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On-Resin Native Chemical Ligation for Cyclic Peptide Synthesis[1,2]

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A novel cysteine derivative, N^{α}-trityl-*S*-(9*H*-xanthen-9-yl)-L-cysteine [Trt-Cys(Xan)-OH] has been introduced for peptide synthesis, specifically for application to a new strategy for the preparation of cyclic peptides. The following steps were carried out to synthesize the cyclic model peptide *cyclo*(Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-Asp-Phe): (i) side-chain anchoring of Fmoc-Asp-OAl via its free β-carboxyl as a *p*-alkoxybenzyl ester to a solid support; (ii) stepwise chain elongation of the peptide by standard Fmoc/*t*Bu solid-phase chemistry; (iii) removal of the N-terminal Fmoc group; (iv) coupling of Trt-Cys(Xan)-OH; (v) selective Pd(0)-promoted cleavage of the C-terminal allyl ester; (vi) coupling of the C-terminal residue, i.e., H-Phe-SBzl, preactivated as a thioester; (vii) selective removal of the N^{α}-Trt and *S*-Xan protecting groups under very mild acid conditions; (viii) on-resin cyclization by native chemical ligation in an aqueous milieu; and (ix) final acidolytic cleavage of the cyclic peptide from the resin. The strategy was evaluated for three supports: poly[*N*,*N*-dimethacrylamide-*co*-poly(ethylene glycol)] (PEGA), cross-linked ethoxylate acrylate resin (CLEAR), and poly(ethylene glycol)−polystyrene (PEG−PS) graft resin supports. For PEGA and CLEAR, the desired cyclic product was obtained in 76−86% overall yield with initial purities of ∼70%, whereas for PEG−PS (which does not swell nearly as well in water), results were inferior. Solid-phase native chemical ligation/cyclization methodology appears to have advantages of convenience and specificity, which make it promising for further generalization.

Introduction

Cyclic peptides, be they from natural sources or designed, are important synthetic targets because of their potential uses as antibiotics and other therapeutic agents.[3] The constraint imposed by cyclization may result in enhanced biological activity, as well as increased resistance to proteolysis, by comparison with linear analogues.[3b,d,4] Moreover, cyclic peptides may be used as mimics for proteins in folding studies and to study ligand−receptor interactions and structure−activity relationships.[3e,4a,b,5]

Numerous strategies, both solution and solid-phase, have been reported for the synthesis of "head-to-tail" cyclic peptides.[3b,d,6] Cyclization needs to be carried out either under high dilution conditions in solution[3b,6b,c] or on a solid support to exemplify the "pseudodilution" principle.[7] In each case, the rationale is to favor intramolecular cyclization over unwanted yield-diminishing dimerization and oligomerization side reactions.

A useful solid-phase approach—which has the added benefit of avoiding tedious purification steps after each individual chemical step—relies on anchoring of the starting amino acid residue to the support via its side chain. This culminates with deprotection steps that free the N- and C-termini so that they can be joined to each

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(2) Abbreviations: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; CLEAR, cross-linked ethoxylate acrylate resin, or poly[trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate-*co*-allylamine]; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; DME, 1,2-dimethoxyethane; DMF, *N*,*N*-dimethylformamide; ESMS, electrospray mass spectroscopy; EtOAc, ethyl acetate; Et_2O, diethyl ether; Et_3SiH, triethylsilane; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; Gdn·HCl, guanidine hydrochloride; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOAt, 1-hydroxy-7-azabenzotriazole (3-hydroxy-3*H*-1,2,3-triazolo[4,5-*b*]pyridine); HOBt, 1-hydroxybenzotriazole; HMPA, hydroxymethylphenoxyacetic acid; HPLC, high-pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight (mass spectrometry); NMR, nuclear magnetic resonance; PAC, peptide acid linker; PEGA, poly[*N*,*N*-dimethacrylamide-*co*-poly(ethylene glycol)]; PEG−PS, poly(ethylene glycol)−polystyrene (graft resin support); $PhSiH_3$, phenylsilane; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; t_R, retention time; Trt, triphenylmethyl or trityl; Xan, 9*H*-xanthen-9-yl. Amino acid symbols denote the L-configuration. Abbreviations used for amino acids follow the IUPAC−IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983.

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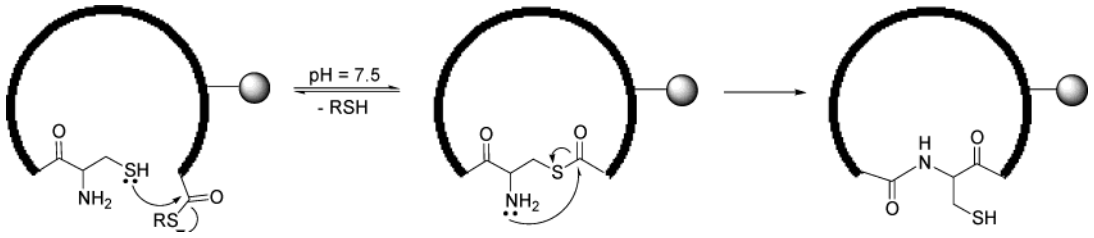
SCHEME 1. Solid-Phase Native Chemical Ligation/Cyclization



other and thereby effect the required cyclization. Amino acids used for solid-phase side-chain anchoring to initiate synthetic routes toward cyclic peptides include Asx/ Glx,[8] Lys/Orn,[8e,9] Ser/Thr,[8e,10] Tyr,[8e,10,11] His,[12] Phe,[13] and Cys.[14] It should be noted in passing that the most general approach to cyclic peptides, which does not require side-chain functionality, is the backbone anchor linkage (BAL) approach.[15] Indeed, some of the transformations needed for the current study have already been demonstrated for BAL;[15a,c] conversely, cyclizations on BAL follow logically from a seminal side-chain anchoring precedent.[8d]

