Affinity purification of synthetic peptides

[solid-phase peptide synthesis/Cys-Met-peptides/organomercurial-agarose/ribonuclease A-(111-124)tetradecapeptide/histone H4-(1-37)-heptatriacontapeptide]

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ABSTRACT A general strategy and a specific tactic for affinity purification of polypeptides synthesized on solid supports are described and demonstrated. The desired peptide chains were distinguished from terminated peptide chains before removal from the support by attachment of an affinity reagent (cysteinyl-methionine) bearing an affinity group (thiol) and a binding group (carboxylic acid). After cleavage from the synthetic support, the affinity-labeled peptides (Cys-Met-peptides) were bound to an affinity receptor (organomercurial-agarose) and thus separated from terminated peptides and all other peptides lacking the affinity group. The desired synthetic peptide was obtained by separation of the affinity-labeled peptides from the affinity receptor (displacement by cysteine or other thiol) followed by removal of the affinity reagent (loss of Cys-Met by cyanogen bromide cleavage).

This general affinity purification strategy is independent of the length or amino acid sequence of the desired peptide. After assembly of ribonuclease-(111-124)tetradecapeptide, using radiolabeled acetic anhydride for termination of uncoupled intermediates, essentially all (>98.5%) of the acetylated deletion peptides were removed by employing the organomercurial Cys-Met tactic. Similarly, the purity of crude synthetic histone H4-(1-37)-heptatriacontapeptide was increased six-fold by using this tactic to remove terminated peptides. A related dimeric Cys-Met tactic is outlined for affinity purification of peptides containing internal cysteine and methionine residues.

Purification of synthetic peptides is important if proper conclusions are to be drawn from subsequent biological experiments. Small peptides can often be separated from the major byproducts by recrystallization or by standard biochemical techniques, but purification of larger synthetic peptides or proteins is more difficult to achieve because the differences in physical or chemical properties are relatively smaller. These difficulties are magnified with the stepwise solid-phase method (1), where extensive purification is possible only at the end of the synthesis. In this case, highly selective affinity purification procedures can be extremely useful.

Synthetic peptide byproducts are conveniently classified by the nature of the structural defect (Table 1) as modified peptides, which contain one or more chemically altered residues; addition peptides, which contain one or more extra residues; deletion peptides, which lack one or more residues; and terminated peptides, which have permanently stopped growing during the synthesis for either chemical or physical reasons. This paper describes an affinity strategy for the separation of the desired product of a solid-phase synthesis from the general class of terminated peptides.

The affinity purification strategy

After solid-phase assembly of a peptide is complete and before the peptide is cleaved from the solid support, the desired peptide and other growing peptides each bear a reactive free amino group, which terminated peptides lack. The affinity purification strategy (Fig. 1) uses this chemical distinction to separate the growing peptides from nongrowing peptides. An affinity reagent A-B, bearing an affinity group A and a binding group B, is selectively attached to the free amino groups of the growing chains. After cleavage from the solid support, the affinity-labeled peptides are separated from terminated peptides and other peptides lacking the affinity reagent by selective binding to an affinity receptor R. Once the affinity-labeled peptides are separated from the affinity receptor, the affinity reagent is removed to obtain the desired peptide.

This strategy is independent of the length or sequence of the desired peptide. It permits separation of the desired peptide from all terminated peptides and other chains lacking the affinity reagent, but modified or addition peptides will not be removed. Terminated peptides include chains inadvertently blocked during the synthesis by such processes as acetylation (2), trifluoroacetylation (3), or pyroglutamyl formation (4, 5). In addition, chains that have failed to couple can be intentionally blocked by a terminating agent, which prevents the formation of internal deletion peptides. Finally, some nongrowing chains might be chemically reactive but physically inaccessible due to their location in a crowded region of the solid support (6). The affinity purification method should efficiently remove all such nongrowing chains.

The organomercurial Cys-Met tactic

One suitable affinity reagent is the dipeptide cysteinyl-methionine. The carboxyl group of methionine functions as the binding group and the thiol group of cysteine as the affinity group. Cys-Met can be selectively and efficiently cleaved by cyanogen bromide (7, 8) to restore the free terminal amino group. Organomercurial-agarose (9) can serve as the affinity receptor to bind the thiol group selectively and reversibly through a covalent mercury-sulfur bond. A column of organomercurial-agarose has often been used to bind proteins or peptides bearing thiol groups (10–15).

