lam et al., 1991, 1993; Lam and Lebl, 1992]. Alternatively, using an orthogonal two-stage release process, compounds can be released from the beads for use in a solution phase assay [Kočiš et al., 1993; Lebl et al., 1993a,b; Salmon et al., 1993]. Positively reacting beads can be identified and the

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structure of the ligand can be determined. We describe here the application of this approach to construct a non-peptide library consisting of acylated and alkylated amino acids.

MATERIALS AND METHODS

Library synthesis was performed on TentaGel Resin S OH (Rapp Polymere, Tubingen, Germany). Fmoc-amino acids, with standard side chain protecting groups were obtained from Advanced ChemTech (Louisville, KY), Bachem (Torrance, CA), or Propeptide (Vert-le-Petit, France). N-Hydroxybenzotriazole (HOBt), diisopropylethylamine (DIEA), piperidine, and diisopropylcarbodiimide (DIC) were obtained from Advanced ChemTech; aldehydes and carboxylic acids were from Aldrich Chemical Co. (Milwaukee, WI). Commercial-grade solvents were used without further purification. 2-Bromo-3-chloro-indolyl phosphate (BCIP) was obtained from Ameresco (Solon, Ohio).

Both analytical and preparative HPLC were carried out on a Waters 625 LC system with a Waters 490E Programmable Multiwavelength Detector. UV/VIS absorption spectra were recorded on a Hewlett Packard HP 8452A Diode-Array spectrophotometer using a 1-cm quartz cuvette. Ion-spray mass spectra were obtained on a triple quadrupole PE-Sciex API III+ mass spectrometer with an articulated ion spray sample inlet system. For MS/MS experiments argon was used as the collision gas.

Synthesis of Library of Small Organic Molecules

Fmoc-Pro was coupled to the resin (TentaGel S OH, 1 g) using DIC (3 equivalents [eq]), HOBt (3 eq), and N-methylimidazole (6 eq) in DMF. Boc-Lys-(Fmoc)-Pro linker was assembled using DIC and HOBt. After cleavage of the Fmoc group, the peptide-resin was divided into eighteen equal portions, and eighteen (excluding cysteine and proline) Fmoc-protected L-amino acids and glycine (one in each reaction vessel, 3 eq each) were coupled using DIC and HOBt in DMF (3 eq). Fmoc protection of the α-amino group was removed by piperidine in DMF (50%, 10 min) and after washing with DMF (5×) and DMF containing 2% of acetic acid, the resin was divided into twenty reaction vessels. Free amino groups were exposed to a solution of aldehydes in methanol/dichloromethane/1% AcOH (2 to 30 eq according to the aldehyde reactivity). After 20 min preincubation, cyanoborohydride (1 mmol/mL DMF, 2 to 30 eq) was added and the reaction proceeded for 40 min. The resin was washed with DMF (5×) and a ninhydrin test was performed to check the completeness of the primary amino group transformation to secondary amino group. In the case of a positive test, the coupling was repeated. The resin was pooled, carefully washed, and redistributed to twenty reaction vessels and twenty carboxylic acids were coupled either by the DIC/HOBt method, or by using symmetrical anhydrides. After thorough washing, the side chain protecting groups were removed with reagent K [King et al., 1990]. The resin was washed, divided into two parts, and one half was acetylated with a mixture of acetic anhydride and pyridine. The non-acetylated part of the library was stored in acidic aqueous solution.

Screening of the Library

An aliquot (50,000 beads) of the acetylated library was gently mixed with an incremental increase of doubly-distilled water to remove all DMF. The beads were thoroughly washed with doubly-distilled water. Gelatin (0.1% w/v) was then used to block any non-specific binding. The beads were then mixed with the streptavidin-alkaline phosphatase complex (as dilute as possible to minimize non-specific binding) in PBS with 0.1% gelatin and 0.1% Tween 20 with gentle mixing for 1 h. The beads were then thoroughly washed with PBS with 0.1% Tween 20 followed by TBS (Tris Buffered Saline: 8 g NaCl, 0.2 g KCl, and 3 g Tris base in 1 liter water; pH 8.0). The standard substrate BCIP (5-bromo-4-chloro-3-indolylphosphate) in an alkaline phosphatase buffer (5.85 g NaCl, 12.1 g Tris base, 0.476 g $MgCl_2$; pH 8.5) was then added and the beads were transferred to a polystyrene Petri dish (100 × 20 mm). The reaction was carried out for up to 2 h. The positive beads became turquoise in color while the majority of the beads remained colorless.

