CHAPTER 6

Bromoacetylated Synthetic Peptides

Starting Materials for Cyclic Peptides, Peptomers, and Peptide Conjugates

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1. Introduction

Modern peptide synthesis techniques make it straightforward to synthesize linear peptides that are based on amino acid sequences of parent proteins. It is well known that the linear peptide itself often will not share the same activity as the peptide in its original biological environment provided by the native protein. In addition, the scientific reasons for making a peptide will often not be simply to mimic an activity of a protein—whatever it may be—with a small synthetic substitute, but the goal may be to enhance or decrease an activity found in a native protein. Therefore, it is important to be able to modify the conformations and configurations of a linear peptide by cyclizing, polymerizing, or conjugating the peptide.

The main obstacle to modification approaches is the lack of available techniques for synthesizing these peptide-containing materials. However, methods are being developed to use automated peptide synthesizers for additional chemistry in order to perform controlled modifications on the peptide after synthesis.

Until recently, there were three obvious limitations to the available methods in peptide chemistry for the design and synthesis of cyclic, polymeric, and/or conjugated peptides. First, it was not demonstrated that leaving groups could be incorporated into a peptide backbone prior to

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deprotection. It was never demonstrated that the placement of a leaving group at a defined position in the peptide backbone could be accomplished, and this is the second limitation. Finally, for peptide conjugates, there were very few accurate quantitation methods for determining the amounts of peptide covalently linked to other molecules, especially to carrier proteins. All of these limitations are overcome by the use of bromoacetyl-derivatized peptides as described in this chapter. The basic finding was that bromoacetyl and chloroacetyl moieties are stable under the reactions conditions used in *t*-butoxycarbonyl (*t*BOC)-based peptide chemistry, including the deprotection steps using anhydrous hydrogen fluoride at –5 to 0°C (*see* Chapter 4). Bromo- and chloroacetyls are expected to be stable also under the conditions of fluorenylmethoxycarbonyl (Fmoc)-based peptide synthesis when the haloacetyl is added at the N^{α}-position.

Examples of haloacetyl-derivatized peptides as starting materials for new peptide-based configurations have appeared from several laboratories. Peptide polymers that were shown to induce T-cell-independent antibody production (*see* Chapters 10–12, *PAP*) in nude mice were synthesized from haloacetyl-modified peptides *(1)*, a chemically well-defined sugar-peptide conjugate from *Neisseria meningitidis* as a synthetic vaccine candidate has been made using a bromoacetylated peptide conjugated to a thiol-derivatized sugar *(2)*, multiple antigenic peptides (MAPS) are based on a chloroacetyl-derivatized backbone *(3)*, and an N^{α}-bromoacetyl peptide has been ligated to a *C*-terminally placed α-thiocarboxylic acid of another peptide to form a backbone-engineered HIV protease *(4)*.

The detailed descriptions that follow are from previously published methods based on *t*BOC chemistry *(5–7)*. The original work introducing haloacetyl-derivatized peptides was developed using an automated peptide synthesizer, and therefore, the assumption is made here that the reader is familiar with the routine uses of automated peptide synthesizers. However, manual syntheses of haloacetyl-derivatized peptides are readily accomplished by anyone with experience in those techniques and are also described below.

2. Materials

1. Bromoacetic acid, anisole, *S*-carboxymethylcysteine (CMC), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), bromoacetylbromide, dithiothreitol (DTT), tri-*n*-butylphosphene (Bu_3P), and *N,N'* diisopropylcarbodiimide

were from Aldrich Chemical Co. (Milwaukee, WI). Reagent-grade chemicals and solvents used in the synthesis of BBAL were obtained from Fisher Scientific (Pittsburgh, PA). β-Alanine, *N*-hydroxysuccinimide, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). N^{α}-BOC-L-lysine was obtained from Vega Biochemicals (Tucson, AZ). 2-Iminothiolane was from Pierce Chemical Co. (Rockford, IL).

2. All reagents, except bromoacetic acid and BBAL *(vide infra)*, used for the automated syntheses of peptides were purchased from Applied Biosystems, Inc. (Foster City, CA).
3. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the gel electrophoresis system sold by Novex (Encinitas, CA) (*see also* vol. 32 of this series).
4. Two amino acid analysis systems were used as described by the manufacturers for the analyses of products: Amino Quant® by Hewlett Packard, Inc. (Gaithersburg, MD) and Picotag® by Waters Associates (Millipore Corp., Milford, MA).
5. N^{α}-(*tert*-butoxycarbonyl)-N^{ε}-[*N*-(bromoacetyl)-β-alanyl]-L-lysine (BBAL) was synthesized using the reagents listed above and the procedure as described (*see* Section 3.3.) by Inman et al. *(8)*.