A specific protocol using these components is shown in Fig. 2. Although Cys-Met could be added as a protected dipeptide, it is more easily added by using the conventional procedures of solid-phase synthesis to couple first methionine as Boc-Met and then cysteine as Boc-Cys(4-CH3OBzl). After cleavage from the solid support with liquid hydrogen fluoride, which liberates the protected thiol group of cysteine, the crude peptide is gel filtered to remove the by-products of low molecular weight and treated with dithiothreitol to reduce the thiol group. After removal of the reducing agent by gel filtration, the peptide is applied to a column of organomercurial-agarose, a beaded carbohydrate matrix bearing covalently bound 4-(chloromercuri)benzoyl sites. Each Cys-Met-peptide molecule is selectively bound through its thiol affinity group to an organomercurial site on the affinity receptor. The column is eluted with aqueous buffer until all peptides lacking a thiol group are removed.

Abbreviation: Acn, acetamidomethyl.
Table 1. Examples of peptide by-products

<table>
<thead>
<tr>
<th>Class</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desired peptide</td>
<td>H-Gly-Ala-Thr-Val-OH</td>
</tr>
<tr>
<td>Modified peptide</td>
<td>H-Gly-Ala-O</td>
</tr>
<tr>
<td>Addition peptide</td>
<td>H-Thr-Val-O</td>
</tr>
<tr>
<td>Deletion peptide</td>
<td>H-Gly-Thr-Val-O</td>
</tr>
<tr>
<td>Terminated peptide</td>
<td>CH₃CO-Thr-Val-O</td>
</tr>
</tbody>
</table>

Then the Cys-Met-peptides are displaced from the column by elution with an organic thiol such as cysteine. After removal of this thiol by gel filtration, the Cys-Met affinity reagent is selectively and efficiently removed from the desired peptide by treatment with cyanogen bromide, which converts the methionine residue into homoserine and liberates the amino group of the desired peptide. A final gel filtration to remove excess reagent and the cysteinyl-homoserine dipeptide furnishes the desired peptide plus any other peptides derived from chains still growing at the end of solid-phase assembly. Peptides that still bear the Cys-Met affinity reagent are removed during a second pass through the organomercurial-agarose column.

This organomercurial Cys-Met tactic has been used successfully to purify two biologically active peptides, bovine pancreatic ribonuclease A-(111-124)-tetradecapeptide and [12-N⁶-[¹⁴C]acetyllysine,16-N⁶-[³H]acetyllysine]histone H4-(1-37)-heptatriacontapeptide.

**MATERIALS AND METHODS**

Organomercurial-Agarose. This affinity support (9) was prepared as described (10). Sepharose 4B was activated with cyanogen bromide and treated with ethylenediamine hydrochloride to form 2-aminoethylisourea-Sepharose, which was coupled with sodium 4-(hydroxymercuri)benzoate using N-ethyl-N’-(3-dimethylaninoethyl)carbodiimide. In several later experiments, a related commercial preparation of organomercurial-agarose (Affinosil 501, Bio-Rad) was used with comparable results. When packed in a column, the affinity supports bound 0.9–1.2 μmol of thiol per ml of settled support. The binding capacity was measured (11) by reaction of the column with a solution of 5,5’-dithio-bis(2-nitrobenzoic acid) (Ellman’s reagent). If the capacity test is omitted, thiol-containing proteins are reported (11, 12) to bind in an irreversible manner. The column was regenerated after use by elution with 0.05 M sodium acetate (pH 5) containing 2 mM mercaptoethanol.

