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Extending Synthetic Access to Proteins with a Removable Acyl Transfer Auxiliary

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Abstract: A chemo- and regioselective auxiliary-mediated peptide ligation has been developed that is effective under nonidealized conditions for the synthesis of proteins. This general amide bond ligation utilizes a removable auxiliary that is analogous to the role of cysteine in native chemical ligation, combining chemoselective thioester exchange with efficient regioselective intramolecular acyl transfer. Acid lability and improved ligation efficiency were introduced into the 2-mercaptobenzyl auxiliary by increasing the electron density of the aromatic ring. The 62 amino acid SH3 domain from α-spectrin was synthesized using the auxiliary-mediated ligation at a Lys-Gly sequence. The auxiliary was removed with TFA and scavengers from the ligated product. This methodology enables unprotected peptides to be coupled at noncysteine ligation sites expanding the scope of protein synthesis and semisynthesis.

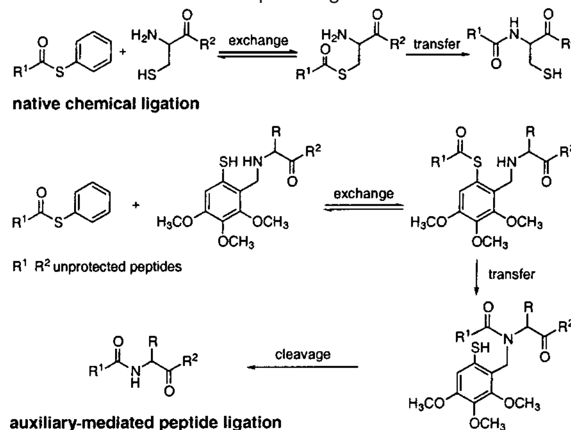
Introduction

Native chemical ligation of a peptide thioester onto an N^{α}-terminal cysteine peptide is an established methodology for the routine synthesis of many proteins using synthetic and expressed peptides.[1] The practical advantages of native chemical ligation come from its ability to generate amide bonds in a single pot reaction using unprotected peptides in neutral aqueous solution at low concentration. Here we describe a method for the chemo- and regioselective ligation of unprotected peptides at non-cysteine ligation sites that maintains many of these useful features (Scheme 1).

Intramolecular acyl transfer has been investigated by Kemp and co-workers as a synthetic route to peptides[2] and used as a method to quantitatively acylate secondary amines during solid-phase peptide synthesis (SPPS).[3] A general amide bond ligation strategy can be modeled after native chemical ligation by utilizing an auxiliary that mimics cysteine, combining thioester exchange with efficient intramolecular acyl transfer to the N^{α}-terminus of the peptide.

The auxiliary must meet stringent specifications. There must be a robust method for the introduction of the auxiliary onto the N^{α}-terminus of the peptide that is compatible with standard SPPS. The ligation step must be rapid at ∼1 mM peptide concentrations in denaturing aqueous buffer. These concentration and solvent restrictions are a result of the unpredictable and often poor solubility of large peptides. The final step must leave a native amide bond at the ligation junction. Several N^{α}-linked auxiliaries based on cysteine have been investigated for the synthesis of peptides.[4] Our auxiliary, based on the 2-mercaptobenzyl system,[5] provisionally addressed these requirements for a general synthesis of proteins.[6] However, this preliminary study was limited to a simple model system and did not demonstrate the removal of the auxiliary. Herein we modify this original system to improve ligation rates and introduce acid lability. This

Scheme 1. Mechanism of Peptide Ligations



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(6) This auxiliary does not introduce a new stereogenic center and its associated difficulties into the peptide.[4b−d]

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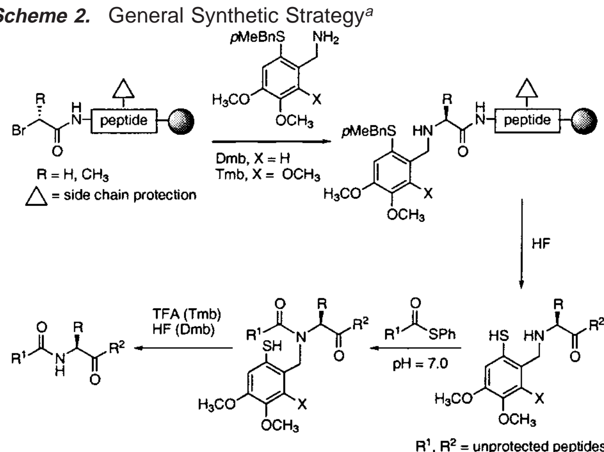
Table 1. Half-Lives for the Ligation of Unprotected Peptide Segments from the SH3 Domain of α-Spectrin and Protein G B1 Domain (PGB1)

