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Articles

Backbone Amide Linker (BAL) Strategy for N^{α}-9-Fluorenylmethoxycarbonyl (Fmoc) Solid-Phase Synthesis of Unprotected Peptide *p*-Nitroanilides and Thioesters[1]

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A novel and general backbone amide linker (BAL) strategy has been devised for preparation of *C*-terminal modified peptides containing hindered, unreactive, and/or sensitive moieties, in concert with N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis protocols. This strategy comprises (i) start of peptide synthesis by anchoring the penultimate residue, with its carboxyl group orthogonally protected, through the backbone nitrogen, (ii) continuation with standard protocols for peptide chain elongation in the $C \rightarrow N$ direction, (iii) selective orthogonal removal of the carboxyl protecting group, (iv) solid-phase activation of the pendant carboxyl and coupling with the desired *C*-terminal residue, and (v) final cleavage/deprotection to release the free peptide product into solution. To illustrate this approach, several model peptide *p*-nitroanilides and thioesters have been prepared in excellent yields and purities, with minimal racemization. Such compounds are very difficult to prepare by standard Fmoc chemistry, including the BAL strategy as originally envisaged.

Introduction

Synthetic peptides are often desired with *C*-terminal end groups other than the carboxylic acid and carboxamide functionalities that are the most common. Recently, we described the *B*ackbone *A*mide *L*inker (BAL) strategy for N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis (SPS) of *C*-terminal-modified and cyclic peptides, whereby the growing peptide is anchored through a backbone nitrogen instead of through a terminal C^{α}-carboxyl group.[3] The BAL strategy thus allows considerable flexibility in management of the termini. For our initial studies, the C^{α}-carboxyl group of the prospective *C*-terminal amino acid was protected as a *tert*-butyl ester (synthesis directed toward peptide acids) or allyl ester (directed toward cyclic peptides); alternatively, an appropriately *C*-terminal modified amino acid derivative was used, e.g., aliphatic ester (directed toward peptide esters), *tert*-butyl ether (directed toward peptide alcohols), or dimethyl acetal (directed toward peptide aldehydes). In all of the cited cases, the *C*-terminal protecting and/or modifying groups used were entirely stable under the various conditions of standard Fmoc protocols.

A goal of the present work was to extend the BAL methodology to accommodate *C*-terminal modifications that are labile to bases, e.g., piperidine (used at each deprotection step in Fmoc chemistry), or to circumvent other synthetic challenges. To do this, BAL anchoring was applied to the *penultimate* residue of the target peptide. An orthogonally removable allyl ester made it possible to selectively liberate, and then activate, a carboxyl group for coupling with the *C*-terminal species. During the latter step, a readily epimerizable oxazolo-

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nium ion[4] may form by attack of the oxygen from the BAL−amide function onto the activated carboxyl;[5,6] despite this risk, we were able to develop *effective protocols which are found empirically to proceed with minimal racemization*.

To illustrate our general approach, we report here Fmoc SPS of unprotected peptide *p*-nitroanilides[7] and peptide thioesters.[8] In the first case, the *C*-terminal modification is particularly challenging due to the low nucleophilicity of the aromatic amino nitrogen of *p*-nitroaniline.[9−11] To the best of our knowledge, the only prior reports of Fmoc SPS of peptide *p*-nitroanilides[12] depend on side-chain anchoring (which requires an appropriately positioned trifunctional amino acid residue; moreover highly variable yields are observed), and use modified deprotection conditions [morpholine−NMP (1:1) instead of the usual piperidine−DMF (1:4)] due to the apparent partial instability of the *p*-nitroanilide to standard Fmoc removal chemistry.[13] In the case of peptide thioesters, standard Fmoc SPS protocols have been inappropriate because of the lability of the sulfur−carbonyl bond to prolonged exposure to secondary amines.[14] These compounds have been previously accessed in concert with Fmoc chemistry either by modifying Fmoc deprotection conditions [i.e., use of 1-methylpyrrolidine (25%), hexamethyleneimine (2%), and 1-hydroxybenzotriazole (HOBt) (2%, w/v) in NMP−DMSO (1:1)],[15] or by an indirect route whereby an otherwise protected peptide with a free C^{α}-carboxyl group, as made by stepwise Fmoc chemistry on a 2-chlorotrityl (Cl-Trt) resin[16] and then released from the support by dilute acid, was extended by a solution coupling to either an alkanethiol or an amino acid *S*-alkyl thioester, followed by final deprotection.[17]

Results and Discussion

Solid-Phase Assembly of Tris(alkoxy)benzylamide BAL-Anchored *C*-Terminal Allyl Ester Peptide-Resins. The target for the first phase of this work was Boc-Val-Tyr(*t*Bu)-Phe-(BAL-Ile-PEG-PS)Ala-OAllyl, although the method used is likely to be successful for most sequences. As already described,[3] 4-[(4 or 2)-formyl-3,5-dimethoxyphenoxy]butyric acid (*o*,*p*-PALdehyde, 4 equiv)[3,18] was added to an amino-functionalized PEG-PS-

(1) Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983. The following additional abbreviations are used: BAL, *B*ackbone *A*mide *L*inker; Bzl, benzyl; Ddz, 2-(3,5-dimethyloxyphenyl)propyl(2)oxycarbonyl; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; EDC, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride; ESMS, electrospray mass spectrometry; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*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; IRAA, "internal reference" amino acid (ref 19); NMM, *N*-methylmorpholine; *o*,*p*-PALdehyde, mixture of 4-(4-formyl-3,5-dimethoxyphenoxy)butyric acid and 4-(2-formyl-3,5-dimethoxyphenoxy)butyric acid; PEG−PS, poly(ethylene glycol)−polystyrene (graft resin support); Ph, phenyl; pNA, *p*-nitroanilide; Py, pyrrolidino; PyAOP, 7-azabenzotriazol-1-yl-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate; SPS, solid-phase synthesis; TFFH, 1,1,3,3-tetramethyl-2-fluoroformamidinium hexafluorophosphate; TMP, 2,4,6-trimethylpyridine (collidine). Amino acid symbols denote the L-configuration unless stated otherwise. All solvent ratios are volume/volume unless stated otherwise.

(2) (a) University of Minnesota. (b) University of Barcelona.

(3) Jensen, K. J.; Alsina, J.; Songster, M. F.; Vágner, J.; Albericio, F.; Barany, G. *J. Am. Chem. Soc.* **1998**, *120*, 5441−5452 and references therein.

(4) As described to account for racemization in the coupling of *N*-acyl-*N*-methylamino acids; see: (a) McDermott, J. R.; Benoiton, N. L. *Can. J. Chem.* **1973**, *51*, 2562−2570. (b) Davies, J. S.; Mohammed, A. K. *J. Chem. Soc., Perkin Trans. 1* **1981**, 2982−2990.

(5) Compare to discussions of the mechanism of racemization via 5(4*H*)-oxazolone mechanisms upon activation of *N*-acylamino acids. Reviews: (a) Kemp, D. S. In *The Peptides: Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 1, pp 315−383. (b) Barany, G.; Merrifield, R. B. In *The Peptides: Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1980; Vol, 2, pp 1−284, especially pp 122−123. (c) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*; CRC: Boca Raton, FL, 1997; pp 116−119.

(6) It is precisely for this reason that stepwise SPPS is rarely carried out in the $N \rightarrow C$ direction, as has been reviewed in ref 5b. For a few examples of $N \rightarrow C$ SPPS, see: (a) Letsinger, R. L.; Kornet, M. J. *J. Am. Chem. Soc.* **1963**, *85*, 3045−3046. (b) Felix, A. M.; Merrifield, R. B. *J. Am. Chem. Soc.* **1970**, *92*, 1385−1391. (c) Henkel, B.; Zhang, L.; Bayer, E. *Liebigs Ann. Recl.* **1997**, 2161−2168. (d) Léger, R.; Yen, R.; She, M. W.; Lee, V. J.; Hecker, S. J. *Tetrahedron Lett.* **1998**, *39*, 4171−4174.

(7) Amino acid and peptide *p*-nitroanilides are needed as chromogenic substrates to monitor the activity of numerous proteolytic enzymes. See: *Handbook of Synthetic Substrates for the Coagulation and Fibrinolytic System*; Hemker, H. C., Ed.; Martinus Nijhof Publishers: Boston, 1983.