Ribonuclease-(111-124)-tetradecapeptide. The desired peptide-resin was prepared by the solid-phase method (16, 17) using a Beckman model 990 automated peptide synthesizer and reagent-grade or purified reagents and solvents (18). Boc-Val-OCH₂-bromoresin (2.5 g, 0.50 mmol of Val) was converted into the tetradecapeptide-resin (3.0 g) by successive addition of 13 Boc-amino acids. The side-chain protecting groups (1) were O-benzyl for serine, aspartic acid, and glutamic acid; O-2,6-dichlorobenzyl for tyrosine; N⁴,4’,4”-dimethoxybenzhydryl for asparagine, and N⁶,N⁶’,2,4-dinitrophenyl for histidine. Each residue was added through the following seven-step cycle: (1) deprotection with 50% (vol/vol) CF₃CO₂H/CH₂Cl₂ for 1 min and 30 min; (2) neutralization three times with 5% (vol/vol) diisopropylethylamine/CH₂Cl₂ for 2 min each, (3) coupling with Boc-amino acid and N,N’-dicyclohexylcarbodiimide (each 1.5 mmol, 3 eq) for 30 min, (4) repetition of neutralization step (2), (5) repetition of the coupling step (3), (6) monitoring (19), and (7) acetylation with [³H]acetic anhydride (0.365 mmol, 2.00×10⁶ cpm) and imidazole (1.0 mmol) in CH₂Cl₂ (10 ml) for 60 min.

**Attachment of Affinity Reagent.** Boc-Methionine and then...
Boc-S-(4-methoxybenzyl)cysteine were coupled to the ribonuclease-(111-124)-resin using the solid-phase procedure outlined above. Nonradioactive acetic anhydride and imidazole were used to terminate any unreacted amino groups after each double coupling. The N^{im}-dinitrophenyl group was removed by thiolysis for 30 min with 2% thiophenol in dimethylformamide.

**Cleavage from Synthetic Support.** The peptide-resin (0.50 g) was treated with 9:1 (vol/vol) anhydrous HF/anisole for 60 min at 0°.

**Binding to Affinity Receptor.** The crude peptide (4.38 × 10^6 cpm) was dissolved in 0.8 M urea buffered to pH 9.0 with 0.1 M Tris and stirred with dithiothreitol (150 eq) for 18 hr under nitrogen, which was freed of oxygen by bubbling through 10% pyrogallol in 1 M NaOH. The reduced mixture was gel filtered on a 1.5 × 30-cm column of Bio-Gel P-2 and eluted with 1% aqueous acetic acid at 20 ml/hr. The eluate absorbance was monitored continuously at both 206 and 280 nm with an LKB UVicord III UV monitor and the radioactivity was measured with a Beckman model 355 liquid scintillation counter. Fractions comprising the first peak were pooled and lyophilized to furnish a mixture of the larger peptides (23.5 mg; 2.72 × 10^6 cpm). Part of this peptide mixture (17.1 mg; 1.6 × 10^6 cpm) was dissolved in 0.05 M sodium phosphate buffer (pH 6.0; 2.0 ml) without stirring, flushed with oxygen-free nitrogen, and applied to a 1.5 × 23-cm column of organomercurial-agarose (38 ml; 35 μmol of Hg) equilibrated with 0.01 M sodium phosphate buffer (pH 6.0; 250 ml). The column was eluted with this buffer for 16 hr at 60 ml/hr and then with 0.01 M sodium acetate (pH 5.0) for 2.8 hr at 30 ml/hr to minimize nonspecific binding.

**Separation from Affinity Receptor.** The Cys-Met-tetradecapeptide was eluted with freshly prepared 0.4 M mercapteno ethanol at 6 ml/hr. The absorbance at 280 nm and the relative radioactivity were measured. After elution of the Cys-Met-peptide, the baseline was higher due to the absorbance of mercaptoethanol. The peptide fractions were pooled, lyophilized, and desalted by gel filtration to furnish the Cys-Met-tetradecapeptide (6.0 mg; 2.28 × 10^4 cpm).

**Removal of Affinity Reagent.** The peptide was dissolved in 70% formic acid (1 ml) and a solution of cyanogen bromide (26 mg; 100 eq) in 70% formic acid (0.6 ml) was added. The mixture was stirred for 22 hr, dialyzed 50-fold with distilled water, lyophilized, and desalted by gel filtration as described above to afford the purified tetradecapeptide (4.7 mg; 1.53 × 10^4 cpm).