3. Methods

All of the bromoacetyl-derivatized peptides were synthesized using an automated solid-phase peptide synthesizer with the exception of the early work where manual bromoacetylations were performed to optimize the method. Both manual (Section 3.1.) and automated (Section 3.2.) approaches using *t*BOC-based peptide chemistry are presented. In addition, *t*BOC-based or Fmoc-based peptide synthesis (for N^{α}-amines) methods can be used, although the *t*BOC approach is outlined in this chapter.

To obtain synthetic peptides that are bromoacetylated at the N^{α}-amino terminus, it is necessary to add the bromoacetyl moiety to the peptide while the peptide is fully protected and, if solid-phase peptide chemistry is being used, covalently linked to the resin provided for normal peptide synthesis. The general step-wise procedure for solid-state peptide bromoacetylation is as follows:

1. Remove the *t*BOC- (*see* Chapter 4) or Fmoc- (*see* Chapter 5) protecting group from the N^{α}-terminus.
2. Neutralize the N^{α}-amine by washing with 10% TEA or DIEA.
3. Add bromoacetic acid anhydride (twofold excess) to the peptide mixture, and stir for 15 min to 1 h.

4. Filter and wash the resin with DMF and DCM to remove soluble, unwanted byproducts.
5. Deprotect the entire peptide with HF (for *t*BOC, *see* Chapter 4) or TFA (for Fmoc, *see* Chapter 5). It is important to omit any thiol-bearing scavengers that may be used here. Other related protocols for deprotection may use thiol-bearing scavengers.
6. Extract the released protecting groups with an organic solvent, generally a 2/1 mixture of ethyl acetate and ether.
7. Filter through a scintered glass filter, and discard the filtrate.
8. Dissolve the peptide in cold aqueous acetic acid.
9. Filter to separate the soluble peptide from the remaining resin.
10. Lyophilize the filtrate to obtain the crude bromoacetylated peptide.

3.1. Manual N^{α}*-Bromoacetylation*

The chemistry presented here describes in detail the bromoacetylation of a protected peptide at the amino terminal α-amine. The basic procedure calls for the simple formation of bromoacetic acid anhydride from commercially available bromoacetic acid and reacting the anhydride in *N,N'*-dimethylformamide (DMF) with a primary amine on the protected peptide.

The following procedure can be used when the amount of peptide being synthesized is on the 0.5-mmol scale:

1. Two hundred and ninety-eight milligrams of bromoacetic acid (2 mmol) in 5 mL CH_2Cl_2 are treated with 2 mL of a 0.5*M* solution of dicyclohexylcarbodiimide (DCC).
2. The solution is stirred for 15 min at 25°C, and during this time, a white precipitate of dicyclohexylurea (DCU) forms. The DCU is filtered using a scintered glass filter with vacuum suction, and the filtrate is evaporated to approx 2.5 mL using a stream of N_2.
3. The volume is then adjusted to 6 mL with DMF, and the solution is further evaporated to approx 4 mL by bubbling N_2 gas into the solution. The temperature remains unadjusted throughout the procedure. The solution is filtered a second time to remove trace amounts of DCU, which form during the evaporation process. The bromoacetic acid anhydride remains in the DMF for coupling to the protected peptide as outlined below.
4. Prior to adding the bromoacetic acid anhydride to the protected peptide, the *t*BOC group is removed from the N^{α} amine of the protected peptide, and the amine is then deprotonated with a suitable base, such as diisopropylethylamine. To a solution of 0.5 mmol protected peptide on the resin in 10 mL DMF is added 4 mL DMF that contains 1 mmol bromoacetic

acid anhydride. The reaction is allowed to proceed at 25°C for 60 min with stirring, after which time the reaction is complete.

5. The resin containing the N^{α}-bromoacetylated-protected peptide is filtered, washed five times with 50-mL portions of CH_2Cl_2 and air-dried. Alternatively, when solution-phase peptide synthesis is used, the solvent can be removed by vacuum evaporation using a rotary evaporator equipped with a mechanical vacuum pump and an acetone-dry ice trap.
6. Deprotection of the bromoacetylated peptide and release from the resin are described below in Section 3.4.

3.2. Automated Nα-Bromoacetylation

In addition to manual bromoacetylation, N^{α}-bromoacetylation using an automated solid-phase peptide synthesizer can be accomplished readily. This is based on the original solid-phase peptide synthesis procedures described in 1963 by R. B. Merrifield *(9)*.

The description given here is for use with a Model 430A peptide synthesizer, but should be adaptable for any automated peptide synthesizer. As a final step in the synthesis, bromoacetic acid anhydride is reacted with the amino terminal amino acid to form the N^{α}-bromoacetyl-derivatized fully protected peptide. This is carried out on a 0.5-mmol scale simply by substituting 2.0 mmol of bromoacetic acid (277.9 mg) for 2.0 mmol of glycine in an empty glycine cartridge and using the preprogrammed run file of the automated synthesizer for glycine coupling.

