Protein Synthesis by Solid-Phase Chemical Ligation Using a Safety Catch Linker

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The native chemical ligation reaction has been used extensively for the synthesis of the large polypeptides that correspond to folded proteins and domains. The efficiency of the synthesis of the target protein is highly dependent on the number of peptide segments in the synthesis. Assembly of proteins from multiple components requires repeated purification and lyophilization steps that give rise to considerable handling losses. In principle, performing the ligation reactions on a solid support would eliminate these inefficient steps and increase the yield of the protein assembly. A new strategy is described for the assembly of large polypeptides on a solid support that utilizes a highly stable safety catch acid-labile linker. This amide generating linker is compatible with a wide range of N-terminal protecting groups and ligation chemistries. The utility of the methodology is demonstrated by a three-segment synthesis of vMIP I, a chemokine that contains all 20 natural amino acids and has two disulfide bonds. The crude polypeptide product was recovered quantitatively from the solid support and purified in 20%-recovered yield. This strategy should facilitate the synthesis of large polypeptides and should find useful applications in the assembly of protein libraries.

Introduction

The development of solid-phase methods has transformed the field of peptide synthesis[1] and, more recently, organic chemistry in general.[2] Although solid-phase synthesis of peptides is dependable up to ∼50 amino acids, synthesis of the proteins is difficult as a result of the accumulation of side products during chain assembly and deprotection.[3] Consequently, protein synthesis is dominated by approaches that depend on the assembly of smaller peptide fragments.[4−6] One such technique, native chemical ligation,[7,8] utilizes a highly chemoselective coupling of two unprotected peptides, one of which bears an N-terminal cysteine residue while the other contains a C-terminal thioester group. These reactions are generally performed in solution, where as many as four peptide segments have been linked together sequentially.[9,10]

Typical ligation reactions proceed with high efficiency (>90%), and a single ligation step produces the desired polypeptide in high purity and reasonable yields (40−60% recovered). Unfortunately, performing multiple ligation reactions in solution requires repeated HPLC separations. These additional steps result in considerable handling losses when synthesizing polypeptides from several segments. As a result, recovered yields of <15% for a three-segment ligation[11] and <10% for a four-segment ligation[10] have been recently reported. In principle, performing ligation reactions on a solid phase would eliminate the need for chromatographic separations after each ligation reaction and would increase the yield and reduce the time required for the assembly of the target protein.[4]

Despite the obvious advantages of the solid-phase ligation method, the general applicability of the method has been restrained by the complexity of selecting a compatible set of ligation chemistries, resin linker, and protecting groups. As illustrated in Scheme 1, the solid-phase ligation approach requires (1) a solid support that is chemically stable and has good swelling characteristics for all ligation, deprotection, and cleavage conditions; (2) a chemoselective reaction to link the first peptide to the solid support; (3) a cleavable linker to remove the polypeptide from the solid support after completion of the peptide assembly; and (4) an N-terminal protecting group to prevent cyclization/polymerization reactions with the internal (N-terminal Cys and thioester-containing) segments during the assembly.

The primary limitation to this approach is that both the cleavable linker and the N-terminal protecting group have to be stable under a variety of chemical conditions.

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(1) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149−2154.
(2) Früchtel, J. S.; Günther, J. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 17−42.
(3) Kent, S. B. H. *Annu. Rev. Biochem.* **1988**, *57*, 957−984.
(4) Wilken, J.; Kent, S. B. H. *Curr. Opin. Biotechnol.* **1998**, *9*, 412−426.
(5) Sakakibara, S. *Biopolymers* **1995**, *37*, 17−28.
(6) Quibell, M.; Packman, L. C.; Johnson, T. *J. Am. Chem. Soc.* **1995**, *117*, 11656−11668.
(7) Dawson, P. E.; Muir, T. W.; Clarklewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779.
(8) Tam, J. P.; Lu, Y.-A.; Liu, C.-F.; Shao, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12485.
(9) Muir, T. W.; Dawson, P. E.; Kent, S. B. H. *Methods Enzymol.* **1997**, *289*, 266−298.
(10) Hackeng, T. M.; Griffin, J. H.; Dawson, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10068−10073.
(11) Beligere, G. S.; Dawson, P. E. *Biopolymers* **2000**, *51*, 363−371.

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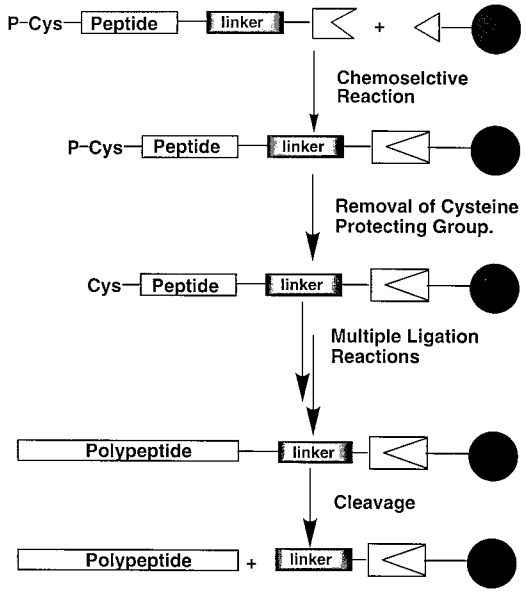
Scheme 1 . Principle of Solid-Phase Chemical Ligation