Positively reacting beads were separated and treated with 8 M guanidine hydrochloride, pH 2.0 for 20 min, destained by an intensive wash with DMF, and the above-described experiment was repeated with the competing ligand (biotin) added to the incubation medium. Colorless beads were selected, stripped by guanidine hydrochloride, and reincubated with the acceptor-alkaline phosphatase complex in the absence of competitor. Three beads were stained (confirmed positive) and isolated for sequencing.

Structure Determination of Positively Reacting Beads

The positive beads were physically removed with the micropipette and treated with 8 M guanidine hydrochloride, pH 2.0, for 20 min to 1 h. The beads were then washed with doubly-distilled water. Cleavage of compounds from a single bead was achieved by

a 20-min treatment with 30 μl of 0.5% NaOH solution, with subsequent quenching of hydrolysis by 5 μl of glacial acetic acid. One half of the resultant solution was injected into the mass spectrometer through a desalting column to determine the molecular weights of the individual components of the mixture. In the second run, selected compounds were fragmented by MS/MS using the remaining solution.

Resynthesis of the Putative Structures

Fmoc-His(Trt) was coupled to the Boc-Lys-Pro linker which was attached onto resin (TentaGel S OH, 2 g). The Fmoc group was removed by piperidine in DMF (20%, 10 min), and after washing with DMF the free amino groups were exposed to the solution of 2,4,6-trimethyoxybenzaldehyde (30 eq) in a solvent system consisting of methanol/methylene chloride/acetic acid (77:20:3). After 20-min preincubation, cyanoborohydride (1 mmol/1 ml DMF, 30 eq) was added and the reaction proceeded for 120 min. After washing with DMF, a ninhydrin test was performed and the resin was divided into two portions (3:2). The side chain protecting groups in the smaller portion were removed using reagent K for 45 min. The resin was then washed and acetylated with a mixture of acetic anhydride and triethylamine in DMF for 50 min (compound I; Fig. 1). Fmoc-Gly was coupled by the DIC/HOBt method to the second portion. Coupling was monitored with bromophenol blue [Krchňák et al., 1988]. Side chain protecting groups were removed with reagent K, and after the resin was washed, it was divided in half. The Fmoc group was removed from the first half by piperidine in DMF (20%, 10 min) and the resin was acetylated (compound II; Fig. 1). The second half was acetylated and then the Fmoc group was removed (compound III; Fig. 1).

Compounds were cleaved from beads by an 0.5% NaOH solution. After 1 h, the solutions were neutralized by acetic acid and salts were removed by filtration through "Sep-pak" filters. Prepared compounds were then lyophilized. The structures without the Lys-Pro linker (compounds IV–VI; Fig. 1) were prepared using the same procedure, beginning with the coupling of His directly to the resin by an ester bond, as well as the structures not containing an aldehyde moiety (compounds VII–IX; Fig. 1). All synthesized structures were tested (bound to the resin) for their binding to streptavidin. Those that showed binding (compounds I–IV; Fig. 1) were cleaved from the beads, purified by preparative HPLC, and characterized.

The synthetic product from the preparation of compound II was analyzed by mass spectroscopy and afforded a nearly identical spectrum to the mixture obtained from the single positively reacting beads (Fig. 2a). Individual components of the mixture were separated by HPLC and analysed by MS and NMR. Retention times (min): 8.2 (VIII), 14.4 (X), 15.0 (II), 15.5 (I) (Microsorb MV C-18, 5 μm, 25 × 0.4 cm, 5 to 65% CH_3CN in 0.1% TFA in 30 min); 14.2 (X), 16.9 (II), 19.9 (I) (Vydac C-18, 10 μm, 25 × 1 cm, 15 to 25% CH3CN in 0.1% TFA in 50 min). HPLC characteristics of compounds I and VIII were identical to those of authentic samples. Compound X: NMR: Ac: 1.834, 1.855; Lys: NH 8.033; $C_\alpha H_2$ 4.396; $C_\beta H_2$ 1.469, 1.332; $C_\gamma H_2$ 0.950; $C_\delta H_2$ 1.157, 1.034; $C_\epsilon H_2$ 2.230, 3.268; Pro: $C_\alpha H$ 4.233; $C_\beta H_2$ 2.148, 1.816; $C_\gamma H_2$ 1.899; $C_\delta H_2$ 3.440, 3.673; His: $C_\alpha H$ 4.506; $C_\beta H_2$ 2.762, 3.734; CH(2) 7.551; CH(4) 8.982; Gly: NH 8.165; CH2 2.762, 3.734; trimethoxybenzylidene: CH 6.301; CH(Ar)3,5 6.331, 6.291; OMe 3.702, 3.805, 3.852. MS fragmentation: Fig. 3.