N-terminal thioester piece[a]	*C*-terminal piece[a]	ligation junction	$t_{1/2}$	obsd mass (calcd)/Da[b]
α-spectrin[1−28]	Dmb α-spectrin[28−62]	Gly/Gly	0.2 h	7459.0 ± 1.0 (7458.6)
α-spectrin[1−27]	Dmb α-spectrin[28−62]	Lys/Gly	2.0 h	7400.0 ± 1.0 (7401.5)
α-spectrin[1−27]	Tmb α-spectrin[28−62]	Lys/Gly	2.0 h	7432.0 ± 1.0 (7431.6)
α-spectrin[1−28]	Dmb PGB1[24−56]	Gly/Ala	5.0 h	7139.0 ± 1.0 (7139.9)
PGB1[1−23]	Dmb PGB1[24−56]	Ala/Ala	no rxn	

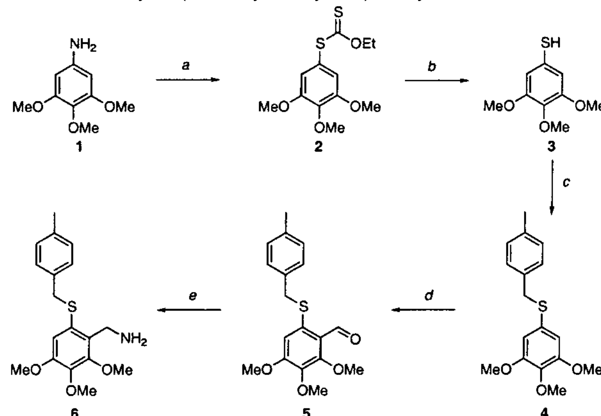
[a] Concentrations are approximately 1 mM. [b] Characterized by ESI-MS.

Scheme 2. General Synthetic Strategy[a]



[a] This general approach should also be compatible with Fmoc SPPS. The auxiliary is acid stable until the amine is converted to the amide by ligation.

Scheme 3. Synthesis of the 2,3,4-Trimethoxyl-6-(4-methylbenzylthio)benzylamine[a]



[a] Key: (a) $NaNO_2$, HCl, 0 °C then KS_2COEt, 65 °C (56%). (b) NaOH, 65 °C (90%). (c) 4-MeBnBr, NaH, PPh_3, (97%). (d) $POCl_3$, DMF, 150 °C (97%). (e) NH_2OH, $LiAlH_4$ (45%).

new removable acyl transfer auxiliary is used to synthesize the 62 amino acid SH3 domain from α-spectrin.

Results and Discussion

Auxiliary Design, Synthesis, and Introduction. Acid lability and improved ligation efficiency were introduced into the original 2-mercaptobenzyl auxiliary[5] by increasing the electron density of the aromatic ring. Methoxy substitution at the 4- and 6-positions of the benzyl group increases its acid lability. Methoxy substitution at the 5-position raises the nucleophilicity of the thiol and increases the efficiency of thioester exchange, the putative rate-limiting step.[5,7,8] Of the possible substitution patterns, we initially investigated the 4,5-dimethoxy-2-mercapto-benzyl (Dmb) (subsequently reported for a ligation at a Gly-Gly ligation site on a small peptide)[9] and the 4,5,6-trimethoxy-2-mercaptobenzyl (Tmb) auxiliaries. The Tmb auxiliary was expected to combine the ligation properties of Dmb with greater acid lability.

The submonomer approach was used to introduce the auxiliaries onto the *N*-terminus of the *C*-terminal peptide fragments. The displacement of an α-bromo-peptide resin with a primary amine results in inversion of configuration at the α-carbon[4c,10] (Scheme 2). As expected no epimerization (<2%) of the *N*-terminal C^{α} carbon was observed in small model peptides using this preparative method. Protection of the thiol group was necessary to prevent *S*-alkylation. The *S*-4-methyl-benzyl protected Dmb amine[11] was synthesized directly from commercially available material. The similarly protected Tmb amine was synthesized in five steps from 3,4,5-trimethoxyaniline as shown in Scheme 3.