A further relevant theme for the present work is the use of native chemical ligation,[16] a method that under aqueous conditions serves to join two essentially unprotected peptide chains, one with a cysteine residue at the N-terminus and the other with a thioester moiety at the C-terminus (Scheme 1). As elegantly shown both in solution[17] and on the solid phase,[18] when the N-terminal Cys and the C-terminal thioester are within the same chain, native chemical ligation becomes intramolecular, and cyclic peptides (or even proteins) can form, especially when there is a conformational bias to do so.[19] Native chemical ligation/cyclization has been generalized by using selenocysteine in place of Cys and relying on an ultimate deselenation step to produce an Ala residue at the cyclization site.[20] We reasoned that side-chain anchoring as described in the previous paragraph, together with intramolecular native chain ligation, could be elaborated into an advantageous solid-phase method to synthesize cyclic peptides. In this sense, our proposed approach differs from the sole literature precedent[18] for on-resin native chemical ligation/cyclization, where the thioester was part of the linkage to the support, with the consequence that cyclization was concomitant with release from the support.

The present work was motivated by our interest in preparing cyclic analogues of proteins, specifically sequences related to bovine pancreatic trypsin inhibitor (BPTI). Due to our commitment to mild orthogonal Fmoc/*t*Bu solid-phase chemistry,[21] a modified protection scheme needed to be worked out to manage the key N-terminal Cys.[22] In addition, the required C-terminal thioester had to be introduced at a late stage of the synthesis, due to the base lability of thioesters (vide infra). The overall side-chain anchoring/native ligation strategy was illustrated on the model peptide *cyclo*(Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-Asp-Phe), which in the native protein encompasses residues 53−58, continues in a circle to 1−4, and includes two modifications [Cys55 to Abu and Arg53 to Cys]. Because native chemical ligation occurs under aqueous conditions, poly[*N*,*N*-dimethacrylamide-*co*-poly(ethylene glycol)] (PEGA)[23] and cross-linked ethoxylate acrylate resin (CLEAR)[24] were expected to be useful supports due to their known excellent swelling in water; this was borne out experimentally. Poly(ethylene glycol)−polystyrene

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(PEG−PS) graft resin supports,[25] which also swell with a range of hydrophilic and hydrophobic solvents but perhaps not as well in water, were included to complete the comparison.

Results and Discussion

Side-Chain Anchoring/Native Chemical Ligation Strategy. Based on considerations outlined in the Introduction to this paper, the overall plan for cyclic peptide synthesis entailed: (i) side-chain anchoring of Fmoc-Asp-OAl via its free β-carboxyl as a *p*-alkoxybenzyl ester to a solid support [PEGA, CLEAR, and PEG−PS were investigated and compared]; (ii) stepwise chain elongation by standard Fmoc/*t*Bu solid-phase chemistry; (iii) removal of the N-terminal Fmoc group; (iv) coupling of N^{α}-trityl-*S*-(9*H*-xanthen-9-yl)-L-cysteine [Trt-Cys(Xan)-OH]; (v) selective Pd(0)-promoted cleavage of the C-terminal allyl ester; (vi) coupling of the C-terminal residue, i.e., H-Phe-SBzl, preactivated as a thioester; (vii) selective removal of the N^{α}-Trt and *S*-Xan protecting groups under very mild acid conditions; (viii) on-resin cyclization by native chemical ligation in an aqueous milieu; and (ix) final acidolytic cleavage of the cyclic peptide from the resin.

