An Affinity Chromatographic Method for the Purification of Water-Insoluble Peptides

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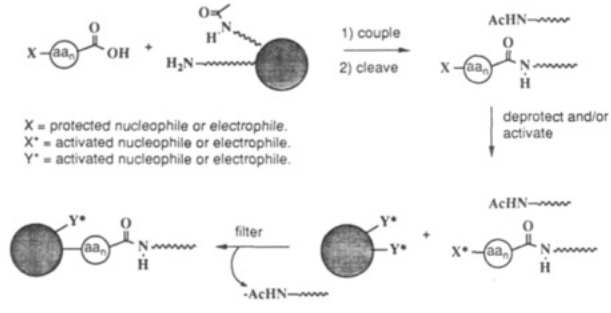
A simple and efficient affinity chromatographic method is described for the purification of hydrophobic and/or protected synthetic peptides in organic solvent. This affinity method entails the use of a new protecting group that contains a UV-active disulfide moiety ([2-[(2-nitrophenyl)dithio]-1-phenylethoxy]carbonyl, NpSSPeoc). NpSSPeoc is electrophilic and can be immobilized by a thiolate–disulfide interchange reaction on a thiol-containing solid support. Alternatively, NpSSPeoc can be converted into a nucleophilic thiol ((2-mercapto-1-phenylethoxy)carbonyl, HSPeoc) and immobilized by an electrophilic solid support. NpSSPeoc can be quantified via an Ellman-type assay to measure the amount of full-length peptide at the completion of a solid-phase synthesis before and/or after cleavage from the synthesis resin. NpSSPeoc can be removed with 25% trifluoroacetic acid in methylene chloride. Four peptides incorporating NpSSPeoc at the N-terminus were synthesized. Affinity chromatography was performed on these peptides using 1% cross-linked polystyrene supports which incorporate a mercury(II) trifluoroacetate, disulfide, alkyl thiol, or iodoacetamide functionality. The results indicate that the highest and most consistent yields of purified peptide are obtained using the combination of HSPeoc and a polystyrene support incorporating the iodoacetamide functionality.

Introduction

Thanks to the development of solid-phase synthetic methods, peptides of 20–30 amino acids can be routinely prepared.[1] However, these advances have uncovered shortcomings in existing purification methodology. Since the standard purification protocols depend on the solubility of the product in aqueous media, hydrophobic sequences present additional difficulties. We have observed that a nine amino acid hydrophobic fragment of the β amyloid protein is extremely difficult to purify in the standard aqueous chromatographic solvents because of its β-sheet forming properties.[2] Protecting groups can dramatically decrease the water solubility of peptides. We have developed a solid-phase fragment coupling strategy which requires the purification of protected peptide fragments.[3,4] The development of a fast and simple affinity chromatographic method for the purification of these fragments as well as unprotected hydrophobic peptides in nonaqueous organic solvents would expand the capabilities of our fragment coupling method and of peptide synthesis in general.

Several affinity methods have been applied to peptide purification.[5–10] These methods all require an acetylation step after each amino acid coupling, which "caps" any unreacted amine groups and prevents the extension of deletion impurities. In most cases,[6–10] the N-terminal amino acid incorporates a protected functional group that can be converted to a reactive moiety (nucleophile or electrophile). Only the target sequence contains this affinity group which allows it to be isolated from the crude synthetic product by reaction with a solid support to form a covalent bond (Scheme I).

Scheme I



The identity of the reactive functional group which is incorporated into the peptide (X*) and the affinity support (Y*) differentiates the various literature methods (Scheme I). Merrifield uses organomercurial agarose as the electrophilic affinity support (Y* = HgOH) and a 4-methoxybenzyl-protected cysteinethiol as the protected nucleophile on the peptide (X = S(MeOBzl), X* = SH).[8] The approach of Andreu is based on a reaction between the nucleophilic mercaptopropylsepharose affinity support (Y* = $CH_2CH_2CH_2SH$) and the electrophilic 3-nitro-2-pyridinesulfenyl-protected cysteine on the peptide (X* = NpysS).[9] Finally, the method of Funakoshi utilizes the electrophilic iodoacetamide-modified Kieselguhr resin for the affinity support (Y* = iodoacetamide) and a linker containing the protected nucleophile 4-methoxybenzenethiol on the peptide (X = S(MeOBzl), X* = SH).[10]

These methods were designed for aqueous purifications and therefore are not generally useful for the purification of water-insoluble hydrophobic peptides and protected

(1) Barany, G.; Merrifield, R. B. Solid Phase Peptide Synthesis. In *the Peptides Vol. 2*; Gross, E., Meienhofer, J., Eds.; Academic Press, New York, 1980, pp 1–284.
(2) Halverson, K.; Fraser, P. E.; Kirschner, D. A.; Lansbury, P. T. *Biochemistry* **1990**, *29*, 2639.
(3) (a) Hendrix, J. C.; Jarrett, J. T.; Anisfeld, S. T.; Lansbury, P. T. *J. Org. Chem.* **1992**, *57*, 3414. (b) Hendrix, J. C.; Lansbury, P. T. *J. Org. Chem.* **1992**, *57*, 3421.
(4) Hendrix, J. C.; Halverson, K. J.; Lansbury, P. T. *J. Am. Chem. Soc.* In press.
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(6) Lobl, T. J.; Deibel, M. R.; Yem, A. W. *Anal. Biochem.* **1988**, *170*, 502.
(7) Merrifield, R. B.; Bach, A. E. *J. Org. Chem.* **1978**, *43*, 4808.
(8) Krieger, D. E.; Erickson, B. W.; Merrifield, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73* (9), 3160.
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(10) Funakoshi, S.; Fukuda, H.; Fujii, N. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6981.

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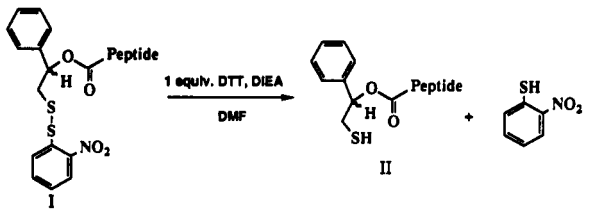
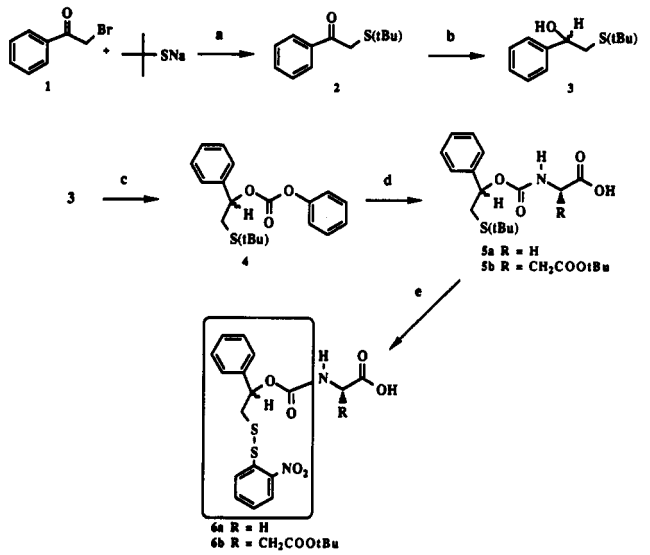
Scheme II