RESULTS AND DISCUSSION

Synthetic combinatorial techniques offer broad opportunities for the construction of peptidomimetic and other non-peptide libraries. These libraries may include unnatural amino acids, cyclic structures, scaffolds, reduced peptide bonds, or other non-peptide coupling chemistries, as well as completely non-peptide moieties [Bunin and Ellman, 1992; Simon et al., 1992; Cho et al., 1993; DeWitt et al., 1993; Kerr et al., 1993; Nikolaiev et al., 1993; Zuckermann, 1993; Chen et al., 1994; Lebl et al., 1994a,b]. To demonstrate the flexibility of this approach, we have synthesized a small model library based on Nα-alkylated and acylated amino acids.

As building blocks in this synthesis, we have used 17 L-amino acids and glycine for the first randomization. The free amino group was reacted with twenty different aldehydes to form the Schiff base, and cyanoborohydride reduction provided alkylated amino acids. The newly formed secondary amino group was then acylated by twenty carboxylic acids. The synthetic scheme is illustrated in Figure 4 and building blocks used in the library synthesis are listed in Table 1. Analysis of the random beads from the library have shown that some structures were generated only in low yield and that each bead contains more than one compound. It is unrealistic to expect that the reactions applied in the construction of non-peptidic libraries would yield a single product. Since the goal of combinatorial libraries is to generate diversity to identify leads, multiple (identifiable) structures on a single bead may be advantageous provided that one can identify the structures of all compounds