(8) Chemical ligation of unprotected peptides in aqueous solution has emerged as an extremely powerful method of peptide/protein assembly. Thioesters are often the reactive *C*-terminal functionality used in these chemical ligations. See: (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779. (b) Tam, J. P.; Lu, Y.-A.; Liu, C. F.; Shao, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12485−12489. (c) Zhang, L.; Tam, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 2363−2370. Review: (d) Muir, T. W.; Dawson, P. E.; Kent, S. B. H. *Methods Enzymol.* **1997**, *289*, 266−298.

(9) For example, the R(PAL) anchoring strategy for SPPS of *N*-alkylamides was successful with R = Me, Et, and $C_6H_5CH_2CH_2$, but when R = $C_6H_4NO_2$, although the reductive amination step gave the expected *N*-PAL-*p*-nitroaniline intermediate, this compound could not be acylated further. See: Songster, M. F.; Vágner, J.; Barany, G. *Lett. Pept. Sci.* **1996**, *2*, 265−270.

(10) An indirect Boc SPS route to *p*-nitroanilides involves a urethane-linked *p*-aminoanilide resin, and a late-stage solution oxidation step to convert an aromatic amine to a nitro group. See: Burdick, D. J.; Struble, M. E.; Burnier, J. P. *Tetrahedron Lett.* **1993**, *34*, 2589−2592.

(11) Another Boc SPS chemistry route involves oxime ester anchoring, but suffers from low yields during the cleavage step using *p*-nitroaniline as the attacking nucleophile. See: Voyer, N.; Lavoie, A.; Pinette, M.; Bernier, J. *Tetrahedron Lett.* **1994**, *35*, 355−358.

(12) (a) Kaspari, A.; Schierhorn, A.; Schutkowski, M. *Int. J. Pept. Protein Res.* **1996**, *48*, 486-494. (b) Bernhardt, A.; Drewello, M.; Schutkowski, M. *J. Pept. Res.* **1997**, *50*, 143−152.

(13) Such instability is reported in ref 12a, which states that about half of the *p*-nitroanilide moieties are lost after 1 h of treatment of Fmoc-Glu-pNA with piperidine−NMP (1:4). However, this result is in contrast to a claim that Boc-Ala-pNA is completely stable toward piperidine−DMF (1:1) and pyrrolidine−DMF (1:3); see: Rijkers, D. T. S.; Adams, H. P. H. M.; Hemker, H. C.; Tesser, G. I. *Tetrahedron* **1995**, *51*, 11235−11250.

(14) On the other hand, thioester intermediates required for chemical ligation are readily prepared by Boc chemistry, as reviewed in ref 8d. See also: (a) Blake, J.; Li, C. H. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4055−4058. (b) Hojo, H.; Kwon, Y.; Kakuta, Y.; Tsuda, S.; Tanaka, I.; Hikichi, K.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2700−2706. (c) Canne, L. E.; Walker, S. M.; Kent, S. B. H. *Tetrahedron Lett.* **1995**, *36*, 1217−1220.

(15) Li, X.; Kawakami, T.; Aimoto, S. *Tetrahedron Lett.* **1998**, *39*, 8669−8672.

(16) Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 513−520.

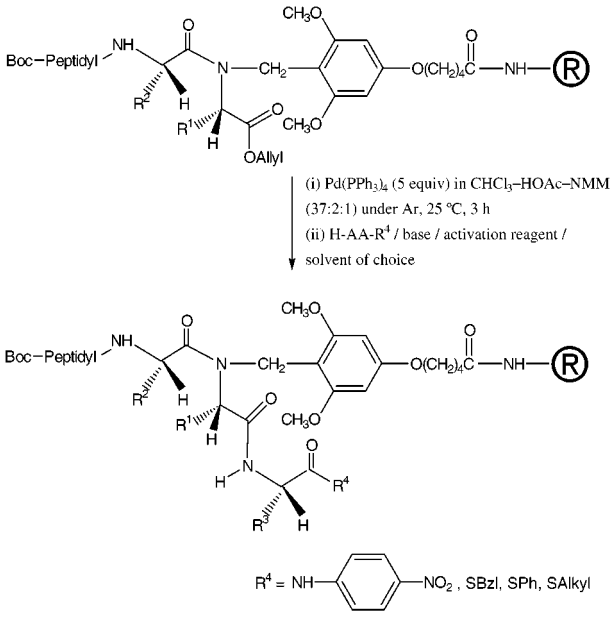
(17) Futaki, S.; Sogawa, K.; Maruyama, J.; Asahara, T.; Niwa, M.; Hojo, H. *Tetrahedron Lett.* **1997**, *38*, 6237−6240.

(18) For all structural formulas in this paper, BAL handles are depicted as the isomer in which the aminomethyl group is *para* to the valeryl linking group. However, many of the experiments described herein start with a mixture (1:2) of the *ortho* and *para* isomers of PALdehyde, i.e., *o*,*p*-PALdehyde; furthermore, the side-chain moiety had one fewer carbon (substitution of butyryl for valeryl). The latter compound is commercially available from PE Biosystems (Framingham, MA).

resin (also containing Ile as IRAA[19]) via an (*N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide[20] (HATU) (4 equiv)/DIEA (8 equiv) mediated coupling for 2 h in DMF. This was followed by on-resin reductive amination with alanine allyl ester hydrochloride salt (10 equiv), mediated by $NaBH_3CN$ (10 equiv) for 15 h in DMF. The next residue was incorporated with N^{α}-2-(3,5-dimethoxyphenyl)propyl[2]oxycarbonyl (Ddz) protection by earlier precedented protocols that optimize acylation of the BAL secondary α-amino group and circumvent diketopiperazine formation upon addition of the third residue.[3,21] Coupling of Ddz-Phe-OH (10 equiv) was mediated by HATU (10 equiv) and DIEA (20 equiv) in CH_2Cl_2−DMF (9:1) for 2 + 2 h (second coupling with fresh reagents), and selective removal of the Ddz group was accomplished without cleavage of the BAL anchor by treatment with TFA−H_2O−CH_2Cl_2 (3:1:96, 6 × 1 min). The third residue, Fmoc-Tyr(*t*Bu)-OH (10 equiv), was introduced by an in situ neutralization/coupling protocol mediated by 7-azabenzotriazol-1-yl-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP)[22] (10 equiv) and DIEA (20 equiv) in DMF for 75 min. Finally, after removal of the N^{α}-Fmoc protecting group, addition of Boc-Val-OH was mediated by DIPCDI/HOBt for 1 h in DMF.[23] To verify success of this synthesis, final acidolytic cleavage of the anchoring linkage, the *t*Bu ether protection of Tyr, and the N^{α}-Boc group occurred upon treatment of an aliquot of completed peptide-resin with TFA−H_2O (19:1) for 90 min; the desired product H-Val-Tyr-Phe-Ala-OAllyl was observed as a single homogeneous component upon analytical HPLC (68% cleavage yield, 92% purity).

Low-Racemization Introduction of *C*-Terminal Amino Acid *p*-Nitroanilide Residues. The just-described allyl ester-protected peptide-resin, Boc-Val-Tyr-(*t*Bu)-Phe-(BAL-Ile-PEG-PS)Ala-OAllyl, was converted into a number of peptide *p*-nitroanilides, with differing *C*-terminal residues, by a novel, generalizable strategy (Scheme 1 and Table 1). First, clean and selective removal of the *C*-terminal allyl ester was achieved by treatment with $Pd(PPh_3)_4$ (5 equiv) in $CHCl_3$−HOAc−NMM (37:2:1)[24] under argon at 25 °C for 3 h. Next, the liberated pendant carboxyl was activated in a variety of ways and reacted with the appropriate amino acid *p*-nitroanilide, as either the free amine or the hydrochloride salt. In situ activating agents tested include HATU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide)] (HBTU),[25] PyAOP, benzotriazol-1-yl-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP),[26] and 1,1,3,3-tetramethyl-2-fluoroformamidinium hexafluorophosphate (TFFH).[27] The challenges were to achieve quantitative conversion without affecting the chiral integrity of the *C*-terminal residue (Ala in this case) that becomes activated.