Scheme III[a]



[a] (a) THF, 0 °C; (b) $NaBH_4$, EtOH; (c) phenyl chloroformate/pyridine, CH_2Cl_2; (d) amino acid/Triton B, DMF, 50 °C; (e) 2-nitrobenzenesulfenyl chloride, DMF, acetic acid, H_2O.

peptide fragments. We set out to design an affinity group that could be applied to an organic solvent-based affinity system. The ideal affinity reagent should be capable of reacting as a nucleophile or as an electrophile. This capability would give the reagent greater versatility with regard to the chemistry of the covalent attachment to the solid support. The affinity protecting group must also be resistant to conditions required for cleavage of the synthetic peptide from the peptide synthesis resin. Finally, the group should have a reporter functionality, which allows its quantification. The group I ([2-[(2-nitrophenyl)-dithio]-1-phenylethoxy]carbonyl, NpSSPeoc, Scheme II) fulfills these criteria. This paper reports the synthesis of NpSSPeoc-derivatized amino acids, their use in peptide synthesis on several solid supports, and the purification of derivatized peptides using four different affinity resins.

Results and Discussion

Synthesis of NpSSPeoc-Amino Acids and Peptides. Bromoacetophenone **1** was treated with the sodium salt of 2-methyl-2-propanethiol in anhydrous tetrahydrofuran to produce the thioether **2** which was then reduced to the secondary alcohol **3** in 54% overall yield (Scheme III).[11] The alcohol **3** was treated with phenyl chloroformate in the presence of pyridine to give the phenyl carbonate **4** in 79% yield. The phenyl carbonate was treated with the desired amino acid in the presence of Triton B in dimethylformamide (DMF) to give the corresponding carbamate which, without further purification, was treated with 1 equiv of 2-nitrobenzenesulfenyl chloride to give the corresponding disulfide **6a** (R = H) or **6b** (R = CH_2-COOtBu).[12,13,14] The NpSSPeoc-amino acid can then be coupled to an amino acid or peptide in solution or to a peptide on a solid support using standard *N,N'*-dicyclohexylcarbodiimide (DCC) or (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) coupling methods.

The disulfide portion of NpSSPeoc is stable to acids and hindered bases such as diisopropylethylamine (DIEA). Treatment of the disulfide with 1 equiv of dithiothreitol (DTT) or triethylphosphine cleaves the disulfide to afford the free thiol, HSPeoc, which can then act as a nucleophile (Scheme II).[15] In addition to the HSPeoc-containing peptide, the 2-nitrobenzenethiolate anion is produced. This species has a distinct UV spectrum (λmax at 490 nm) which can be used to quantify the amount of peptide incorporated at the end of a solid-phase synthesis and after cleavage from the solid support. The modified Ellman assay involves treating the NpSSPeoc-containing peptide with excess β-mercaptoethanol (BME) and DIEA in DMF and monitoring the absorbance at 490 nm.[16] The UV absorbance of NpSSPeoc itself is very large at 225 nm, a feature which can be useful for distinguishing the desired peptide containing NpSSPeoc from the truncated ones that do not.

The NpSSPeoc group is stable in 1% trifluoroacetic acid (TFA) in methylene chloride for at least 1 h at room temperature. When the concentration of TFA is increased to 5%, roughly half of the carbamate is cleaved in 25 min, as assayed by reversed-phase HPLC. At a concentration of 25% TFA in methylene chloride, complete cleavage of the carbamate occurs within 30 min. Substituted analogs of NpSSPeoc have been prepared which are more acid-stable.[17]

Synthesis of Peptides. Four peptides were synthesized incorporating NpSSPeoc I (Table I). Compound **6a** was coupled to glycine benzyl ester in solution using BOP and DIEA to give the protected dipeptide **7** after chromatography (Table I). The pure dipeptide was used as a model compound to determine the efficiency of binding and cleavage from the various affinity supports. Peptide **8** was synthesized on the Kaiser oxime resin following the standard tBOC synthesis protocol.[18] The tetrapeptide was then cleaved from the Kaiser oxime resin using *N*-hydroxypiperidine to give peptide **8** as its piperidyl ester.[19] Peptide **9** was synthesized on the (hydroxymethyl)-

(11) Fujisawa, T.; Takemura, I.; Ukaji, Y. *Tetrahedron Lett.* **1990**, *31*, 5479.

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

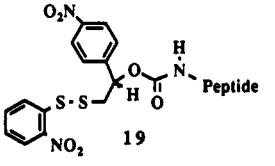
(13) Pastuszak, J. J.; Chimiak, A. Roczniki Chemii **1977**, *51*, 1567.

(14) Fontana, A.; Scoffone, E.; Benassi, C. A. *Biochemistry* **1968**, *7*, 980.

(15) Matsueda, R.; Kimura, T.; Kaiser, E. T.; Matsueda, G. R. *Chem. Lett.* **1981**, 737.

(16) Glaser, C. B.; Maeda, H.; Meienhofer, J. *J. Chromatogr.* **1970**, *50*, 151.

(17) Incorporating a nitro group on the phenylethoxy portion of NpSSPeoc (**19**) causes the carbamate to be stable to 100% TFA over 3 h. Cleavage of the carbamate is accomplished by using 10% methanetrifluorosulfonic acid in TFA, at 0 °C, over 30 min.



(18) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, *47*, 3258.

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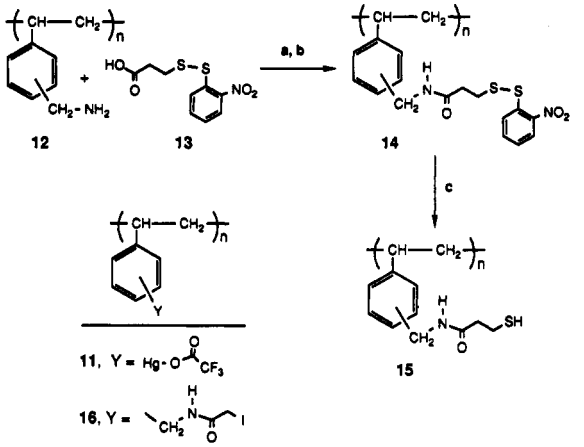
Table I. Synthetic Peptides Incorporating Compounds 6a or 6b at the N-Terminus

peptide	synthetic method
NpSSPeoc-GG-OBn 7	solution coupling
NpSSPeoc-GAIA-OPip 8	oxime resin[a]
NpSSPeoc-GAIA-OH 9	HMPB resin[b]
NpSSPeoc-D(tBu)AE(tBu)FR(Pmc)H(Trt)-D(tBu)S(tBu)G-OH 10	HMPB resin

[a] References 18, 19, 29. [b] HMPB = (hydroxymethyl)methoxyphenoxybutyric acid, ref 20; OPip = hydroxypiperidine ester; Pmc = pentamethylchroman; Trt = trityl.