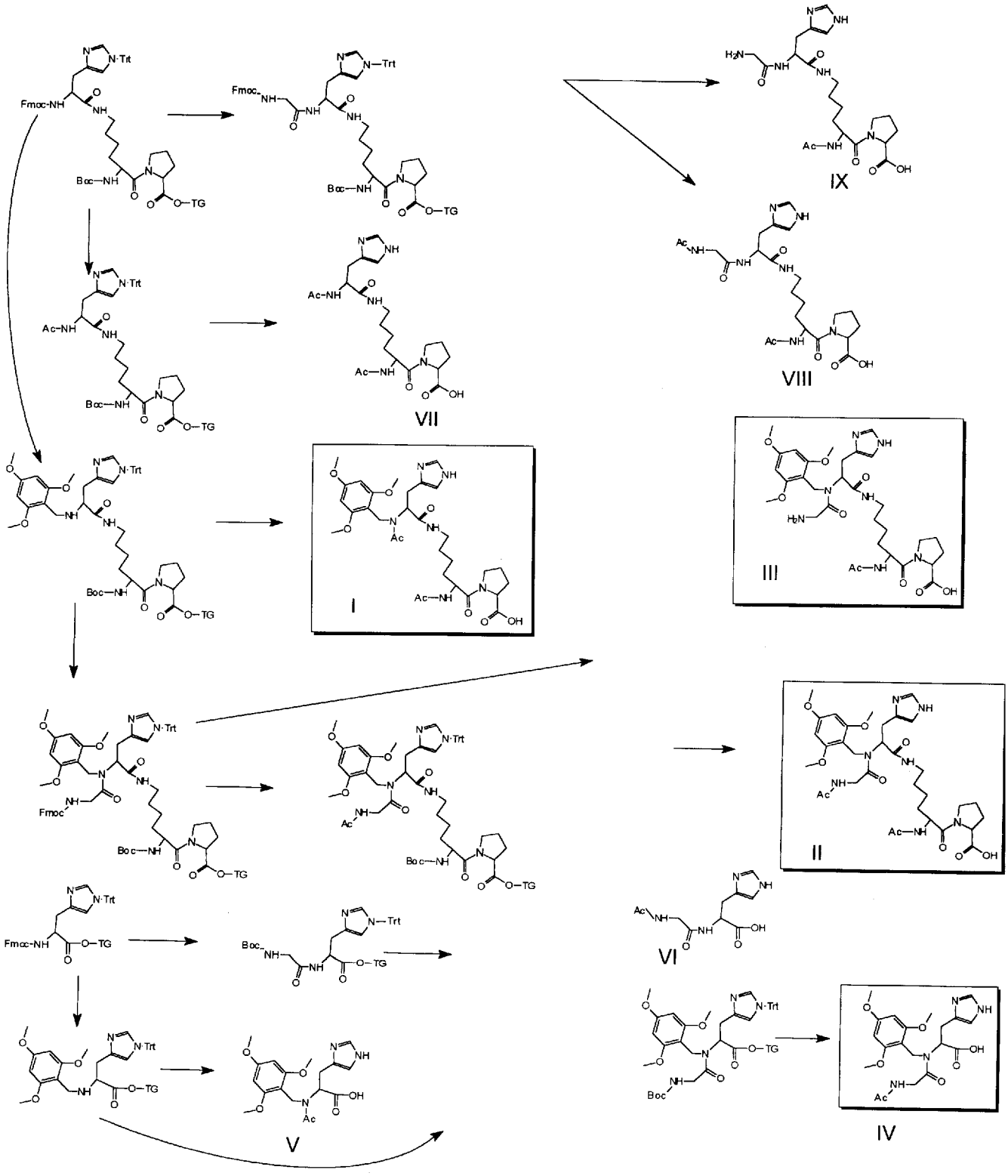


Figure 1. Scheme for the synthesis of some permutations of identified compound. Compounds in the boxes show the binding to streptavidin.

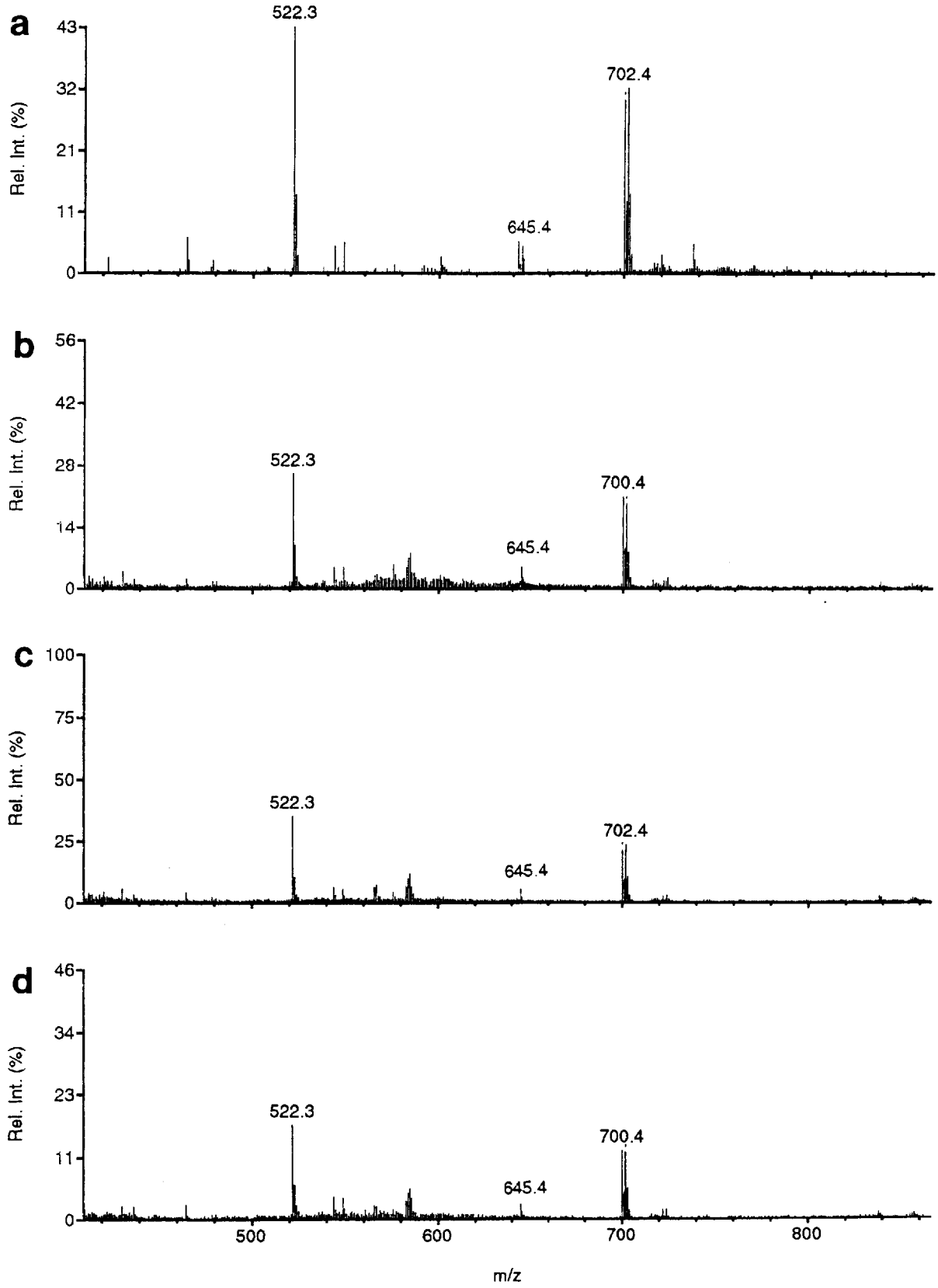
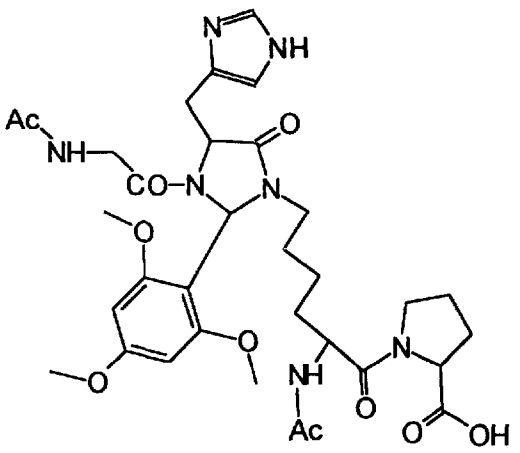