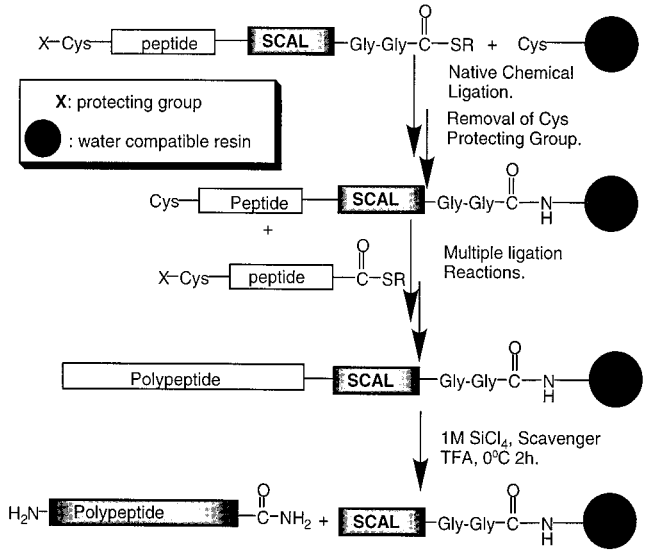
First, they must be stable to TFA and anhydrous HF during the assembly and cleavage reactions of Boc-based solid-phase peptide synthesis (SPPS).[12] Second, they must be stable to thiol nucleophiles under the native chemical ligation conditions: 6 M Gdm.Cl, 2% thiophenol, 2% benzyl mercaptan, pH 7.0.[13] Third, they must be chemically unreactive toward each other. Finally, all reaction conditions must keep the unprotected peptide/solid support network fully solvated.[14]

A recently described system for solid-phase ligation utilizes an oxime-forming ligation to attach the first peptide to the resin, a selectively cleavable ester link to remove the peptide from the resin as a C-terminal carboxylic acid, and the Acm group to protect the N-terminal cysteine residue.[15] Here, a complimentary approach for the assembly of unprotected peptides on a solid support is described. This approach utilizes a highly stable, safety catch acid-labile (SCAL) linker[16] that produces an amide C-terminus after cleavage and is compatible with a wide range of N-terminal protecting groups and ligation chemistries. The utility of our methodology is demonstrated by the synthesis of vMIP I from three peptide segments. The crude polypeptide product was recovered quantitatively from the solid support and purified in 20% recovered yield.

Results and Discussion

A robust and synthetically flexible strategy for chemical ligation on a solid support is described in Scheme 2. This C-to-N terminus approach begins with a sepharose solid support bearing an N-terminal cysteine residue. The initial peptide segment contains a thioester C-terminus[17,18,10] to allow straightforward attachment to the solid

Scheme 2 . Solid-Phase Chemical Ligation Using a Safety Catch Linker and Native Chemical Ligation



support by native chemical ligation. In addition, a SCAL linker is incorporated into the C-terminal peptide. The SCAL linker is stable to both SPPS and ligation conditions but is selectively cleaved by TFA following reduction of the linker sulfoxide groups.[16] Finally, The N-terminal cysteine residue is protected on either the side chain thiol and/or the terminal amine using two different protecting groups, N^{α}Msc[19] and S^{β}Acm.

Solid Support. The ideal support for solid-phase ligation must remain swollen in a variety of solvents and be stable to a broad range of pH values and to both oxidizing and reducing conditions. Sepharose resin consisting of 4% cross-linked agarose functionalized with ~20 μmol primary amine/mL of gel has proven to be an excellent solid support. DADPA gel (diaminodipropylamine 4% cross-linked beaded agarose) is functionalized with free amines at 16−20 μmol/mL and is commercially available from Pierce. This support can be acylated using standard solid-phase synthesis procedures;[21] in this example, Boc-Cys(Trt)-OH was coupled in using HBTU/DIEA in DMF.[22]

The sepharose support is compatible with basic conditions (pH 13, 20 min combined reaction times) and for over 2 h in TFA. The support also remains highly solvated throughout the synthesis, even when it is derivatized with long polypeptide chains. As observed during standard SPPS on polystyrene resins,[14] the sepharose solid support swells significantly (greater than 2-fold in volume) as the polypeptide length increases. This mutual solvation of peptide and resin allows the attached peptide to be soluble at high (~10 mM) concentrations and should allow for large (>200 aa) polypeptides to be assembled.

The choice of a polymeric support is an essential factor for the success of solid-phase ligation. To examine the role of the solid support on the ligation chemistry, several

(12) Merrifield, R. B. *Science* **1986**, *232*, 341−347.
(13) Dawson, P. E. *Methods Enzymol.* **1997**, *287*, 34−45.
(14) Sarin, V. K.; Kent, S. B. H.; Merrifield, R. B. *J. Am. Chem. Soc.* **1980**, *102*, 5463−5470.
(15) Canne, L. E.; Botti, P.; Simon, R. J.; Chen, Y.; Dennis, E. A.; Kent, S. B. H. *J. Am. Chem. Soc.* **1999**, *121*, 8720−8727.
(16) Patek, M.; Lebl, M. *Tetrahedron Lett.* **1991**, *32*, 3891−3894.
(17) Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 111−117.
(18) Hojo, H.; Kwon, Y.; Kakuta, Y.; Tsuda, S.; Tanaka, I.; Hikichi, K.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2700−2706.
(19) Tesser, G. I.; Balvert-Geers, I. C. *Int. J. Pept. Protein Res.* **1975**, *7*, 295.
(20) Veber, D. F.; Milkowski, J. D.; Varga, S. L.; Denkewalter, R. G.; Hirschmann, R. *J. Am. Chem. Soc.* **1972**, *94*, 5456−5461.
(21) Tegge, W.; Frank, R. *Int. J. Pept. Protein Res.* **1997**, *49*, 355−362.
(22) Schnolzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180−193.