Scheme IV[a]



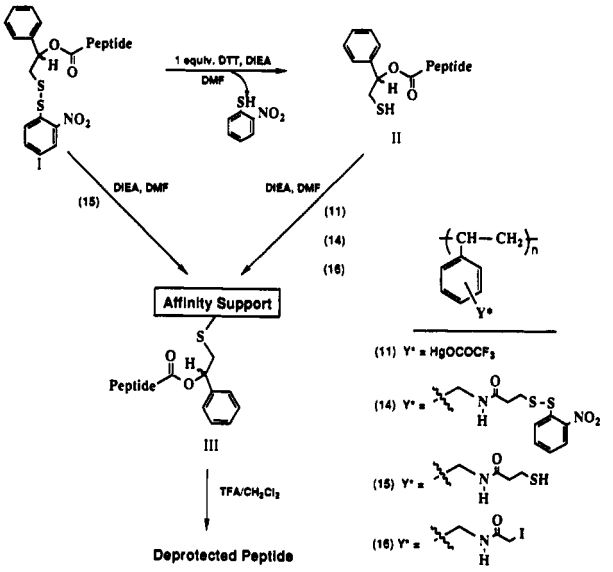
[a] (a) BOP/DIEA, DMF; (b) acetic anhydride/DIEA, CH_2Cl_2; (c) BME/DIEA, DMF.

methoxyphenoxybutyric acid resin (HMPB) using the standard Fmoc chemistry protocol and cleaved using 1% TFA in CH_2Cl_2.[20,21] Peptide **10** (β-amyloid 1–9)[3b] was also synthesized on the HMPB resin, using compound **6b** as the N-terminal amino acid. This peptide represents the N-terminal nine amino acid portion of the β-amyloid protein.[4] Cleavage of the β-amyloid 1–9 peptide from the HMPB resin was accomplished by successive washes with 1% TFA in CH_2Cl_2.

Synthesis of Affinity Supports. Four affinity supports, each incorporating a potential electrophile or nucleophile, were synthesized (Scheme IV). All were derivatives of 1% cross-linked polystyrene. Following the method of Bullen, polystyrene was treated with excess mercury(II) trifluoroacetate in anhydrous THF to give mercurated polystyrene **11**.[22] The amount of mercury incorporated into the polystyrene was determined by elemental analysis (ca. 2 mmol of Hg/g of resin).

Affinity support **14** was synthesized by treating (aminomethyl)polystyrene resin **12** with excess disulfide **13**, which was prepared by treating 3-mercaptopropionic acid with 2-nitrobenzenesulfenyl chloride (Scheme IV). This resin was then treated with acetic anhydride to acetylate any free amines. The disulfide substitution level of affinity support **14** was measured by monitoring the release of 2-nitrobenzenethiolate anion in the presence of excess

Scheme V



BME and DIEA at 490 nm (ca. 0.26 mmol/g of resin). Reaction of the disulfide affinity support **14** with excess BME and DIEA gave the corresponding free thiol resin **15** (Scheme IV).

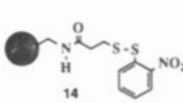
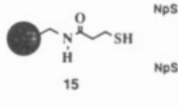
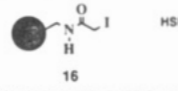
The iodoacetamide affinity support **16** was synthesized following the procedure of Funakoshi.[10] Iodoacetic acid was treated with DCC to give the anhydride, which was then added in excess to the (aminomethyl)polystyrene resin **12** to give the resulting iodoacetamide resin **16** (0.4 mmol of iodoacetamide/g of resin).[23]

Affinity Chromatography. A series of affinity experiments were undertaken involving an electrophilic resin (**11**, **14**, or **16**) with the HSPeoc-containing peptide (II, Scheme V) or a nucleophilic resin (**15**) with the NpSSPeoc-containing peptide (I, Scheme V). The protocol for the former involves first treating the NpSSPeoc-containing peptide (I) with 1 equiv of DTT and excess DIEA in DMF to form the HSPeoc-containing peptide (II). This reaction mixture was applied to the affinity support III (**11**, **14**, or **16**). In the case where the free thiol resin **15** is used, the NpSSPeoc-peptide (I) is added directly in the presence of excess DIEA to the affinity support. Reversed-phase HPLC was used to monitor (at 225 nm) the formation of the HSPeoc-peptide (II) as well as its incorporation onto the affinity support. In order to monitor the formation of the 2-nitrobenzenethiolate anion, the UV absorbance (490 nm) of the reaction mixture was measured. Amino acid analysis was used to determine the amount of peptide bound on the affinity support (III). Cleavage of the peptide (III) from the resin was achieved with 25% TFA in CH_2Cl_2. In the case of peptide **10**, cleavage was accomplished with 75–90% TFA in order to simultaneously deprotect the peptide.

When the HSPeoc-peptide **7** and the mercurated polystyrene resin **11** were shaken together (40 min), 64% of the dipeptide was covalently bound (by amino acid analysis of resin) to the affinity support. Unfortunately, cleavage of the bound peptide (25% TFA) was found to cause protolysis of the C–Hg bond. The liberated product was found to contain Hg(I) and Hg(II) dipeptide complexes (FABMS). This is in accordance with results obtained in

(20) Kamber, B.; Riniker, B.; Poster, *The Solid Phase Synthesis of Protected Peptides, Combined with Fragment Coupling in Solution*, 12th American Peptide Symposium, Boston, MA, 1991.
(21) Sieber, P. *Tetrahedron Lett.* **1987**, *28*, 6147.
(22) Bullen, N. P. B.; Hodge, P.; Thorpe, F. G. *J. Chem. Soc., Perkin Trans. 1* **1981**, *7*, 1863.
(23) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147.

Table II. Percent Yield of Deprotected Peptide after Affinity Chromatography

affinity support	peptide	DIEA (equiv)	reactn time (min)	% yield[a]
 14	HSPeoc-GGOBn 7	4.8	6	81
	HSPeoc-GAIA-OPip 8	4.6	7	59
	HSPeoc-GAIA-OH 9	5.7	10	67
	HSPeoc-D(tBu)AE(tBu)FR(Pmc)H(trityl)D(tBu)S(tBu)G-OH 10	44	10	0[b]
 15	NpSSPeoc-GGOBn 7	6.2	60	32
	NpSSPeoc-GAIA-OPip 8	5.3	10	44
	NpSSPeoc-D(tBu)AE(tBu)FR(Pmc)H(trityl)D(tBu)S(tBu)G-OH 10	28	10	26
 16	HSPeoc-GGOBn 7	9.7	60	75
	HSPeoc-D(tBu)AE(tBu)FR(Pmc)H(trityl)D(tBu)S(tBu)G-OH 10	35	120	45

[a] Isolated yield of deprotected peptide by weight. [b] Five binding experiments were performed. Shaded circles = polystyrene resin, 1% cross-linked.