Several aspects of this plan require commentary, by way of comparison to related literature approaches. For one, C-terminal peptide thioesters are usually made by Boc chemistry and C → N chain assembly, due to the lability of the thioester moiety to nucleophilic displacement by piperidine, the base generally used for repetitive removal of the N^{α}-amino 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group. To prepare thioesters by Fmoc chemistry, approaches include (i) use of carefully attenuated Fmoc removal conditions, e.g., a cocktail of 2% (w/v) 1-hydroxybenzotriazole (HOBt) in hexamethylenimine−1-methylpyrrolidine−1-methyl-2-pyrrolidinone−dimethyl sulfoxide (1.6:20:40:40);[26] (ii) use of a "safety-catch" type handle that is first activated (e.g., alkylation reaction) and then cleaved by the appropriate thiolate nucleophile;[27] (iii) use of standard [e.g., 4-(hydroxymethyl)phenylacetamidomethyl (PAM)] resins during chain assembly, followed by organoaluminum compound-catalyzed transthioesterification for cleavage;[28] (iv) use of an indirect "backing-off" procedure[15c] [featuring one step in the N → C direction], as shown previously with BAL and chosen in the present work with side-chain anchoring; (v) an acid-labile BAL strategy [to date demonstrated only for C-terminal glycine] wherein the C-terminal residue from the start is a base-stable, acid-labile trithioester;[29] and (vi) a strategy with side-chain anchoring of a residue with a C-terminal allyl ester, selective deprotection, and direct on-resin modification with isothiouronium salts, or the solution counterpart in which the partially protected peptide is modified after cleavage from a chlorotrityl resin.[27c] A number of the aforementioned approaches have been reported to give low or modest yields,[27c,30] and in some cases specific side reactions such as aspartimide formation,[28b] hydrolysis/spontaneous cyclization,[27c] thioester migration,[27c] and racemization[27c,28b] have been mentioned—all the more reason to favor the "backing-off" approach. Second, the issue of providing selectively removable protection simultaneously to the N^{α}-amino and the β-thiol groups of cysteine has been tackled before, for example by use of thiazolidine derivatives that are labile to acid, metals, and electrophiles.[31] (In some tandem ligations, the sulfhydryl is allowed to remain unprotected, because thiol−disulfide exchange equilibrations continue until the thermodynamic end point of the desired native ligation is reached.[16b,c,e]) In the present work, we take advantage of two highly acid-sensitive protecting groups, *S*-9*H*-xanthen-9-yl (Xan)[32] for a sulfhydryl function and *N*-trityl (Trt) for the N^{α}-amine.[33]

Preparation of Linear Protected Resin-Bound Peptide-Thioester. The overall specific procedure is delineated in Scheme 2. First, a *p*-alkoxybenzyl alcohol-type handle[34] was loaded onto the free amine functionality of a commercially available CLEAR support. Standard *N*,*N*′-diisopropylcarbodiimide (DIPCDI)/1-hydroxy-7-azabenzotriazole (3-hydroxy-3*H*-1,2,3-triazolo-[4,5-*b*]pyridine) (HOAt) activation, in DMF, was applied. Meanwhile, HMPA−PEGA was already available. Following that, Fmoc-Asp-OAl was attached via its free β-carboxyl side chain to both HMPA−CLEAR and HMPA−PEGA, by use of DIPCDI-mediated esterifications catalyzed by 4-(*N*,*N*-dimethylamino)pyridine (DMAP). For PEG−PS, a corresponding sequence of steps[8d] has been carried out to provide a commercial product which is a chemically equivalent starting point for the synthesis.

The linear sequence was assembled by standard Fmoc solid-phase peptide synthesis, via *N*-[(dimethylamino)-

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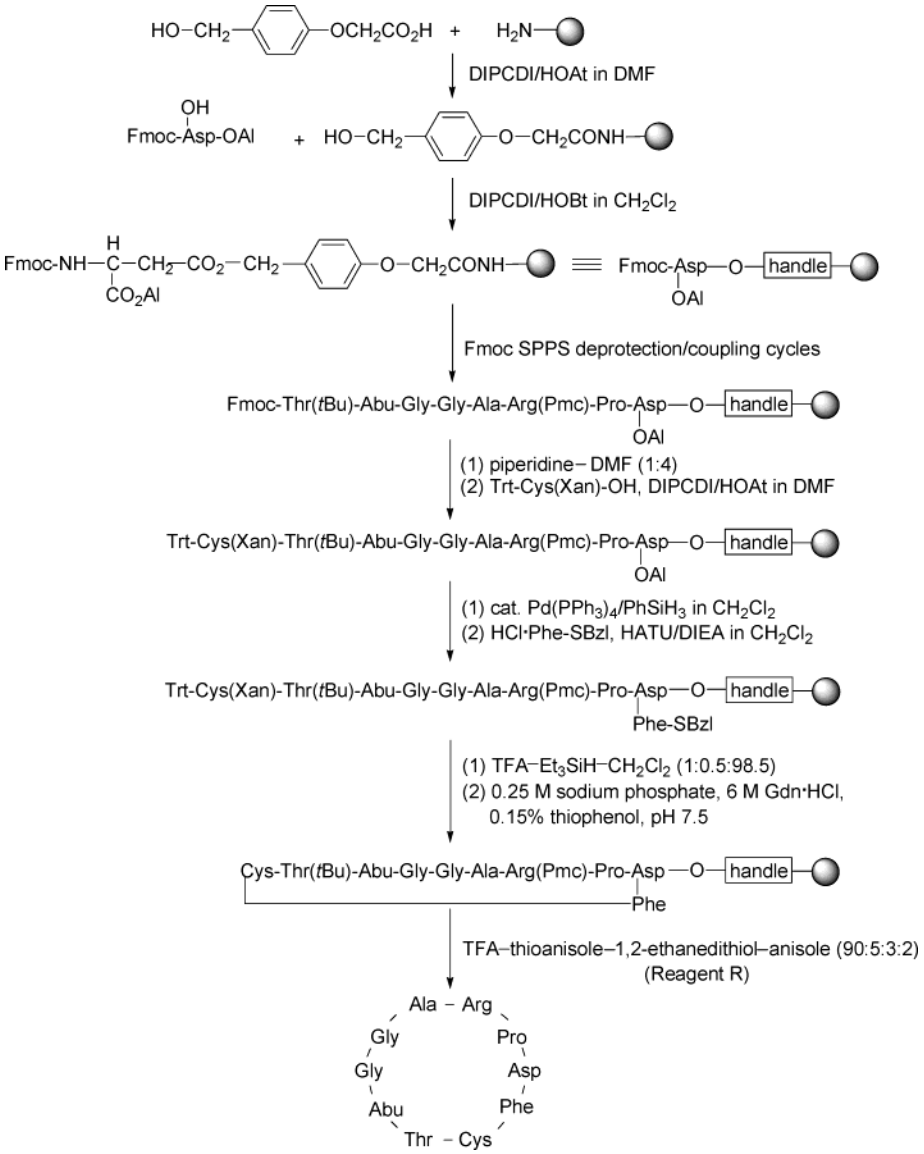
SCHEME 2. Preparation of *cyclo*(Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-Asp-Phe)



