Synthesis of a 39-Peptide and a 25-Peptide by Thiol Capture Ligations: Observation of a 40-Fold Rate Acceleration of the Intramolecular *O,N*-Acyl-Transfer Reaction between Peptide Fragments Bearing Only Cysteine Protective Groups

D. S. Kemp* and Robert I. Carey

Room 18-584, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received August 25, 1992

In this paper we report the syntheses of the 39-peptide C(Acm)LNELDADEQADL-CESLHDHADE-LYRSCLARFGDDGENL, **1**, and of the 25-peptide C(Acm)LNELDADEQADL-CLARFGDDGENL, **2**, by means of thiol capture ligations using precursor peptides bearing blocking groups only on cysteine residues. The ligations were made in each case at the underlined cysteine, cleanly and in high yield. For each of the above syntheses, an acidolytically deblocked 13-peptide dibenzofuranyl ester, 6-[C(Acm)LNELDADEQADLeucinyloxy]-4-mercaptodibenzofuran, **3**, was prepared in pure form in 52% overall yield through three stages: (1) stepwise synthesis on a solid-phase resin loaded with the dibenzofuran template, (2) acidolytic removal of the *tert*-butyl esters of the resin-bound peptide, and (3) preparative cleavage of the deblocked peptidyloxydibenzofuran ester from the resin. In the case of both the 39-peptide and the 25-peptide, significant rate enhancements were seen for the *O,N*-acyl-transfer step of the thiol capture sequence when both the N-terminal and C-terminal fragments had been previously side-chain deblocked, in comparison with the cases when only the C-terminal fragment had been side-chain deblocked. In the 13-peptide + 12-peptide ligation to form the 25-peptide **2**, a $t_{1/2}$ = 5 min was seen for the leucine–cysteine amide bond-forming reaction. A model leucine–cysteine *O,N*-acyl transfer as well as leucine–cysteine *O,N*-acyl transfers between protected peptide fragments, however, showed the expected $t_{1/2}$ = 4 h. Rationalization of this observed 40-fold rate enhancement is offered that identifies the aspartic acid side chain carboxylate, 12 residues in sequence from the N-terminus and penultimate to the amide ligation site, as a possible intramolecular general base catalyst for the proton-transfer step during the *O,N*-acyl transfer.

The efficiency of intramolecular catalysis is a subject that has increasingly attracted the attention of organic chemists in recent years. The comprehensive compilation of effective molarity "EM" values by Kirby[1] contained many examples of intramolecular reactions that display dramatically increased reactivity in comparison with their corresponding intermolecular reactions, and the origin of this increased reactivity has been treated in detail in a recent review.[2] The thiol capture method of peptide synthesis,[3,4] now under development in these laboratories, is a procedure that harnesses this efficiency for the purpose of effecting amide ligation between medium- to large-sized peptide fragments, a process for which the conventional (bimolecular) methodology is highly unreliable. As noted in Scheme I, a thiol capture ligation involves forming a readily cleaved bond prior to the amide-forming step, which then occurs intramolecularly. Complete, rapid capture chemistry at high dilution in protic, peptide-solubilizing media and efficient intramolecular *O,N*-acyl transfer are the two chemical problems that had to be solved definitively to achieve a practical methodology. Previously we have reported the design principles[5] that led to the identification[6,7] of 4,6-substituted dibenzofuran as an optimal spacer with an EM = 5 M for the acyl transfer in Scheme I, and we have reported the use of unsymmetrical disulfide formation in solvent mixtures of hexafluoro-2-propanol and water to successfully capture N-terminal cysteine peptides.[4]

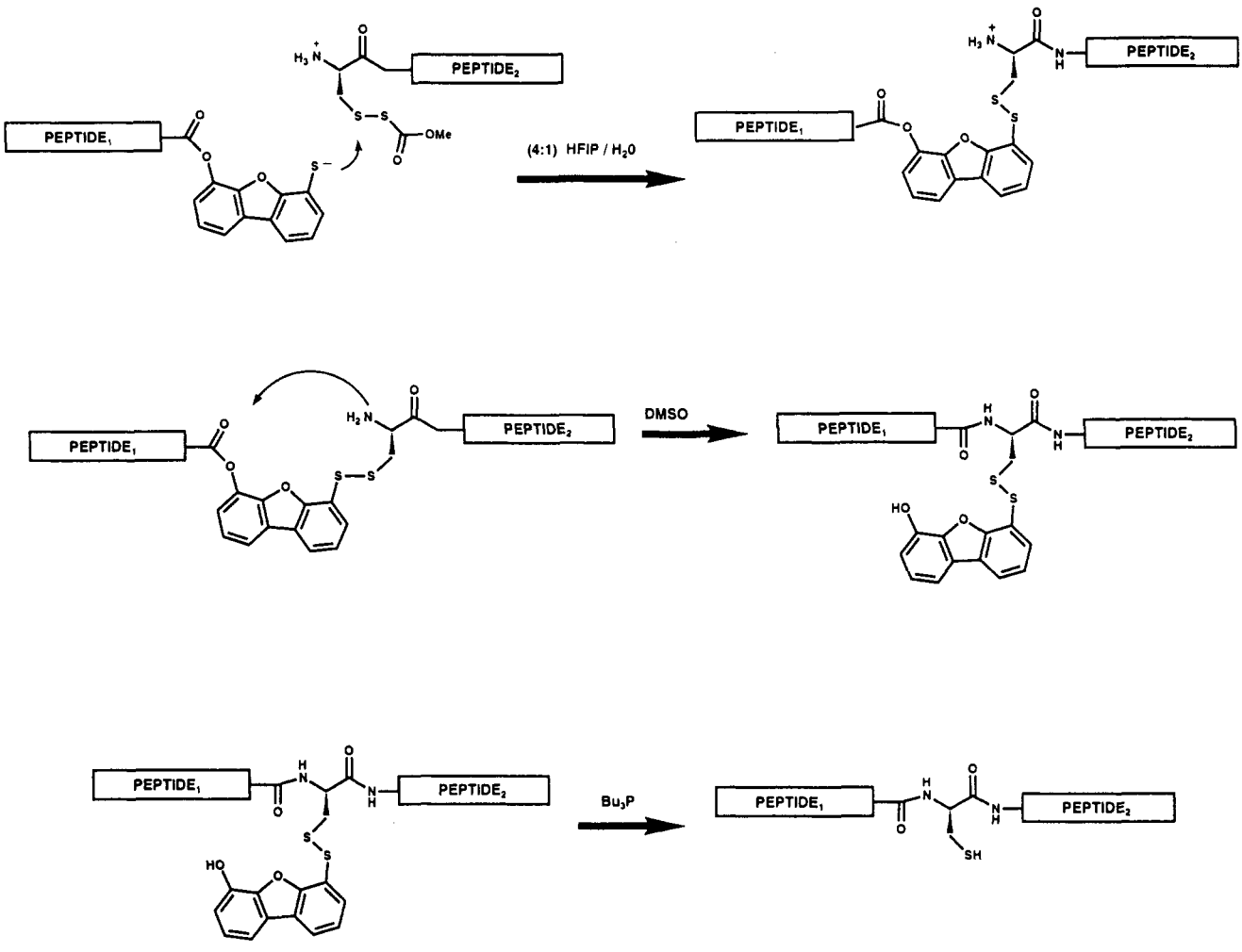
The ultimate aim of the thiol capture methodology is to achieve clean, reliable amide bond formation between peptide fragments that bear few or no side-chain blocking groups. A strategy for synthesis that is based on the coupling of *deprotected* peptides, rather than the fully or partially side-chain blocked derivatives, offers numerous advantages. Most notably, the severe problems associated with synthesis, solubility, and purification of protected fragments can be avoided, and there is no final step of global acidolytic deprotection to reduce the yield and purity of the ligation product.