resins were studied. For example, CLEAR resin (cross-linked ethoxylate-acrylate resin), developed by Barany, has been successfully used in solid-phase synthesis.[23] This resin has excellent swelling properties in both organic solvent and water. Although this resin allowed for efficient ligation of the first segment to the solid support, upon Acm removal with $Hg(II)(OAc)_2$ in acetic acid, the peptide−resin completely dissolved, presumably as a result of ester hydrolysis. Furthermore, this polyester resin is known to be hydrolyzed under the basic conditions (pH 13) needed for the removal of Msc groups.

PEGA resin (poly(ethylene glycol)-poly-(*N*,*N*-dimethylacrylamide))[24] was also investigated. This support has used been successfully for peptide cylization using native chemical ligation.[25] As with CLEAR resin, PEGA had good swelling properties and allowed efficient ligation of the first segment. Successful removal of both the Msc and Acm protecting groups was verified by HPLC/MS analysis of the peptide product following cleavage of a sample of the resin. Ligation of the second segment, however, was incomplete, resulting in <50% ligation in each case. Yields were not improved by adding a large (>10 equiv) excess of peptide-thioester, increasing the temperature, or increasing the ligation time. This poor coupling could be a result of peptide aggregation on the support due to the high loading of amine groups (300−400 μmol/g) on the resin (10- to 20-fold higher than the sepharose supports).

Attachment of the C-Terminal Peptide to the Solid Support. Since the chemical ligation approach requires that all peptide side chains be unprotected, a highly chemoselective reaction must be used to link the first peptide to the solid support. Many ligation chemistries have been developed that form thioester,[26] thioether,[27] oxime/hydrazone,[28,29] thiazoidine,[30] and amide bonds[30] at the C-terminus. In this study, the amide bond forming "native chemical ligation"[7,8] was selected. As described in Figure 1, attachment of the first segment to the solid support proceeds rapidly in denaturing aqueous solution with only a small excess (1.1 equiv) of peptide. If desired, a limiting amount of peptide could be used, followed by capping the remaining active groups on the resin with small molecules bearing a thioester group. One advantage of using native ligation to attach the C-terminal peptide to the solid support is that the same chemical reaction is used for the subsequent assembly of the target polypeptide from its segments. Limiting the number of chemoselective reactions used in the synthesis enables other reactive groups including aminooxy or ketone functionalities to be incorporated into the peptides without protection. Such reactive groups have been used for the chemoselective attachment of sugars or fluorophores using oxime linkages.[31,32]

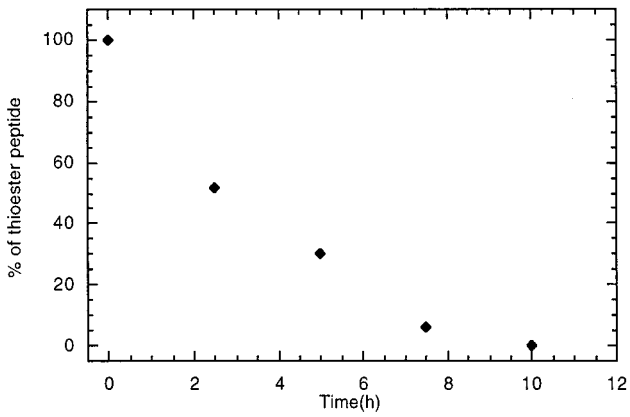


Figure 1. Consumption of thioester peptide during ligation reaction. The ligation of vMIP-I (Msc-(36−71)-SCAL-Gly-Gly-SR) to Cys-Sepharose was monitored by the diappearance of the peptide from solution. Samples were taken at 2.5 h intervals and analyzed by HPLC. The amount of peptide was quantitated by integration of the HPLC peaks detected at 214 nm.

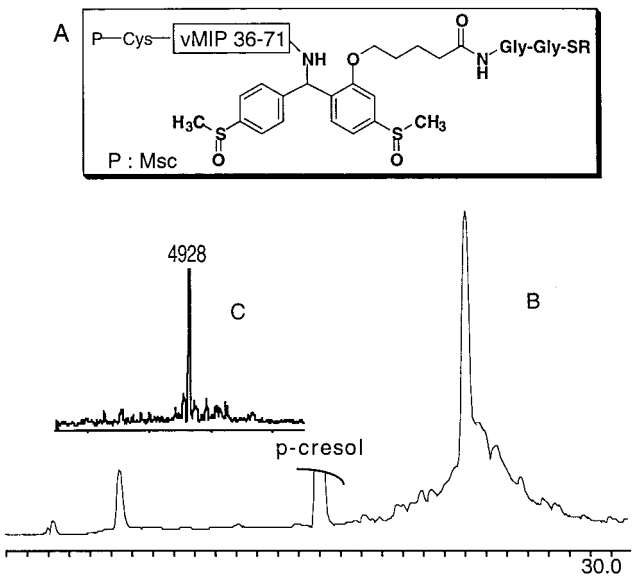


Figure 2. (A) vMIP-I (Msc-(36−71)-SCAL-Gly-Gly-SR). The structure of the SCAL linker is shown. (B) Analytical HPLC (C4 reversed-phase 0−60% acetonitrile/30 min, monitored at 214 nm) of crude peptide vMIP-I (Msc-(36−71)-Gly-Gly-SCAL-SR) after HF cleavage. (C) Electrospray mass spectrum (raw data displayed reconstructed to a single charge state): observed molecular mass, 4928.0 Da; calculated molecular mass (average isotope composition), 4928.8 Da.