Scheme 1. Preparation of Unprotected Peptide *p*-Nitroanilides and Peptide Thioesters Using Backbone Amide (BAL) Approach for N^{α}-Fluorenylmethoxycarbonyl (Fmoc) SPPS



Initial studies showed that protocols involving preactivation led to unacceptably high levels of undesired epimers (see Table 1, entries 1.1, 2.1, 3.1, 4.1, and 4.2).[28] Therefore, subsequent experiments were conducted by using solutions of each amino acid *p*-nitroanilide (H-AA-pNA) (10 equiv, AA was Ala, Phe, or Val) in the appropriate solvent (DMF or CH_2Cl_2), prepared in the presence of base [DIEA or 2,4,6-trimethylpyridine (collidine) (TMP)] (2, 10, or 20 equiv), then adding these solutions to the peptide-resin (1 equiv), and, finally, initiating coupling by addition of the activating reagent (10 equiv) in solid form. Reactions were studied as a function of time (30 vs 60 min) and temperature (4 vs 25 °C), and monitored after acidolytic cleavage [70−85% yield after treatment with TFA−H_2O (19:1) for 2 h at 25 °C] by HPLC−ESMS analysis of the crude products. In most cases, the desired all-L-peptide *p*-nitroanilides were the major products (>90%), with negligible starting material remaining. The racemic byproduct H-Val-Tyr-Phe-D-Ala-AA-pNA was also identified (Figure 1) and quantified (Table 1). To ensure that all peptide components were accounted for, FABMS analysis was carried out on the unfractionated crude material.

These studies led to the following findings and conclusions: (a) Use for coupling of a less polar solvent such as CH_2Cl_2 resulted in lower racemization levels, by comparison to use of the more polar DMF (e.g., Table 1, entries 1.4 vs 1.3, 1.6 vs 1.5, and 2.4 vs 2.2). (b) Aminium/

(19) (a) Atherton, E.; Clive, D. L.; Sheppard, R. C. *J. Am. Chem. Soc.* **1975**, *97*, 6584−6585. (b) Matsueda, G. R.; Haber, E. *Anal. Biochem.* **1980**, *104*, 215−227. (c) Albericio, F.; Barany, G. *Int. J. Pept. Protein Res.* **1993**, *41*, 307−312.

(20) Carpino, L. A.; El-Faham, A.; Minor, C. A.; Albericio, F. *J. Chem. Soc., Chem. Commun.* **1994**, 201−203.

(21) Built into the BAL approach (see structure in Scheme 1), the anchored first amino acid allyl ester is *N*-alkylated, and therefore more likely to form a diketopiperazine at the dipeptidyl ester stage.

(22) Albericio, F.; Cases, M.; Alsina, J.; Triolo, S. A.; Carpino, L. A.; Kates, S. A. *Tetrahedron Lett.* **1997**, *38*, 4853−4856.

(23) For longer peptides, the strategy from this point on would be to use standard cycles of Fmoc chemistry to achieve chain growth in the $C \rightarrow N$ direction. The second phase of this study uses a longer model peptide-resin.

(24) Kates, S. A.; Daniels, S. B.; Albericio, F. *Anal. Biochem.* **1993**, *212*, 303−310.

(25) (a) Dourtoglou, V.; Ziegler, J.-C.; Gross, B. *Tetrahedron Lett.* **1978**, *19*, 1269−1272. (b) Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30*, 1927−1930.

(26) Coste, J.; Le-Nguyen, D.; Castro, B. *Tetrahedron Lett.* **1990**, *31*, 205−208.

(27) Carpino, L. A.; El-Faham, A. *J. Am. Chem. Soc.* **1995**, *117*, 5401−5402.

(28) From these experiments, both diastereomers (L-Ala and D-Ala at penultimate position) were formed, isolated, and characterized.

Table 1. Preparation of Peptide *p*-Nitroanilides[a]

entry	coupling method	preact time (min)	coupling time (min)	starting material (%)	LLLDL-isomer[b] (%)	final product purity (%)
			HCl·H-Ala-pNA (10)			
1.1	HATU/DIEA (10:20) in DMF	10	60	1.6	52.1	43
1.2	TFFH/DIEA (10:20) in DMF	0	60	41.4	2.0	51
1.3	HATU/DIEA (10:20) in DMF	0	60	0.2	2.1	91
1.4	**HATU/DIEA (10:20) in CH_2Cl_2**	**0**	**30**	**0.3**	**1.3**	**93**
1.5	PyAOP/DIEA (10:20) in DMF	0	30	nd	2.3	92
1.6	**PyAOP/DIEA (10:20) in CH_2Cl_2**	**0**	**30**	**0.2**	**1.5[c]**	**86**
			HCl·H-Val-pNA (10)			
2.1	HATU/DIEA (10:20) in DMF	10	60	2.4	52.5	42
2.2	PyAOP/DIEA (10:20) in DMF	0	30	0.3	2.7	90
2.3	PyAOP/HOAt/DIEA (10:10:20) in DMF	0	30	0.2	7.0	88
2.4	PyAOP/DIEA (10:20) in CH_2Cl_2	0	30	0.4	2.0[c]	89
2.5	PyBOP/DIEA (10:20) in CH_2Cl_2	0	30	0.3	2.3	94
2.6	**HATU/DIEA (10:20) in CH_2Cl_2**	**0**	**30**	**0.2**	**1.9**	**94**
2.7	HATU/TMP (10:20) in CH_2Cl_2	0	30	nd	2.1	93
2.8	**HBTU/DIEA (10:20) in CH_2Cl_2**	**0**	**30**	**0.2**	**1.8**	**94**
			H-Phe-pNA (10)			
3.1	HATU/DIEA (10:20) in DMF	10	60	2.2	52.3	42
3.2	PyAOP/DIEA (10:20) in DMF	0	30	0.2	3.5[c]	88
3.3	HATU/DIEA (10:10) in CH_2Cl_2	0	30	0.2	2.8	93
3.4	HBTU/DIEA (10:10) in CH_2Cl_2	0	30	0.2	3.1	93
3.5	**HATU/DIEA (10:2) in CH_2Cl_2**	**0**	**30**	**0.2**	**2.6**	**94**
3.6	**HATU/DIEA (10:2) in CH_2Cl_2 at 4 °C**	**0**	**60**	**0.2**	**1.6**	**95**
			2HCl·H-Arg-pNA (10)			
4.1	HBTU/DIEA (10:30) in DMF	10	60	1.0	15.6	79
4.2	HATU/DIEA (10:30) in DMF	10	60	2.1	53.7	41
4.3	PyAOP/DIEA (10:30) in DMF	0	30	0.5	10.0[c]	79
4.4	PyBOP/DIEA (10:30) in DMF	0	30	1.3	8.7	86
4.5	HATU/DIEA (10:30) in DMSO	0	30	nd	11.4	84
4.6	HATU/DIEA (10:30) in NMP	0	30	0.3	9.3	86
4.7	HATU/DIEA (10:30) in DMF	0	30	0.2	6.4	88
4.8	**HBTU/DIEA (10:30) in DMF**	**0**	**30**	**1.2**	**5.2**	**89**
4.9	**HBTU/DIEA (10:12) in DMF**	**0**	**30**	**1.2**	**5.1**	**89**
4.10	**HBTU/DIEA (10:30) in DMF at 4 °C**	**0**	**90**	**2.1**	**2.4**	**91**

[a] Details regarding the design and execution of these studies are provided in the Experimental Section. nd means "not detected". The number of equivalents used, with respect to pendant carboxyl on the BAL support, are indicated in parentheses. Conditions that are considered "optimal" are highlighted in bold. [b] LLLDL-peptide × 100/(LLLDL-peptide + LLLLL-peptide). [c] The pyrrolidide derivative H-Val-Tyr-Phe-Ala-Py was found (~1−7%); see ref 29 for further information.

uronium salts [HATU and HBTU] were slightly superior in terms of reducing racemization than phosphonium salts [PyAOP and PyBOP] (e.g., compare Table 1, entries 1.3 vs 1.5 and 2.8 vs 2.5). Both aminium/uronium salts gave similar results (Table 1, entries 2.6 vs 2.8 and 3.3 vs 3.4). (c) The presence of excess HOAt during PyAOP-mediated coupling reactions was not beneficial, insofar as racemization was enhanced (Table 1, entry 2.2 vs 2.3). (d) In some cases, when PyAOP was the coupling reagent, the pyrrolidide derivative H-Val-Tyr-Phe-Ala-Py was noted as an unexpected byproduct (~1−7%; e.g., Table 1, entries 1.6, 2.4, and 3.2).[29] (e) Use of TFFH is not advisable despite the acceptably low level of racemization, because excess TFFH presumably reacts with the amino group of the amino acid *p*-nitroanilide to give the corresponding guanidino derivative (Table 1, entry 1.2).[30] (f) Use of collidine (weaker base) instead of DIEA did not improve the results (Table 1, entry 2.7 vs 2.6). (g) Slightly less racemization was obtained by reducing the excess of base from 10-fold to 2-fold, the minimal acceptable amount (Table 1, entry 3.5 vs 3.3). (h) In more demanding cases, the level of racemization was reduced considerably by maintaining the temperature at 4 °C (Table 1, entry 3.6 vs 3.5, and Figure 1). (i) For each amino acid example studied, we were able to establish conditions where the level of racemization was <2% D, and the amount of starting material was <0.3%.