1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU)/HOBt/*N*,*N*-diisopropylethylamine (DIEA) couplings in DMF. For the last cycle, Trt-Cys(Xan)-OH was combined with DIPCDI/HOAt (4:4, 0.18 M), with 5 min preactivation, to minimize racemization.[35] Next, the C-terminal allyl group ester was cleaved in the presence of catalytic Pd(0) under neutral conditions first described by Albericio and co-workers;[36] note that literature alternatives for removal of allyl-type protection[37] were found to be not applicable to the highly acid-sensitive substrate for the present studies (details not shown). Finally, HATU/DIEA coupling was used to add Phe-SBzl and thereby provide the desired resin-bound peptide thioester.[15c]

Solid-Phase Ligation/Cyclization. With the C-terminal thioester established in the linear resin-bound sequence, it was next necessary to establish an N-terminal cysteine with both the N^{α}-amine and the β-thiol in free form. This simultaneous removal of both the *N*-Trt and *S*-Xan protecting groups was achieved by treatment of the peptide–resin with TFA–Et_3SiH–CH_2Cl_2 (1.0:0.5:98.5) for 2 h at 25 °C. There was a relatively small and readily tolerated loss (~7%) of chains from the support, due to premature cleavage of the C^{β}-*p*-alkoxybenzyl ester. Next, native chemical ligation was effected by swelling the peptide–resin in a buffer of 0.25 M sodium phosphate, 6 M guanidinium hydrochloride (Gdn·HCl), and 0.15% thiophenol, made fresh and adjusted to pH 7.5. As is commonly done, the Gdn·HCl was included to serve as a denaturant; the idea was that reasonably stable partially folded conformations where the cyclization sites were not near to each other would be forced to equilibrate with conformations more favorable to cyclization when in the presence of Gdn·HCl. Furthermore, the thiophenol was added to promote thiol exchange, as first proposed

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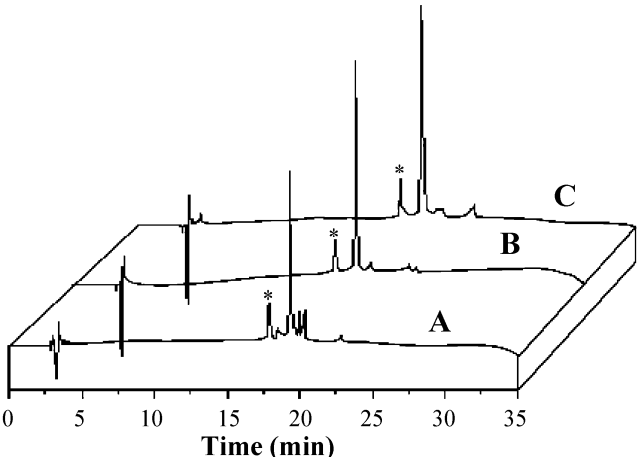

FIGURE 1. Analytical HPLC of H-Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-Asp-Phe-SBzl, synthesized on (A) PEGA, (B) CLEAR, and (C) PEG−PS. HPLC was on a C-18 column, developed at 1.0 mL/min, with a linear gradient of 0.1% aqueous TFA−0.1% TFA in CH_3CN, from 9:1 to 2:3 for 30 min. The title peptide is the main peak (t_R = 19.1 min) and the asterisk indicates the racemized peptide: H-Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-D-Asp-Phe-SBzl (t_R = 17.8 min).

by Dawson and coworkers.[38] Final release of the cyclic peptide, with concomitant removal of side-chain protecting groups, was accomplished by exposure of the peptide−resin to TFA−thioanisole−1,2-ethanedithiol−anisole (90:5:3:2) (reagent R)[39] for 1 h.

Chemistry as a Function of Resin Support. Purities and yields of the *linear* precursor H-Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-Asp-Phe-SBzl [analyzed after full deprotection and cleavage from the support] were comparable with all three resins studied (Figure 1). In all cases, the main byproduct observed was Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-D-Asp-Phe-SBzl, in 6−8% of the amount of the main species. Given that a peptidyl C-terminal residue (Asp) is activated when a C-terminal thioester (Phe-SBzl) is installed, a certain level of racemization is inevitable.[6c,7b,40] The level observed is acceptable since the product diastereomers are well separated, but could probably be reduced with careful attention to experimental details.