3.3. Selective Placement of a Bromoacetyl Moiety at Any Position in a Peptide Using BBAL

3.3.1. Synthesis of BBAL

Synthesizing BBAL involves coupling *N*-(bromoacetyl)-β-alanine to N^{α}-BOC-L-lysine as outlined below and taken from ref. *8*. This is performed by using the succinimidyl ester of the bromoacetic acid β-alanine to react with the free amine in the epsilon position of the N^{α}-BOC-L-lysine.

3.3.1.1. *N*-(Bromoacetyl)-β-alanine

A solution of β-alanine (53.5 g, 0.60 mol) in 600 mL of water was cooled to 5°C with an ice-alcohol bath. Bromoacetyl bromide (60.0 mL, 0.66 mol) was added under efficient stirring at such a rate as to maintain the temperature below 12°C. Concurrently, 5*M* NaOH were added at a rate needed to keep the pH near 7. These conditions were maintained for

45 min after completing addition of bromoacetyl bromide. The pH of the solution was then adjusted to 1.9–2.0 using 48% HBr, and its volume was reduced using a rotary evaporator to ca. 150 mL using a 60°C water bath and aspirator vacuum. The heavy precipitate of NaBr was removed by suction filtration and washed with ca. 15 mL water.

The filtrate was treated with a small amount of water to dissolve the NaBr and then shaken once with hexane-ethyl ether 1:1 v/v (450 mL), once with ethyl ether 1:5 v/v (450 mL each time). The upper phase (rich in bromoacetic acid) and final lower phase were discarded. The next five upper phases were pooled, filtered, and rotary evaporated to remove solvent. The residue was dried under vacuum and crystallized from hot ethyl acetate (81 mL) by addition of hexane (about 12 mL) and cooling at 4°C. The dried product (31.4 g) was similarly recrystallized from ethyl acetate plus hexane and dried under vacuum. The yield was 23.8%.

3.3.1.2. Succinimidyl 3-(bromoacetamido)propionate (SBAP)

To a solution of *N*-(bromoacetyl)-β-alanine (21.00 g, 100 mmol) and *N*-hydroxysuccinimide (13.01 g, 113 mmol) in 2-propanol (280 mL) at room temperature was added 1,3-diisopropylcarbodiimide (16.0 mL, 101 mmol). After 8–12 min, an oily precipitate of the product began to appear, and the walls of the container were scratched to induce crystallization. The mixture was allowed to stand for 1 h at room temperature and overnight at 4°C. The crystals were collected, washed with 2-propanol (30 mL), and redissolved in 2-propanol (200 mL brought to reflux). After an overnight stand at 4°C, the crystals were collected, washed with 2-propanol and then hexane, and dried under vacuum/$CaCl_2$. The yield was 74.6%.

3.3.1.3. N^{α}-(*tert*-butoxycarbonyl)-N^{ε}-[*N*-(bromoacetyl)-β-alanyl]-L-lysine (BBAL)

N^{α}-BOC-L-lysine (17.73 g, 72 mmol) was ground to a fine powder and suspended in DMF (600 mL). SBAP (18.43 g, 60.0 mmol) was added to the suspension in five portions at 10-min intervals. The reaction mixture was stirred for 2 h at room temperature, allowed to stand overnight at 4°C, filtered, and rotary evaporated to remove DMF (bath 30°C, vacuum pump). The residue was shaken with a mixture of ethyl acetate (960 mL), 1-butanol (240 mL), and aqueous 0.2*M* $KHSO_4$ (300 and 150 mL, respectively), filtered (Whatman #1 paper), and rotary evaporated to remove solvent (32°C water bath, pump vacuum). Vacuum was applied

for at least 2 h to remove traces of solvent. The oily residue was dissolved in 1,2 dichloroethane (400 mL) by gentle warming and swirling. The solution was slowly cooled to 15–20°C, during which time the product initially precipitated as an oil, but was induced to crystallize by scratching. After an overnight stand (4°C), the product was collected, washed in dichloroethane, and dried in vacuum. The yield was 65.8%.

The chemistry for adding BBAL to a synthetic peptide at any desired position involves the formation of the 1-hydroxybenzotriazole (HOBt) ester (Fig. 1) of BBAL in DMF; BBAL is sparingly soluble in CH_2Cl_2, the solvent used to dissolve many of the other *t*BOC-derivatized amino acids in the formation of symmetric anhydrides. BBAL has been used with *t*BOC chemistry only; theoretically, the nucleophiles that remove Fmoc-protecting groups in Fmoc-based peptide synthesis also might react with the bromoacetyl moiety of BBAL, although we have no absolute proof of this occurring.