Special Considerations with Arginine. Peptide *p*-nitroanilides with an arginine residue at the *C*-terminus are important synthetic targets, since they are chromogenic substrates for serine proteases with a primary specificity pocket for a guanidinium moiety.[7] Nevertheless, these compounds cannot be prepared directly by some of the previously described methods, e.g., side-chain anchoring.[12] Furthermore, coupling conditions that can be tried are limited by the low solubility of the dihydrochloride salt arginine *p*-nitroanilide (2HCl·H-Arg-pNA) in solvent mixtures that are less polar than DMF [e.g., neat CH_2Cl_2, $CHCl_3$−TFE (7:3), or DMF−CH_2Cl_2 (1:1)], with or without the presence of base.

Our methodology extends successfully to *C*-terminal arginine *p*-nitroanilides. The principal conclusions are (a) again, aminium/uronium salts [HATU and HBTU] were superior to phosphonium salts [PyAOP and PyBOP] (e.g.,

(29) The structure of the byproduct was proven by HPLC (t_R 16.6 min; condition A) and ESMS (*m*/*z* calcd for $C_{30}H_{41}N_5O_5$ 551.7, found 552.0 $[M + H]^+$, 574.0 $[M + Na]^+$). The formation of this carboxyl-capped derivative is attributed to the presence of small amounts (e.g., 0.4%, w/w) of pyrrolidine as a contaminant to commercial phosphonium salts. For more on this side reaction, which is avoided entirely by using purified coupling reagent, see: Alsina, J.; Barany, G.; Albericio, F.; Kates, S. A. *Lett. Pept. Sci.* **1999**, *6*, 243−245.

(30) Formation of the guanidino side product consumes the nucleophilic moiety, and as a result, it is more difficult to carry out the desired amide formation to completion. Therefore, in the solid-phase mode, the side reaction leads to lower yields (unreacted starting material observed), although the purity of the product on the support is not affected. Interestingly, the side reaction was *not* observed when aminium/uronium salts based on HOXt were used.

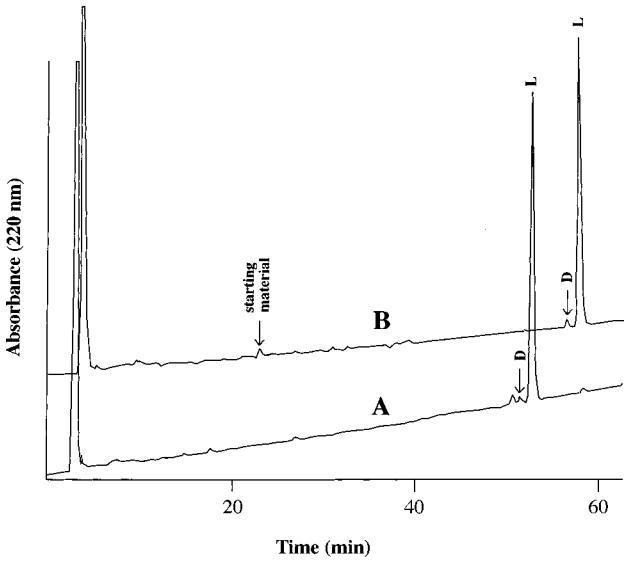


Figure 1. Analytical HPLC of crude (A) H-Val-Tyr-Phe-Ala-Phe-pNA (Table 1, entry 3.6) and (B) H-Val-Tyr-Phe-Ala-Arg-pNA (Table 1, entry 4.10), as obtained following acidolytic cleavage with TFA−H_2O (19:1). Details regarding the execution of these syntheses are provided in the Experimental Section. Chromatography followed conditions C and D, respectively, as listed in the Experimental Section, General Procedures. Identification of the D/L-containing diastereomers (same molecular mass) was based on standards that have been evaluated by HPLC−ESMS (see ref 34).

Table 1, compare entries 4.7 vs 4.3 and 4.8 vs 4.4), (b) although reaction with HBTU was slower, this reagent provides somewhat superior results by comparison to HATU in terms of reducing racemization (Table 1, entry 4.8 vs 4.7), (c) DMF was clearly the solvent of choice, as opposed to other polar solvents such as NMP or DMSO (Table 1, entry 4.7 vs 4.6 vs 4.5), (d) the excess of base used was not an important factor (Table 1, entry 4.9 vs 4.8), and (e) again, when the temperature was kept at 4 °C, the level of racemization was reduced dramatically to acceptable levels (Table 1, entry 4.10 vs 4.8, and Figure 1).

Low-Racemization Introduction of *C*-Terminal Amino Acid Thioester Residues. Building on our experiences from the *p*-nitroanilide system, the allyl ester-protected peptide-resin Boc-Val-Tyr(*t*Bu)-Gly-Gly-Phe-(BAL-Ile-PEG-PS)Ala-OAllyl[31] was converted by optimized routes to peptide thioesters. This involved (i) removal of the allyl protecting group, (ii) addition to the peptide-resin (1 equiv) of a solution containing amino acid thioester H-AA-SR·HCl[32] [10 equiv; AA = Ala; R = Ph, Bzl, or $-(CH_2)_2CO_2Et$] and DIEA (20 equiv) in CH_2Cl_2, and (iii) initiation of couplings by addition of HATU (10 equiv) in solid form. The reactions were allowed to proceed for 30 min at 25 °C, and monitored by HPLC after acidolytic cleavage with TFA−H_2O (19:1) (65−72% cleavage yields). In all experiments, the desired L-peptide thioesters (i.e., the product where the Ala residue did not epimerize upon activation of its carboxyl group) were obtained as major products, as indicated by HPLC (85−93% final purities) and ESMS (Table 2 and Figure 2). The yields of the reactions were almost quantitative, after single couplings.[33] Minor byproducts detected and characterizedby HPLC−ESMS were (a) H-Val-Tyr-Gly-Gly-Phe-D-Ala-Ala-SR (∼2%, Table 2), the product where the Ala residue did epimerize upon activation of its carboxyl group,[34] (b) H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-OH (∼2−7%), peptide acid possibly generated by hydrolysis of the corresponding peptide thioesters during acidolytic cleavage with TFA−H_2O (19:1), and (c) H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-Ala-SPh (∼1%), L-peptide thioesters containing an additional residue [these were only detected in the experiments directed at *S*-phenyl thioesters]. The unfractionated crude samples were also analyzed by FABMS, and only [M + H^+] and expected fragmentations were observed, ensuring that all peptide components were accounted for.

In view of the successful strategy described above, it is natural to wonder whether the route could be streamlined further by *direct* thioesterification after the allyl removal step (i). This would follow the originally envisaged BAL strategy.[3] However, after extensive evaluation of coupling conditions, we were never able to achieve better than 65% reaction yield, and the level of racemization was routinely 12% D or worse.[35]

With solid-phase peptide assembly and on-resin establishment of the *C*-terminal thioester moiety successfully accomplished, there remains the final cleavage step to detach the peptide thioester into solution and concurrently remove side-chain protecting groups. For the model peptide thioester explored in this work, TFA−H_2O (19:1) was an adequate cleavage reagent, but for complete generality in the synthesis of complex multifunctional peptide thioesters, more sophisticated acid cleavage cocktails must be used. Two widely favored cocktails in Fmoc peptide synthesis are reagent B (TFA−phenol−H_2O−Et_3SiH (88:5:5:2))[36] and reagent R (TFA−thioanisole−1,2-ethanedithiol−anisole (90:5:3:2)).[37] An obvious concern was that the nucleophilic scavengers might affect the thioester, but gratifyingly, we found just the opposite to be true. Thus, the model peptide thioesters (R = Ph, Bzl, alkyl) were stable, with very little additional hydrolysis, upon treatment with either reagent B or reagent R (Table 2 and Figure 2).

Conclusions

We have shown herein how the BAL strategy can be extended for Fmoc SPS of peptide *p*-nitroanilides and peptide *S*-benzyl, *S*-phenyl, and *S*-alkyl thioesters. The principal new step was a solid-phase coupling connecting a free α-carboxyl group at the *C*-terminus of a protected

(31) Preparation of this model peptide-resin was along the same lines already described (see the text and ref 23), as detailed in the Experimental Section.