Peptide Ligation. To demonstrate the effectiveness of this methodology we used the auxiliary-mediated ligation to synthesize the 62 amino acid SH3 domain from α-spectrin at a Lys-Gly sequence (entry 3, Table 1). Since the rate of ligation is putatively controlled by thioester exchange,[5,7] the nature of the *C*-terminal amino acid of the thioester peptide has a major effect on the ligation rate. For example, thioester exchange with glycine and histidine thioester peptides is relatively rapid while the β-branched amino acids exchange slowly.[4,7] These extreme cases are not representative of the ligation properties of the majority of amino acids such as leucine and lysine. As a result, the Lys-Gly ligation junction was selected to best represent a typical ligation site in a protein.[7]

To perform the ligation reaction, a single equivalent of SH3 α-spectrin[1−27] thiophenol thioester[13,14] was added to the Tmb *N*-terminally modified [28−62] peptide (Figure 1).[15,16] The major product peak possessed the expected mass of the target material. Preparative HPLC of the ligation mixture yielded the SH3 sequence with attached auxiliary in 66% yield. The successful construction of the SH3 Lys-Gly bond demonstrates the broad scope of our method.

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(8) Model studies suggest that the 5-methoxy substitution increases ligation rates (data not shown).
(9) Kawakami, T.; Akaji, K.; Aimoto, S. *Org. Lett.* **2001**, *3*, 1403−1405.
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(12) HPLC conditions: Vydac C_{18} (100 × 4.6 mm) column, 25−50% B over 30 min (1 mL min^{-1}). Buffer A = 0.1% aqueous TFA. Buffer B = 10% water and 0.09% TFA in acetonitrile.
(13) Muir, T. W.; Dawson, P. E.; Kent, S. B. H. *Methods Enzymol.* **1997**, *289*, 266−298. Available from Fmoc/*t*-Bu chemistry: Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369−11374.
(14) Ligation was observed for Gly-Gly (8 h) and Lys-Gly (48 h) with *C*-terminal alkyl thioester in the absence of thiol additives.
(15) Ligation can be completely suppressed by the addition of thiols.

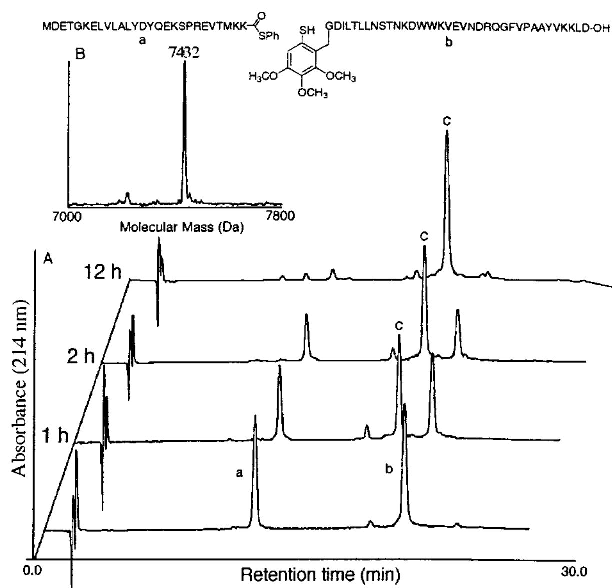

Figure 1. (A) Analytical HPLC[12] of the time-course for a ligation reaction between SH3 α-spectrin[1−27] thiophenol thioester (a, 4.0 mg, 1.2 mM) and SH3 α-spectrin Tmb[28−62] (b, 5.0 mg, 1.18 mM) with TCEP (5 mg, 17.4 mM) in degassed 200 mM NaH_2PO_4 and 6 M GdmCl (1 mL) at pH 7.0. The product was isolated in 66% yield (c, 5.8 mg). (B) Reconstructed mass of product c.

The scope of this ligation reaction was further investigated by attempting ligation reactions at three additional ligation sites, Gly-Gly, Gly-Ala, and Ala-Ala. Because of facile thioester exchange onto *C*-terminal glycine thioester peptides, the half-life for the Gly-Gly coupling was very rapid (entry 1, Table 1). Ligation onto non-glycine sites has been particularly challenging.[4] The successful ligation of the sterically demanding *N*-terminal Dmb-alanine with the synthetically convenient *C*-terminal glycine thioester (entry 4, Table 1) was thus an important result. However, no product was observed for the more challenging alanine thioester to Dmb-alanine ligation (entry 5, Table 1). Table 1 shows the range of the auxiliary-mediated ligation indicating the broad applicability of this methodology and its limitations.