Safety Catch Amide Linker (SCAL). The SCAL linker (Figure 2) developed by Lebl[16] has many advantages for solid-phase chemical ligation. In the oxidized form, SCAL is stable to a wide range of chemical conditions. It is stable to both TFA and HF used during Boc-chemistry SPPS and is compatible with Fmoc and Alloc protection strategies. It is also compatible with aqueous thiophenol, reducing agents such as TCEP and DTT, basic conditions (pH 13), and $Hg(II)(OAc)_2$. However, following reduction of the aryl sulfoxide to the corresponding aryl sufide, the SCAL linker becomes labile to TFA. These properties of the SCAL linker makes it compatible with the variety of subsequent chemical manipulations in the course of the polypeptide synthesis.

The SCAL linker has been used for the synthesis of

(23) Kempe, M.; Barany, G. *J. Am. Chem. Soc.* **1996**, *118*, 7083.
(24) Meldal, M. *Tetrahedron Lett.* **1992**, *33*, 3077−3080.
(25) Camarero, J. A.; Cotton, G. J.; Adeva, A.; Muir, T. W. *J. Pept. Res.* **1998**, *51*, 303−16.
(26) Schnolzer, M.; Kent, S. B. H. *Science* **1992**, *256*, 221−225.
(27) Englebretsen, D. R.; Garnham, B. G.; Bergman, D. A.; Alewood, P. F. *Tetrahedron Lett.* **1995**, *36*, 8871−8874.
(28) Gaertner, H. F.; Rose, K.; Cotton, R.; Timms, D.; Camble, R.; Offord, R. E. *Bioconjugate Chem.* **1992**, *3*, 262−268.
(29) Rose, K. *J. Am. Chem. Soc.* **1994**, *116*, 30−33.
(30) Liu, C. F.; Tam, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6584−6588.
(31) Rodriguez, E. C.; Winans, K. A.; King, D. S.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1997**, *119*, 9905−9906.
(32) Cervigni, S. E.; Dumy, P.; Mutter, M. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1230−1232.

short peptides and peptide libraries but has not been utilized for the synthesis of large peptides. Although many procedures for SCAL cleavage have been reported,[16,33] the use of $SiCl_4$/TFA gave the cleanest results with large peptides on the sepharose support. The cleavage reaction reached completion within 2 h at 0 °C and could be visually monitored by the blue color of the benzhydrylium ion[33] that is slowly trapped by scavengers (ethanedithiol, thioanisol, and *m*-cresol). This blue color disappeared after 1 h, at which point the cleavage was found to be greater than 90%.

Cleavage of the SCAL linker yields a C-terminal amide peptide, in contrast to the methodology described by Canne et al. that yields a C-terminal acid.[15] Generation of an amide is highly desirable for the synthesis of protein domains because it mimics the peptide bond more accurately than a carboxylic acid. Consequently, this linker is ideal for the syntheses of single- or multiple-protein domains such as SH3, SH2, EGF, and others. In addition, although most full-length proteins have a C-terminal carboxylate, this group is rarely involved in direct protein contacts. As a result, the SCAL linker should be compatible with most proteins of interest.

N-Terminal Cysteine Protecting Groups. Multiple component syntheses using native chemical ligation require protection of the N-terminal cysteine residue for all "middle" segments to prevent polymerization or cyclization. In this study we protected the N-terminal cysteine either at the amine group using Msc or at the thiol side chain using Acm. The Msc group has been used in the synthesis of several multiple-segment proteins with great success despite the necessity for brief (5 min) treatments at pH 13.[9] The addition of the Acm group to the methodology is particularly advantageous for the synthesis of proteins with base-sensitive groups. The Acm group is removed by $Hg(II)(OAc)_2$ at pH 4. Protecting groups such as isonicotinyloxycarbonyl (iNoc),[34] 6-nitroveratryloxycarbonyl (nVoc),[35] N-terminal Factor X sequences,[36,37] and other enzymatically labile strategies could also be compatible with ligation on the solid support. The use of enzymes, however, could be limited if denaturants are needed for solubility of the peptide−agarose network. In principle, N-terminal protecting groups can be removed by acidic, basic, photolytic, reductive, or oxidative conditions.

Synthesis, Attachment, and Cleavage of vMIP-(35−71)-SCAL-Gly-Gly-thioester. To test the compatibility of the elements described above, we designed a model peptide corresponding to the C-terminus of vMIP I on a thioester resin, incorporating the SCAL linker and a Gly-Gly spacer between the SCAL linker and the thioester (Figure 2). This peptide was synthesized using in situ neutralization protocols for Boc SPPS on a TAMPAL thioester resin using a C-terminal Ala residue.[10] The synthesis of this peptide proved to be straightforward (Figure 2), and purification resulted in a 30% yield of highly homogeneous product by HPLC and ESMS. The resulting peptide was comparable in purity