Figure 2. Mass spectra of compound mixture cleaved from three library beads (b–d) reacting positively with streptavidin compared with the mass spectrum of mixture cleaved from the beads synthesized using identified building blocks (histidine, trimethoxybenzaldehyde, and acetylglycine, a).



X

Figure 3. Structure of compound X.

present. We have optimized reaction conditions and building block selection (Flegelova et al., submitted for publication), but have tested the prepared library to demonstrate that even unoptimized chemistries can provide reasonable lead structures. Incomplete reactions in the randomization steps simplified the analysis of the library structures by mass spectroscopy. We have written a program which can generate the building block composition of a particular molecular weight, taking into consideration incomplete reactions. The composition of all compounds (resulting from incomplete or alternative reactions) generated on one bead must correspond to each other since the bead is exposed to only one reagent in each step. Information from fragmentation patterns of individual compounds may confirm the structural conclusions.

The library contained 7,600 compounds (i.e., a complete library may be represented by 7.6 mg of resin). Samples of the library were screened against streptavidin, anti-β-endorphin monoclonal antibody, and thrombin. Of the three targets screened, noteworthy ligands were identified only in the streptavidin assay. Specificity of binding was demonstrated in a binding assay by competition with biotin.

The structures of compounds from the positively reacting beads were determined by mass spectroscopy. Compounds were cleaved from the beads by alkaline hydrolysis and analyzed by MS and MS/MS. Figure 2 presents the MS spectra of compounds cleaved from each positive bead. All three of the selected beads showed nearly identical mass spectra. We concluded that all positive beads contained the same mixture of compounds. The smallest molecular weight (considering incomplete coupling of one building block) of the compound which could have been synthesized in the library is 384, and therefore we have taken only peaks with higher mass into consideration (see Fig. 2). Mass 407 does not correspond to any combination of building blocks. Figure 5 shows the daughter spectrum of peak 522 (upper panel), in which fragment 407 can be clearly identified. Therefore we can suggest that appearance of peak 407 in the original spectrum is the result of inlet fragmentation of peak 522. The difference of 115 U can be explained by cleavage of the C-terminal proline. There are only two combinations containing an incomplete set of building blocks which fit molecular ion 522: R1, His; R2(aldehyde), not coupled; R3, Ac-Gly or succinamic acid. Daughter spectra of molecular ion 522 (Fig. 6) showed intensive cleavage of the Lys-Pro amide bond (peak 407), as well as cleavage of the peptide bond between an unknown amino acid and the Lys-Pro linker (peak 237); neither of these peaks is informative for structure elucidation, since the linker structure was known. But the presence of peak 110 (immonium ion of His) suggests the presence of His in the molecule. Peak 700 does not correspond to any combination of building blocks identified in peak 522 with the aldehyde building block (assuming a normal path of alkylation and acylation). Calculation of the possible combinations of building blocks which fit MH^+ 702 demonstrated that there are only two combinations which include previously defined components: R1 (amino acid), His; R2(aldehyde), trimethoxybenzyl; R3, Ac-Gly or succinamic acid. The presence of a small peak 644 (explained by incomplete acylation of sterically hindered secondary amino group by tertbutyloxycarbonylglycine followed by acetylation: R1, His; R2, trimethoxybenzyl; R3, acetyl) allows us to distinguish between two possible carboxylic acids (R3, Ac-Gly).