On a 0.5-mmol scale:

1. A mixture of BBAL and HOBt is made by dissolving 2.0 mmol BBAL (878 mg) in a solution containing 2.0 mmol of HOBt in 4.0 mL DMF and 0.3 mL CH_2Cl_2.
2. A solution containing 4.0 mL of 0.5*M* DCC in CH_2Cl_2 is added to the BBAL-HOBt mixture.
3. Agitate the mixture by bubbling N_2 gas through it for a period of at least 30 min at 25°C.
4. Filter off the DCU byproduct.
5. React the BBAL-HOBt ester that is in the filtrate with a free amine on the protected peptide or resin to couple the BBAL to the peptide.
6. If continuing with peptide elongation, *t*BOC is removed from the coupled BBAL with TFA in CH_2Cl_2 and coupling the remaining amino acids proceeds as usual.

3.4. Deprotection of Bromoacetylated Peptides

As mentioned above, bromoacetyl groups remain intact during the routine deprotection protocols for *t*BOC-based peptide synthesis. The only precaution is that sulfur-containing scavengers, such as thiocresol, thiophenol, or thioanisole, should be avoided.

When a bromoacetylated peptide contains an SH-bearing amino acid (or other strong nucleophile), extra precautions following HF (*see* Chapter 4) or TFA (*see* Chapter 5) deprotection should be taken to prevent undesirable reactions. These include the use of ice-cold extraction solu-

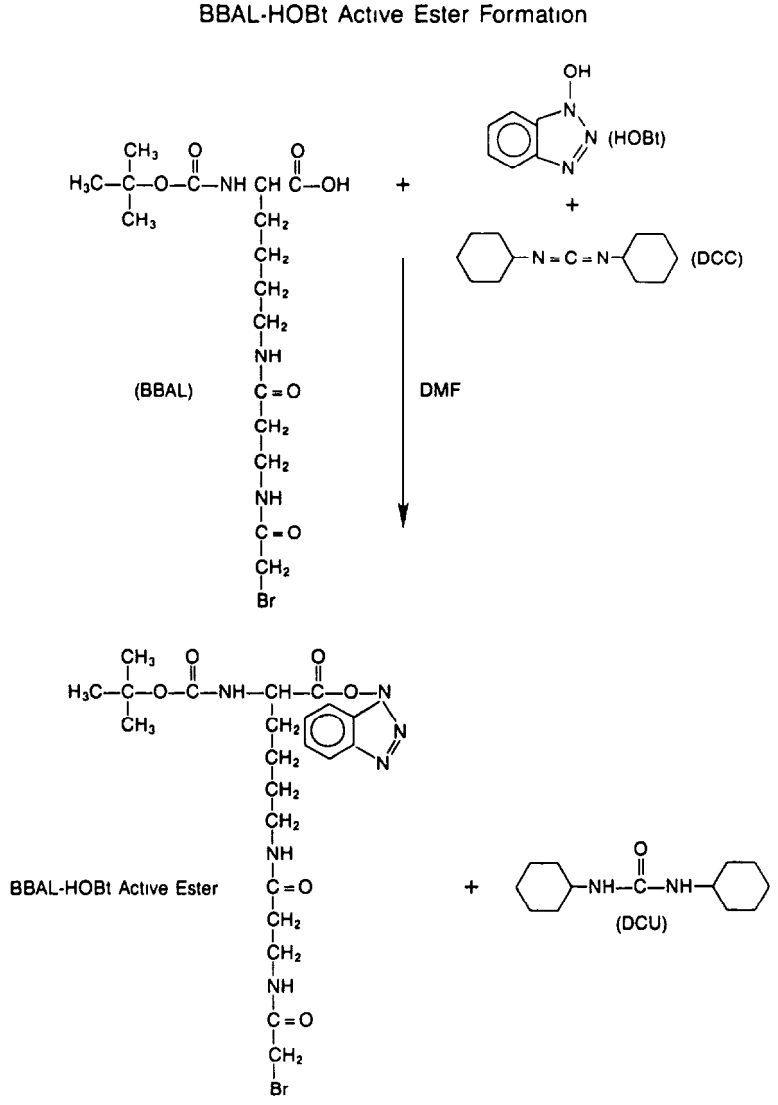

Fig. 1. Reaction scheme for the formation of the HOBt ester of BBAL. The active ester is formed as a result of DCC-mediated coupling of BBAL to HOBt. The ester is then used to couple BBAL to the protected peptide as outlined in Section 3.3.

tions and ice-cold, aqueous acid solvents (such as 10% aq. acetic acid) in which the peptide is dissolved prior to further purification. Bromoacetylated synthetic peptides should be stored dry and frozen in the dark to

prevent decomposition, although storage at room temperature in a closed container appears to be suitable for up to 1 wk. Longer periods of time have not been investigated (*see* Note 1).

Finally, it may be necessary to use chloroacetyl moieties instead of bromoacetyl moieties if the bromoacetyl appears to be unacceptably reactive. We have notice that, unpredictably, certain bromoacetylated peptides react with the thiol in a cysteine even in cold solutions of anhydrous hydrogen fluoride (HF) (*see* Chapter 4).