The primary concern for thiol capture ligations with *deprotected* peptide fragments is that the EM of the acyl transfer step must be efficient enough to preclude the competition of intra- and intermolecular side reactions resulting from involvement of reactive side-chain functional groups. To establish this point, Fotouhi et al.[4] have demonstrated a successful amide ligation under buffered conditions in the presence of ϵ-lysine amino groups, and in a prior paper[8] we have reported the initial step in the present study, which was to resolve the three unique problems posed by the presence of deblocked histidines during the individual steps of the thiol capture strategy: (1) the conversion of N-terminal cysteine blocking groups such as Acm ($SCH_2NHCOCH_3$) or 2,2-dimethylthiazolidine[9] to an activated S-Scm (Scm = SCOOMe) without

(1) Kirby, A. *Adv. Phys. Org. Chem.* **1980**, *17*, 183.
(2) Menger, F. *Acc. Chem. Res.* **1985**, *18*, 128.
(3) Kemp, D. S. *Biopolymers* **1981**, *20*, 1793.
(4) Fotouhi, N.; Galakatos, N.; Kemp, D. S. *J. Org. Chem.* **1989**, *54*, 2803.
(5) Kemp, D. S.; Carey, R. I.; Dewan, J.; Galakatos, N. G.; Kerkman, D.; Leung, S.-L. *J. Org. Chem.* **1989**, *54*, 1589.
(6) Kemp, D. S.; Galakatos, N. *J. Org. Chem.* **1986**, *51*, 1821.
(7) Kemp, D. S.; Galakatos, N.; Tan, K.; Bowen, B. *J. Org. Chem.* **1986**, *51*, 1829.
(8) Kemp, D. S.; Carey, R. I. *Tetrahedron Let.* **1991**, *32*, 2845.

0022-3263/93/1958-2216$04.00/0

Scheme I. Steps of the Prior Thiol Capture Strategy for Peptide Ligation[a]



[a] Steps: (1) unsymmetrical disulfide formation, (2) intramolecular *O,N*-acyl transfer across a rigid spacer template in the dipolar aprotic solvent DMSO, and (3) reductive cleavage of the cysteine–template disulfide to release the newly formed peptide and to regenerate the template.

incurring side reactions at the histidine imidazole, (2) the high nucleophilicity of imidazole nitrogen toward simple phenyl esters, and (3) interference of the imidazole in the permanent blocking scheme of the internal cysteine thiols.

Here we present the syntheses of the 39-peptide C(Acm)-LNELDADEQADL-CESLHDHADELYRSCLARFGDD-GENL, **1**, and of the 25-peptide C(Acm)LNELDADE-QADL-CLARFGDDGENL, **2**, by means of thiol capture ligations using precursor peptides bearing blocking groups only on cysteine residues. The ligations were made in each case at the underlined cysteine, cleanly and in high yield. The target sequence is taken from the 63 amino acid residue ColE1 repressor of primer protein (sequence shown in Figure 1), which in the folded state consists of two nearly parallel helical regions that dimerize easily to form a water-soluble four-helix bundle.[10] The sequence is of an attractive size for a thiol capture ligation, and the presence of two histidines provided us with the opportunity to stringently test the compatibility of each of the steps of the thiol capture sequence with unprotected imidazoles. In addition, we report our observation that in the case of both the 39-peptide and the 25-peptide, significant rate

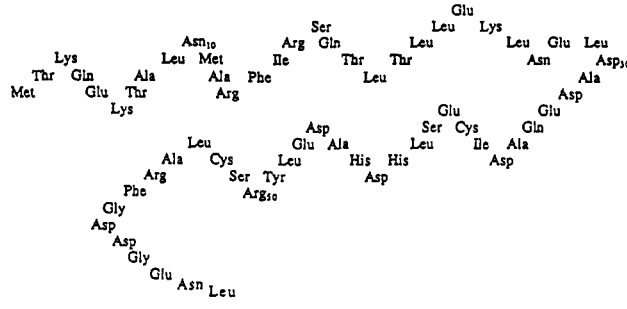


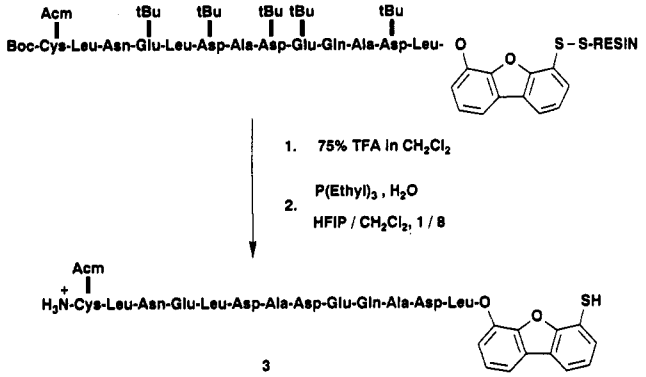
Figure 1. Sequence of the ColE1 ROP protein.[10]

enhancements were seen for the *O,N*-acyl-transfer step of the thiol capture sequence when both the N-terminal and C-terminal fragments had been previously side-chain deblocked, in comparison with the cases in which only the C-terminal fragment had been deprotected and the N-terminal (peptidyloxy)dibenzofuranyl ester still bore side-chain blocking groups.