(32) The required amino acid thioester building blocks were synthesized by EDC (1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride)/HOBt-mediated coupling of RSH to the Boc-AA-OH, followed by Boc removal with 4 N HCl−dioxane. The products were obtained in 63−71% yields, and suitable for direct use, as detailed in Experimental Section.

(33) When TFFH was used to mediate couplings, the desired peptide thioesters were formed with little racemization or other byproducts, but due to the guanidino side reaction already discussed (ref 30), reactions seldom went to completion and unreacted starting material was observed. See Table 3 in the Supporting Information.

(34) As was precedented in the *p*-nitroanilide system, substantial formation of the diastereomer occurred when a preactivation protocol was used. Therefore, when such a protocol was carried out intentionally, sufficient levels of the racemized species formed to allow their characterization.

(35) These experiments are described in the Supporting Information.

(36) Solé, N. A.; Barany, G. *J. Org. Chem.* **1992**, *57*, 5399−5403.

(37) Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R. I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, *55*, 3730−3743.

Table 2. Preparation of Peptide Thioesters[a]

entry	amino acid thioester	cleavage reagent	final products (%) detected by HPLC: starting material	L-peptide thioester	D-peptide thioester	hydrolysis product	L-peptide thioester with additional AA
1	HCl·H-Ala-SPh	TFA−H_2O	0.3	93.6	2.2	3.0	0.9
		rgt B	0.5	96.3	2.0	0.3	0.9
		rgt R	0.2	96.6	2.0	0.3	0.9
2	HCl·H-Ala-SBzl	TFA−H_2O	0.4	90.8	1.9	6.9	nd
		rgt B	0.4	97.0	2.0	0.6	nd
		rgt R	0.2	97.3	2.0	0.5	nd
3	HCl·H-Ala-$S(CH_2)_2$-COOEt	TFA−H_2O	0.6	97.7[b]	[b]	1.7	nd
		rgt B	0.6	98.1[b]	[b]	1.3	nd
		rgt R	0.2	98.8[b]	[b]	1.0	nd

[a] Details regarding the design and execution of these studies are provided in the Experimental Section. nd means "not detected". The coupling reagents were HATU/DIEA, and the solvent was CH_2Cl_2. See Table 3 in the Supporting Information for similar studies using TFFH/DIEA in DMF for coupling. [b] The reported values correspond to the mixture of D- and L-peptide thioesters, because these diastereomers were not separable by HPLC.

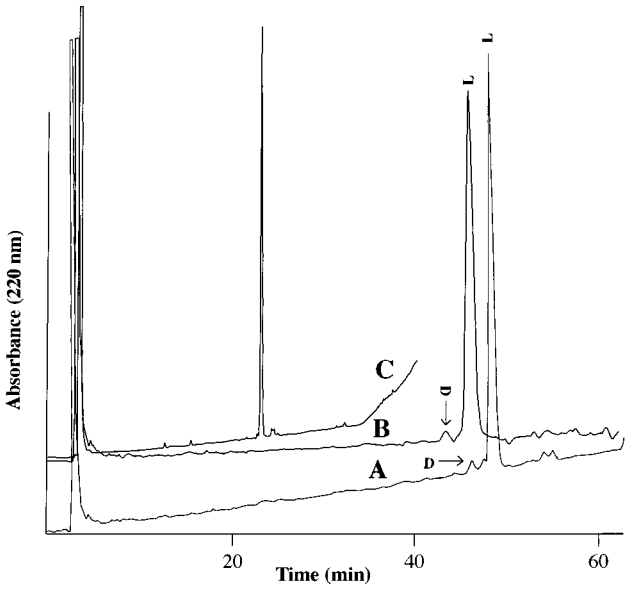


Figure 2. Analytical HPLC of crude (A) H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-SBzl (Table 2, entry 2), (B) H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-SPh (Table 2, entry 1), and (C) H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-S-$(CH_2)_2$-COOEt (Table 2, entry 3), as obtained following acidolytic cleavage with reagent R. Details regarding the execution of these syntheses are provided in the Experimental Section. Chromatography followed conditions G, F, and A, respectively, as listed in the Experimental Section, General Procedures. Identification of the D/L-containing diastereomers (same molecular mass) was based on standards that have been evaluated by HPLC−ESMS (see ref 34).

BAL-anchored peptide-resin with an α-amino group of an amino acid *p*-nitroanilide or an amino acid thioester. Yields of these coupling reactions were essentially quantitative, and the levels of side reactions (e.g., racemization, hydrolysis, and polymerization) were acceptably low. In addition, the peptide thioesters were found to be stable to cleavage cocktails that are commonly used in Fmoc chemistry, thus opening the door to the general application of the methodology for preparation of complicated unprotected peptide thioesters, key intermediates for chemical ligation methods of protein synthesis. We believe that the modified BAL strategy presented here may be useful for other cases where the presence of hindered, unreactive, or sensitive *C*-terminal residues or conjugates compromises the originally envisaged strategy.

Experimental Section

General Procedures. Materials, solvents, instrumentation, and general methods were essentially as described in previous publications from our laboratory.[3] Organic and peptide synthesis transformations and washes were at 25 °C unless indicated otherwise. PEG-PS resin (0.17 mmol/g) and 4-[(4 or 2)-formyl-3,5-dimethoxyphenoxy]butyric acid (*ortho* and *para* mixture; *o,p*-PALdehyde) were obtained from PE Biosystems (Framingham, MA), Ddz-Phe-OH was obtained from Advanced ChemTech (Louisville, KY), and HCl·H-Ala-pNA, HCl·H-Val-pNA, H-Phe-pNA, and 2HCl·H-Arg-pNA were from Bachem Bioscience (Philadelphia, PA). To accurately determine anchoring, coupling, and cleavage yields in this work, resins were extended further with an "internal reference" amino acid (IRAA; Ile was used), introduced as its Fmoc derivative by standard coupling methods, at a point before introduction of the handle. Peptide-resin samples were hydrolyzed in 12 N aqueous HCl−propionic acid (1:1) with a drop of phenol, at 155 °C for 3 h, peptide samples were hydrolyzed in 6 N aqueous HCl with a drop of phenol, at 155 °C for 1 h, and amino acid analyses were carried out on a Beckman 6300 analyzer. Organic solvent extracts were dried over anhydrous $MgSO_4$, followed by solvent removal at reduced pressures and <40 °C. ^1H NMR spectra were obtained at ambient temperature on a Varian VXR-300 spectrometer. Electrospray mass spectrometry (ESMS) was performed on a Perkin-Elmer Sciex API III triple quadrupole mass spectrometer equipped with an ionspray interface. Low-resolution fast atom bombardment mass spectroscopy (FABMS) was carried out in glycerol−H_2O matrix on a VG 11/250 data system, operated at a resolution of 2000. Molecular masses were calculated with the Sciex MacSpec 3.22 program. Analytical HPLC was performed using a Vydac C_{18} reversed-phase column (0.46 × 25 cm) on a Beckman instrument, configured with two 112 pumps and a 165 variable-wavelength detector. UV detection was at 220 nm, and linear gradients of 0.1% TFA in CH_3CN and 0.1% aqueous TFA were run at a 1.0 mL/min flow rate from (condition A) 1:9 to 2:3 over 30 min and then to 1:0 over the next 10 min, (condition B) 1:9 to 9:16 over 60 min and then to 1:0 over the next 10 min, (condition C) 2:8 to 19:31 over 60 min and then to 1:0 over the next 10 min, (condition D) 1:9 to 1:3 over 60 min and then to 1:0 over the next 10 min, (condition E) 1:9 to 2:3 over 50 min, (condition F) 1:4 to 6:19 over 60 min and then to 1:0 over the next 10 min, and (condition G) 1:9 to 7:13 over 60 min and then to 1:0 over the next 10 min.