With essentially comparable resin-bound linear precursors, the final stages of the overall ligation/cyclization strategy (Scheme 2) could be studied as a function of time (Figure 2). For PEGA, reaction reached 74% completion within a few hours. Reaction on CLEAR was several-fold slower but reached 74% after a day. However, the reaction on PEG−PS gave only 27% of the desired cyclic peptide on the same time scale, with substantial levels of unreacted starting linear peptide (~52%). In all cases, reaction products at the end points (Figure 2) were analyzed further by MALDI-TOF. For PEG−PS, cyclodimer appeared after 6 h of reaction and reached the level of 11% after 24 h. For CLEAR, cyclodimerization was only 1% after 24 h, whereas for PEGA the side reaction was detected from the beginning and reached 11% after 2 h.

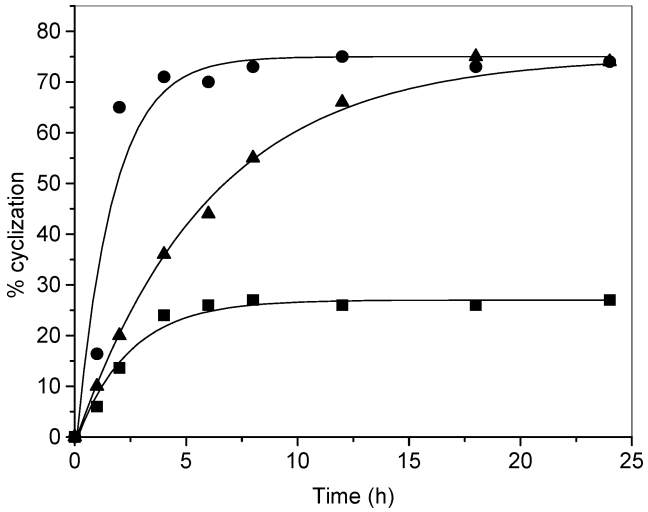

FIGURE 2. Formation of cyclic peptide as a function of time for each of the three resin supports: PEGA (●), CLEAR (▲), and PEG−PS (■). Peptide−resin aliquots were taken at the indicated times, cleaved with reagent R, and studied by HPLC (compare to Figure 3 for typical data). Percentages indicate area of desired cyclic peptide divided by total area of all peptide material, with no further corrections.

Conclusions

A new strategy has been developed for synthesis of cyclic peptides by on-resin native chemical ligation. Key aspects of this strategy include Fmoc/*t*Bu chemistry, side-chain anchoring, allyl protection of the penultimate residue for later introduction of the C-terminal thioester, and a new derivative to manage N-terminal cysteine, i.e., Trt-Cys(Xan)-OH, that allows simultaneous, selective, and mild removal of both protecting groups. A model peptide was prepared successfully with either PEGA or CLEAR resin supports, which have good aqueous compatibility properties; PEG−PS did not give as good results but the question remains open for all three of these supports how ligation outcomes would differ with mixed aqueous/organic or even polar organic solvent milieus. Future work will also establish the generality of this strategy to additional cyclic peptides, including those that do not use a Cys residue for ligation/cyclization. In the latter regard, further developments in the area of temporary auxiliaries[41] and in the applications of selenocysteine[20] ought to be generalizable to ligation/cyclization. Another issue that needs to be addressed relates to whether, for some longer target sequences that could potentially aggregate under aqueous conditions if still protected, all other side-chain protecting groups [not just that on the β-thiol of N-terminal Cys] should be removed before the ligation/cyclization step. This could be done easily, either by changing to more acid-sensitive groups or by using a more acid-stable (perhaps orthogonally cleavable) anchor.

Experimental Section

General. Materials, solvents, instrumentation, and general methods, as well as procedures for peptide synthesis, followed the literature from our laboratory[8d,15a,c,32,35] or others.[36] Fmoc-

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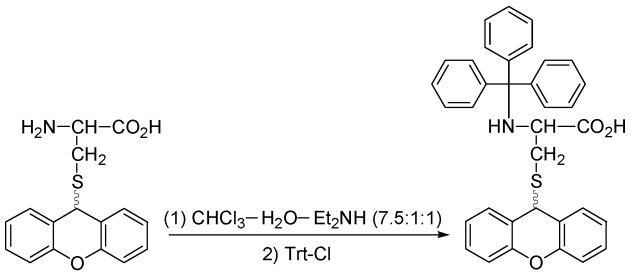
SCHEME 3. Preparation of N^{α}-Trityl-*S*-(9*H*-xanthen-9-yl)-L-cysteine



Asp-(PAC-PEG-PS)-OAllyl, CLEAR, and HMPA−PEGA were obtained from Applied Biosystems (Foster City, CA), Peptides International (Louisville, KY), and Novabiochem (Läufelfingen, Switzerland), respectively. Analytical HPLC was performed on a two-pump instrument with a C18 analytical reversed-phase column (5 μm particle size; 0.46 × 25 cm) with UV detection at 220 nm.

***S*-(9*H*-Xanthen-9-yl)-L-cysteine** (left structure in Scheme 3). This starting material was made in 87% yield on a 10 mmol scale, exactly following Han and Barany.[32] Analytical data matched what was published, and in addition, the following were recorded: ^{13}C NMR (CD_3OD) δ 172.6, 154.0, 130.9, 130.6, 130.2, 130.1, 124.9, 117.6, 55.3, 43.1, 32.5. ESMS: *m*/*z* calcd for $C_{16}H_{15}NNaO_3S$, 324.1; found, 324.1 $[M + Na]^+$.