Preparation of a Deprotected 6-(Peptidyloxy)-4-mercaptodibenzofuran: 6-[C(Acm)LNELDADE-QADLeucinyloxy]-4-mercaptodibenzofuran. The above 13-peptide dibenzofuran ester was prepared by thiol capture solid-phase synthesis[4] with N^{α}-Bpoc/*t*-Bu side-chain protection on a disulfide resin-linked dibenzofuran. The couplings were effected using preformed symmetrical

(9) Kemp, D. S.; Carey, R. I. *J. Org. Chem.* **1989**, *54*, 3640.
(10) (a) Banner, D.; Kokkinidis, M.; Tsernoglou, D. *J. Mol. Biol.* **1987**, *196*, 657. (b) Castagnoli, L.; Scarpa, M.; Kokkinidis, M., Banner, D.; Tsernoglou, D.; Cesareni, G. *EMBO J.* **1989**, *8*, 621.

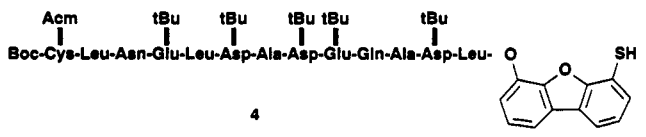
Scheme II. Preparation of Deprotected 4-Mercapto-6-(peptidyloxy)dibenzofuran Esters via *tert*-Butyl Side-Chain Removal of the Protecting Groups from the Resin-Bound Peptide Followed by Reductive Cleavage of the Disulfide Resin Linker.



anhydrides, with the exception of Asn, which necessitates the use of a carbodiimide/*N*-hydroxybenzotriazole procedure.[11] Assays of the yields following each coupling step were assessed by Bu_3P disulfide cleavage and subsequent HPLC analysis of the growing peptide chain. All couplings were judged to proceed with ≫99% efficiency, except for Asn (97%) and Gln (98%). The five *tert*-butyl esters were cleaved from the resin-bound peptide by shaking the beads with four portions of 3:1 TFA in dichloromethane for 30 min each in the presence of the cation scavenger thioanisole (2.5% for the first and 0.25% for the succeeding portions). No peptide- or dibenzofuran-containing products could be detected in the effluent from the deblocking steps, and the α-amino content of the resin was found by quantitative ninhydrin assay to be within 10% of the value at the start of the synthesis.

Cleavage of the peptide from the resin, as shown in Scheme II, was carried out by swelling in 8:1 CH_2Cl_2/$(CF_3)_2CHOH$ followed by treatment with 1 equiv of Et_3P and several equivalents of H_2O in the same solvent. A crude weight yield of 90% was obtained of product that was 87% pure by HPLC at 214- and 254-nm detection. The product was purified to homogeneity by preparative HPLC to give a 52% weight yield of material, based on initial loading, characterized by amino acid analysis, ^1H NMR, and amino acid sequencing. Thus, it has been shown that side-chain deblocking can be carried out while the peptide is resin bound by its template disulfide and that after cleavage from the resin the resulting deblocked peptide dibenzofuran ester can be purified efficiently by RP-HPLC to yield a homogeneous product.

The fully protected 13-peptide ester **4** was prepared in analogous fashion to **3**, except that the resin-bound acidolytic deprotection step was omitted.



Thiol Capture Ligation with Unblocked Peptide Residues. Two N-terminal cysteine-functionalized peptides were prepared on *p*-alkoxybenzyl alcohol ester-linked resins using a mixture of N^{α}-Fmoc- and N^{α}-Bpoc-protected amino acid residues with *tert*-butyl-derived side chain blocking.[12] The guanidino function of arginine was protected with the TFA-labile Pmc group (Pmc = pentamethylchromansulfonyl),[13] and the sulfhydryl of the internal cysteine was protected as the *tert*-butylthio disulfide. The N-terminal cysteine residues were introduced in activated form as Boc-Cys(Scm)-OH,[14] or as the protected derivatives Boc-Dmt-OH[9] or Boc-Cys(Acm)-OH, which were subsequently converted to the N-terminal Cys(Scm). The 12-peptide C(Scm)LARFGDDGENL, **5**, was obtained in 45% overall yield after purification to homogeneity. The 26-peptide C(Scm)ESLHDHADELYRSC(*tert*-butylthio)LARFGDDGENL, **6**, was obtained in lower yield owing to problems of the acylation step with the second arginine. After preparative HPLC each structure was established by FAB MS, amino acid analysis, ^1H NMR, and amino acid sequencing.

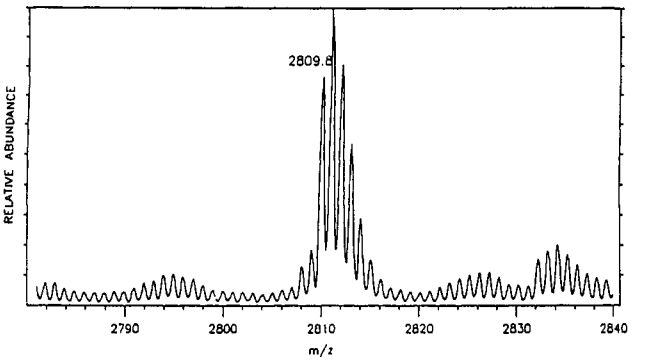


Figure 2. FAB mass spectrum of the 25-peptide C(Acm)-LNELDADEQADL-CLARFGDDGENL, **2**, prepared by means of a thiol capture ligation at the underlined cysteine.

A thiol capture ligation of the 13-peptide Dbf-SH **3** and the Scm-12-peptide **5** (1.2-fold excess) was carried out in HFIP/H_2O (4:1) for 30 min. Evaporation and removal of excess Scm-peptide by HPLC afforded the thiol capture product **7** as a homogenous white powder (1 μmol) that was dissolved in DMSO (25 mL) containing 0.01 μM $AgNO_3$ and 15 μL of DIEA. Clean acyl transfer was observed; after 2 h the solvent was removed, and the residue was dissolved in dioxane/water containing 15 μL of Et_3P. The 25-peptide **2** was purified by HPLC to give a weight yield of 82% of material, homogeneous by HPLC in two linear solvent gradients. A FAB MS of this product (Figure 2)is noteworthy for the presence of isotopic ^{13}C peaks in their expected abundances.