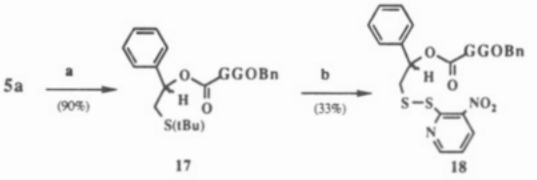
earlier studies which show that electrophilic substitution reactions carried out under acidic conditions cause varying degrees of protodemercuration of mercury-bound polystyrene.[24,25] Due to the instability of the mercury–carbon bond to strong acids, work with affinity support **11** was discontinued.

When the disulfide affinity resin **14** was used in combination with the HSPeoc-containing di- and tetrapeptides **7–9** (Table II), good recoveries of pure peptide were obtained. On the other hand, no binding of the peptide **10** occurred. During this experiment, the UV absorbance due to the 2-nitrobenzenethiolate anion, which normally increases as resin-bound disulfide is cleaved, rapidly disappeared. This implies that the 2-nitrobenzenethiolate leaving group is competing with the free thiol-containing peptide in displacing the disulfide on the resin. With small thiol-containing peptides the peptide thiolate–disulfide interchange reaction occurs fast enough that this side reaction is not observed.

When the free thiol affinity resin **15** was used, in combination with either peptide **7** or peptide **8**, poor yields were obtained. Further examination of this system revealed the binding to be time- and base-dependent. For example, in binding peptide **7**, a 40% drop in the amount of resin-bound peptide was observed (by amino acid analysis of the resin) when the amount of base was increased from 0.6 to 1 equiv. In other experiments, it was observed that with a constant amount of base, the binding of peptide **8** dropped by 65% when the reaction time was extended from 9 min to 1 h. The time- and base-dependency of the binding process may be due to competing side reactions involving thiolate–disulfide interchange.

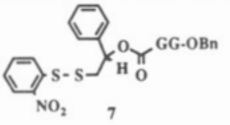
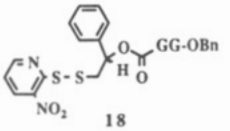
In order to reduce the likelihood of cleavage of resin-bound peptide by disulfide interchange involving the thiolate leaving group of the affinity protecting group, the NpySSPeoc-peptide ([2-[(3-nitro-2-pyridinyl)dithio]-1-phenylethoxy]carbonyl) **18** was synthesized (Scheme VI).

Scheme VI[a]



[a] (a) Glycine benzyl ester/BOP/DIEA, DMF; (b) 3-nitro-2-mercaptopyridinesulfenyl bromide/DIEA, MeOH, $CHCl_3$.

Table III. Reaction of Free Thiol Affinity Resin 15 with Peptides 7 and 18 as a Function of Time[a]

dipeptide	reaction time (min)	H-Gly-Gly-OBn isolated (%)[b]
 7	5	72
	60	16
 18	5	70
	60	57

[a] Using 2.8 equiv. of DIEA. [b] Isolated as the trifluoroacetate salt.

This dipeptide incorporates a 3-nitro-2-mercaptopyridine leaving group which is a much weaker base (pK_a = 2.2) than 4-nitrobenzenethiolate (pK_a = 4.7).[26,27] Therefore, the leaving group of **18** should be less nucleophilic. The reaction of NpySSPeoc-peptide **18** with the affinity support **15** was compared to the reaction of NpSSPeoc-peptide **7** with **15** (Table III). The isolated yield of the cleaved peptide GlyGly(OBn) was measured as a function of the reaction time. The results show comparable yields of GlyGly(OBn) using either of the peptides **7** or **18**, at short reaction time (5 min, Table III). However, at longer reaction time (1 h), the isolated yield using the NpSSPeoc-containing peptide **7** was reduced significantly as compared to the NpySSPeoc-containing peptide **18**, which incorporates the less nucleophilic leaving group. These results are consistent with the suggestion that the 2-nitrobenzenethiolate leaving group of NpSSPeoc can cleave resin-bound peptide via a slow disulfide interchange reaction. The further development of NpySSPeoc is in progress, with the goal of utilizing affinity support **15**.

When the iodoacetamide resin **16** was treated with the HSPeoc-containing peptides **7** and **10**, good yields of purified peptide were obtained (Table II). This combination gave the most consistent yields of any of the methods discussed herein. The 2-nitrobenzenethiolate leaving group was covalently bound to the resin within 5 min (UV) while 78% of the HSPeoc-peptide **10** was bound to resin **16** after 3 h (amino acid analysis). The average yield of the cleaved, deprotected peptide β1-9 was 45% (Table II, final entry). This recovery is comparable to that achieved by the traditional approach, i.e., deprotection followed by reversed-phase HPLC (62%). One must bear in mind that the water-soluble peptide (β1–9) is easily purified by the standard method (RPHPLC); therefore, the stated comparison is the worst case for the NpSSPeoc

(24) Taylor, R. T.; Cassell, R. A.; Flood, L. A. *Ind. Eng. Chem. Prod. Res. Dev.* **1982**, *21*, 462.
(25) Stamberg, J.; Petrus, V.; Gregor, P. *J. Appl. Polym. Sci.* **1979**, *23*, 503.
(26) Ponsati, B.; Ruiz-Gayo, M.; Giralt, E.; Albericio, F.; Andreu, D. *J. Am. Chem. Soc.* **1990**, *112*, 5345.
(27) De Maria, P.; Fini, A.; Hall, F. M. *J. Chem. Soc., Perkin Trans. 2* **1973**, 1969.

strategy. The yield of RPHPLC purification decreases dramatically for sparingly soluble hydrophobic peptides or protected peptides; however, these compounds are expected to be easily purified in organic solvents by our affinity approach (NpSSPeoc).

The discrepancy between the 78% yield of resin-bound **10** and the 45% yield of purified peptide is due primarily to incomplete cleavage from the affinity support. We observed that after repeated TFA washes of the affinity support, roughly 30% of the peptide was still bound to the resin. The addition of thiol scavengers such as 1,2-ethanedithiol to the cleavage and deprotection protocol has no effect on the isolated yield. A series of model experiments were conducted in which various Fmoc-protected amino acids were treated with the iodoacetamide resin **16** under reaction conditions similar to those used for peptide **10**. The results indicate that a carboxylic acid can slowly react ($t_{1/2}$ = ca. 7 h) with the resin.[28] By reducing the number of equivalents of iodoacetamide resin used in the affinity protocol and shortening the reaction time, this side reaction can be minimized.

Conclusions

The N-terminal protecting group/affinity reagent NpSS-Peoc has a variety of properties which make it very useful for peptide synthesis. It is versatile in that it can be used with a nucleophilic or electrophilic affinity support. The highly UV active nature of the disulfide portion of the molecule allows for easy differentiation of a peptide containing this group from acetylated peptide impurities. In addition, the UV-active leaving group allows one to quantify the amount of the full-length resin-bound peptide at the end of a synthesis and of the free peptide after cleavage from the synthesis resin.

The thiolate–disulfide interchange affinity method (Scheme V, I + **15** or II + **14**) has the advantage that one may be able to cleave the resin-bound peptide with a free thiol to regenerate the N-terminally protected peptide. This strategy would be useful for purification of protected peptide fragments for fragment coupling. Our preliminary results with this combination demonstrated that the leaving group could cleave resin-bound peptide and that the modification of NpSSPeoc yields an analog for which this undesired side reaction is minimized.