Determination of building blocks used in the synthesis of the particular positively reacting bead in the library format allows us to repeat the synthesis of the same putative structure on a scale affording enough material for structural analysis. We have repeated the synthetic scheme used in library synthesis and analyzed the products. As can be seen from Figure 2, the mass spectrum of the mixture matched very closely the spectra obtained from the individual positive beads, supporting the conclusion about the building blocks present in the positive compounds. We have analyzed the mixture by reversed phase HPLC and were able to isolate four major components of the mixture. The first peak was identified by MS (molecular weight [m.w.] 521) analysis as compound VIII, the result of incomplete alkylation of the histidine amino group, or acidolytic cleavage of the

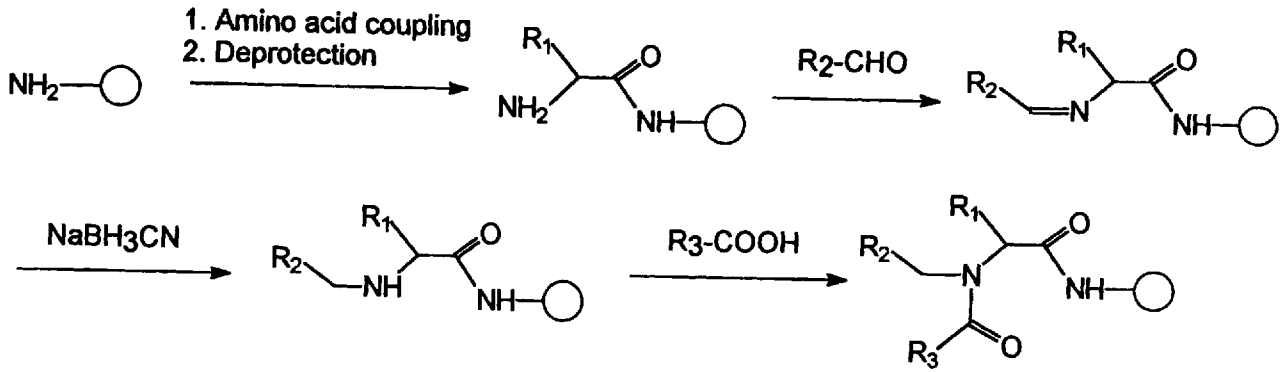


Figure 4. Scheme of the reactions used in library synthesis.

TABLE 1. Building Blocks Used in the Construction of the Model Non-Peptidic Library

Amino acids	Aldehydes	Carboxylic acids
Alanine	Butyraldehyde	Acetic
Arginine	Isobutyraldehyde	Butyric
Asparagine	2-Methyl-2-butenal	Succinic
Aspartic acid	3-Methylbutanal	Hexanoic
Glutamic acid	3-Methylthiopropionaldehyde	Pivalic
Glutamine	3,3,5-Trimethylhexanal	Phenylacetic
Glycine	3-Dimethylaminoacrolein	4-Phenylbutyric
Histidine	5-Norbornene-2-carboxaldehyde	Diphenylacetic
Isoleucine	Cyclohexanecarboxaldehyde	1-Naphthylacetic
Leucine	2,4,6-Trimethoxybenzaldehyde	2-Phenylpropionic
Lysine	Benzaldehyde	Succinamic
Methionine	4-Nitrobenzaldehyde	Glutaric
Phenylalanine	4-Dimethylaminobenzaldehyde	2-Furoic
Serine	4-Methoxybenzaldehyde	Cyclohexylacetic
Threonine	4-Hydroxybenzaldehyde	2-Bromopentanoic
Tyrosine	Hydrocinnamaldehyde	Alanine
Tryptophan	4-Dimethylaminocinnamaldehyde	Glycine
Valine	2-Imidazolecarboxaldehyde	Guanidinoacetic
	2-Pyridinecarboxaldehyde	Guanidinobutyric
	2-Thiophenecarboxaldehyde	4-Bromophenylacetic

trimethoxybenzyl group during the final deprotection. The structure of the second peak (m.w. 699) was elucidated by NMR spectroscopy and confirmed by MS fragmentation. It was found to contain an imidazolidinone ring (X; Fig. 3). Speculation about the formation of this product may include formation of imidazolidinone structure during the step of reductive amination followed by acylation of the amino group. The third peak (m.w. 701) was found to be the correct structure II. The fourth peak (m.w. 643) was found to be structure I, in which the glycine moiety was not attached to the secondary amino group. Fragmentation of authentic materials (see below) was found to be identical to that of the compounds that were found in the library (lower panels in Figs. 5 and 6 show fragmentation of compounds VIII and X).