In an analogous fashion, unsymmetrical disulfide formation between **3** and **6** afforded the thiol capture product **8**, which underwent acyl transfer and after phosphine reduction yielded the 39-peptide C(Acm)LNELDADE-QADLCESLHDHADELYRSCLARFGDDGENL, **1**. As with the 25-peptide synthesis, ligation occurred cleanly in a yield of 80%. Neither the FAB mass spectrum nor the HPLC, shown in Figure 3, indicates the presence of impurities. Edman sequencing was carried out for both the 25- and the 39-peptide and showed no evidence of preview sequences even at full instrument attenuation.[15]

(11) Mojsov, S.; Mitchell, A.; Merrifield, R. *J. Org. Chem.* **1980**, *45*, 555.

(12) Kemp, D. S., Fotouhi, N.; Boyd, J. G.; Carey, R. I.; Ashton, C.; Hoare, J. *Int. J. Pept. Prot. Res.* **1988**, *31*, 359.

(13) Ramage, R.; Green, J. *Tetrahedron Let.* **1987**, *28*, 2287.

(14) Schroll, A.; Berany, G. *J. Org. Chem.* **1989**, *54*, 244.

(15) Brown, A. J.; Bennet, J. C. In *Peptide and Protein Reviews*; Hearn, M. T. W., Ed.; Marcel Dekker; New York, 1983; Vol. 1, pp 1–40.

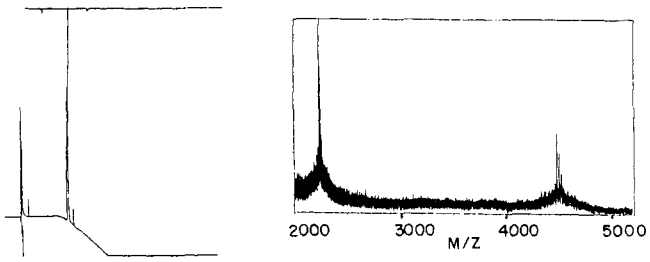
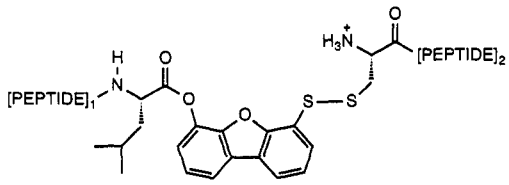


Figure 3. Analytical HPLC (left) and FAB mass spectrum of the 39-peptide C(Acm)LNELDADEQADLCESLHDHADELYRSCLARFGDDGENL, 1, prepared by means of a thiol capture ligation at the underlined cysteine.



1,2 PEPTIDE$_1$ = [C(Acm)LNELDADEQAD]

1 PEPTIDE$_2$ = [LARFGDDGENL]

2 PEPTIDE$_2$ = [ESLHDHADELYRSC(StBu)LARFGDDGENL]

Rates of the *O,N*-Acyl-Transfer Step: Comparison of Deprotected Fragments with Protected Fragments. During the course of this investigation the noteworthy observation was made that the acyl-transfer step with the deprotected fragments proceeded at a rate much faster than had been expected. In the synthesis of the 25-peptide the half-life of the acyl-transfer step was found to be $t_{1/2}$ = 5 min, and in the case of the 39-peptide $t_{1/2}$ = 53 min. Each of the above examples involves an *O,N*-acyl transfer between leucine and cysteine across the dibenzofuran framework. In comparison, a model leucine–cysteine transfer had been shown in a previous study to have a $t_{1/2}$ = 4 h,[16] a result that was repeated and confirmed during the course of this work. For the sake of further comparison, the acyl transfers to make the 39-peptide and the 25-peptide were repeated except that 4, the fully protected N-terminal 13-peptidyloxydibenzofuranyl ester, was used rather than the deprotected fragment 3. The result was that the $t_{1/2}$ = 3.4 h in each case, essentially the same as in the model leucine–cysteine transfer and substantially slower than with the rates observed with 3. Clearly, these results, which are summarized in Table I, establish two points: (1) that the observed rate enhancement manifests itself only in the presence of the deblocked side chains of 3 and (2) that the rates for *O,N*-acyl transfers in both 7 and 8 are fast but not equally so.

To rationalize this observation we first turned our attention to the reaction solvent DMSO and to the nature of the amino acid side chains that were present in each case. DMSO is a dipolar aprotic medium in which pH is defined and acidity scales have been measured.[17] DMSO is also an aggressive hydrogen bond forming solvent, and the large solvent effect (DMSO > DMF > MeCN) that we have observed[4,6] for the *O,N*-acyl transfer reaction is consistent with our model for the transition-state structure

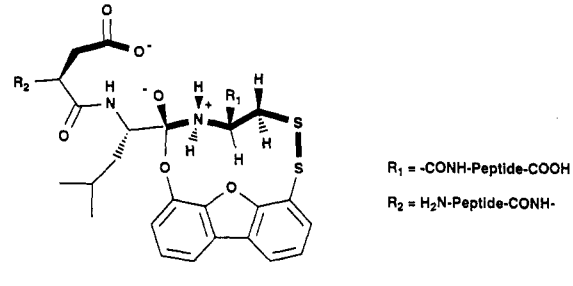
Table I. Rates of Intramolecular *O,N*-Acyl Transfer[a]

reactants in thiol capture	acyl transfer product	10^3k_2, min^{-1}	$t_{1/2}$, h	relative rate
3 + 5	2[b]	140	0.1	40
3 + 6	1[b]	13	0.88	3.9
4 + 5	2[c]	3.4	3.4	1.0
4 + 6	1[c]	3.4	3.4	1.0
9 + 10	Z-Leu-Cys-OMe	2.9	4.0	1.2

[a] Rates were measured as previously described.[5] [b] Product after phosphine reduction of the template–peptide disulfide. [c] Product after acidolytic deprotection and phosphine reduction of the template–peptide disulfide, 9 = 6-[[*N*-(Benzyloxycarbonyl)-L-leucinyl]-oxy]-4-dibenzofuranthiol, 10 = Boc-Cys(Scm)-OMe.

in which DMSO is strongly hydrogen bonded to the breaking N–H bond and in which DMSO assists in the proton transfer to the leaving phenolic oxygen. Examination of the amino acid sequences revealed that aspartic acid and glutamic acid are present in abundance in both 7 and 8. Under the reaction conditions, these residues will exist to a substantial extent as negatively charged carboxylates.