***N*$^{\alpha}$-Trityl-*S*-(9*H*-xanthen-9-yl)-L-cysteine** (right structure in Scheme 3). In a tritylation procedure modeled on several in the literature,[33] *S*-(9*H*-xanthen-9-yl)cysteine (3.01 g, 10.0 mmol) was dissolved in $CHCl_3$ (30 mL), and then H_2O (4 mL) and Et_2NH (4 mL). were added. The heterogeneous mixture was cooled to 4 °C (ice bath), and trityl chloride (2.93 g, 10.5 mmol) was added with magnetic stirring over 30 min. After a further 2 h of stirring at 25 °C, the layers were separated, and the organic phase was washed twice with 4% aqueous Et_2NH, dried ($MgSO_4$), and evaporated to dryness. The resulting residue was converted to its diethylammonium salt by refluxing in Et_2NH−EtOH (1:30, 31 mL) for 10 min. The salt was then partitioned between EtOAc (30 mL) and 5% aqueous $KHSO_4$ (20 mL), and the organic layer was washed with 5% aqueous $KHSO_4$ (20 mL) and H_2O (20 mL) and then dried ($MgSO_4$) and concentrated to provide a white residue, which was recrystallized from toluene−petroleum ether to give a white crystalline solid (3.9 g, 72%). ^{1}H NMR ($CDCl_3$) δ 7.39 (m, 2H), 7.18−7.34 (m, 17H), 7.05−7.12 (m, 4H), 5.20 (s, 1H), 3.52 (dd, J = 5.5 and 3.5 Hz, 1H), 2.81 (dd, J = 13.5 and 3.5 Hz, 1H), 1.82 (dd, J = 13.5 and 5.5 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 176.3, 152.7, 152.2, 144.9, 129.9, 129.7, 129.4, 129.2, 129.0, 128.7, 128.5, 128.1, 128.0, 127.9, 127.5, 123.9, 123.7, 116.9, 67.3, 55.8, 42.2, 32.9. HR-FABMS: *m*/*z* calcd for $C_{35}H_{29}NaO_3S$, 566.1766; found, 566.1781 $[M + Na]^+$.

L-Phenylalanine *S*-Benzyl Ester, Hydrochloride Salt (H-Phe-SBzl·HCl). Following a general procedure from our laboratory,[15c] a solution of Boc-Phe-OH (1.77 g, 8.0 mmol) in CH_2Cl_2 (50 mL) was cooled to 0 °C and HOBt (1.23 g, 8.0 mmol), DCC (1.65 g, 8 mmol), and DIEA (2.79 mL, 16.0 mmol) were added sequentially. After 10 min of stirring, benzyl mercaptan (0.94 mL, 8.0 mmol) was added and the resultant mixture was stirred for 2 h at 0 °C and then overnight at 25 °C. The reaction mixture was washed with 1 N aqueous HCl (2 × 150 mL), 10% (w/v) aqueous Na_2CO_3 (2 × 150 mL), and H_2O (2 × 150 mL), dried ($MgSO_4$), and concentrated in vacuo. The resultant oil was taken up in 4 N HCl−dioxane and stirred for 90 min to remove the Boc group. The mixture was concentrated and chased with Et_2O (8 × 50 mL) to obtain the title product as a white solid (1.86 g, 86%). ^{1}H NMR (DMSO-d_6) δ 8.84 (br s, 3H), 7.22−7.32 (m, 8H), 7.12−7.18 (m, 2H), 4.48 (br s, 1H), 3.71 (s, 2H), 3.23 (dd, J = 14.5 and 5.5 Hz, 1H), 3.08 (dd, J = 14.5 and 8.0 Hz, 1H). ^{13}C NMR (DMSO-d_6) δ 195.6, 136.3, 134.2, 129.6, 128.9, 128.5, 128.4, 127.4, 127.3, 59.2, 36.9, 32.4. ESMS: *m*/*z* calcd for $C_{16}H_{17}NOS$, 271.10; found, 271.94 $[M + H]^+$.

HMPA−CLEAR. CLEAR resin (0.3 g, 0.45 mmol of NH_2/g) was washed thoroughly with DMF (3 × 1 min) and (3 × 1 min). Next, a solution of HMPA (0.20 g, 1.12 mmol) in DMF (0.7 mL) was added to the resin, followed in turn by HOAt (0.15 g, 1.12 mmol) in DMF (0.5 mL) and then DIPCDI (170 μL, 1.12 mmol). After 12 h of reaction, the resin was washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min), and then dried in vacuo.

Fmoc-Asp(HMPA−CLEAR)-OAllyl. HMPA−CLEAR (0.3 g, 0.45 mmol/g) was washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Fmoc-Asp-OAllyl (0.21 g, 0.54 mmol) plus HOBt (83 mg, 0.54 mmol) were dissolved in CH_2Cl_2 (0.5 mL), and added to the resin, followed by DIPCDI (95 μL, 0.54 mmol) and DMAP (28 mg, 0.23 mmol). Coupling was carried out for 90 min, following which the resin was washed with CH_2Cl_2 (3 × 2 min) and dried in vacuo.