To determine the importance of the individual building blocks and the linker used for the releasable attachment of this library to the solid support for binding to streptavidin, some possible permutations of the suggested structure were synthesized, both on the cleavable linker and directly, without any linker (see Fig. 1). Binding of streptavidin was observed for the structures marked by the box in Figure 1. As can be seen, the presence of a trimethoxybenzyl group and the imidazole ring of histidine is critical for binding, but the linker or acetylglycine does not have to be present in the streptavidin ligand. The binding structures were synthesized in larger quantity, and their binding to streptavidin was confirmed in solution. We have found that the observed binding is of greater affinity than that observed with previously identified peptides [Lam et al., 1991, 1994; Lam and Lebl, 1992]. Precise evaluation of binding constants is in progress.

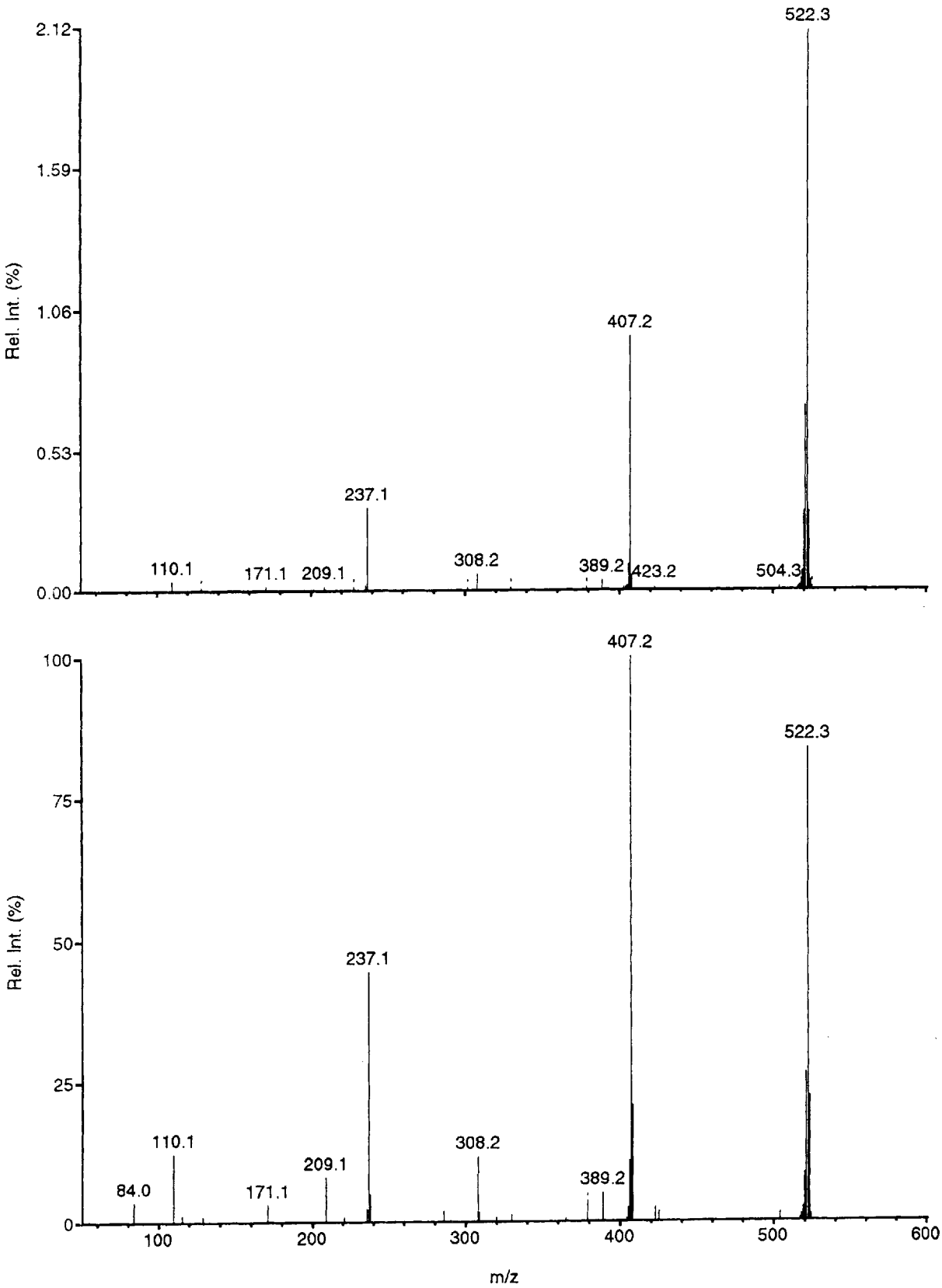


Figure 5. MS/MS of peak containing compound of molecular weight 522 (top) in comparison with the spectrum of resynthesized compound VIII (bottom).