The presence of unprotected side chain carboxyl groups is of mechanistic significance due to the results of a prior study[16] in which we have shown that the rate of *O,N*-acyl transfer across the dibenzofuran framework depends largely upon the nature of the side-chain substituent of the amino acid that forms the dibenzofuran ester. Typically, half-lives in the range of 2–4 h have been observed either directly or by analogy for the natural amino acids. Proline and the sterically hindered β-branched amino acids valine and isoleucine are exceptions that are 5- to 10-fold slower. The most striking exception, however, is that of aspartic acid, which is much faster when it is side-chain deprotected ($t_{1/2}$ < 5 min) than when it is protected with a β-*tert*-butyl ester ($t_{1/2}$ = 70 min). In this case the rate enhancement that is at least 14-fold is almost certainly the result of the formation of an intramolecular salt bridge and/or the formation of a strong intramolecular hydrogen bond. Although the dibenzofuranyl ester in the cases of 7 and 8 is formed by a leucine residue, the penultimate residue is an aspartic acid, and the 40-fold and 4-fold rate enhancements seen in 7 and 8, respectively, are most likely of the same origin; i.e., the $CH_2CO_2^-$ of the aspartate side chain proximate to the transition state 11 assists the



11

necessary proton transfer more efficiently than the solvent DMSO acting alone. That 7 proceeds 10-fold faster than 8 can be attributed to the presence of a glutamic acid residue that is penultimate in sequence to the cysteine and that is therefore capable of providing a repulsive partial negative charge in the environment of the transition state, thus preventing the optimum interaction necessary for the aspartate to assist in the proton transfer.

There are other possible explanations for our observations, specifically productive binding of the peptide

(16) Kemp, D. S.; Galakatos, N. G.; Dranginis, S.; Ashton, C.; Fotouhi, N.; Curran, T. P. *J. Org. Chem.* **1986**, *51*, 3320.
(17) Ritchie, C. D. *J. Am. Chem. Soc.* **1969**, *91*, 6749.

fragments or unspecific electrostatic catalysis. However, we have no evidence for productive binding of the fragments, and due to the large number of negative charges throughout the sequence, this is very unlikely for electrostatic reasons as well. Unspecific electrostatic catalysis is unlikely to produce the rate enhancements that we observed, and furthermore, there is no precedent that it should. The most likely source of our observed dramatic rate enhancements (40-fold in one case and 4-fold in the other) is through the general base catalysis by the aspartate residue as described above. Although in this case the catalysis came serendipitously from the peptide sequence that was being ligated, the rate enhancement that was observed gives strong encouragement for us to proceed with one of our long-standing goals in thiol capture, which is to incorporate such a hydrogen-bonding site into the dibenzofuran template (or other acyl-transfer template).[18]

Summary

We have shown here for the first time that medium-sized, *deprotected* 4-mercapto-6-(peptidyloxy)dibenzofuran esters can be prepared via acidolytic deprotection of the resin-bound peptide, followed by resin cleavage and preparative HPLC purification. These steps were carried out cleanly, without detection of either phenyl ester hydrolysis or modification of the dibenzofuran thiol. Also, this study marked the first demonstration that each of the steps of the thiol capture sequence can be carried out in the presence of deblocked imidazole side chains of histidine residues. Through the synthesis of the 25- and 39-peptides, we established that the thiol capture methodology can achieve ligation of medium- to large-sized *unblocked* polypeptide fragments efficiently, in high yield, and in a well-characterized, homogenous state. Further, we have found that in addition to the entropic activation of the 4,6-substituted dibenzofuran template, we can achieve significant catalysis through a carboxylate function properly spaced in proximity to the transition state of the of the intramolecular *O,N*-acyl transfer reaction that is the centerpiece of our methodology.

Experimental Section

Samples for amino acid analysis were prepared in the gas phase by propionic acid/HCl at 150 °C for 24 h. For peptides containing tyrosine, the hydrolyses were carried out in the presence of phenol. The coupling efficiency of each step of solid-phase synthesis on the dibenzofuran prior thiol capture method solid-phase synthesis resin[4] was obtained by the following procedure. A sample of <1 mg of resin was removed, and the growing peptide chain was cleaved from this resin sample by treatment with a solution of tributylphosphine in 8:1 CH_2Cl_2/HFIP containing several μL of water. An aliquot was eluted through a C18 analytical HPLC column, and the extent of coupling was quantitated from the integration at 254 and 214 nm of the HPLC chromatogram.

Analytical high-pressure liquid chromatography (HPLC) was performed on a Vydac 218TP54 reversed-phase C18 column. Preparative HPLC was performed on a Vydac 218TP1022 reversed-phase C-18 column. HPLC solvent gradients are always linear and composed of two buffers A and B. Buffer B is 90% MeCN/water containing 0.1% TFA. Buffer A is 0.1% TFA in water.

Unless otherwise specified all amino acids are of the L configuration and were purchased from Calbiochem corporation. Scm-Cl was prepared from $COCl_2S$ (Aldrich) by a literature procedure,[19] purified by fractional distillation at reduced pressure, and stored at −20 °C in sealed ampules (stable for 1–2 months). N^{α}-Bpoc amino acids were prepared by a literature procedure[12] and stored as their CHA or DCHA salts in amber bottles at −20 °C. Boc-Cys(Acm)-OH was purchased from Bachem, Torrence, CA, and used without further purification.

The following abbreviations are used throughout the text: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(*p*-biphenylyl)-2-isopropoxycarbonyl; CHA, cyclohexylamine; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; EtOAc, ethyl acetate; MeCN, acetonitrile; HFIP, hexafluoro-2-propanol; Scm, carboxymethoxysulfenyl; TFA, trifluoroacetic acid.

N^{α}-(*tert*-Butyloxycarbonyl)-*S*-(methoxycarbonylsulfenyl)-L-cysteine (Boc-Cys(Scm)-OH) Dicyclohexylammonium Salt. To a slightly turbid solution/suspension of Boc-Cys(Acm)-OH (800 mg, 2.7 mmol) in $CHCl_3$ (15 mL) was added at 4 °C Scm-Cl (274 μL, 3.0 mmol). A clear yellow solution resulted that after 30 min was concentrated in vacuo to a yellow oil. This oil was placed under high vacuum (<1 Torr) for 15 min, redissolved in $CHCl_3$, extracted with 0.1 N HCl (2×) and water (1×), dried ($MgSO_4$), and concentrated to a pale yellow oil. This material may be crystallized directly as its DCHA salt (see below) or it may be equally facilely purified at this point by flash chromatography (eluent, $CHCl_3$/AcOH (19:1); product R_f = 0.3 and the single impurity R_f = 0.15).