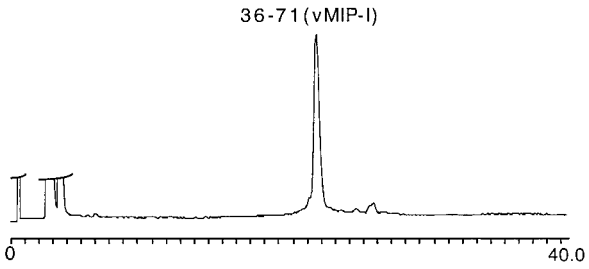


Figure 3. Model study to test attachment, deprotection, and cleavage of a peptide on the sepharose support. vMIP-I (Msc-(36−71)-SCAL-Gly-Gly-SR) was ligated to Cys-sepharose resin, deprotected at pH 13, 5 min, and then cleaved from the support by TFA/scavenger, 0 °C/2 h. The crude cleavage product was analyzed by HPLC (22−45% acetonitrile/30 min). The peptide was recovered in quantitative yield.

to a similar peptide (vMIP-I-(36−71)) synthesized on traditional PAM resin. This synthesis demonstrates the compatibility of the SCAL linker with Boc chemistry and thioester linkages.

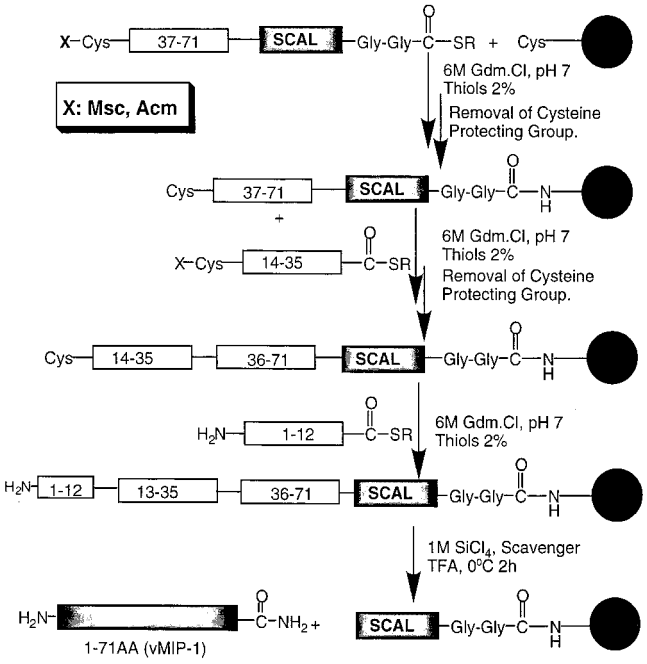
The peptide was then attached to the N-terminal Cys-bearing solid support by adding 1.1 equiv (6 mM) of peptide to 1.0 equiv of Cys-sepharose under standard ligation conditions. The reaction was monitored by measuring the peptide consumption over time using HPLC (Figure 1). The ligation was complete in less than 8 h, as expected for ligation reactions at similar concentrations in solution. The N-terminal protecting group of the resultant Msc-(36−71)-SCAL-Gly-Gly-Cys-resin was removed by exposing the ligation mixture to pH 13 for 5 min, followed by a flow wash with 6 M Gdm.Cl at pH 4. Finally, the peptide was cleaved from the resin by reductive acidolysis using 1 M $SiCl_4$, 10% thioanisol, 6% EDT, and 10% *m*-cresol in TFA at 0 °C for 2 h. The peptide was collected by filtering the TFA from the resin and washing it with additional TFA. The pooled TFA fractions were added dropwise to ether to precipitate the peptide. The peptide was recovered quantitatively and found to be clean by HPLC, Figure 3.

Synthesis of vMIP I. To demonstrate the applicability of the solid-phase ligation approach to protein synthesis, vMIP-1, a 71 amino acid chemokine was synthesized. This target was chosen since it contains all 20 natural amino acids and has four cysteine residues that form two disulfide bonds upon oxidative folding. As shown in Scheme 3, the sequence can be divided into three segments, 1−12, 13−35, and 36−71. The resulting Cys12−Cys13, and Ala35−Cys36 ligation sites are fully compatible with the native chemical ligation approach.

The C-terminal segment was synthesized on a thioester resin with a Gly-Gly spacer and the SCAL linker as described previously. The N-terminal Cys36 was protected with a base-labile N^{α}Msc group. Similarly, for peptides corresponding to vMIP-I-(1−12)-COSR and vMIP-I-(13−35)-COSR, N^{α}Msc was synthesized directly on a thioester resin without the SCAL linker or Gly-Gly sequence. Assembly of the vMIP-1 polypeptide was performed as follows. The C-terminal segment, Msc-(36−71)-SCAL-Gly-Gly-COSR was attached to the Cys-sepharose support by adding a slight excess of peptide (1.2 equiv, 6 mM) to Cys-resin under standard ligation conditions for 8 h. Following deprotection and washing of the support, the next segment, Msc-(13−35)-COSR, was added (1.2 equiv, 8 mM) and allowed to react for 12 h. Finally, after deprotection and washing steps, the

(33) Pétek, M.; Lebl, M. *Collect. Czech. Chem. Commun.* **1992**, *57*, 508−524.
(34) Veber, D.; Paleveda, W. J.; Lee, Y. C.; Hirschmann, R. *J. Org. Chem.* **1977**, *42*, 3286−3288.
(35) Amit, B.; Zehavi, U.; Patchornik, A. *J. Org. Chem.* **1974**, *39*, 192−196.
(36) Erlanson, D. A.; Verdine, G. L. *Protein Eng.* **1997**, *10*, 47.
(37) Xu, R.; Ayers, B.; Cowburn, D.; Muir, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 388−93.