The results in Table 1 are consistent with known data. The order of magnitude rate increase between lysine (entry 2 and 3) and glycine thioesters (entry 1) is consistent with known thioester exchange behavior[7] and supports thioester exchange as the rate-limiting step for these ligations. The dramatic rate decrease between Dmb-glycine and Dmb-alanine ligations with glycine thioester (entries 1 and 4) suggests a change in the rate-determining step from thioester exchange to acyl migration. This hypothesis is consistent with the known acyl migration behavior of the related 2-hydroxybenzylamines where secondary amines of glycine rearrange much faster than secondary amines of other amino acids.[2,3] On the basis of these preliminary observations we propose that thioester exchange is the rate-limiting step for ligation reactions where the auxiliary is attached to a glycine residue. These results also suggest that the rate-limiting step for the ligation reaction changes to acyl migration when non-glycine residues are modified with the auxiliary.

Cleavage of the Auxiliary. Because amines are poor leaving groups, benzylamines are stable to the strong acid conditions used for peptide cleavage and side chain deprotection (Scheme 2). The Dmb and Tmb auxiliaries were completely stable to anhydrous HF prior to their conversion from amine to amide substituents. Ligation transforms the benzylamine into a benzylamide, which is more electron withdrawing. This increases the leaving group propensity of the system rendering it acid sensitive.[3b,4,5,17] The Tmb auxiliary can be removed with TFA and scavengers, while the Dmb auxiliary can be removed with either TFMSA[9] or anhydrous HF (Scheme 2 and Figure 2). In our hands TFMSA did not give clean results on large peptides. The practical difficulties associated with TFMSA or HF significantly limit the use of the Dmb auxiliary. The Tmb auxiliary has the same ligation properties and has a simple and general cleavage.

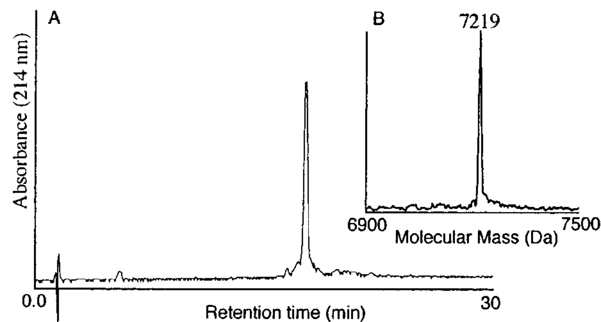

Figure 2. (A) Typical analytical HPLC[12] of crude α-spectrin SH3 after removal of ligation auxiliary. Tmb: The lyophilized crude ligation mixture was treated with TFA and 5% triisopropylsilane for 2 h at 25 °C. Dmb: The ligation product was treated with 1% v/v *p*-cresol and 1% v/v ethanedithiol in HF for 0.5 h at 0 °C. (B) Reconstructed mass of the SH3 domain.

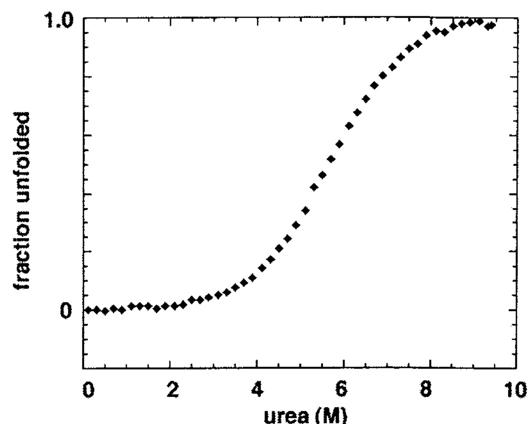

Figure 3. Equilibrium urea denaturation of folded α-spectrin SH3 in 0.1 M phosphate at pH 7.0 (intrinsic fluorescence was monitored at 315 nm with an excitation wavelength of 280 nm).

To complete the synthesis of the SH3 domain of α-spectrin, it was necessary to remove the auxiliary. This was easily achieved under acidic conditions (Figure 2) providing the final product. The CD spectrum of the folded material was consistent with the published spectrum of the SH3 domain of α-spectrin.[18] The denaturation profile (Figure 3) was determined by monitoring the fluorescence of the two Trp residues as a function of urea concentration; $\Delta G°_{H_2O} = 16 \pm 0.46$ kJ mol^{-1},

(16) This ligation was also carried our using the Dmb auxiliary. The time course for ligation was identical with that of the Tmb auxiliary as monitored by HPLC (entry 2, Table 1).