The DCHA salt was prepared by dissolving the crude or purified product in a minimum of EtOAc (6 mL), cooling to 5 °C, and adding the DCHA (2.7 mmol) as a solution in EtOAc (500 μL). Crystallization occurred at ambient temperature in a sealed evaporation chamber. A white solid (935 mg, 70%) was collected by filtration and washed with cold EtOAc/ether (3:1): mp = 138–141 °C (lit.[14] mp 140–145 °C).

The DCHA salt prepared above was converted to its free acid by partitioning between DCM and 0.5 M citrate (2×). The aqueous phase was backwashed with DCM, and the DCM layers were pooled and washed with brine (3×), dried over $MgSO_4$, filtered through Celite, and concentrated in vacuo to an oil that was placed under high vacuum for at least 1 h. This oil was of suitable purity (>99% by NMR and TLC) for use in coupling during solid-phase peptide synthesis. TLC: R_f = 0.73, one spot ($CHCl_3$/MeOH/acetic acid (10:1:0.5)). ^{1}H NMR (300 MHz, $CDCl_3$); δ 1.49 (s, 9 H, *t*-Bu), 3.44 (br-m, 2 H, C_{β}-H_2), 3.95 (s, 3 H, Scm-OMe), 4.41 (m, 1 H, C_{α}-H).

Note: Schroll and Barany[14] have also reported a synthesis of this compound in which they use a Dowex 50X80-400 ion-exchange resin to prepare the free acid from the DCHA salt.

Solid-Phase Synthesis of the Scm-12mer-peptide 5 and Scm-26mer-peptide 6. All solid-phase synthesis reactions were carried out manually with an in-house designed shaker vessel. For both peptides, the synthesis was started out on an alkoxybenzyl alcohol "Wang" resin[20] loaded with Fmoc-Leucine (0.25 g, 0.39 mmol/g). The couplings and washes for the Fmoc-amino acids were carried out using standard methodology,[21] carbodiimide/HOBt-mediated couplings in DMF, 20% piperidine in DMF for Fmoc cleavage, and all washing steps utilizing only DMF. Arg(Pmc) and Cys(*tert*-butylthio) were coupled as N^{α}-Bpoc derivatives.

The side-chain protecting groups were removed, and the peptide was released from the resin by successive treatments with TFA/DCM/thioanisole/water [(75:24:0.5:0.5) 1 × 15 min, 2 × 30 min, 1 × 60 min, total deprotection time of 2.25 h]. The deprotection mixtures were pooled and concentrated in vacuo to an oil that was precipitated with ether. The crude peptide was then triturated with ether (6× with centrifugation) to yield a tan powder, which was immediately analyzed and purified by preparative HPLC. The Scm-12-peptide was obtained in 45% yield after purification and the 26-Scm-peptide was obtained in

(18) Kemp, D. S.; Buckler, D. R. *Tetrahedron Let.* **1991**, *32*, 3009 and 3013.

(19) Brois, J.; Pilot, J.; Barnum, H. *J. Am. Chem. Soc.* **1970**, *92*, 7629.

(20) (a) Wang, S. *J. Am. Chem. Soc.* **1973**, *95*, 1328. (b) Lu, G.; Mojsov, S.; Tam, J.; Merrifield, R. *J. Org. Chem.* **1981**, *46*, 3433.

(21) Barany, G.; Knieb-Cordonier, N.; Mullen, D. *Int. J. Pept. Prot. Res.* **1987**, *30*, 705.

lower yield due to difficulties in coupling the second arginine in the sequence.

Cys(Scm)LeuAlaArg-Phe-Gly-Asp-Asp-Gly-Glu-Asn-Leu, 5. HPLC: t_R = 15.4 min (20%–35% in 15 min). ^{1}H NMR (300 MHz, D_2O): δ 0.92 (dd, 6 H, Leu C_δ-H_3), 0.97 (dd, 6 H, Leu C_δ-H_3), 1.37 (d, 3 H, Ala CH_3), 1.53 (m, 2 H, Leu C_γ-H), 1.6–1.8 (m, 8 H, Arg C_β-H_2, C_γ-H_2, and Leu-C_β-H_2), 2.04 (m, 1 H, Glu C_β-H_2), 2.17 (m, 1 H, Glu C_β-H_2), 2.50 (m, 2 H, Glu C_γ-H_2), 2.73–3.28 (m, 12 H, Asx, Phe, and Cys C_β-H_2, and Arg C_δ-H_2), 3.95 (s, 3 H, Scm OCH_3), 3.92 (dd, 2 H, Gly), 4.02 (dd, 2 H, Gly), 4.33 (m, 4 H, Ala, Leu, Glu C_α-H), 4.45 (m, 2 H, Arg and Phe C_α-H), 4.79 (m, 4 H, Cys and Asx C_α-H), 7.29–7.45 (m, 5 H, Phe). FAB mass spectrum (nitrobenzyl alcohol matrix): 1400 (MH^+, 100). Amino acid analysis: $Ala_{(1.0)}$ 1.05, $Arg_{(1.0)}$ 1.01, $Asp_{(3.0)}$ 2.77, $Glu_{(1.0)}$ 0.97, $Gly_{(2.0)}$ 2.12, $Leu_{(2.0)}$ 1.99, $Phe_{(1.0)}$ 1.00. Amino acid sequence (mass spectrum): expected, C(Scm), L, A, R, F, G, D, D, G, E, N, L; found, C(Scm), L, A, R, F, G, D, D, G, E, N, L.