**Characterization.** Amino acid analysis was performed by hydrolysis in 6 M HCl at 110° for 18 hr followed by ion-exchange separation with a Beckman model 121 analyzer. The affinity-purified tetradecapeptide gave the expected analysis (theoretical yield in parentheses): His: 0.8 (1), Asp: 1.8 (2), Ser: 1.1 (1), Glu: 1.0 (1), Pro: 1.9 (2), Gly: 1.0 (1), Ala: 1.1 (1), Val: 3.1 (3), Tyr: 1.0 (1), Phe: 1.1 (1). Enzymatic assay was performed by combining the purified peptide with natural ribonuclease-(1-118) and measuring the ribonuclease activity of the resulting binary complex towards cyclic cytidine 2′,3′-phosphate (17). A parallel assay using ribonuclease-(111-124) highly purified by ion-exchange chromatography (17) served as the positive control.

**Histone H4-(1-37)-heptatriacontapeptide.** The protected Cys-Met-(1-37)-peptide was assembled on an unbrominated polystyrene support as described for Cys-Metribonuclease-(111-124). Arginine was protected by the N^6-4-toluenesulfonyl group and lysine was masked with the N^6-2,4-dichlorobenzoyloxy carbonyl group (18) except for lysines 12 and 16, which were blocked by N^6-[14]Clacetyl and N^6-[3]Hacetyl groups, respectively. Uncoupled amino groups were terminated with nonradiolabeled N-acetylimidazole (19). The neutralization and coupling steps were repeated one to four times as judged necessary by automated picrate monitoring (20).

The peptide was cleaved in HF/anisole containing 20 eq of methionine. Part of the crude peptide (5.8 × 10^6 cpm) in a buffer (pH 8.9; 4 ml) 6 M in guanidine-HCl, 0.5 M in Tris, and 2 mM in ethylenediaminetetraacetic acid was flushed with oxygen-free nitrogen and stirred with dithiothreitol (200 mg, 150 eq) for 10 hr. After more dithiothreitol (50 mg) was added, the solution was stirred at 40° for 6 hr and gel filtered on a 1.5 × 85-cm column of Bio-Gel P-2 with 1% acetic acid to remove the dithiothreitol. Exposure to oxygen was minimized to prevent reoxidation of the thiol groups. The radioactive eluate was lyophilized and dissolved in a nitrogen-flushed buffer (pH 5; 4.5 ml) 0.05 M in sodium acetate and 0.1 M in potassium chloride. This solution was applied to a 1.5 × 23-cm column of organomercurial-agarose (40 ml; 36 μmol of Hg) equilibrated with the sodium acetate buffer. Elution with this buffer for 15 hr at 60 ml/hr removed the nonthiol peptides. Subsequent elution of the organomercurial-agarose column with 0.5 M cysteine for 6 hr at 10.4 ml/hr displaced the Cys-Met-peptides (1.8 × 10^6 cpm).

By lyophilization and gel filtration, the Cys-Met-peptide (1.5 × 10^6 cpm) was stirred with cyanogen bromide (160 mg) in aqueous 70% formic acid (6.5 ml) for 11 hr. More cyanogen bromide (105 mg) in 70% formic acid (2 ml) was added, and the mixture was stirred for 10 hr and filtered on a 1.5 × 85-cm column of Bio-Gel P-2 with 1% acetic acid to give a single radioactive peak at the void volume. Part of this peptide (4.6 × 10^6 cpm) was lyophilized, dissolved in the reducing buffer, reduced with dithiothreitol (75 mg; then 15 mg), gel filtered, and lyophilized. Part of the lyophilized peptide (2.9 × 10^6 cpm) was dissolved in a buffer (pH 5) 0.05 M in sodium acetate and 0.1 M in potassium chloride and reapplied to the organomercurial-agarose column. Elution with this buffer for 11.5 hr at 9.9 ml/hr gave histone H4-(1-37) (2.5 × 10^6 cpm; 87% yield). Subsequent elution with 0.5 M cysteine afforded uncleaved Cys-Met-histone H4-(1-37) (0.4 × 10^6 cpm; 13%). The histone H4-(1-37) gave the expected analysis after acid hydrolysis: Lys: 6.1 (6), His: 0.8 (1), Arg: 5.9 (6), Asp: 2.0 (2), Thr: 1.1 (1), Ser: 1.0 (1), Glu: 1.2 (1), Pro: 1.0 (1), Gly: 8.5 (9), Ala: 2.1 (2), Val: 0.9 (1), Cys: 0.0 (0), Met: 0.0 (0), Ile: 3.2 (3), Leu: 3.1 (3).