Fmoc-Asp(HMPA−PEGA)-OAllyl. HMPA−PEGA (0.3 g, 0.55 mmol/g) was washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Fmoc-Asp-OAllyl (0.26 g, 0.66 mmol) plus HOBt (100 mg, 0.66 mmol) were dissolved in CH_2Cl_2 (0.5 mL) and added to the resin, followed by DIPCDI (117 μL, 0.66 mmol) and DMAP (28 mg, 0.23 mmol). Coupling was carried out for 90 min, following which the resin was washed with CH_2Cl_2 (3 × 2 min) and dried in vacuo.

H-Thr(*t*Bu)-Abu-Gly-Gly-Ala-Arg(Pmc)-Pro-Asp(PAC−PEG−PS)-OAllyl. Automated Fmoc solid-phase synthesis was carried out on an Applied Biosystems Pioneer synthesizer. Side-chain protection was provided by 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg and *tert*-butyl (*t*Bu) for Thr. Fmoc removal was achieved with piperidine−DMF (1:4, 5 min), and 1 h couplings of Fmoc-amino acids (4 equiv) were mediated by *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) (4 equiv)/HOBt (4 equiv)/DIEA (8 equiv) in DMF at 25 °C. When chain assembly was complete, the fully protected peptide−resin was dried in vacuo [and stored as such, at 4 °C]. An aliquot of the resin (10 mg) was washed with CH_2Cl_2 (5 × 2 min) and with DMF (10 × 0.5 min), and the Fmoc group was cleaved by treatment with piperidine−DMF (1:4) (3 × 1 min, 2 × 5 min, 3 × 1 min, and 2 × 5 min). After the peptide−resin was washed with DMF (5 × 1 min) and CH_2Cl_2 (3 × 1 min), freshly prepared reagent R, TFA−thioanisole−1,2-ethanedithiol−anisole (90:5:3:2) was added (1 mL), and the filtrate after 1 h of cleavage was concentrated under N_2. The peptide was precipitated with cold Et_2O (0.5 mL), vortexed, centrifuged, and decanted, and the vortex/centrifugation/decantation cycle was repeated twice more with fresh cold Et_2O (0.5 mL). The remaining ether was evaporated with a stream of N_2, and the dried peptide was dissolved in 0.01 N aqueous HCl (0.5 mL) and lyophilized. The peptide was characterized by analytical HPLC (t_R = 7.5 min for main peak, ~91% of total) and MALDI-TOF (*m*/*z* calcd for $C_{33}H_{55}N_{11}O_{12}$, 797.42; found, 798.43 $[M + H]^+$).

H-Thr(*t*Bu)-Abu-Gly-Gly-Ala-Arg(Pmc)-Pro-Asp(HMPA−PEGA)-OAllyl and H-Thr(*t*Bu)-Abu-Gly-Gly-Ala-Arg-(Pmc)-Pro-Asp(HMPA−CLEAR)-OAllyl were prepared as described immediately above for the corresponding synthesis on PEG−PS. Portions of the peptide−resin were cleaved and shown to give the same major product by analytical HPLC (t_R = 7.5 min) with the following purities: 90% for CLEAR and 84% for PEGA.

Trt-Cys(Xan)-Thr(*t*Bu)-Abu-Gly-Gly-Ala-Arg(Pmc)-Pro-Asp(PAC−PEG−PS)-OAllyl. Coupling of N^{α}-trityl-*S*-(9*H*-xanthen-9-yl)-L-cysteine was carried out for 1 h, in the presence of DIPCDI/HOBt (4 equiv each with respect to resin, 0.18 M, 5-min preactivation) in DMF to minimize racemization.[35a] An aliquot of the resin (10 mg) was cleaved as described earlier and characterized by analytical HPLC (t_R = 8.1 min) and

MALDI-TOF (*m/z* calcd for $C_{36}H_{60}N_{12}O_{13}S$, 900.43; found, 901.4 $[M + H]^+$). Procedures and results were the same for synthesis on PEGA and on CLEAR.

Trt-Cys(Xan)-Thr(*t*Bu)-Abu-Gly-Gly-Ala-Arg(Pmc)-Pro-Asp(PAC−PEG−PS)-OH. The fully protected nonapeptide−resin just described (0.1 g, 23 μmol) was washed with CH_2Cl_2 (5 × 0.5 min) under argon. Next, a solution of $PhSiH_3$ (68 μL, 0.55 mmol) in CH_2Cl_2 (0.4 mL) was added, and the peptide−resin was stirred manually for 1 min. Following that, a solution of $Pd(PPh_3)_4$ (2.7 mg, 2.3 μmol) in CH_2Cl_2 (0.2 mL) was added, and the peptide−resin was gently agitated on a rotary mixer for 10 min under Ar. The resin was then washed with CH_2Cl_2 (3 × 1 min), and the deprotection step was repeated. An aliquot of peptide−resin was cleaved and characterized by analytical HPLC (t_R = 3.8 min) and MALDI-TOF (*m/z* calcd for $C_{33}H_{56}N_{12}O_{13}S$, 860.43; found, 862.43 $[M + H]^+$). Procedures and results were the same for synthesis on PEGA and on CLEAR.