3.5. Synthesis of Cyclic Peptides from Bromoacetylated Peptides

At neutral pH and room temperature, bromoacetyl moieties in buffered aqueous solutions are very reactive toward SH-containing materials, such as the thiol group in cysteine. Thus, if a cysteine is present in a bromoacetyl-containing peptide, it is very likely that the SH will attack the bromoacetyl to form inter- and/or intramolecular thioether bridges. Intramolecular crosslinking of peptides results in a cyclic peptide that will often have activities different from those found in the linear analog. A general reaction scheme to summarize the formation of cyclic peptides starting with a bromoacetyl, cysteine-containing peptide is given in Fig. 2. The ability of a peptide to cyclize cannot be predicted at the present time, but it is strictly dependent on the sequence of amino acids that are present between the bromoacetyl and thiol moieties and to a lesser extent on those outside of these boundaries. Thus, a few pilot experiments suggested below should be performed with a bromoacetylated, thiol-containing peptide to evaluate its ability to cyclize.

A major property of a peptide that is simple to evaluate is its elution time from a reverse-phase (C18, C8, or C4) column. As a general rule, a cyclic peptide will appear in the eluant from a reverse-phase column (*see* Chapter 3, *PAP*) earlier than the noncyclized peptide when the column is eluted with a linear gradient of increasing amounts of an organic solvent, such as CH_3CN. The reason is most likely that the cyclic peptide has less available hydrophobic surface area to bind to the resin than the linear, unfolded peptide.

To determine by reverse-phase HPLC if a bromoacetylated, cysteine-containing peptide can be cyclized:

1. The pure peptide is dissolved at 1 mg/mL in an ice-cold aqueous acidic buffer (such as 0.1% TFA), and an aliquot is immediately analyzed by HPLC. Evaluate the elution time of the peptide from a reverse-phase HPLC

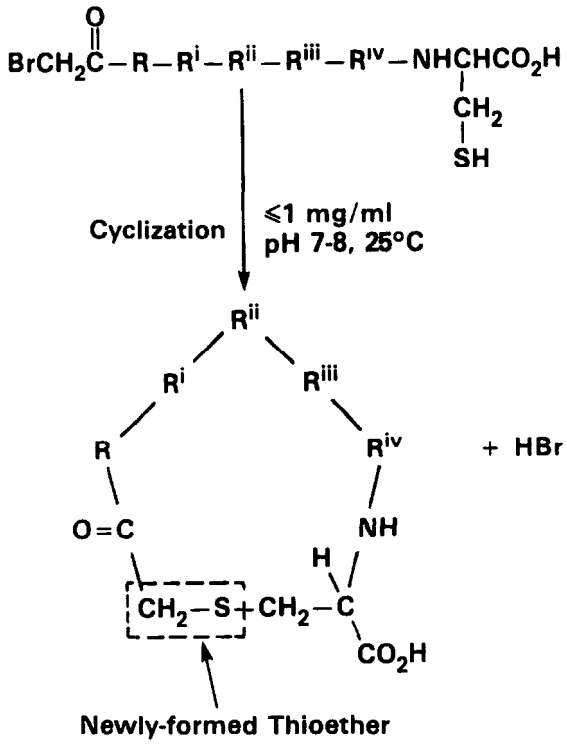

Fig. 2. Reaction scheme for the formation of cyclic peptides using a theoretical bromoacetylated, cysteine-containing peptide as the starting material. When this reaction is buffered with a sodium-containing buffer, such as $NaHCO_3$, the only byproduct of the cyclization reaction is NaBr, an innocuous salt.

column using a linear gradient of 0–70% CH_3CN over 20 min. Use 100 µL/run of peptide-containing solutions having a concentration of 1 mg/mL with detection at 210 nm.

2. Place some of the dry peptide into a buffer at pH 7–8 at a concentration of ≤1 mg/mL.
3. After 1 h at 25°C, run an analytical HPLC of the peptide incubated at a neutral pH, and compare this analysis to that from step 1 above. If there is a peak in front of the original linear peptide peak in the HPLC chromatograph, this is most likely the cyclized peptide.
4. Continue the incubation as in step 2 above until the HPLC profile is no longer changing.

In addition to the elution time by reverse-phase HPLC, further proof for the existence of a cyclic peptide can be obtained by testing the puri-

fied materials with DTNB for the presence of detectable SH groups *(10)*, and amino acid analysis for the presence of CMC and the other expected amino acids that would be present in acid hydrolysates of cyclic peptides containing the thioether formed as outlined above *(5–7)*. The most conclusive proof is mass spectrometry to verify an expected mass from the parent molecular ion of the cyclized peptide. Following the proteolysis of a cyclic peptide by enzymatic digestion, products that could only be formed by having the cyclic materials present at the start could be isolated and characterized (*see* Note 2).