**RESULTS AND DISCUSSION**

Ribonuclease-(111-124). At the end of each synthetic cycle during assembly of this carboxyl-terminal tetradecapeptide (17, 21), the amino groups of any peptide chains that had failed to couple were permanently blocked by acetylation with [3H]acetic anhydride. Thus potential deletion peptides were converted into radiolabeled terminated peptides. After assembly of the desired peptide was complete, Boc-Met and then Boc-Cys(4-CH_2OBzl) were added to the growing chains and the protocol of Fig. 2 was carried out. Most of the terminated peptides were eluted near the void volume of the organomercurial-agarose column (Fig. 3), but some bound nonspecifically to the affinity column and were eluted with a different buffer. Elution with 2-mercaptoethanol displaced the Cys-Met-peptides. The small amount of [3H]acetylated peptide observed in this peak (0.13 mol %) may be explained by slow acid cleavage (22) of the benzyl group from serine-123 followed by acetylation of its free hydroxyl group. Thus, the organomercurial Cys-Met tacit removed essentially all of the terminated peptides, which amounted to about 5 mol % of the peptide chains. After removal of Cys-Met, the tetradecapeptide was combined
with natural ribonuclease-(1-118) to reconstitute 85% of the ribonuclease activity observed with highly purified synthetic tetradecapeptide (17, 21).

Histone H4-(1-37). The amino-terminal 37-residue peptide from histone H4 (23) was synthesized by the stepwise solid-phase method. The radiolabels in this synthesis were in the NaOAc groups of lysine 12 and 16, not in the terminating agent. Since known synthetic difficulties were encountered at several points during this particular synthesis, the organomercurial Cys-Met procedure was considered to be a stringent test of the affinity purification strategy as well as an important initial step in purification of the synthetic peptide. About 70% of the radiolabeled peptides lacked Cys-Met and passed directly through the affinity column (Fig. 4). The Cys-Met-peptide was eluted with aqueous cytoine. The purification achieved is shown by the gel filtration profiles of the synthetic peptide before and after the affinity column (Fig. 5). Since the first radiolabeled residue was Nα[3H]acetlysine-16, most radiolabeled peptides should be longer than 21 residues. Before the affinity column three UV-absorbing peaks were seen in front of the salt peak. Most of the radiolabeled peptides were present in the void-volume peak and thus were longer than about 25 residues. The Cys-Met-peptides retained by the affinity column, however, eluted as a single peak at the void volume. All peptides shorter than 25 residues and even some longer peptides were removed.

The histone peptide contains a single serine residue at the amino terminus and a single threonine residue at position 50 near the carboxyl terminus. The ratio of these diagnostic residues provides an independent criterion of homogeneity. All peptide chains longer than seven residues will contain threo-
nine, but only chains still growing at the end of chain assembly will contain serine. By amino acid analysis (Table 2) the Thr:Ser ratio was 6.0:1 before the affinity column but was 1.0:1 after. Thus, although less than 20% of the chains were still growing at the end of assembly, essentially all terminated peptides and other non growing chains were removed by the organomercurial-agarose column.