Trt-Cys(Xan)-Thr(*t*Bu)-Abu-Gly-Gly-Ala-Arg(Pmc)-Pro-Asp(PAC−PEG−PS)-Phe-SBzl. H-Phe-SBzl·HCl (62 mg, 0.23 mmol) was dissolved in CH_2Cl_2 (1.3 mL), and DIEA (80 μL, 0.46 mmol) was added. The resultant solution was added to the resin described above. Coupling was initiated by addition of HATU (87 mg, 0.23 mmol) and the peptide−resin was stirred for 30 min at 25 °C. The resin was then washed with DMF (5 × 0.5 min) and CH_2Cl_2 (5 × 0.5 min), and an aliquot was taken and cleaved to provide the desired peptide thioester (75% cleavage yield), which was characterized by analytical HPLC (t_R = 19.1 min, 85%) and MALDI-TOF (*m/z* calcd for $C_{49}H_{71}N_{13}O_{13}S_2$, 1113.50; found, 1114.23 $[M + H]^+$). The main byproduct, H-Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-D-Asp-Phe-SBzl, was characterized by HPLC (t_R = 17.8 min, 8%) and MALDI-TOF. Procedures were the same for synthesis on PEGA and on CLEAR. For PEGA, the cleavage yield was 72% for material of 74% purity by analytical HPLC; H-Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-D-Asp-Phe-SBzl was present at 7%. For CLEAR, the corresponding values were 68%, 89%, and 6%.

***Cyclo*(Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-Asp-Phe).** The resin-bound peptide thioester (Scheme 2) was washed with CH_2Cl_2 (3 × 1 min) and then a mixture of CH_2Cl_2−Et_3SiH−TFA (98.5:0.5:1, 2 mL) was added. After 2 h, the resin was washed with CH_2Cl_2 (5 × 1 min) and dried. To effect cyclization, the peptide−resin was treated with a solution containing 0.25 M sodium phosphate, 6 M Gdn·HCl, and 1% thiophenol, at pH 7.5, and reaction progress was followed by HPLC (Figures 2 and 3). After the appropriate time, the peptide−resin was washed with H_2O (3 × 0.5 min) and CH_2Cl_2 (5 × 1 min) and dried. The peptide was cleaved as described previously and checked by analytical HPLC (t_R = 11.5 min) and by MALDI-TOF (*m/z* calcd for $C_{42}H_{63}N_{13}O_{13}S$, 989.50; found, 990.19 $[M + H]^+$). The following data were obtained: PEG−PS, 27% purity, 86% cleavage yield; CLEAR, 74% purity, 76% cleavage yield; PEGA, 74% purity, 77% cleavage yield. In all cases the cyclodimer, *cyclo*(Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-Asp-Phe-Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-Asp-Phe) was observed by analytical HPLC (t_R = 13.5 min) and characterized by MALDI-TOF (*m/z* calcd for $C_{84}H_{126}N_{26}O_{26}S_2$, 1978.9; found, 1980.1 $[M + H]^+$). The following amounts of cyclodimer were obtained: PEG−PS, 11% after 24 h; CLEAR, 1% after 24 h; PEGA, 11% after 2 h.

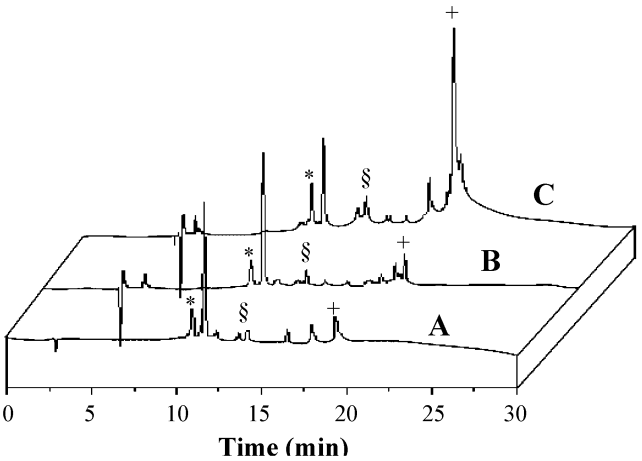


FIGURE 3. Analytical HPLC of *cyclo*(Cys-Thr-Abu-Gly-Gly-Ala-Pro-Asp-Phe) on (A) PEGA, (B) CLEAR, and (C) PEG−PS. HPLC was on a C-18 column, developed at 1.0 mL/min with a linear gradient of 0.1% aqueous TFA−0.1% TFA in CH_3CN from 9:1 to 2:3 for 30 min. The main peak (A, B) is the title peptide (t_R = 11.5 min); (*) racemized cyclic peptide *cyclo*(Cys-Thr-Abu-Gly-Gly-Ala-Pro-D-Asp-Phe) (t_R = 10.8 min); (§) cyclodimer *cyclo*(Cys-Thr-Abu-Gly-Gly-Ala-Pro-Asp-Phe-Cys-Thr-Abu-Gly-Gly-Ala-Pro-Asp-Phe) (t_R = 13.5 min); (+) linear precursor H-Cys-Thr-Abu-Gly-Gly-Ala-Arg-Pro-Asp-Phe-SBzl (t_R = 17.8 min).

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