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m = 2.83 ± 0.09 kJ mol^{-1} M^{-1}, consistent with published values.[18] The presence of both Trp and Met residues in the final product, known to be sensitive to chemical modification during acid deprotection,[19] made this target a good test for the removal of the 2-mercaptobenzyl auxiliary.

Summary

The 4,5,6-trimethoxy-2-mercaptobenzyl (Tmb) auxiliary functions as a cysteine mimic for the ligation of polypeptides. Our results suggest that this auxiliary will be applicable to ligation sites containing a glycine at either side of the ligation junction. This added flexibility in synthetic design is crucial for the synthesis of larger, more challenging, biologically important targets.[20] A variety of approaches for the synthesis of non-cysteine ligation sites have recently been disclosed.[4,5,9] The strength of our approach is that it combines simple auxiliary construction and introduction, practical ligation rates at low concentrations, and removal of the auxiliary by TFA. We anticipate that this approach can be extended to the chemoselective synthesis of a range of amide-containing molecules such as natural products, materials, and bioconjugates.

Experimental Section

Synthesis of 2,3,4-Trimethoxy-6-(4-methyl benzylthio)benzylamine (6). General Techniques. All reactions were carried out under an argon atmosphere with dry, freshly distilled solvents under anhydrous conditions, unless otherwise noted. Tetrahydrofuran (THF) was distilled from sodium benzophenone; methylene chloride (CH_2Cl_2) was freshly distilled from calcium hydride. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials, unless otherwise stated.

Reagents were purchased at the highest commercial quality and used without further purification, unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as a visualizing agent and 7% ethanolic phosphomolybdic acid or *p*-anisaldehyde solution and heat as developing agent. E. Merck silica gel (60, particle size 0.040−0.063 mm) was used for flash column chromatography.

NMR spectra were recorded on Bruker DRX-600 or DRX-500 instruments and calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, m = multiplet, b = broad, bs = broad singlet. IR spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer. High-resolution mass spectra (HRMS) were recorded on a IONSPEC FTMS spectrometer (MALDI) with DHB as matrix.

Xanthate 2: Aniline **1** (9.97 g, 0.0544 mol) was dissolved in MeOH (10 mL) and 10% aqueous HCl and was then cooled to 0 °C. A solution of sodium nitrite (5.0 g, 0.0725 mol) in H_2O (20 mL) was added dropwise over 1 h. The reaction mixture was stirred at 0 °C for an additional 15 min at which time the solution was added to a solution of potassium ethyl xanthate (17.3 g, 0.108 mol) in H_2O (50 mL) at 65 °C. After the mixture was stirred for 15 min, the reaction was cooled to 25 °C. The resulting mixture was extracted with EtOAc (3 × 200 mL) and the combined organic extracts were washed with brine (200 mL), dried (Na_2SO_4), and concentrated in vacuo. Flash column chromatography of the residue (silica gel, 10% EtOAc in hexanes) afforded xanthate **2** (8.80 g, 56%). **2**: R_f = 0.36 (silica gel, 20% EtOAc in hexanes); IR (thin film) ν_{max} 2936, 1580, 1496, 1455, 1405, 1306, 1234, 1125, 1024 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.72 (s, 2 H, ArH), 4.60 (q, J = 7.1 Hz, 2 H, OCH_2), 3.86 (s, 3 H, OCH_3), 3.84 (s, 6 H, OCH_3), 1.33 (t, J = 7.0 Hz, 3 H, OCH_2CH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 214.0, 154.2, 140.4, 125.4, 113.0, 71.2, 61.8, 57.1, 14.6; HRMS (MALDI-FTMS) calcd for $C_{12}H_{16}O_4S_2Na$ (M + Na$^+$) 311.0382, found 311.0382.

Thiophenol 3: Xanthate **2** (7.90 g, 0.02739 mol) was dissolved in EtOH (100 mL). A solution of sodium hydroxide (3 M, 100 mL) was added and the reaction mixture was heated to 65 °C for 2 h. The reaction mixture was cooled to 25 °C and acidified to pH 5 by the addition of 10% aqueous HCl. The resulting mixture was extracted with EtOAc (3 × 200 mL) and the combined organic extracts were washed with brine (200 mL), dried (Na_2SO_4), and concentrated in vacuo to provide thiophenol **3** (4.91 g, 90%), which was used without further purification.