The Cys-Met affinity reagent was removed from the desired peptide by cleavage of the Met-Ser peptide bond with cyanogen bromide. Gel filtration of the product gave a single radioactive peak showing constant 3H:14C ratio across the peak. Thus by the double-label criterion (24) no terminated peptides longer than 22 but shorter than 26 residues were present. The Cys-Met-peptide may not be cleaved if the methionine side chain is sterically inaccessible, if the sulfide group suffered air oxidation to the sulfoxide (25), or if the iminolactone intermediate is intercepted before hydrolysis by the hydroxyl group of an adjacent serine (or threonine) residue (26). Any uncleaved chains were separated from histone H4-(1-37) by a second passage through the organomercurial-agarose column. About

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**Table 2. Amino acid composition of synthetic Cys-Met-histone H4-(1-37) during affinity purification with organomercurial-agarose**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Before</th>
<th>By-products</th>
<th>After</th>
<th>Theory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
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<td>2.2</td>
<td>5.4</td>
<td>6</td>
</tr>
<tr>
<td>His</td>
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<td>0.4</td>
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<td>1</td>
</tr>
<tr>
<td>Arg</td>
<td>4.2</td>
<td>4.0</td>
<td>5.3</td>
<td>6</td>
</tr>
<tr>
<td>Asp</td>
<td>1.9</td>
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<td>2.0</td>
<td>2</td>
</tr>
<tr>
<td>Thr*</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>Ser*</td>
<td>0.2</td>
<td>0.0</td>
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</tr>
<tr>
<td>Glu</td>
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<td>1.0</td>
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</tr>
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<td>Pro</td>
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<tr>
<td>Leu</td>
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<td>2.0</td>
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<tr>
<td>Thr:Ser*</td>
<td>6.0</td>
<td>46.0</td>
<td>1.0</td>
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</table>

*Threonine values were increased 5% and serine values 8% to compensate for destruction during acid hydrolysis in 6 M HCl at 110° for 18 hr. Thr:Ser ratios were calculated with amino acid data containing one more significant figure.
87% of the CNBr-treated peptide passed directly through the column in acetate buffer and thus had undergone removal of the Cys-Met affinity reagent.

**The dimeric Cys-Met tactic**

Application of the organomercurial Cys-Met tactic to polypeptides containing cysteine or methionine residues introduces major complications. If the thiol group of each internal cysteine is not masked during affinity purification, peptides containing cysteine but lacking the amino-terminal Cys-Met moiety will also bind to organomercurial-agarose. If the sulfide group of each internal methionine residue is not blocked, cyanogen bromide treatment will fragment the desired peptide after each methionine. Thus the cysteine and methionine residues should be protected by groups that survive not only assembly of the desired peptide but also each step of the affinity purification procedure.

None of the presently available protecting groups is entirely satisfactory. For example, even the HF-stable S-acetamidomethyl (Acm) group (27) for cysteine and S-oxide group (28) for methionine are not compatible with the organomercurial Cys-Met tactic. The Cys(Acm) residues are stable during solid-phase synthesis, HF cleavage, thiol reduction, and CNBr treatment; the Met(O) residues are stable during synthesis, HF cleavage, binding to organomercurial-agarose and CNBr cleavage. But the organomercurial Cys-Met tactic will generally fail for polypeptides containing Cys(Acm) or Met(O) residues because the Acm group is removed by mercuric reagents and the sulfoxide is reduced to the sulfide by the thiol needed to reduce the Cys-Met-peptides and to elute them from organomercurial-agarose.

This dilemma could be resolved by replacing the organomercurial column by another affinity receptor. One Cys-Met-peptide molecule can effectively act as an affinity receptor for another such molecule by dimerization through formation of a disulfide bond between their amino-terminal cysteine residues. This dimeric Cys-Met tactic uses acetamidomethyl protection for internal cysteine residues and sulfoxide protection for internal methionine residues during assembly and purification. After cleavage from the solid support, the free thiol group of the amino-terminal cysteine is air oxidized to the disulfide. Then the Cys-Met-peptide dimer is separated by gel filtration from the monomer and any smaller by-products lacking Cys-Met. After cyanogen bromide cleavage of the dimer, the Met(O) residues are reduced with thiol and the Cys(Acm) residues are deprotected with mercuric acetate to provide the desired polypeptide. Preliminary experiments have shown that this dimeric Cys-Met tactic is feasible.

Several other promising groups can be envisaged in the role of affinity receptor, affinity group, or binding group. For example, avidin-agarose (29) could be used as the affinity receptor, a biotin derivative such as lipolic acid (30) as the affinity group, and a photolable group as the binding group. The two Cys-Met tactics described above, however, have the distinct advantage of using commercially available components and well-established chemical reactions.

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