Scheme 3 . Synthetic Strategy for Resin Attachment, Solid-Phase Assembly, and Cleavage for the vMIP-I Polypeptide



N-terminal peptide, (1−12)-COSR (1.2 equiv, 8 mM) was ligated to form the desired polypeptide vMIP-(1−71)-SCAL-Gly-Gly-Cys-sepharose. All ligation reactions were performed at room temperature. This procedure was complete in just 30 h, and the observed ligation times for the individual segments onto the solid support were comparable to similar ligation reactions in solution.

Following the assembly of vMIP-1, the polypeptide was washed with 6 M Gdm.Cl, then with DMF, and finally with CH_2Cl_2. The resin was then treated with a mixture of 1 M $SiCl_4$, 10% thioanisol, 10% *m*-cresol, and 6% ethanedithol in TFA at 0 °C for 2 h. The resultant polypeptide was precipitated in cold ether and analyzed by HPLC and ESMS. The crude peptide product was recovered quantitatively, and the polypeptide corresponding to vMIP-1 was found to be the predominant product, Figure 4B. HPLC/MS analysis of the mixture revealed that most of the observed side products were due to incomplete Msc deprotection. In addition, a product with a mass of M + 108 was observed that is presumably a scavenger adduct formed during the cleavage process.

The ability to protect either the N^{α} or S^{β} groups of the N-terminal cysteine allows for greater choice in selecting protecting groups with different cleavage properties. A second synthesis was perfomed using Cys(S^{β}Acm), a Hg(II)$(AcO)_2$ labile group on the side chain thiol of the N-terminal Cys. Starting from the same peptide-resins described above, Boc-Cys(Acm) was coupled to the N-terminus, before cleavage and purification. The peptide-thioesters corresponding to 1−12, Acm-(13−35), and Acm-(36−71)-SCAL-Gly-Gly were assembled in a sequential manner using 15 mg/mL Hg(II)$(AcO)_2$ in 10% acetic acid, pH 4, for 30 min to remove the Acm protecting group.[15,20] This procedure produced the crude vMIP-(1−71) polypeptide in quantitative yield and similar purity compared to the Msc protection approach (Figure 5). Use of the Cys(Acm) group avoids treatment with base during the assembly process. As observed in other studies,[15] the solid support allows the unreacted Hg(II) and quenched Hg$(SCH_2CH_2OH)_2$ to be removed by washing the resin with 6 M Gdm.Cl.

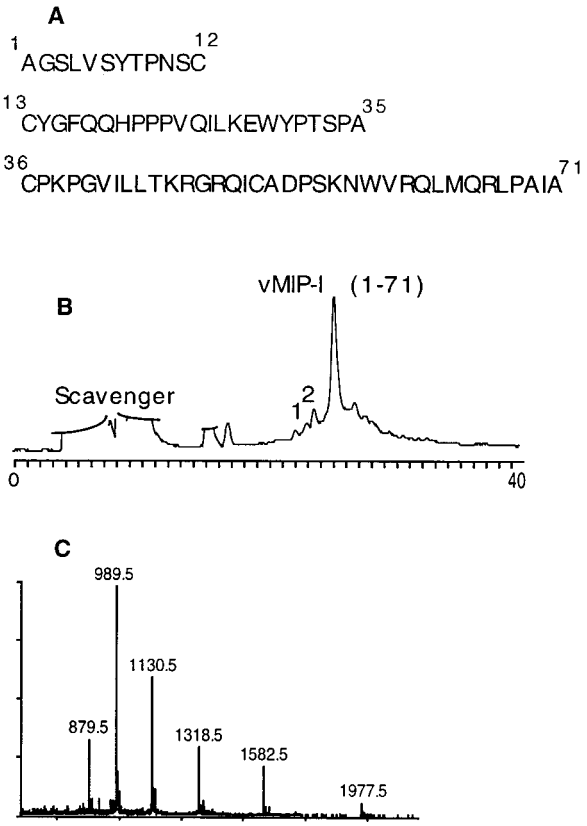


Figure 4. Synthesis of the polypeptide corresponding to vMIP-I-(1−71) using Msc protecting strategy. **(**A**)** The sequence of the target polypeptide. **(**B**)** Analytical HPLC of the crude cleavage product from solid-phase ligation. **1** represents an incomplete removal of the Msc protecting group from the segment vMIP-(36−71) and has a mass of 4209 daltons. **2** represents a deletion of the second peptide segment vMIP-(13−35) and has a mass of 5238 Da. (C) Electrospray ionization mass spectrum of the purified vMIP-I. The $4H^+$ to $9H^+$ charged states of the polypeptide correspond to a mass of 7907 Da; calculated mass (average isotope composition), 7908.3 Da.

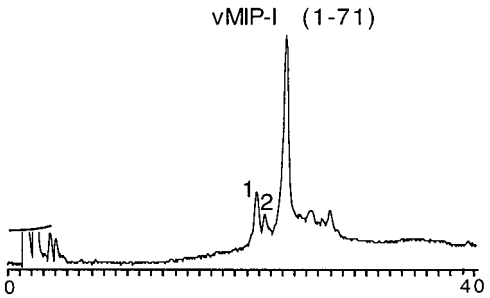


Figure 5. Synthesis of vMIP-I-(1−71) using Acm protecting strategy. Analytical HPLC of the crude polypeptide product from solid-phase ligation after cleavage from the solid support. Peak **1** represent an incomplete removal of the Acm protecting group from the segment vMIP-(36−71) and has a mass of 4130 Da. Peak **2** corresponds to a deletion of the second peptide segment vMIP-13−35) and has a mass of 5238 Da.