Cys(Scm)Glu-Ser-Leu-His-Asp-His-Ala-Asp-Glu-Leu-Tyr-Arg-Ser-Cys(*tert*-butylthio)-Leu-Ala-Arg-Phe-Gly-Asp-Asp-Gly-Glu-Asn-Leu, 6. HPLC: t_R = 19.6 min, 100% (25%–35% B in 15 min); t_R = 8.1 min, 100% (35% B–55% B in 15 min). ^{1}H NMR (300 MHz, D_2O): the spectrum is fully consistent with the proposed sequence. Characteristic resonances for His-C_2 [8.65 (s) and 8.17 (s)], Tyr aromatics [6.75 (d) and 7.05 (d)], His C_4, and Phe [7.20–7.35 (m)] are present in the downfield region in the expected integration ratios. The ratio of the integral regions of the Leucine C_δ-H_3 [0.83–0.93 (dd)] to the glutamic acid C_β-H_2 [1.95–2.1 (m)] was the expected 4:1. The following resonances are also present: δ 1.4 (s, *S-tert*-butylthio), 1.35–1.45 (d's, alanine methyls), 1.5 (m, leucine), 1.65 (m, Arg and Leu), 2.42 (m, Glu C_γ-H_2), 2.7–3.3 (dd's + m, Arg and C_β-region), 3.9–4.0 (dd's, Gly), 3.95 (s, Scm OCH_3), 4.25 (m, C_α-H), 4.35 (m, C_α-H); 4.7 (m, C_α-H). FAB mass spectrum (glycerol matrix): 3144.855 (MH^+). Amino acid analysis (hydrolysis, 1 h, 150 °C): $Asp_{(5.0)}$ 5.0, $Leu_{(4.0)}$ 3.7, $Glu_{(3.0)}$ 2.4, $Arg_{(2.0)}$ 2.2, $His_{(2.0)}$ 1.1, $Ala_{(2.0)}$ 2.0, $Gly_{(2.0)}$ 2.7, $Ser_{(2.0)}$ 1.1, $Phe_{(1.0)}$ 1.4, $Tyr_{(1.0)}$ 0.5, [Cys(Acm) and Cys(S-tert-Butylthio)]$_{(2.0)}$. Amino acid sequencing (Edman): Expected, C(Scm), E, S, L, H, D, H, A, D, E, L, Y, R, S, C, L, A, ...; found, C(Scm) Blank, E, S, L, H, D, H, A, D, E, L, Y, R, S, C, L, A, ...

Representative Conversion of an N-Terminal Cys(Acm)-Peptide to an N-Terminal Cys(Scm)-peptide. Preparation of C(Scm)LARFGDDGNEL from C(Acm)LARFGDDGNEL. Note: The Scm-12-peptide, C(Scm)LARFGDDGNEL, has been prepared not only by introducing the N-terminal cysteine as Boc-Cys(Scm)-OH as described above but also by introducing the N-terminal cysteine as Boc-Dmt-OH[9] or as Boc-Cys(Acm)-OH, either of which were facilely converted to the N-terminal Cys-(Scm)-peptide by treatment with Scm-Cl in an 8:1 acetic acid/DMF solvent mixture containing 1%–3% water. The following procedure has been applied successfully in our laboratory to a several deprotected peptides ranging from 8–21 amino acids in length.[4,9]

To a solution of the Acm-12-peptide (20 mg, 0.0145 mmol) in acetic acid/DMF (8:1) (9 mL containing 1% water) at 6 °C under N_2 was added Scm-Cl (1.95 μL, 1.5 equiv) as a solution in 8:1 acetic acid/DMF (100 μL). The reaction was stirred for 45 min and then concentrated in vacuo to a clear oil that was then taken up in 0.1% TFA. This aqueous solution was lyophilized to a white powder (19.5 mg, 96%). If necessary, this Scm-activated peptide can be purified by preparative HPLC.

Preparation of Boc-Cys(Acm)-Leu-Asn-Glu(*t*-Bu)-Leu-Asp(*t*-Bu)-Ala-Asp(*t*-Bu)-Glu(*t*-Bu)Gln-Ala-Asp(*t*-Bu)-Leu-[Dbf]-S-S-[Resin]. The title 4-mercapto-6-peptidyloxy disulfide resin was prepared by solid-phase synthesis starting with the precursor 6-hydroxydibenzofuran resin (0.26 mmol/g loading), which was prepared by the literature procedure.[4] N^α-Bpoc amino acids were used for the introduction of the first 12 amino acids, and the couplings were performed by means of preformed symmetrical anhydrides, with the exception of Bpoc-Asn-OH which was coupled as a preformed hydroxybenzotriazole ester.[11,22] The N-terminal Boc-Cys(Acm)-OH was also introduced as the preformed symmetrical anhydride. After each coupling, a 5-min acetylation protocol was performed using 500 μL each of DIEA and acetic anhydride in DCM (6 mL).

Acidolytic Side Chain Deblocking of a Peptide on the Thiol Capture Resin. Preparation of Deprotected 4-Mercapto-6-[C(Scm)LNELDADEQADLeucinyloxy]dibenzofuran, 3. The fully protected precursor resin (30 mg, 7.8 μmol) was swelled in DCM (6 mL × 2 min) in a solid-phase shaker vessel. The DCM was forced through the glass frit of the shaker via N_2 pressure, and the process was repeated (3×). The swelled resin was then suspended in a premixed solvent composed of TFA (3 mL), DCM (1 mL), and thioanisole (100 μL) and the resin suspension shaken for 30 min. The solvent was again removed through the glass frit of the shaker and was replaced with a second premixed solvent composed of TFA (3 mL), DCM (1 mL), and thioanisole (10 μL). After 30 min the solvent mixture was removed through the frit and was replaced with a fresh portion of the same mixture, which was shaken for a total time of 1 h, thus giving a total deprotection time of 2 h. The resin was then washed with DCM (8 × 2 min) to remove all excess TFA and thioanisole. Analysis of the peptide resin at this point by a quantitative ninhydrin test indicated a free amine content within 10% of the starting resin (after one amino acid).

The deprotected peptide resin above was immediately swelled in a premixed solvent composed of DCM/HFIP (4:1). After 2 min the swelling solvent was removed and replaced with a 4:1 DCM/HFIP mixture containing triethylphosphine (1.2 μL, 1 equiv). The resin was shaken for 15 min with the phosphine solution and was washed with 4:1 HFIP/DCM (3 × 1 min). The pooled washes were concentrated in vacuo (gently, no heating) to an oily residue that solidified under ether. Trituration with ether yielded a white powder (13.2 mg, 90%). After passage through a preparative HPLC column using the conditions listed for the analytical below, a white powder (7 mg, 52%) was isolated that was >99% pure by analytical HPLC. The only impurity that was present in a significant amount was 4-mercapto-6-acetoxydibenzofuran. HPLC: t_R = 18.2 min (30%–50% B in 15 min); t_R = 14 min (35%–55% B in 15 min). Amino acid analysis: $Asp_{(4.0)}$ 4.0, $Leu_{(3.0)}$ 2.8, $Glu_{(3.0)}$ 3.1, $Ala_{(2.0)}$ 2.0, $Cys(Acm)_{(1.0)}$. Amino acid sequencing (Edman): expected, C(Acm)-L-N-E-L-D-A-D-E-Q-A-D-L; found C(Acm) Blank, L,N,E,L,D,A,D,E,Q,A,D,L. No preview amino acids were detected.