Thioether 4: Thiophenol **3** (1.29 g, 6.45 mmol) was dissolved in THF (50 mL) and MeOH (50 mL). A 60% dispersion of NaH in mineral oil (0.70 g, 17.5 mmol) was added slowly to the reaction and the mixture was stirred for 5 min. 4-Methylbenzyl bromide (1.31 g, 7.08 mmol) and triphenylphosphine (1.71 g, 6.53 mmol) were then added to the reaction. The mixture was stirred for 15 min and then concentrated in vacuo. Flash column chromatography of the residue (silica gel, 10% EtOAc in hexanes) afforded thioether **4** (1.90 g, 97%). **4**: R_f = 0.38 (silica gel, 20% EtOAc in hexanes); IR (thin film) ν_{max} 2935, 2831, 1578, 1498, 1406, 1307, 1233, 1179, 1124, 1007 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.13 (d, J = 7.9 Hz, 2 H, ArH), 7.06 (d, J = 7.4 Hz, 2 H, ArH), 6.50 (s, 2 H, ArH), 4.01 (s, 2 H, SCH_2), 3.79 (s, 3 H, OCH_3), 3.73 (s, 6 H, OCH_3), 2.29 (s, 3 H, CH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 154.0, 138.1, 137.6, 135.5, 131.6, 130.0, 129.8, 109.1, 61.7, 56.9, 40.7, 21.9; HRMS (MALDI-FTMS) calcd for $C_{17}H_{20}O_3S$ (M$^+$) 304.1128, found 304.1136.

Aldehyde 5: Thioether **4** (1.82 g, 5.98 mmol) and *N,N*-dimethylformamide (0.70 mL, 9.04 mmol) were disolved in CH_2Cl_2 (10 mL) at 0 °C. Phosphorus oxychloride (0.95 mL, 10.2 mmol) was added dropwise over 10 min. The reaction was heated to 150 °C for 2 h generating a dark red oil. H_2O (100 mL) was added to the oil and the mixture was refluxed for 1 h. The resulting mixture was extracted with EtOAc (3 × 200 mL) and the combined organic extracts were washed with brine (200 mL), dried (Na_2SO_4), and concentrated in vacuo. Flash column chromatography of the residue (silica gel, 20% EtOAc in hexanes) afforded aldehyde **5** (1.92 g, 97%). **5**: R_f = 0.37 (silica gel, 30% EtOAc in hexanes); IR (thin film) ν_{max} 2936, 2850, 1664, 1580, 1546, 1488, 1369, 1299, 1240, 1117, 1018 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.41 (s, 1 H, CHO) 7.39 (d, J = 8.1 Hz, 2 H, ArH), 7.19 (d, J = 7.7 Hz, 2 H, ArH), 6.65 (s, 1 H, ArH), 4.18 (s, 2 H, SCH_2), 4.04 (s, 3 H, OCH_3), 3.90 (s, 3 H, OCH_3), 3.87 (s, 3 H, OCH_3), 2.39 (s, 3 H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.4, 158.7, 158.5, 153.5, 140.2, 138.7, 137.6, 133.5, 129.8, 129.1, 120.5, 105.0, 62.8, 61.5, 56.5, 37.3, 21.5; HRMS (MALDI-FTMS) calcd for $C_{18}H_{21}O_4S$ (M + H$^+$) 333.1155, found 333.1150.

Amine 6: Aldehyde **5** (0.68 g, 2.05 mmol) was disolved in CH_2Cl_2 (25 mL). To this was added hydroxylamine (0.24 g, 3.45 mmol) and triethylamine (1.0 mL, 13.6 mmol). The reaction mixture was stirred for 18 h and then quenched via the addition of H_2O (50 mL). The resulting mixture was extracted with EtOAc (3 × 100 mL) and the combined organic extracts were washed with brine (100 mL), dried (Na_2SO_4), and concentrated in vacuo. The resulting oil was dissolved in THF (30 mL) and cooled to 0 °C. A solution of lithium aluminum hydride in THF (1.0 M, 6.0 mL) was added dropwise over 5 min. The reaction was heated to reflux for 1 h and then cooled to 0 °C and quenched via the addition of H_2O (0.25 mL), 3 M aqueous NaOH (0.25 mL), and H_2O (0.75 mL). The mixture was dilluted with EtOAc (300 mL) and filtered and concentrated in vacuo. Flash column chromatography of the residue (silica gel, gradient elution 0→20% MeOH in EtOAc) afforded amine **6** (0.31 g, 45%). **6**: R_f = 0.21 (silica gel, 25% MeOH in EtOAc); IR (thin film) ν_{max} 3344, 2934, 1583, 1482, 1397, 1303, 1237, 1195, 1108, 1021 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.14 (s, 4 H, ArH), 6.74 (s, 1 H, ArH), 4.05 (s, 2 H, SCH_2), 3.95 (s, 3 H, OCH_3), 3.93 (s, 3 H, OCH_3), 3.89 (s, 2 H, NCH_2), 3.83 (s, 3 H, OCH_3), 2.38 (s, 3 H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.6,