Boc-Val-Tyr(*t*Bu)-Phe-(BAL-Ile-PEG-PS)Ala-OAllyl. *o,p*-PALdehyde-Ile-PEG-PS resin (1.0 g, 0.17 mmol/g) prepared as described earlier[3] was washed with CH_2Cl_2 (5 × 0.5 min) and DMF (3 × 0.5 min). Next, H-Ala-OAllyl·HCl (281 mg, 10 equiv) and $NaBH_3CN$ (107 mg, 10 equiv) were dissolved separately in DMF (1.9 mL total), combined, and added to the resin; reaction at 25 °C for 15 h gave H-(BAL-Ile-PEG-PS)-Ala-OAllyl resin, which was washed consecutively with DMF (8 × 0.5 min), CH_2Cl_2 (5 × 0.5 min), DMF (3 × 0.5 min), piperidine−DMF (1:4, 3 × 1 min), DMF (8 × 0.5 min), and

CH_2Cl_2 (5 × 0.5 min). Subsequently, Ddz-Phe-OH (658 mg, 10 equiv) was dissolved in CH_2Cl_2−DMF (9:1, 2.2 mL), DIEA (0.58 mL, 20 equiv) was added, the solution was added to the resin, and coupling initiated by addition of solid HATU (646 mg, 10 equiv) was carried out for 2 h. The peptide-resin was then washed with CH_2Cl_2 (5 × 0.5 min), DMF (5 × 0.5 min), and CH_2Cl_2 (3 × 0.5 min), and the coupling procedure (2 h) was repeated. The Ddz group was removed by treatment with TFA−H_2O−CH_2Cl_2 (3:1:96, 6 × 1 min), followed by washing with CH_2Cl_2 (5 × 0.5 min). Next, Fmoc-Tyr(*t*Bu)-OH (782 mg, 10 equiv) and PyAOP (887 mg, 10 equiv) were dissolved separately in DMF (1.9 mL total), combined, added to the resin, and in situ neutralization/coupling initiated by the addition of DIEA (0.58 mL, 20 equiv) was carried out for 75 min [Kaiser ninhydrin test negative after this time].[38] The remaining residue, Boc-Val-OH (185 mg, 5 equiv), was introduced by standard Fmoc chemistry; DIPCDI (132 μL, 5 equiv)/HOBt (115 mg, 5 equiv) mediated coupling in DMF (1.9 mL) was carried out for 1 h to provide the completed peptide-resin. The amino acid composition of the hydrolyzed peptide-resin was Val, 1.00; Tyr, 1.01; Phe, 1.20; Ala, 1.02; and Ile, 1.00. A portion of the completed peptide-resin (20 mg) was cleaved with TFA−H_2O (19:1, 1 mL) at 25 °C for 90 min. The filtrate from the cleavage reaction was collected, combined with TFA washes (1 mL) of the cleaved peptide-resin, dried, and redissolved in HOAc−H_2O (1:4) to give the peptide H-Val-Tyr-Phe-Ala-OAllyl (68% cleavage yield), which was characterized by analytical HPLC (t_R 23.6 min, >92% purity; condition A) and ESMS (*m/z* calcd for $C_{29}H_{38}N_4O_6$ 538.6, found 539.0 $[M + H]^+$).

H-Val-Tyr-Phe-Ala-OH. Different portions (50 mg, 0.14 mmol/g) of the Boc-Val-Tyr(*t*Bu)-Phe-(BAL-Ile-PEG-PS)Ala-OAllyl resin were washed with CH_2Cl_2 (5 × 0.5 min) and DMF (5 × 0.5 min), and the *C*-terminal allyl esters were cleaved by treatment with $Pd(PPh_3)_4$ (40 mg, 5 equiv) in $CHCl_3$−HOAc−NMM (37:2:1, 1.0 mL) under argon at 25 °C for 3 h. The peptide-resins were then washed with THF (3 × 2 min), DMF (3 × 2 min), CH_2Cl_2 (3 × 2 min), DIEA−CH_2Cl_2 (1:19, 3 × 2 min), CH_2Cl_2 (3 × 2 min), sodium *N*,*N*-diethyldithiocarbamate (0.03 M in DMF, 3 × 15 min), DMF (5 × 2 min), CH_2Cl_2 (3 × 2 min), and DMF (3 × 1 min). An aliquot of the Boc-Val-Tyr-(*t*Bu)-Phe-(BAL-Ile-PEG-PS)Ala-OH resin (2 mg) was cleaved with TFA−H_2O (19:1, 1 mL) at 25 °C for 2 h. The filtrate from the cleavage reaction was collected and dried to give the peptide H-Val-Tyr-Phe-Ala-OH, which was characterized by analytical HPLC (t_R 13.2 min, 94% purity; condition A) and ESMS (*m/z* calcd for $C_{26}H_{34}N_4O_6$ 498.6, found 499.0 $[M + H]^+$).

H-Val-Tyr-Phe-Ala-Ala-pNA (Table 1, Entry 1.4). A solution of H-Ala-pNA·HCl (17 mg, 10 equiv) in CH_2Cl_2 (0.28 mL) and DIEA (24 μL, 20 equiv) was added to Boc-Val-Tyr-(*t*Bu)-Phe-(BAL-Ile-PEG-PS)Ala-OH resin (50 mg, 0.14 mmol/g) prepared freshly as described in the preceding experimental entry; next, the coupling was initiated by addition of HATU (27 mg, 10 equiv) in solid form. After 30 min at 25 °C, the peptide-resin was washed with DMF (6 × 0.5 min) and CH_2Cl_2 (4 × 0.5 min). Amino acid analysis on a hydrolyzed aliquot of peptide-resin showed Val, 0.98; Tyr, 0.99; Phe, 1.17; Ala, 1.93; and Ile, 1.00. The peptide-resin was treated with TFA−H_2O (19:1, 1 mL) at 25 °C for 2 h, followed by the same workup as for the H-Val-Tyr-Phe-Ala-OAllyl synthesis, to give the desired peptidyl-*p*-nitroanilide (71% cleavage yield), which was characterized by analytical HPLC (t_R 26.4 min, 93% purity; condition A) and ESMS (*m/z* calcd for $C_{35}H_{43}N_7O_8$ 689.8, found 690.4 $[M + H]^+$). The main byproduct, detected and characterized by analytical HPLC using condition A, was H-Val-Tyr-Phe-D-Ala-Ala-pNA (t_R 25.8 min, 1.2%) (ESMS *m/z* calcd for $C_{35}H_{43}N_7O_8$ 689.8, found 690.4 $[M + H]^+$). The crude (unfractionated) material was checked by amino acid analysis (Val, 0.94; Tyr, 0.87; Phe, 1.03; Ala, 2.16) and FABMS (*m/z* calcd for $C_{35}H_{43}N_7O_8$ 689.8, found 690.3 $[M + H]^+$, 674.3 $[M + H - O]^+$, 660.3 $[M + H - NO]^+$).

H-Val-Tyr-Phe-Ala-Val-pNA (Table 1, entry 2.8). A solution of H-Val-pNA·HCl (19 mg, 10 equiv) in CH_2Cl_2 (0.27 mL) and DIEA (24 μL, 20 equiv) was added to Boc-Val-Tyr-(*t*Bu)-Phe-(BAL-Ile-PEG-PS)Ala-OH resin (50 mg, 0.14 mmol/g); next, the coupling was initiated by addition of HBTU (27 mg, 10 equiv) in solid form. After 30 min at 25 °C, the peptide-resin was washed with DMF (6 × 0.5 min) and CH_2Cl_2 (4 × 0.5 min). Amino acid analysis on a hydrolyzed aliquot of peptide-resin showed Val, 1.90; Tyr, 0.99; Phe, 1.19; Ala, 1.03; and Ile, 1.00. The peptide-resin was treated with TFA−H_2O (19:1, 1 mL) at 25 °C for 2 h, followed by the same workup as for the H-Val-Tyr-Phe-Ala-OAllyl synthesis, to give the desired peptidyl-*p*-nitroanilide (75% cleavage yield), which was characterized by analytical HPLC (t_R 56.3 min, 94% purity; condition B) and ESMS (*m/z* calcd for $C_{37}H_{47}N_7O_8$ 717.8, found 718.4 $[M + H]^+$). The main byproduct, detected and characterized by analytical HPLC using condition B, was H-Val-Tyr-Phe-D-Ala-Val-pNA (t_R 55.2 min, 1.7%) (ESMS *m/z* calcd for $C_{37}H_{47}N_7O_8$ 717.8, found 718.4 $[M + H]^+$). The crude (unfractionated) material was checked by amino acid analysis (Val, 1.98; Tyr, 0.90; Phe, 1.04; Ala, 1.08) and FABMS (*m/z* calcd for $C_{37}H_{47}N_7O_8$ 717.8, found 718.2 $[M + H]^+$, 702.2 $[M + H - O]^+$, 688.2 $[M + H - NO]^+$).