The use of the iodoacetamide resin **16** in combination with the HSPeoc-containing peptides (Scheme V, II + **16**) was shown to be the simplest and highest-yielding affinity method of all the strategies reported herein. This combination proved that it is possible to covalently bind and cleave peptides containing a variety of different protected side chains. In the case of a water-soluble peptide, the yield obtained by this purification strategy is comparable to that obtained by traditional methods. We expect that for insoluble peptides, the yield for this strategy will greatly exceed that of traditional methods. Work is currently underway to apply this affinity system to the purification of large hydrophobic peptides synthesized by a solid-phase stepwise strategy.

(28) The experiments were monitored using reversed-phase HPLC at a wavelength of 301 nm. In a typical experiment, a known quantity of amino acid and naphthalene (used as a standard) were dissolved in dimethylformamide. To the solution was added 2 equiv of iodoacetamide resin and a large excess of diisopropylethylamine (100 equiv). Aliquots were then taken at various intervals and analyzed using reversed-phase HPLC.

Experimental Section

Materials. The syntheses of the Kaiser oxime resin[18,19,29] and *N*-hydroxypiperidine (HOPiP)[30] were done according to published procedures. Diisopropylethylamine (DIEA) was distilled from ninhydrin under reduced pressure. Pyridine was distilled from NaH and stored over 4-Å molecular sieves. Tetrahydrofuran (THF) was distilled from Na/benzophenone. Hydrolysis of peptides and peptide bound resins were performed in HCl/propionic acid (1:1, v/v; Pierce) at 130 °C for 3 h; derivatization and amino acid analyses were performed by the MIT Biopolymers Laboratory, Cambridge, MA. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. DMF was purchased from Baxter Scientific. Flash chromatography was conducted using silica gel (grade 60) purchased from Merck and Co.

2-((2-Methyl-2-propyl)thio)-1-phenylethanol (3). To a 100-mL round-bottom flask containing 1.5 g (0.062 mol) of sodium hydride was added 20 mL of anhydrous THF, and the mixture was stirred under nitrogen at 0 °C. To the stirred mixture was then added 2-methyl-2-propanethiol (6 mL, 0.053 mol) over a period of 4–5 min. The mixture was stirred for 10 min at 0 °C, and then 11 g (0.055 mol) of 2-bromoacetophenone dissolved in 25 mL of anhydrous THF was slowly added over a period of 10 min. The resulting mixture was stirred under a nitrogen atmosphere for 40 min at 0 °C and then for 1.5 h at room temperature, after which time the solvent was removed under reduced pressure. The resulting residue was dissolved in 100 mL of diethyl ether and washed with (2 × 80 mL) H_2O and then with 80 mL of saturated aqueous $NaHCO_3$. The organic layer was dried over $MgSO_4$ and filtered, and the solvent was removed under reduced pressure to give **2** as a brown oil which was used without any further purification.

To the oil (**2**) was added 50 mL of absolute ethanol, and the mixture was stirred until solution was complete. To the solution was added 4.6 g (0.121 mol) of $NaBH_4$ and mixture stirred, under nitrogen atmosphere and at room temperature for 48 h. The solution was then cooled to 0 °C, and 80 mL of a 10% aqueous HCl solution was slowly added. The aqueous mixture was extracted with 100 mL of CH_2Cl_2. The organic layer was separated, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give a yellow oil. The oil was purified by flash chromatography (SiO_2, 1.5- × 19-cm column, 50% hexane–CH_2Cl_2) to isolate 5.8 g (54%) of **3** as a yellow oil: R_f = 0.34 (SiO_2, 50% hexane–CH_2Cl_2); IR (film) 3263, 2960, 2874, 1458, 1363, 1162, 1040, 699 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.35 (s, 9 H), 2.78 (dd, 1 H, J = 2.5 Hz, J = 13.0 Hz), 2.90 (d, 1 H, J = 2.5 Hz), 2.96 (dd, 1 H, J = 2.5 Hz, J = 13.0 Hz), 4.70–4.75 (m, 1 H), 7.35–7.38 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 30.9, 38.3, 42.4, 72.3, 125.5, 127.5, 128.2, 142.7; FAB MS m/z 211 $(M + H)^+$.

2-((2-Methyl-2-propyl)thio)-1-phenylethyl Phenyl Carbonate (4). To a 25-mL round-bottom flask containing alcohol **3** (0.8 g, 3.85 mmol) was added, under nitrogen atmosphere, 4 mL of anhydrous CH_2Cl_2. To the resulting solution was added 0.4 mL of anhydrous pyridine. The solution was stirred at −4 °C for 5–10 min, then 0.5 mL (3.98 mmol) of phenyl chloroformate was added, and the resulting mixture was stirred at 0 °C for 1.5 h. The reaction mixture was slowly warmed to room temperature and stirring was continued for 4 h. After 4 h, the reaction mixture was filtered and the filtrate washed with 15 mL of CH_2Cl_2. The mother liquor was then washed with 30 mL of H_2O, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give a yellow oil. The yellow oil was purified by flash chromatography (SiO_2, 1.5- × 10-cm column, 50% hexane–CH_2Cl_2) to isolate 1.0 g (79%) of **4** as a thick yellow oil: R_f = 0.48 (SiO_2, 50% hexane–CH_2Cl_2); IR (film) 2961, 2898, 2331, 1759, 1592, 1494, 1472 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.33 (s, 9 H), 2.99 (dd, 1 H, J = 6.3 Hz, J = 13.2 Hz), 3.16 (dd, 1 H, J = 7.7 Hz, J = 13.2 Hz), 5.75 (t, 1 H, J = 6.4 Hz), 7.14–7.42 (m, 10 H); ^{13}C NMR ($CDCl_3$) δ 30.8, 34.0, 42.7, 80.4, 120.9, 125.9, 126.7, 128.5, 128.7, 129.3, 138.2, 151.0, 152.9; FAB MS m/z 353 $(M + Na)^+$.

Synthesis of *N*-[[2-[(2-Nitrophenyl)dithio]-1-phenylethoxy]carbonyl]glycine ((±)-6a). To a 100-mL round-bottom

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flask was added 0.35 g (4.66 mmol) of glycine and 2.0 mL of 40% Triton B (4.40 mmol), and mixture was heated at 60 °C until most of the solid was dissolved. The volatile components of the solution were then removed under reduced pressure to give an oil. To the oil was added 2.0 mL of DMF which was removed under reduced pressure at 40–50 °C to give a solid/oil mixture. This process was repeated, and to the resulting solid/oil mixture was added 1.0 g (3.03 mmol) of the phenyl carbonate **4**. The mixture was stirred at 50–60 °C under closed atmosphere for 4 h. The DMF was removed under reduced pressure to give an oily residue. The residue was taken up in 80 mL of H_2O and transferred to a separatory funnel. To the aqueous was added ~100 mg of $MgSO_4$, and the aqueous layer was washed with diethyl ether (2 × 50 mL) and then aqueous acidified with 10% aqueous HCl until pH ≈ 2.0. The aqueous phase was then quickly extracted with (2 × 50 mL) diethyl ether, the organic extracts were combined, dried over $MgSO_4$, and filtered, and the volatile components were removed under reduced pressure to give 1.1 g of **5a** as a light yellow oil. This material was then used without any further purification.