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152.5, 142.3, 137.4, 135.0, 132.2, 129.6, 129.4, 129.2, 112.7, 61.7, 61.3, 56.4, 41.1, 39.3, 21.5; HRMS (MALDI-FTMS) calcd for $C_{18}H_{23}NO_3SNa$ (M + Na$^+$) 356.1291, found 356.1304.

Peptide Synthesis. General Methods. Electrospray ionization mass spectrometry (ESI-MS) was performed on an API-III triple quadrupole mass spectrometer (PE-Sciex). Peptide masses were calculated from the experimental mass to charge (*m/z*) ratios from all the observed protonation states of a peptide using MacSpec software (Sciex). Theoretical masses of peptides and proteins were calculated using MacProMass software (Beckman Research Institute, Duarte, CA).

HPLC. Analytical reversed-phase HPLC was performed on a Hewlett-Packard HPLC 1050 system using Vydac C_{18} columns (5 μm, 0.46 × 15 cm). Semipreparative reversed-phase HPLC was performed on a Rainin HPLC system using a Vydac C-18 column (10 μm, 1.0 × 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 5 mL/min (semipreparative).

Reagents. Boc-amino acids for peptide synthesis were from Midwest Biotech (Fishers, IN), 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) from Quantum-Appligene (Carlsbad, CA), diisopropylethylamine from Applied Biosystem (Foster City, CA), TFA from Halocarbon (River Edge, NJ), and DMF from EM-Science (Gibbstown, NJ).

Solid-Phase Peptide Synthesis (SPPS). Peptides were prepared by manual solid-phase synthesis using the in situ neutralization/HBTU activation for Boc chemistry as previously described;[21] couplings were carried out with a 5-fold excess of activated amino acid for a minimum of 15 min and monitored by quantitative ninhydrin test.[22] *C*-Terminal peptides were synthesized on the appropriate Boc-aminoacyl-OCH_2-Pam preloaded resin (Applied Biosystems).

After chain assembly was complete the peptide-resin was treated with HF containing 5% (v/v) *p*-cresol for 1 h at 0 °C. After evaporation of HF, the crude peptide was precipitated in anhydrous Et_2O, dissolved in HPLC buffer, and lyophilized.

Peptide-α-thioesters. Thio acid peptides were synthesized on the appropriate Boc-aminoacyl-*S*-resins.[23] Alternatively *N*-terminal *β*-mercaptopropionic acid-leucine (MPAL) thioester peptides were synthesized according to a published procedure[7] and converted to thio acids by dissolving the crude peptide in 6 M guanidine·HCl, 0.2 M phosphate, pH 7.2, containing ammonium sulfide. The conversion was monitored to completion by analytical HPLC. Peptide-$COSC_6H_5$ esters were prepared by dissolving the peptide thio acid in 6 M guanidine·HCl, 0.1 M sodium acetate, pH 4.5, and adding an excess of Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)). The mixture was vortexed briefly and after 20 min thiophenol (2% v/v) was added. The exchange reaction was monitored by analytical HPLC to completion.

SH3 α-spectrin[1−27] thiophenol thioester: *H*-MDETGKELVLALYDYQEKSPREVTMKK-SC_6H_5, observed mass 3294 ± 0.6 Da, calculated 3294.7 Da.

SH3 α-spectrin[1−28] thiophenol thioester: *H*-MDETGKELVLALYDYQEKSPREVTMKKG-SC_6H_5, observed mass 3351 ± 0.3 Da, calculated 3351.8 Da.

Protein G B1 domain (PGB1[1−23] thiophenol thioester: *H*-MTYKLILNGKTLKGETTTEAVDA-SC_6H_5, observed mass 2590 ± 0.6 Da, calculated 2589.9.