3.6. Synthesis of Peptomers from Bromoacetylated Peptides

"Peptomer" is a new term we are using for a peptide polymer formed by the crosslinking of a peptide to itself in a specific fashion *(11)*. The biological characteristics of peptomers are generally not known, but they are being studied very closely as immunogenic materials for vaccine candidates, receptor crosslinking agents, enzyme substrates, or as research tools for the understanding of the chemical and conformational effects of specific amino acid sequences.

As mentioned above, a bromoacetylated, cysteine-containing peptide either will cyclize, polymerize, or do both. The chances of favoring a peptomer-forming reaction appear to be best at high concentrations (≥10 mg/mL) of the bromoacetylated, cysteine-containing peptide dissolved or suspended in an aqueous buffer at pH 6–8. At a pH >9, the thioate anion may compete with other nucleophiles, such as the ε-amine of lysine, and when this occurs, selective control of the peptide polymerization reaction is lost. A scheme for polymerizing peptides starting with a bromoacetylated, cysteine-containing peptide is shown in Fig. 3.

As noted above for cyclic peptides, peptomer formation can be followed by reverse-phase HPLC. In contrast to the formation of cyclic peptides where the cyclized form elutes from the column before the noncyclized starting material, peptomers will elute later than the starting material. An example of this is given in Fig. 4, where the reverse-phase HPLC of an incomplete polymerization reaction is shown. The A panel of Fig. 4 shows the purified bromoacetylated, cysteine-containing starting material. The multiple peaks of the peptomer appear in highest quantity along with small amounts of the cyclic peptide and the starting material as shown in panel B. The C panel shows the HPLC of the

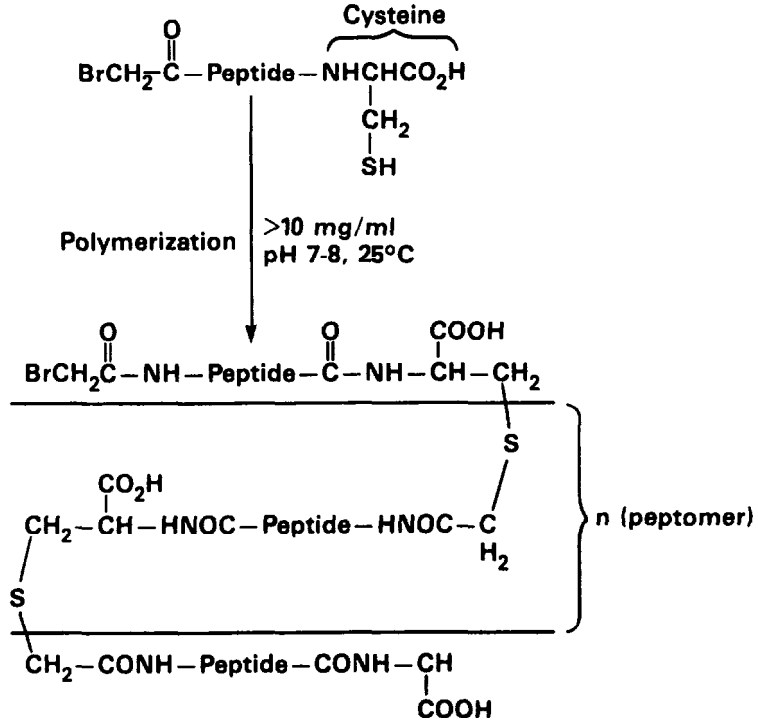

Fig. 3. Reaction scheme for the formation of a peptomer using a hypothetical bromoacetylated, cysteine-containing peptide as detailed in Section 3.6. As with the cyclization reaction shown schematically in Fig. 2, the only byproduct of the polymerization reaction is NaBr.

peptomer after dialysis to remove the low-mol-wt components from the peptomer mixture.

Peptomer formation may be followed using DTNB for the disappearance of the free thiols *(10)* and by SDS-PAGE. In contrast to cyclic peptides, mass spectrometric analyses (*see* Chapter 7, *PAP*) of peptomers may be uninterpretable. The lot-to-lot quality control of peptomers, therefore, would consist of the reverse-phase HPLC and mass spectrometric analysis of the starting peptide, and the reverse-phase HPLC and SDS-PAGE patterns of the peptomer formed. (The key considerations for identity and manufacturing would reside in the lot-to-lot consistency of the starting monomeric peptide and the peptomer formed, but not in the purity of the peptomer itself.) An example of the SDS-PAGE of a sample peptomer (the same as analyzed in Fig. 4) is shown in Fig. 5.