Folding of vMIP. The oxidative folding of chemokines has been well characterized and is known to proceed with high efficiency. The chemokine vMIP-1 was obtained through air oxidation of the vMIP-I-(1−71) polypeptide chain. The lyophilized peptide was dissolved in 1 M

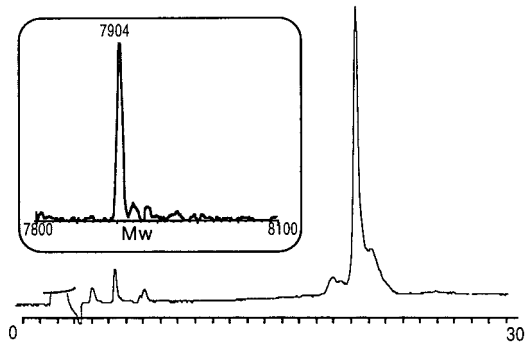


Figure 6. Folding of vMIP-I. Air oxidation of the purified vMIP-I-(1−71) polypeptide forms the folded vMIP-I molecule containing two disulfide bonds. Conditions: 0.3 mg/mL polypeptide, 1 M Gdm.Cl, 0.1 M sodium phosphate, pH 7.3. The solution was stirred open to air at ambient temperature. **(A)** Analytical HPLC (C4 reversed-phase 0−60% acetonitrile/30 min) of the crude oxidized vMIP-I-(1−71). **(B)** Electrospray spectra of the oxidized synthetic vMIP-I (raw data reconstructed to a single charge state): observed molecular mass, 7904 Da; calculated mass (average isotope composition), 7904.3 Da.

Gdm.Cl, 0.1 M sodium phosphaste, pH 7.2 to form a final peptide concentration of 0.03 mM. The solution was stirred gently at ambient temperature for 7 h. Figure 6 shows the HPLC profile of the crude folded product, the mass was lowered by 4 Da, consistent with the formation of two disulfide bonds.

Conclusion

Solid-phase ligation methods promise to greatly simplify the synthesis of proteins from multiple-peptide subunits. Performing ligation reactions on a solid support removes the limitations of solution syntheses and allows the rapid assembly of smaller peptides into the large polypeptides that compose folded proteins. The methodology described in this work utilizes the native chemical ligation reaction for both the initial attachment of the C-terminal peptide and subsequent assembly of the final polypeptide from its segments. A SCAL linker provides a strong basis for solid-phase assembly. In the oxidized form, the linker is stable to most common synthetic procedures but upon reduction becomes labile to TFA. In contrast to the solution syntheses in which large handling losses are common, assembly on the solid support produces quantitative crude polypeptide products. The C-terminal amide product is complementary to the previously described linker that yields a C-terminal carboxylate. These features of native chemical ligation of peptides on a solid support should greatly facilitate the synthesis of large proteins. Additionally, it should allow multiple ligations to be performed in parallel, a prerequisite for combinatorial approaches for protein assembly.

Experimental Section

Materials. Boc-protected amino acids were obtained from Nova Biochem, and Boc-Arg(*p*-toluenesulfonyl)-OH and Boc-Asn(xanthyl)-OH were obtained from Midwest BioTech (Fisher, IN). 2-(*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIEA) were obtained from Quantum Biotechnologies (Montreal, CA). 2-(Methylsulfonyl)ethyl 4-nitrophenyl carbonate was obtained from Fluka (Milwaukee, WI). Fmoc-SCAL linker was obtained from CoshiSoft (La Jolla, CA). 4-Methylbenzylhydrylamine (MBHA) resin was obtained from Peninsula Laboratories. *N*,*N*-Dimethylformamide (DMF) and HPLC-grade acetonitile were purchased from Fischer. Trifluoroacetice acid (TFA) was obtained from Halocarbon (Hackensack, NJ). HF was purchased from Matheson (Cucamonga, CA).

Peptide Synthesis. Peptides were prepared manually on a 0.4-mmol synthesis, using in situ neutralization protocols for Boc chemistry.[22] All peptides were prepared as thioester-peptide on TAMPAL resin.[10] Each synthetic cycle consisted of N$^{\alpha}$Boc removal by 1- to 2-min with TFA, a 1-min DMF flow wash, a 10- to 20-min coupling time with 2.2 mmol of preactivated Boc-amino acid in the presence of excess of DIEA, and a second flow wash. N$^{\alpha}$Boc amino acids (2.2 mmol) were preactivated for 3 min with 2.2 mmol of HBTU (0.5 M in DMF) in the presence of DIEA (6 mmol). After each coupling step, yields were determined by measuring residual free amine with the quantitative ninhydrin assay. After coupling of Gln residues, a DCM flow wash was used before and after deprotection with TFA, to prevent possible high-temperature (TFA/DMF)-catalyzed pyrrolidone formation. After the chain assembly was completed, the peptides were deprotected and cleaved from the resin by treatment with anhydrous HF for 1 h at 0 °C with 4% *p*-cresol as a scavenger. Following the cleavage, peptides were precipitated with ice-cold diethyl ether, dissolved in aqueous acetonitrile, and lyophilized.

vMIP-I Fragments Synthesis. vMIP-I **1** (1−12), **2** (13−35), and **3** (36−71) were synthesized on TAMPAL resin (0.4 mmol scale) to yield C-terminal mercaptopropionic acid-leucine (MPAL) activated thioester. All three segments were N-terminally protected with a 2-(methylsulfonyl)ethyl carbonate (Msc) group by a 2 h incubation with 10-fold excess of activated Msc-nitrophenyl ester in minimal volume of DMF/5% DIEA or with an Acm group using Boc-Cys(Acm)-OH. After deprotection and cleavage from the resin, the crude peptides were purified by HPLC, lyophilized, and stored at −20 °C until use.