To a 25-mL round-bottom flask containing the crude carbamate **5a** was added 3 mL of DMF, 7 mL of glacial acetic acid, and 0.7 mL of H_2O, and mixture was stirred until a clear solution was obtained. To this solution was added 0.68 g (3.66 mmol) of 2-nitrobenzenesulfenyl chloride, and the yellow solution was stirred at room temperature for 2 h. After 2 h, the solution was concentrated under reduced pressure to half the original volume. The crude product was then precipitated from water and the aqueous mixture extracted with (2 × 60 mL) diethyl ether. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give 1.2 g of a thick yellow oil. The oil was purified by flash chromatography (SiO_2, 2- × 16-cm column) first with CH_2Cl_2 then with 50% diethyl ether–CH_2Cl_2 to isolate 0.67 g (54%) of **6a** as a yellow oil: R_f = 0.58 (SiO_2, 50% diethyl ether–CH_2Cl_2); IR (film) 3413, 2848, 1731, 1716, 1589, 1511, 1449 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.11 (dd, 1 H, J = 4.9 Hz, J = 14.0 Hz), 3.25 (dd, 1 H, J = 8.2 Hz, J = 14.0 Hz), 3.98 (d, 2 H, J = 5.4 Hz), 5.86 (q, 1 H, J = 4.9 Hz), 7.32–7.37 (m, 6 H), 7.63 (t, 1 H, J = 8.3 Hz), 8.18 (d, 1 H, J = 8.3 Hz), 8.25 (d, 1 H, J = 9.4 Hz); ^{13}C NMR ($CDCl_3$) δ 42.5, 44.6, 52.2, 74.9, 125.9, 126.1, 126.2, 127.1, 128.5, 134.0, 137.1, 138.4, 145.4, 154.9, 170.1; FAB MS m/z 409 $(M + H)^+$.

Synthesis of *N*-[[2-[(2-Nitrophenyl)dithio]-1-phenylethoxy]carbonyl]aspartic Acid β-*tert*-Butyl Ester ((±)-6b). The synthesis was done in the same manner as the glycine **6a**, starting with 0.65 g (3.43 mmol) of L-aspartic acid (β-tBu ester) to afford a yellow oil which was purified by flash chromatography (SiO_2, 18- × 1.5-cm column) first with CH_2Cl_2 and then with 50% diethyl ether–CH_2Cl_2 to isolate 0.54 g (44%) of two diastereomers of **6b**: R_f = 0.47 (SiO_2, 20% diethyl ether–CH_2Cl_2); IR (film) 2978, 2933, 1737, 1673, 1651, 1633, 1590, 1504, 1450 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.25–1.47 (m, 9 H), 2.65–3.66 (m, 4 H), 4.55 (br s, 1 H), 5.74–5.96 (m, 2 H), 7.31 (br s, 6 H), 7.63 (t, 1 H, J = 1.2 Hz), 8.14–8.26 (m, 2 H), 10.75 (br s, 1 H); ^{13}C NMR ($CDCl_3$) δ 27.9, 30.8, 37.4, 38.1, 44.8, 49.7, 50.3, 52.4, 75.1, 82.1, 82.2, 126.0, 126.1, 126.2, 126.4, 127.2, 128.3, 128.5, 134.0, 137.1, 138.4, 145.5, 154.8, 170.1, 172.1, 175.2; FAB MS m/z 523 $(M + H)^+$.

***N*-[[2-[(2-Nitrophenyl)dithio]-1-phenylethoxy]carbonyl]-glycylglycine Benzyl Ester ((±)-7).** To a 25-mL round-bottom flask containing 0.73 g (1.80 mmol) of **6a** were added 0.8 g of BOP (1.80 mmol) and 0.36 g (1.80 mmol) of glycine benzyl ester, and the mixture was dissolved in 2 mL of DMF. To the solution was then added 0.8 mL (4.59 mmol) of DIEA, and the solution was stirred for 24 h. At the end of 24 h, the volatile components were removed under reduced pressure to give an oil which was purified by flash chromatography (SiO_2, 2- × 20-cm column, 50% diethyl ether–CH_2Cl_2) to isolate 0.87 g (87%) of dipeptide **7** as a thick yellow oil: R_f = 0.6 (SiO_2, 50% diethyl ether–CH_2Cl_2); IR (film) 1730, 1675, 1589, 1566, 1513, 1450, 1387, 1338, 1303 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.09 (dd, 1 H, J = 4.8 Hz, J = 13.7 Hz), 3.23 (dd, 1 H, J = 8.1 Hz, J = 13.7 Hz), 3.88 (br s, 2 H), 4.07 (d, 2 H, J = 5.3 Hz), 5.17 (s, 2 H), 5.43 (br s, 1 H), 5.87 (t, 1 H, J = 4.9 Hz), 6.38 (br s, 1 H), 7.31–7.34 (m, 11 H), 7.63 (t, 1 H, J = 7.5 Hz), 8.17 (d, 1 H, J = 8.1 Hz), 8.25 (d, 1 H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 41.1, 44.1, 44.5, 67.1, 75.0, 126.0, 126.1, 126.2, 127.1, 128.1, 128.5, 134.0, 135.0, 137.0, 138.4, 145.4, 155.3, 169.1, 169.4; FAB MS m/z 556 $(M + H)^+$.

Spectrophotometric Assay for 2-Nitrobenzenethiolate Anion. To a test tube was added a known amount of dry resin-bound peptide (2–3 mg). To the resin was then added 1.0 mL of DMF, 40 μL (0.229 mmol) of DIEA, and 40 μL (0.57 mmole) of BME (total volume = 1.08 mL), and the mixture was vortexed for 1–2 min. The resulting red mixture was filtered through a pipette containing glass wool directly into a cuvette. The absorbance of the mother liquor was then measured at 490 nm (extinction coefficient of standard = 1679.1 cm^{-1} M^{-1}) against a blank consisting of 1.0 mL of DMF, 40 μL of DIEA, and 40 μL of BME. The assay for cleaved peptide was done in the same manner except that the reaction mixture was not filtered. The 3-nitro-2-mercaptopyridine-containing dipeptide **18** was assayed at 490 nm using an extinction coefficient = 1021 cm^{-1} M^{-1}.

Mercurated Polystyrene Resin 11. The synthesis was done following the method of Bullen[22] starting with 2.0 g of Biobeads SX-1 and 8.0 g (18.7 mmol) of mercury(II) trifluoroacetate to give 5.5 g of **11** as a white solid: IR (Nujol mull) 1652. Anal. Found: Hg, 41.35 (2.06 mmol of Hg/g).