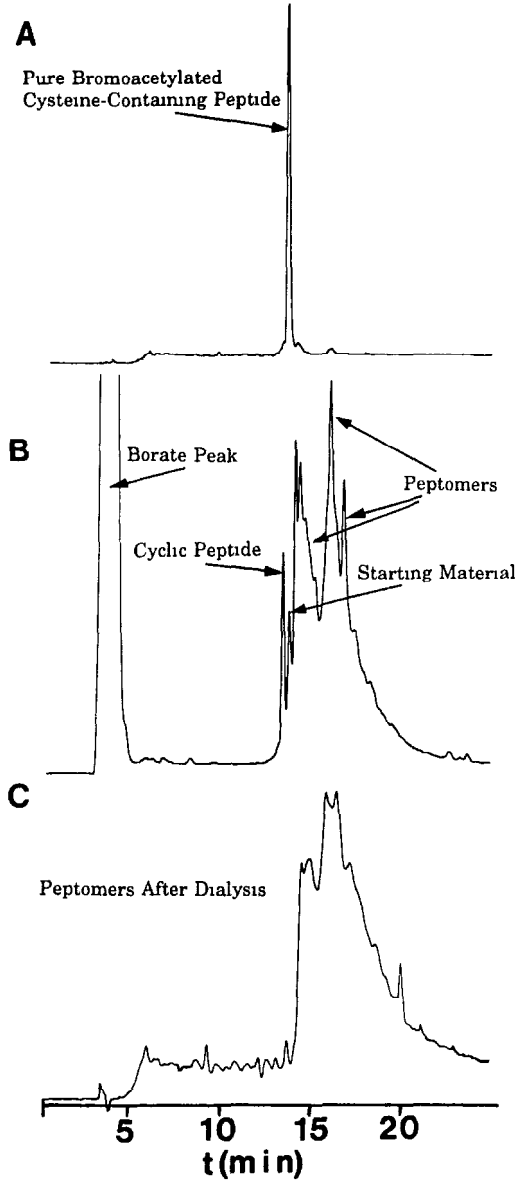

Fig. 4. Reverse-phase HPLC on a Vydac C18 column of: (**A**) pure $BrCH_2CO$-K-R-K-R-I-H-I-G-P-G-R-A-F-C-OH. (**B**) HPLC analysis of the above peptide after stirring at room temperature for 3 h in 0.1*M* $NaHBO_3$, pH 8.5, buffer. The cyclic peptide, starting peptide, and peptomer peaks are noted by the arrows in the figure. (**C**) Chromatogram of the reverse-phase HPLC after dialysis to remove the starting materials and dialyzable byproducts from the peptomer solution.

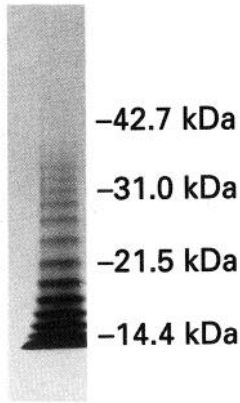

Fig. 5. SDS-PAGE of a representative peptomer from the same peptide given in the legend to Fig. 4. Mol-wt standard values are given to the right of the figure.

3.7. Synthesis of Peptide Conjugates from Bromoacetylated Peptides

There are three very important advantages of synthesizing protein conjugates with bromoacetylated peptides compared with conventional conjugation chemistry (*see* Note 3). First, the position of the bromoacetyl moiety in a peptide can be controlled. Second, by assaying for the amount of thioether present in acid hydrolysates of a protein-peptide conjugate, reliable values for the amount of covalently linked peptide can be determined. Finally, where in vivo uses of the conjugate are intended, it is reassuring to know that the thioether bonds or the long chain of BBAL (cf Fig. 1) appear to be nonimmunogenic (F. A. Robey, unpublished data).

A general strategy for covalently linking bromoacetylated peptides to sulfhydryl-containing proteins has been given in detail *(7)*. The primary starting point is to introduce the free thiol groups into the carrier for subsequent reactions with bromoacetyl moieties. For proteins, there are three ways to achieve this goal. First, the protein naturally contains free sulfhydryls without any modification. Because of the susceptibility of thiols to oxidize on storage, commercial sources of these proteins are rare. In addition, some proteins, such as papain, are stored in a solution or as a suspension containing excessive amounts of thiol-containing preservatives, and these would certainly destroy the bromoacetyl before it could react with the protein's thiol groups. Removal of the preserva-