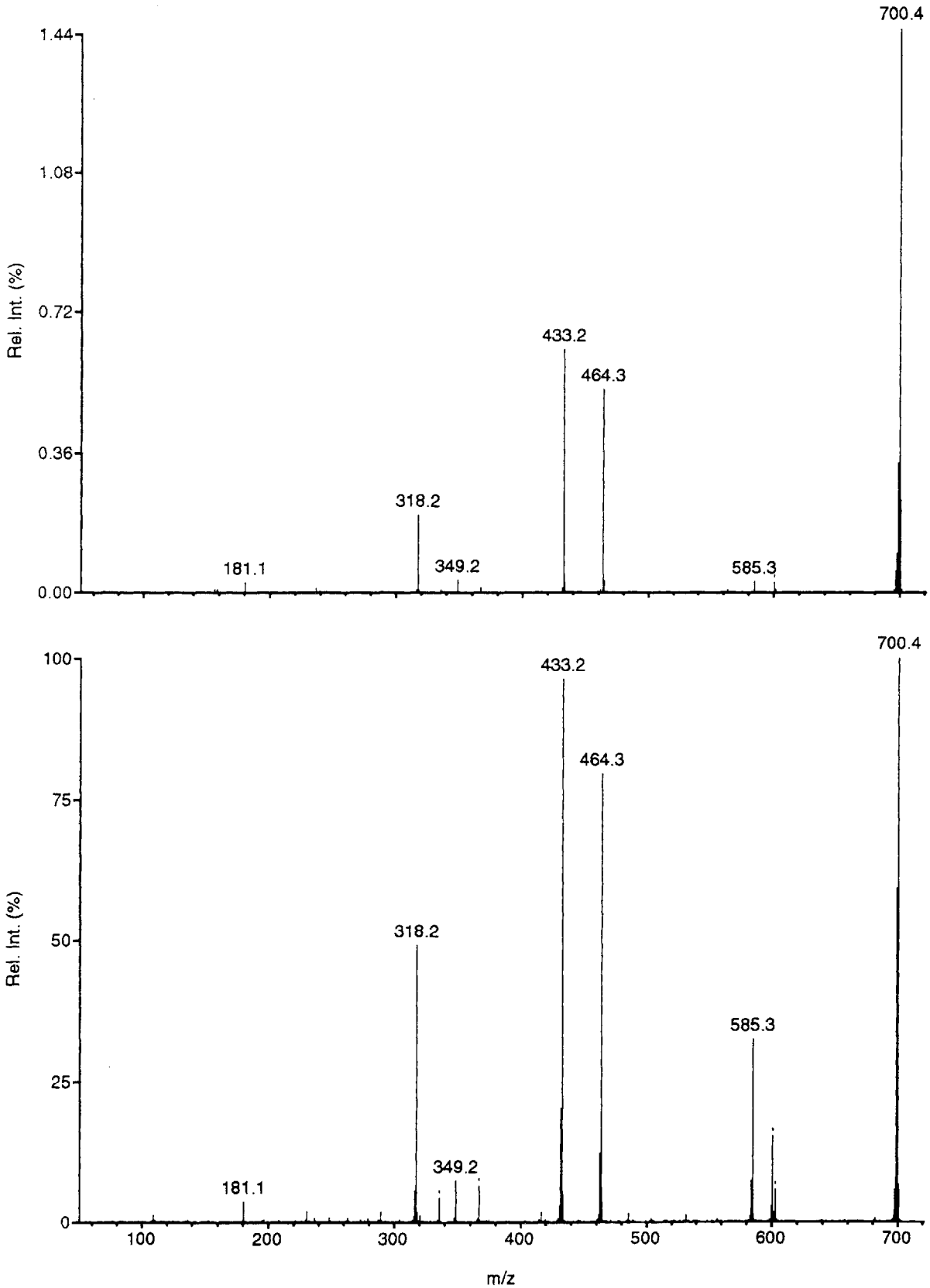


Figure 6. MS/MS of peak containing compound of molecular weight 700 (top) in comparison with the spectrum of compound X (bottom).

CONCLUSIONS

We have demonstrated that a small non-peptidic library can be used to identify ligands of significant affinity for a relatively large protein molecule. In addition, we have illustrated that mass spectroscopy offers a viable alternative to coding methods [Brenner and Lerner, 1992; Kerr et al., 1993; Needels et al., 1993; Nielsen et al., 1993; Nikolaiev et al., 1993; Ohlmeyer et al., 1993; Lebl et al., 1994a,b] for the identification of structures of non-Edman degradable compounds.

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