Preparation of the 25-Peptide by Thiol Capture, 2. To a solution of the Scm-12mer-peptide (3 mg, 1.8×10^{-6} mol, di-TFA salt) in HFIP/water (4:1) (750 μL) was added a solution of the 13mer-Dbf-SH (3 mg, 1.6×10^{-6} mol of the mono-TFA salt) in HFIP/water (4:1) (500 μL and 300 μL for the rinsing of the flask). The reaction was allowed to stir under N_2 for 30 min, at which time it was concentrated in vacuo to a white solid that was left under high vacuum for 0.5 h. HPLC analysis at this point indicated that all of the 13mer-SH had been consumed and that a single new peak had appeared (13 + 12 capture product, strong UV absorbance at both 254 and 214 nm). A small amount of Scm-12mer-peptide was still present.

This solid residue was taken up in 1:1 MeCN/water (500 μL) and passed through a preparative HPLC column from 30%–50% B in 15 min) to separate the capture product from the small amount of unreacted Scm-12mer-peptide. The capture product eluted at 12 min and was collected and lyophilized to a white solid residue that was left under high vacuum for 2 h.

To proceed to the acyl transfer, the purified 13 + 12 capture product was then taken up in DMSO (25 mL) containing $AgNO_3$ (0.01 μM) and DIEA (15 μL, 86 μmol), and the reaction was left under N_2 for 2 h. Aliquots (20 μL) of the reaction mixture were taken at 5-min intervals and analyzed by HPLC (30%–50% B in 15 min) to follow the course of the reaction. The capture product (t_R = 12.5 min) was observed to be converted cleanly to its corresponding product of acyl transfer (t_R = 16.1 min) with an estimated half-life of reaction, $t_{1/2}$ = 5 min.

After 2 h, the DMSO was removed in vacuo using a high-vacuum rotary evaporation apparatus and a 50 °C water bath. The residue was taken up in 4:1 dioxane/water (2 mL) containing

(22) During the coupling of Bpoc-Asn-OH (with DCC, HOBt tertiary base, in DMF) to a growing peptide chain on the prior thiol capture solid-phase synthesis resin, it has been noted[4] that some resin cleavage (at the phenyl ester) does occur. The extent of this problem has been shown to be increasingly worse as the concentrations of tertiary base and DMF increase. Due to reasons of solubility of Bpoc-Asn-OH, some DMF must be used as a cosolvent during the coupling reaction. Therefore, both DMF concentration and coupling times should be kept as low as possible to minimize this problem.

triethylphosphine (15 μL, 100 μmol) and left under N_2 for 5 h to reduce both the *S-tert*-butylthio and the template-cysteine disulfides. HPLC analysis (detection at 214 and 254 nm, 30%–50% B over 15 min) showed the formation of only two products, one of which was the template itself, 4-mercapto-6-hydroxydibenzofuran, t_R = 23 min. The other product (t_R = 5.6 min) had a strong absorbance at 214 nm in comparison (20:1) to its absorbance at 254 nm. The dioxane/water was removed in vacuo, leaving a residue that was taken up in 1:1 MeCN/water and passed through a preparative HPLC column. The product was collected and lyophilized to a white solid residue (3.9 mg, 82% by weight based on starting 13mer-[Dbf]-SH). HPLC: t_R = 5.6 min (30%–50% B in 15 min). FAB mass spectrum (glycerol/3-nitrobenzyl alcohol matrix): 2810.9 (MH^+, 100%). A clear centroid showing isotopic ^{13}C peaks in their expected abundance was observed (see Figure 2). Amino acid analysis: $Asp_{(7.0)}$ 7.2, $Leu_{(5.0)}$ 4.7, $Glu_{(4.0)}$ 3.9, $Ala_{(3.0)}$ 3.0, $Gly_{(2.0)}$ 2.2, $Arg_{(1.0)}$ 1.3, $Phe_{(1.0)}$ 1.0, [Cys(Acm) and $Cys(SH)]_{(2.0)}$. Amino acid sequencing (Edman): expected, C(Acm)-L-N-E-L-D-A-D-E-Q-A-D-L-C-L-A-R...; found, C(Acm) (Blank), L, N, E, L, D, A, D, E, Q, A, D, L, C(Blank), L, A, R. Sequencing was remarkably clean with no evidence of preview at full instrument attenuation through the twenty cycles that were run.

Preparation of the 39-Peptide by Thiol Capture, 1. Starting with the 13mer-[Dbf]-SH and the Scm-26mer-peptide the thiol capture sequence was carried through here exactly as in the case for the 13 + 12 described in detail above. The half-life for the acyl-transfer step was determined to be $t_{1/2}$ = 53 min. After capture, transfer, and reductive cleavage of the template cysteine disulfide and the *S-tert*-butylthio disulfide, the 39-peptide was passed through a preparative HPLC column (33%–53% B in 15 min). The product was collected and lyophilized to a white solid residue (ca. 1 mg). As was the case with the 25-peptide, all of the reactions in the thiol capture sequence proceeded cleanly and to completion (as seen by HPLC profiles at each step). HPLC: t_R = 11.8 min, 100% (33%–53% B in 15 min). FAB mass spectrum (glycerol matrix): 4468.375 (MH^+), the doubly charged species was also observed. Amino acid analysis: $Asp_{(9.0)}$ 8.6, $Leu_{(7.0)}$ 6.4, $Glu_{(6.0)}$ 6.0, $Ala_{(4.0)}$ 4.0, $Gly_{(2.0)}$ 2.5, $His_{(2.0)}$ 1.9, $Arg_{(2.0)}$ 2.2, $Ser_{(2.0)}$ 2.2, $Tyr_{(1.0)}$ 0.5, $Phe_{(1.0)}$ 1.1, [Cys(Acm) and $Cys(SH)]_{(2.0)}$. Amino acid sequencing (Edman): expected, C(Acm)-L-N-E-L-D-A-D-E-Q-A-D-L-C-E-S-L-H...; found, Cys(Acm) (Blank), L, N, E, L, D, A, D, E, Q, A, D, L, C(Blank), E, S, L, H.... Sequencing was remarkably clean with no evidence of preview at full instrument attenuation through the 20 cycles that were run.

Acknowledgment. Financial support from Pfizer, Inc., and from the National Institutes of Health, Grant GM R37 13453, is gratefully acknowledged. FAB mass spectra were obtained by Dr. I. Papayannopoulos and Prof. K. Biemann. Amino acid sequencing was performed by Mr. C. Burkins of the Whitehead Institute, Cambridge, MA, and amino acid analysis was obtained by Mr. Richard Cooke of the MIT biopolymers laboratory.