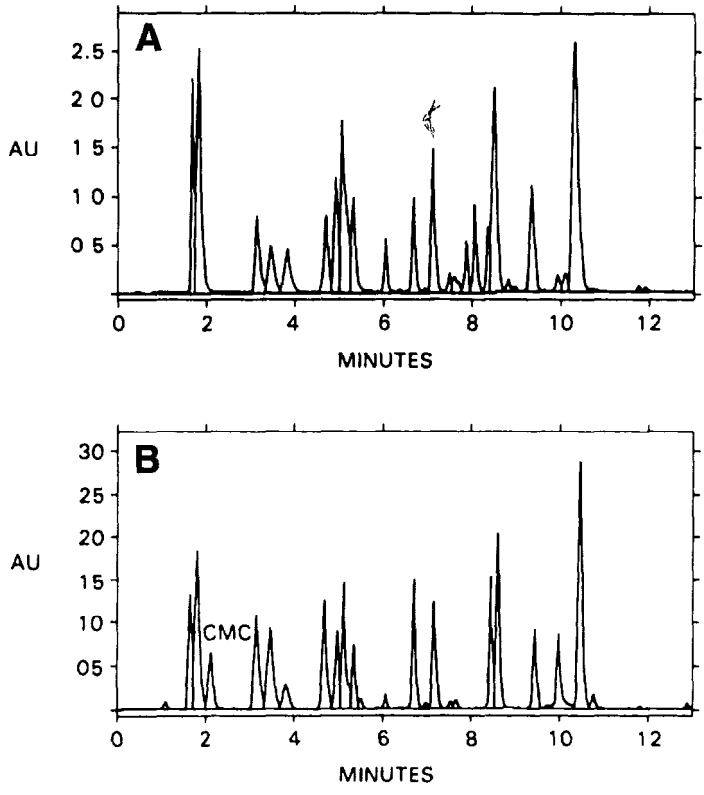

Fig. 6. Amino acid analysis chromatogram of (**A**) BSA and (**B**) the YIGSR conjugated to BSA. Bromoacetyl-YIGSR was reacted with Bu_3P-reduced BSA to form the conjugate. The procedure is outlined in Section 3.7., and the details of the conjugation are taken from ref. *7*. The value taken from the integrated area for the CMC is representative of the minimum amount of YIGSR covalently linked to BSA. With permission, Academic Press, Inc.

tives would probably sacrifice the protein's thiols because of auto-oxidation reactions.

Second, disulfide-rich proteins can be reduced to offer free thiols. Examples are serum albumin and immunoglobulin. Prior to the reaction with the bromoacetylated peptides, the disulfides are reduced with DTT or Bu_3P *(7)*. An amino acid analysis chromatogram for the YIGSR peptide conjugated to BSA is given in Fig. 6. To make the YIGSR-BSA conjugate, the bromoacetylated YIGSR was reacted with Bu_3P-reduced BSA as described in *(7)*. Finally, new thiols may be introduced into a

protein by the reaction of a functional group on a protein with certain compounds, such as 2-iminothiolane *(12)*, *N*-acetylhomocysteine thiolactone *(13)*, or the *N*- hydroxysuccinimide ester of *S*-acetylthioacetic acid (SATA) *(14)*. These compounds covalently attach to the α-amine of proteins and the ε-amines of lysine residues in proteins. Although the chemistry of these thiol-producing compounds is very efficient and reproducible, they do not allow for the convenient quantitative analysis of covalently coupled peptide as can be done when a peptide's own cysteines are used for the conjugation *(7)*.

An example of introducing free thiols into bovine serum albumin using 2-iminothiolane and conjugating to a haloacetyl-derivatized peptide is outlined below as reported previously *(5)*.

1. Thirty milligrams of bovine serum albumin is dissolved in 2 mL 0.1*M* $NaHCO_3$ (pH 8.3). To this solution are added 4 mg 2-iminothiolane, and the reaction mixture is stirred for 15 min at room temperature.
2. The protein is separated from the unreacted reagents and byproducts by gel filtration chromatography (Sephadex G-25 (fine); 1.5 × 10 cm column, 0.1*M* $NaHCO_3$ buffer).
3. Fractions containing the modified protein as judged by the 280-nm absorbance and a positive DTNB reaction *(10)* for free thiols are pooled.
4. Protein in solution is reacted with a haloacetyl-containing peptide by adding the solid haloacetyl-containing peptide (20–50 mg) to the solution and stirring the mixture for 3 h at room temperature or until there are no detectable SH groups as tested with DTNB *(10)*.

For the above example, the degree of reaction can be estimated by following the amount of sulfhydryl that is consumed by the haloacetyl-containing peptide. However, it must again be stressed that this does not give a value for the covalently bound peptide.

4. Notes

1. The stability of bromoacetyl peptides is not known, but lyophilized peptides that are stored desiccated should be stable for 1 wk. Because of the lack of information available concerning storage conditions suitable for longer than 1 wk, it is recommended that storage in a desiccator at –20°C be used.
2. Thioether-containing peptides that are formed by reacting bromoacetyl- or chloroacetyl-containing peptides with free SH-containing materials are very stable because of the stability of the thioether bond. However, the sulfur atom can be oxidized, and the peptide material, which is often

hygroscopic, could act as a source of nutrient for microorganisms. Therefore, the storage of the cyclic peptides, peptomers, and peptide conjugates should be under a dry, oxygen-free atmosphere.

3. This chapter emphasized the reactivity of free thiols with haloacetyl moieties. However, there are other nucleophiles, such as histidine, methionine, homocysteine, and thiocarboxylic acid, that could be present on a synthetic peptide and that have the ability to react with the haloacetyl group. During the work-up of haloacetyl-containing peptides that contain various strong nucleophiles, one should be aware of possible side reactions of the nucleophiles with the haloacetyl moiety. This could occur even if they do not occur with cysteine as described here.

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