Attachment of N^αTmb and N^αDmb to Peptides. After chain assembly was completed the N^α-Boc group was removed and the peptide-resin neutralized with DIEA, washed with DMF and CH_2Cl_2. The appropriate α-bromo symmetric anhydride prepared from the α-bromo acid (bromoacetic acid for *N*-terminal glycine and (*R*)-2-bromopropionic acid for *N*-terminal (*S*)-alanine) in CH_2Cl_2 was added to the peptide-resin. The peptide-resin was washed with DMF, and 2,3,4-trimethoxy-6-(4-methylbenzylthio)benzylamine (3 equiv) dissolved in a minimum of DMF was added and left for 18 h. Inspection of analytical HPLC of peptide showed quantitative addition of Dmb and Tmb to *N*-terminal bromoacetylated peptide-resin with no loss of Dmb or Tmb with HF treatment. Similarly addition of Dmb to (*R*)-2-bromopropionyl terminal peptide proceeded with an estimated unoptimized yield of 50%. Peptides were purified by semipreparative HPLC after cleavage from resin.

SH3 α-spectrin Dmb[28−62]: *Dmb*GDILTLLNSTNKDWWKVEVNDRQGFVPAAYVKKLD-*OH,* observed mass 4216 ± 1 Da, calculated 4216.8 Da.

SH3 α-spectrin Tmb[28−62]: *Tmb*GDILTLLNSTNKDWWKVEVNDRQGFVPAAYVKKLD-*OH*, observed mass 4246.2 ± 1 Da, calculated 4246.9 Da.

PGB1 Dmb[24−56]: *Dmb*ATAEKVFKQYANDNGVDGEWTYDDATKTFTVTE-*OH*, observed mass 3898.0 ± 0.3 Da, calculated 3898.2 Da.

Ligation of Peptide Segments. 6 M guanidine·HCl, 200 mM sodium phosphate, pH 8.5, was degassed under vacuum and flushed with argon. TCEP (5 mg mL^{-1}) was added. Typically peptides were added to ligating buffer at concentrations of approximately 5 mg mL^{-1} of each peptide. The pH of the ligation mixture after the addition of peptides was 7.0. The ligation reactions were monitored to completion by analytical HPLC.

SH3 α-spectrin 28-*N*-Dmb: *H*-MDETGKELVLALYDYQEKSPREVTMKK-*Dmb*G-DILTLLNSTNKDWWKVEVNDRQGFVPAAYVKKLD-*OH*, observed mass 7400 ± 1.0 Da, calculated 7401.5 Da.

SH3 α-spectrin 28-*N*-Tmb: *H*-MDETGKELVLALYDYQEKSPREVTMKK-*Tmb*G-DILTLLNSTNKDWWKVEVNDRQGFVPAAYVKKLD-*OH*, observed mass 7432 ± 1.0 Da, calculated 7431.6.

SH3 α-spectrin [1−28-TmbG-29−62]: *H*-MDETGKELVLALYDYQEKSPREVTMKKG-*Tmb*G-DILTLLNSTNKDWWKVEVNDRQGFVPAAYVKKLD-*OH*, observed mass 7459 ± 1.0 Da, calculated 7458.6 Da.

Condensation product of SH3 α-spectrin[1−28] and PGB1 Dmb-[24−56]: *H*-MDETGKELVLALYDYQEKSPREVTMKKG-*Dmb*A-TAEKVFKQYANDNGVDGEWTYDDATKTFTVTE-*OH*, observed mass 7139 ± 1 Da, calculated 7139.9 Da.

Removal of Auxiliary. The peptide was placed in a 15 mL Falcon tube with *p*-cresol (10 μL) and ethanedithiol (10 μL). After brief centrifugation the Falcon tube was inserted into a Teflon reaction vessel and HF (approximately 1 mL) was condensed into the Falcon tube. The cleavage was left for 30 min at 0 °C. HF was evaporated and peptide precipitated with ice-cold anhydrous Et_2O (10 mL) and left in the CO_2/acetone bath for 5 min. Et_2O was decanted off and the Et_2O wash was repeated three times.

The Tmb auxiliary was removed by treating the lyophilized crude ligation reaction product with a cocktail containing 5% (v/v) triisopropylsilane in TFA for 2 h at 23 °C. The bulk of TFA was removed by rapid sparging with a stream of dry argon. The peptide was precipitated with ice-cold anhydrous Et_2O and washed as for the HF cleavage.

SH3 α-spectrin: *H*-MDETGKELVLALYDYQEKSPREVTMKKGDILTLLNSTNKDWWKVEVNDRQGFVPAAYVKKLD-*OH*, observed mass 7219 ± 1 Da, calculated 7219.3 Da.

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