H-Val-Tyr-Phe-Ala-Phe-pNA (Table 1, Entry 3.6). A solution of H-Phe-pNA (20 mg, 10 equiv) in CH_2Cl_2 (0.26 mL) and DIEA (2.4 μL, 2 equiv) was added to Boc-Val-Tyr(*t*Bu)-Phe-(BAL-Ile-PEG-PS)Ala-OH resin (50 mg, 0.14 mmol/g); next, the coupling was initiated by addition of HATU (27 mg, 10 equiv) in solid form. After 1 h at 4 °C, the peptide-resin was washed with DMF (6 × 0.5 min) and CH_2Cl_2 (4 × 0.5 min). Amino acid analysis on a hydrolyzed aliquot of peptide-resin showed Val, 1.02; Tyr, 0.99; Phe, 1.97; Ala, 1.05; and Ile, 1.00. The peptide-resin was treated with TFA−H_2O (19:1, 1 mL) at 25 °C for 2 h, followed by the same workup as for the H-Val-Tyr-Phe-Ala-OAllyl synthesis, to give the desired peptidyl-*p*-nitroanilide (83% cleavage yield), which was characterized by analytical HPLC (t_R 52.8 min, 95% purity; condition C) and ESMS (*m/z* calcd for $C_{41}H_{47}N_7O_8$ 765.9, found 766.4 $[M + H]^+$). The main byproduct, detected and characterized by analytical HPLC using condition C, was H-Val-Tyr-Phe-D-Ala-Phe-pNA (t_R 51.6 min, 1.5%) (ESMS *m/z* calcd for $C_{41}H_{47}N_7O_8$ 765.9, found 766.4 $[M + H]^+$). The crude (unfractionated) material was checked by amino acid analysis (Val, 0.96; Tyr, 0.92; Phe, 2.04; Ala, 1.09) and FABMS (*m/z* calcd for $C_{41}H_{47}N_7O_8$ 765.9, found 766.2 $[M + H]^+$, 750.2 $[M + H - O]^+$, 736.3 $[M + H - NO]^+$).

H-Val-Tyr-Phe-Ala-Arg-pNA (Table 1, Entry 4.10). A solution of H-Arg-pNA·2HCl (26 mg, 10 equiv) in DMF (0.22 mL) was added to Boc-Val-Tyr(*t*Bu)-Phe-(BAL-Ile-PEG-PS)-Ala-OH resin (50 mg, 0.14 mmol/g), next, DIEA (36 μL, 30 equiv) was added to the resin, and finally, the coupling was initiated by addition of HBTU (27 mg, 10 equiv) in solid form. After 90 min at 4 °C, the peptide-resin was washed with DMF (6 × 0.5 min) and CH_2Cl_2 (4 × 0.5 min). Amino acid analysis on a hydrolyzed aliquot of peptide-resin showed Arg, 1.05; Val, 1.00; Tyr, 0.88; Phe, 0.88; Ala, 1.07; and Ile, 1.00. The peptide-resin was treated with TFA−H_2O (19:1, 1 mL) at 25 °C for 2 h, followed by the same workup as for the H-Val-Tyr-Phe-Ala-OAllyl synthesis, to give the desired peptidyl-*p*-nitroanilide (78% cleavage yield), which was characterized by analytical HPLC (t_R 58.1 min, 91% purity; condition D) (ESMS *m/z* calcd for $C_{38}H_{50}N_{10}O_8$ 774.9, found 775.4 $[M + H]^+$, 387.7 $[M + 2H/2]^+$). The main byproducts, detected and characterized by analytical HPLC using condition D, were H-Val-Tyr-Phe-D-Ala-Arg-pNA (t_R 56.8 min, 2.3%) (ESMS *m/z* calcd for $C_{38}H_{50}N_{10}O_8$ 774.9, found 775.4 $[M + H]^+$, 387.7 $[M + 2H/2]^+$) and H-Val-Tyr-Phe-Ala-OH (t_R 22.8 min, 2.1%) (ESMS *m/z* calcd for $C_{26}H_{34}N_4O_6$ 498.6, found 499.0 $[M + H]^+$). The crude (unfractionated) material was checked by amino acid analysis (Val, 0.96; Tyr, 0.92; Phe, 1.07; Ala, 1.10; Arg, 0.95) and FABMS (*m/z* calcd for $C_{38}H_{50}N_{10}O_8$ 774.9, found 775.3 $[M + H]^+$, 759.3 $[M + H - O]^+$).

Boc-Val-Tyr(*t*Bu)-Gly-Gly-Phe-(BAL-Ile-PEG-PS)Ala-OAllyl[31] was prepared starting from *o*,*p*-PALdehyde-Ile-PEG-PS resin (1.0 g, 0.17 mmol/g) as described above for Boc-Val-Tyr(*t*Bu)-Phe-(BAL-Ile-PEG-PS)Ala-OAllyl. The amino acid composition of the hydrolyzed peptide-resin was Val, 0.89; Tyr,

(38) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. *Anal. Biochem.* **1970**, *34*, 595−598.

0.85; Gly, 1.76; Phe, 1.14; Ala, 1.02; and Ile, 1.00. A portion of the completed peptide-resin (20 mg) was cleaved with TFA−H_2O (19:1, 1 mL) at 25 °C for 90 min. The filtrate from the cleavage reaction was collected, combined with TFA washes (1 mL) of the cleaved peptide-resin, dried, and redissolved in HOAc−H_2O−CH_3CN (1:1:1) to give the peptide H-Val-Tyr-Gly-Gly-Phe-Ala-OAllyl (67% cleavage yield), which was characterized by analytical HPLC (t_R 21.2 min, >92% purity; condition A) (ESMS *m/z* calcd for $C_{33}H_{44}N_6O_8$ 652.8, found 653.0 $[M + H]^+$).

H-Val-Tyr-Gly-Gly-Phe-Ala-OH was prepared as described earlier for H-Val-Tyr-Phe-Ala-OH, and characterized by analytical HPLC (t_R 12.5 min, >93% purity; condition A) (ESMS *m/z* calcd for $C_{30}H_{40}N_6O_8$ 612.7, found 613.0 $[M + H]^+$).

Alanine *S*-Phenyl Ester, Hydrochloride Salt (H-Ala-SPh·HCl). Boc-Ala-OH (1.51 g, 8.0 mmol) was dissolved in CH_2Cl_2 (50 mL) and cooled to 0 °C. HOBt·H_2O (1.37 g, 8.0 mmol), EDC (1.53 g, 8.0 mmol), and DIEA (2.80 mL, 16.0 mmol) were added sequentially to the cooled solution, and the resulting mixture was allowed to stir for 10 min. At this point, thiophenol (0.82 mL, 8.0 mmol) was added in one portion, and the homogeneous reaction was stirred for 2 h at 0 °C and overnight at 25 °C. The organic reaction mixture was washed with 1 N aqueous HCl (2 × 150 mL), 10% aqueous Na_2CO_3 (2 × 150 mL), and H_2O (1 × 150 mL), dried ($MgSO_4$), and concentrated in vacuo. The resultant yellow oil was taken up in a minimal amount of CH_2Cl_2 and precipitated with pentane to give a white solid, which was collected and washed thoroughly with cold pentane. This Boc-Ala-SPh intermediate was dissolved in 4 N HCl−dioxane (20 mL), and the deprotection reaction mixture was stirred at 25 °C for 90 min, at which point TLC [$CHCl_3$−MeOH−HOAc (75:25:3)] indicated complete Boc removal. The homogeneous reaction was concentrated in vacuo and chased with Et_2O (6 × 50 mL, followed by reconcentration) to provide the title product as a white solid (1.15 g, 66%): ^{1}H NMR (DMSO-d_6) δ 8.86 (br s, 3H), 7.46 (m, 5H), 4.38 (q, J = 7.2 Hz, 1H), 1.54 (d, J = 7.2 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 195.9, 135.1, 130.6, 130.1, 125.8, 54.8, 17.3; ESMS *m/z* calcd for $C_9H_{11}NOS$ 181.2, found 181.8 $[M + H]^+$.

Alanine *S*-Benzyl Ester, Hydrochloride Salt (H-Ala-SBzl·HCl) was prepared as described above for H-Ala-SPh·HCl, but starting with Boc-Ala-OH (1.51 g, 8.0 mmol) and using benzyl mercaptan (0.94 mL, 8.0 mmol), to provide 1.17 g (63%) of the title product as a white solid: ^{1}H NMR (DMSO-d_6) δ 8.82 (br s, 3H), 7.30 (m, 5H), 4.25 (q, J = 7.2 Hz, 1H), 4.21 (s, 2H), 1.45 (d, J = 7.2 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 197.2, 137.4, 129.3, 129.1, 127.9, 54.8, 32.7, 17.4; ESMS *m/z* calcd for $C_{10}H_{13}NOS$ 195.3, found 195.8 $[M + H]^+$.