Disulfide 13. To a 25-mL round-bottom flask containing 1.67 g (9.0 mmol) of 2-nitrobenzenesulfenyl chloride was added 6 mL of anhydrous CH_2Cl_2, and the mixture was stirred and sonicated, under a nitrogen atmosphere, until solution was complete. To the yellow solution was added, at 0 °C, 0.65 mL (7.45 mmol) of 3-mercaptopropionic acid, and the mixture was stirred at 0 °C under a nitrogen atmosphere for 10 min. A yellow solid precipitated out which was filtered through a medium-porosity fritted-glass funnel. The yellow solid was washed with a few milliliters of CH_2Cl_2 and then with methanol and placed under vacuum to give 1.2 g (62% crude yield) of **13** as a yellow solid: R_f = 0.16 (SiO_2, CH_2Cl_2); ^1H NMR ($CDCl_3$) δ 2.77 (t, 2 H, J = 6.5 Hz), 2.97 (t, 2 H, J = 6.5 Hz), 7.38 (t, 1 H, J = 6.98 Hz), 7.70 (t, 1 H, J = 6.98 Hz), 8.28 (d, 2 H, J = 8.28 Hz); FAB MS m/z 260 $(M + H)^+$.

Disulfide Polystyrene Resin 14. To a peptide synthesis vessel were added 4.5 g of (aminomethyl)polystyrene resin (subst. = 0.6 mmol/g), 2.0 g (4.52 mmol) of BOP dissolved in 10 mL of DMF, and 0.56 g (2.16 mmol) of disulfide **13** dissolved in 10 mL of DMF, and the mixture was shaken for 1 min. To the mixture was added 2.0 mL (11.5 mmol) of DIEA, and the mixture was shaken at room temperature for 2 h. The mixture was washed six times with DMF, once with CH_2Cl_2, twice with methanol, and then three times with CH_2Cl_2 in that order. The yellow resin was acetylated with 0.6 mL of acetic anhydride (6.3 mmol) and 1.0 mL (5.7 mmol) of DIEA in CH_2Cl_2 over a period of 40 min. The resulting solid was washed with methanol and then with CH_2Cl_2, and the solid was placed under vacuum to give 4.79 g of **14** as a yellow solid. Spectrophotometric assay at 490 nm gave a substitution of 0.26 mmol/g. Cleavage of the disulfide with excess BME and DIEA in DMF followed by repeated washings with DMF, methanol, and CH_2Cl_2 gave the free thiol affinity resin **15**.

Iodoacetamide Polystyrene Resin 16. The synthesis was done following the method of Funakoshi with the exception that the Kieselguhr resin was replaced by (aminomethyl)polystyrene (subst. = 0.6 mmol/g).[10] The amount of resin-bound amine after derivatization was measured by quantitative ninhydrin (0.4 mmol of iodoacetamide/g of resin).

Synthesis of Thioether Dipeptide 17. To a 100-mL round-bottom flask containing 2.2 g (4.9 mmol) of BOP and 0.69 g (3.42 mmol) of the hydrochloride salt of glycine benzyl ester were added 7 mL of DMF and 2.2 mL (12.6 mmole) of DIEA. To the stirred mixture was then added 1.1 g (3.38 mmol) of crude **5a**, and the solution was stirred under closed atmosphere at 25 °C for 2 h. At the end of 2 h, the DMF was removed under reduced pressure and the resulting oil taken up in 100 mL of CH_2Cl_2. The organic layer was washed with 100 mL of 0.1 N HCl. The organic layer was then separated, dried over $MgSO_4$, filtered, and CH_2Cl_2 concentrated under reduced pressure. The resultant oil was purified by flash chromatography (SiO_2, 3- × 15-cm column, 30% diethyl ether in CH_2Cl_2), to isolate 1.4 g (90%) of **17** as a thick oil: R_f = 0.53 (SiO_2, 50% diethyl ether in CH_2Cl_2); IR (film) 2960, 2940, 1726, 1677, 1605, 1585, 1529, 1498 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.30 (s, 9 H), 2.88 (d, 2 H, J = 5.6 Hz), 4.04 (d, 2 H, J = 5.3 Hz), 5.16 (s, 2 H), 5.45 (br s, 1 H), 5.76 (t, 1 H, J = 2.5 Hz), 6.55 (br

s, 1 H), 7.28–7.35 (m, 10 H); ^{13}C NMR ($CDCl_3$) δ 30.7, 34.3, 41.0, 42.4, 44.1, 53.3, 67.0, 76.6, 115.2, 126.4, 128.1, 128.3, 128.4, 129.3, 135.0, 129.4, 155.7, 169.4; FAB MS *m/z* 459 $(M + H)^+$.

Synthesis of *N*-[[2-[(3-Nitro-2-pyridinyl)dithio]-1-phenylethoxy]carbonyl]glycylglycine Benzyl Ester ((±)-18). To a 25-mL round-bottom flask containing 0.44 g (0.96 mmol) of 17 was added 2 mL of methanol, 2 mL of $CHCl_3$, and 0.167 mL (0.95 mmol) of DIEA, and the mixture was stirred until solution was complete. To the solution was added 0.29 g (1.57 mmol) of 3-nitro-2-pyridinesulfenyl bromide which was synthesized following the method of Matsueda and Walter.[31,32] The mixture was stirred for 1.5 h at 25 °C and then filtered through a medium-porosity fritted-glass funnel. The mother liquor was concentrated under reduced pressure, and the resulting oil was purified by flash chromatography (SiO_2) first with CH_2Cl_2 and then with 50% methanol in CH_2Cl_2 to isolate 0.3 g of a yellow foam consisting of 60% of the desired product 18 (33% yield) and recovered starting material. A portion of the foam was recrystallized, first with CH_2Cl_2–2-propanol and then with 50% hexafluoro-2-propanol in ethanol, to give 0.1 g of 18 as a yellow crystalline solid: R_f = 0.36 (SiO_2, 50% diethyl ether–CH_2Cl_2); IR (film from $CHCl_3$) 2939, 1726, 1673, 1580, 1556, 1515, 1395 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.19 (dd, 1 H, *J* = 4.7 Hz, *J* = 14.0 Hz), 3.34 (dd, 1 H, *J* = 4.7 Hz, *J* = 12.0 Hz), 3.87 (br s, 2 H), 4.03 (d, 2 H, *J* = 4.9 Hz), 5.14 (s, 2 H), 5.61 (br s, 1 H), 5.95 (br s, 1 H), 6.65 (br s, 1 H), 7.26–7.33 (m, 11 H), 8.47 (dd, 1 H, *J* = 1.4 Hz, *J* = 8.1 Hz), 8.83 (dd, 1 H, *J* = 1.4 Hz, *J* = 4.5 Hz); ^{13}C NMR ($CDCl_3$) δ 41.2, 44.3, 44.6, 67.2, 75.6, 120.8, 126.4, 128.2, 128.6, 133.6, 135.1, 138.9, 142.7, 153.7, 157.1, 169.1, 169.4, 186.2; FAB MS *m/z* 557 $(M + H)^+$.