HPLC. Analytical reversed-phase HPLC was performed on a Hewlett-Packard 1050 instrument using Vydac C-18 columns (5 μm, 0.46 cm × 15 cm). Preparative reversed-phase HPLC was performed on a rainin HPLC system using a Vydac C-18 column (10 μm, 2.5 cm × 25 cm). Linear gradients of acetonitrile in water/0.1% TFA were used to elute bound peptides. The flow rates used were 1 mL/min (analytical) and 15 mL/min (preparative).

MS. Electrospray ionization MS was performed on an API-III triple quadruple mass spectrometer (Sciex, Thornhill, ON, CA). Peptide masses were calculated from the experimental *m*/*z* from all of the observed protonation states of a peptide by using MACSPEC software (Sciex). Theoretical masses of peptide and proteins were calculated by using MACPROMASS software (Beckman research Institute, Duarte, CA).

Polymer Support Preparation. Cross-linked agarose (4%) derivitized with a diaminodipropyl group (16−20 μmol available amino group per mL of gel) was used as a polymer support (Pierce, Rockford, IL). The resin was allowed to swell in DMF, followed by normal coupling of Boc-Cys(Trt)-OH (6 equiv) for 30min. The modified resin was then treated with 1 N NaOH for 5 min to get rid of any esterification of the hydroxyl function of the agarose with cysteine. The Boc and the trityl groups were removed by treating the modified resin with a mixture of diisopropylsilane/water/TFA (2.5/2.5/95) for 1 min, followed by DMF and DCM flow washes. The resin then was allowed to swell in 6 M Gdm.Cl, pH 7, and was ready for the first ligation.

Linker. Fmoc-SCAL linker was obtained from CoshiSoft. The Fmoc protecting group was replaced by the Boc protecting group. Removal of the Fmoc was achieved using DBU (1.2 equiv) in $CHCl_3$. The free amine was protected as a Boc derivative using di-*tert*-butyl dicarbonate (2 equiv) and DIEA (2 equiv) in $CHCl_3$ to give Boc-SCAL in >95% yield for the two steps. The SCAL linker was introduced into the vMIP-I-(36−71) segment (Figure 3) by coupling Boc-SCAL using 1.5 equiv (6 mmol, 315 mg, for 0.4 mmol scale synthesis) for 20 min (99.9% coupling).

Ligation Reactions. The modified resin (0.4 μmol, 25 μL) was allowed to swell in 6 M Gdm.Cl pH 7. The C-terminal segment of vMIP-I {Msc-(36−71)-SCAL-Gly-Gly-SR} (0.4 μmol, 2 mg) was dissolved in 6 M Gdm.Cl (60 μL, 6 mM peptide), containing thiophenol and benzylmercptan (2% v/v, each), and added to the polymer support. The ligation reaction was performed at room temperature and was vortexed periodically to equilibrate the thiol additives. The reaction was monitored using HPLC; every 2.5 h a 1-μL sample of ligation mixture was diluted in 9 μL of 6 M Gdm.Cl and injected into the HPLC to follow the consumption of the peptide (Figure 1). Following completion of the ligation (8−10 h) the Msc protecting group was removed by exposing the ligation mixture to pH 13 for 5 min (by adding 1 N NaOH), followed by 6 M Gdm.Cl flow wash. The same steps were repeated for the other vMIP-I segments, however, using 1.5 equiv of peptides relative to the Cys modified gel. When the N-terminal Cys of vMIP-I segments were protected with an Acm group, the Acm was removed as has been reported in the literature.[20] The resin was washed with acetic acid/water/acetonitrile (10/45/45) and treated for 30 min with a solution of mercury(II) acetate (15 mg/mL) in 10% acetic acid solution pH 4 and thorough washing of the resin with the acetic acid solution followed by 6 M Gdm.Cl pH 8 and 0.1 M sodium phosphate. The resin was treated with 20% mercaptoethanol in the same pH 7.0 buffer for 30 min.

Cleavage of vMIP Polypeptide from the Support. Once the assembly of the polypeptide was completed, the resin was washed several time with 6 M Gdm.Cl, DMF, and CH_2Cl_2. A mixture of TFA (315 μL), thioanisol (50 μL), *m*-cresol (50 μL), EDT (25 μL) ,and $SiCl_4$ (60 μL) was added to the resin, and the reaction was kept at 0 °C for 2 h. Finally the TFA mixture was titrated into 5 mL of cold ether, and the product was precipitated, isolated, and dissolved in acetonitrile water (1:1) containing tris carboxyethylphoshine (TCEP) to reduce any oxidized product. The polypeptide was then purified using analytical reversed-phase HPLC column to give pure polypeptide.

vMIP-I Polypeptide Oxidation. Pure vMIP-I polypeptide was dissolved in 1 M Gdm.Cl, 0.1 M sodium phosphate buffer pH 7.2 (0.03 mM) and was stirred gently in air at ambient temperature. HPLC showed the oxidation reaction to be complete within 7 h (Figure 6).

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