Example Affinity Procedure: Disulfide Affinity Resin 14 with Free Thiol Form of Peptide 8. To a test tube containing a small stir bar was added 60 mg (1.02 mmol/g by spectrophotometric assay = 0.061 mmol) of crude 8 RX-GAIA-OPip. To the test tube was then added 3 mL of DMF, and the mixture was stirred until solution was complete. To the yellow solution were then added 11.0 mg (0.072 mmol) of dithiothreitol (DTT) and 0.05 mL (0.287 mmol) of DIEA. The resulting dark red solution was stirred under closed atmosphere for 17 min, diluted with 1 mL of DMF, and transferred to a 15-mL peptide synthesis vessel containing 0.26 g (0.264 mmol/g by spectrophotometric assay = 0.068 mmol) of disulfide affinity resin 14. The red mixture was shaken for 6 min and filtered, and the resin was washed three times with DMF, twice with methanol, and three times with CH_2Cl_2. To the resin was added 4 mL of 25% TFA in CH_2Cl_2, and the mixture was shaken for 30 min. After 30 min the contents were filtered, the resin was washed with 2 mL of hexafluoro-2-propanol, the organics were combined, and the volatile components were removed under reduced pressure to give an oily residue. The residue was triturated with 2–3 mL of anhydrous diethyl ether, and the resulting solid was placed under vacuum to give 19 mg (59%) of GAIA-OPip as the TFA salt: ^{1}H NMR (D_2O) δ 0.67–0.79 (m, 6 H), 0.96–1.02 (m, 2 H), 1.19 (d, 3 H, *J* = 7.2 Hz), 1.23 (d, 3 H, *J* = 8.5 Hz), 1.30–1.50 (m, 4 H), 1.61–1.65 (m, 3 H), 2.50 (br t, 2 H, *J* = 25 Hz), 3.15 (br s, 2 H), 3.65 (s, 2 H), 3.80–4.03 (m, 1 H), 4.15–4.20 (m, 2 H), 8.09 (d, exch., *J* = 8.5 Hz), 8.41 (d, exch., *J* = 5.6 Hz), 8.50 (d, exch., *J* = 5.6 Hz); amino acid analysis G (1.0), A (1.26), I (0.79); FAB MS *m/z* 413 $(M + H)^+$.

Affinity Purification of Peptide 9. The purification was done following the same procedure as above to isolate GAIA as the TFA salt: ^{1}H NMR (D_2O) δ 0.69 (t, 3 H, *J* = 7.2 Hz), 0.77 (d, 3 H, *J* = 6.8 Hz), 1.00–1.08 (m, 1 H), 1.19 (d, 3 H, *J* = 7.16 Hz), 1.24 (d, 3 H, *J* = 7.3 Hz), 1.19–1.35 (m, 1 H), 1.60–1.70 (m, 1 H), 3.66 (s, 2 H), 3.98 (d, 1 H, *J* = 8.2 Hz), 4.13–4.21 (m, 2 H), 8.10 (d, exch., *J* = 5.5 Hz); amino acid analysis G (1.0), A (2.0), I (1.0); FAB MS *m/z* 331 $(M + H)^+$.

Example Affinity Procedure: Free Thiol Affinity Resin 15 with Peptide 7. To a 15-mL vessel containing 0.41 g (0.20 mmol/g = 0.083 mmol) of free thiol affinity resin in 2 mL of DMF was added a solution of 19 mg (1.729 mmol/g = 0.032 mmol) of peptide 7 in 1 mL of DMF and 0.015 mL (0.086 mmol) of DIEA. The mixture was shaken for 5 min, filtered, and washed five times with DMF, twice with methanol, and then five times with CH_2Cl_2. To the resin was added 4 mL of 25% TFA in CH_2Cl_2, and the mixture was shaken for 1 h. After 1 h the mixture was filtered, the resin was washed with 2 mL of HFIP, the organics were combined, and the volatile components were removed under reduced pressure to give a oil. The oil was triturated with 2–3 mL of diethyl ether and residue placed under vacuum to give 8.0 mg (72%) of GGOBn as the TFA salt: ^{1}H NMR (D_2O) δ 3.69 (s, 2 H), 3.93 (s, 2 H), 5.05 (s, 2 H), 7.26 (s, 5 H); FAB MS *m/z* 223 $(M + H)^+$.

Example Affinity Purification Procedure: Iodoacetamide Resin 16 with Peptide 10. To a test tube containing a tiny stir bar was added 0.2 g (0.261 mmol/g by spectrophotometric assay) of crude peptide 10, and the solid was dissolved in 2 mL of DMF. To the solution was then added 13 mg (0.085 mmol) of DTT and 0.32 mL (1.83 mmol)[33] of DIEA, and the resulting red solution was stirred under closed atmosphere for 15 min. After 15 min the red solution was diluted with 3 mL of DMF and transferred to a 15-mL peptide synthesis vessel containing 0.56 g (0.39 mmol/g by quantitative ninhydrin) of iodoacetamide resin. The reaction mixture was shaken for 3 h at room temperature. After 3 h, the reaction mixture was filtered and the resin was washed five times with DMF, three times with methanol, and five times with CH_2Cl_2. To the resin was then added a solution of 5.2 mL of TFA, 0.3 mL of H_2O, and 0.3 mL of 1,2-ethanedithiol, and the mixture was shaken for 2 h. After 2 h, the mixture was filtered, the resin was washed with (2 × 3 mL) TFA, (1 × 3 mL) HFIP, and (1 × 3 mL) CH_2Cl_2, the organics were combined, and the volatile components were removed under reduced pressure to give a oil. The oil was then triturated with CH_2Cl_2 and placed under vacuum to give a white solid. The solid was taken up in H_2O and lyophilized to give 32 mg (45%) of DAEFRHDSG as a fluffy solid: ^{1}H NMR (DMSO-d_6) δ 1.14–1.28 (m, 3 H), 1.40–1.80 (m, 6 H), 2.16 (br s, 2 H), 2.59–2.98 (m, 8 H), 3.60 (d, 2 H, *J* = 5.4 Hz), 3.77 (d, 2 H, *J* = 5.7 Hz), 4.05–4.35 (m, 7 H), 4.45–4.65 (m, 3 H), 7.19–7.31 (m, 8 H), 7.69 (br s, 1 H), 7.94 (d, 1 H, *J* = 6.4 Hz), 8.10–8.16 (m, 3 H), 8.25–8.35 (m, 2 H), 8.44 (d, 1 H, *J* = 6.4 Hz), 8.62 (d, 1 H, *J* = 6.9 Hz), 8.79 (br s, 1 H); amino acid analysis D (2.0), A (1.0), E (1.0), F (0.9), R (1.0), H (1.0), S (0.8), G (0.9); FAB MS *m/z* 1033 $(M + H)^+$.

Anal. Calcd for $C_{42}H_{60}N_{14}O_{17}\cdot 5H_2O\cdot 3TFA$: C, 39.25; H, 4.90; N, 13.31; F, 10.74. Found: C, 39.43; H, 4.82; N, 13.41; F, 11.69.

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(33) Repeated washes with 1% TFA in CH_2Cl_2 are required to cleave the peptide from the HMPB resin. Each of the acid washes is then neutralized with excess pyridine, and the combined organics are concentrated under reduced pressure to give an oil which is then precipitated from water. Enough DIEA must be added in order to deprotonate the alkanethiol. A large excess of DIEA is required in order to neutralize any remaining pyridinium trifluoroacetate salt that was not removed when the crude, cleaved peptide was precipitated from H_2O.