Alanine *S*-(ethyl 3-mercaptopropionate) ester, hydrochloride salt (H-Ala-S(CH_2)$_2$-COOEt·HCl) was prepared as described above for H-Ala-SPh·HCl, but starting with Boc-Ala-OH (1.51 g, 8.0 mmol) and using ethyl 3-mercaptopropionate (1.03 mL, 8.0 mmol), to provide 1.37 g (71%) of the title product as a white solid: ^{1}H NMR (DMSO-d_6) δ 8.78 (br s, 3H), 4.20 (q, J = 7.2 Hz, 1H), 4.04 (q, J = 6.9 Hz, 2H), 3.10 (t, J = 6.9 Hz, 2H), 2.61 (t, J = 6.9 Hz, 2H), 1.43 (d, J = 7.2 Hz, 3H), 1.15 (t, J = 6.9 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 197.6, 171.5, 60.8, 54.9, 33.9, 24.1, 17.4, 14.6; ESMS *m/z* calcd for $C_8H_{15}NO_3S$ 205.3, found 205.8 $[M + H]^+$.

H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-SPh (Table 2, Entry 1). H-Ala-SPh·HCl (15.2 mg, 10 equiv) and DIEA (24 μL, 20 equiv) were dissolved together in CH_2Cl_2 (0.28 mL), and this solution was added to Boc-Val-Tyr(*t*Bu)-Gly-Gly-Phe-(BAL-Ile-PEG-PS)Ala-OH resin (50 mg, 0.14 mmol/g) prepared freshly as described previously. Next, coupling was initiated by addition of HATU (26.6 mg, 10 equiv) in solid form. After 30 min at 25 °C, the peptide-resin was washed with DMF (6 × 0.5 min) and CH_2Cl_2 (4 × 0.5 min). Amino acid analysis on a hydrolyzed aliquot of peptide-resin showed Val, 0.90; Tyr, 0.80; Gly, 1.96; Phe, 0.99; Ala, 1.97; and Ile, 1.00. Treatment of an aliquot of the resin with TFA−H_2O (19:1, 1 mL) at 25 °C for 2 h, followed by the same workup as for the H-Val-Tyr-Gly-Gly-Phe-Ala-OAllyl synthesis, gave the desired peptide thioester (59% cleavage yield), which was characterized by analytical HPLC (t_R 25.7 min, 88% purity; condition A) (ESMS *m/z* calcd for $C_{39}H_{49}N_7O_8S$ 775.9, found 776.2 $[M + H]^+$). The crude (unfractionated) material was checked by amino acid analysis (Val, 1.03; Tyr, 0.90; Gly, 2.09; Phe, 0.98; Ala, 2.00) and FABMS (*m/z* calcd for $C_{39}H_{49}N_7O_8S$ 775.9, found 776.3 $[M + H]^+$). The main byproducts detected and characterized by analytical HPLC−ESMS using condition A were H-Val-Tyr-Gly-Gly-Phe-Ala-OH (t_R 11.8 min, 0.2%; ESMS *m/z* calcd for $C_{30}H_{40}N_6O_8$ 612.7, found 613.0 $[M + H]^+$), H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-OH (t_R 12.5 min, 2.6%; ESMS *m/z* calcd for $C_{33}H_{45}N_7O_9$ 683.8, found 684.2 $[M + H]^+$), and a mixture of H-Val-Tyr-Gly-Gly-Phe-D-Ala-Ala-SPh (t_R 25.4 min, 3.1%; ESMS *m/z* calcd for $C_{39}H_{49}N_7O_8S$ 775.9, found 776.2 $[M + H]^+$) and H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-Ala-SPh (ESMS *m/z* calcd for $C_{42}H_{54}N_8O_9S$ 847.0, found 847.2 $[M + H]^+$). The two components were resolved by analytical HPLC−ESMS using condition F: H-Val-Tyr-Gly-Gly-Phe-D-Ala-Ala-SPh (t_R 52.5 min, 2.2%) and H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-Ala-SPh (t_R 53.5 min, 0.9%).

Another aliquot of the resin was cleaved with reagent B (TFA−phenol−H_2O−Et_3SiH, 88:5:5:2, 1 mL) at 25 °C for 2 h. The filtrate from the cleavage reaction was collected, combined with TFA washes (1 mL) of the cleaved peptide-resin, concentrated, dissolved in HOAc−H_2O (1:4), lyophilized, and redissolved in HOAc−H_2O (1:4) to give the desired peptide thioester (58% cleavage yield), which was characterized by analytical HPLC−ESMS (t_R 25.7 min, 90% purity; condition A). The main byproducts were detected and characterized by analytical HPLC−ESMS using condition A (see Table 2 for product distribution).

Finally, another aliquot of the resin was cleaved with reagent R (TFA−thioanisole−1,2-ethanedithiol−anisole, 90:5:3:2, 1 mL) at 25 °C for 2 h. The filtrate from the cleavage reaction was collected, combined with TFA washes (1 mL) of the cleaved peptide-resin, concentrated under a stream of N_2, precipitated and washed with diethyl ether (4 × 5 mL), dissolved in HOAc−H_2O (1:4), lyophilized, and redissolved in HOAc−H_2O (1:4) to give the desired peptide thioester (70% cleavage yield), which was characterized by analytical HPLC−ESMS (t_R 25.7 min, 92% purity; condition A). The main byproducts were detected and characterized by analytical HPLC−ESMS using condition A (see Table 2 for product distribution).

H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-SBzl (Table 2, entry 2) was prepared essentially as for the H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-SPh (Table 2, entry 1) but using H-Ala-SBzl·HCl (16.2 mg, 10 equiv) instead of H-Ala-SPh·HCl. Amino acid analysis on a hydrolyzed aliquot of peptide-resin showed Val, 0.95; Tyr, 0.78; Gly, 1.91; Phe, 1.00; Ala, 1.89; and Ile, 1.00. Cleavage with TFA−H_2O (19:1, 1 mL) at 25 °C for 2 h of an aliquot of the resin, followed by the same workup as for the H-Val-Tyr-Gly-Gly-Phe-Ala-OAllyl synthesis, gave the desired peptide thioester (68% cleavage yield), which was characterized by analytical HPLC (t_R 54.9 min, 84% purity; condition G) and ESMS (*m/z* calcd for $C_{40}H_{51}N_7O_8S$ 789.9, found 790.2 $[M + H]^+$). The crude (unfractionated) material was checked by amino acid analysis (Val, 0.99; Tyr, 0.83; Gly, 2.20; Phe, 0.96; Ala, 2.02) and FABMS (calcd for $C_{40}H_{51}N_7O_8S$ 789.9, found 790.3 $[M + H]^+$). The main byproducts detected and characterized by analytical HPLC−ESMS using condition G were H-Val-Tyr-Gly-Gly-Phe-Ala-OH (t_R 17.7 min, 0.4%; ESMS *m/z* calcd for $C_{30}H_{40}N_6O_8$ 612.7, found 613.0 $[M + H]^+$), H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-OH (t_R 19.2 min, 6.5%; ESMS *m/z* calcd for $C_{33}H_{45}N_7O_9$ 683.8, found 684.2 $[M + H]^+$), and H-Val-Tyr-Gly-Gly-Phe-D-Ala-Ala-SBzl (t_R 54.0 min, 1.7%; ESMS *m/z* calcd for $C_{40}H_{51}N_7O_8S$ 789.9, found 790.2 $[M + H]^+$).

Another aliquot of the resin was cleaved with reagent B at 25 °C for 2 h, followed by the same workup as described previously for this reagent. The desired peptide thioester (67% cleavage yield) was characterized by analytical HPLC−ESMS (t_R 54.9 min, 91% purity; condition G). The main byproducts were detected and characterized by analytical HPLC−ESMS using condition G (see Table 2 for product distribution).

Finally, another aliquot of the resin was cleaved with reagent R at 25 °C for 2 h, followed by the same workup as described previously for this reagent. The desired peptide

thioester (72% cleavage yield) was characterized by analytical HPLC−ESMS (t_R 54.9 min, 91% purity; condition G). The main byproducts were detected and characterized by analytical HPLC−ESMS using condition G (see Table 2 for product distribution).

H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-S(CH$_2$)$_2$-COOEt (Table 2, entry 3) was prepared essentially as for the H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-SPh (Table 2, entry 1) but using H-Ala-S(CH$_2$)$_2$-COOEt·HCl (16.9 mg, 10 equiv) instead of H-Ala-SPh·HCl. Amino acid analysis on a hydrolyzed aliquot of peptide-resin showed Val, 0.83; Tyr, 0.68; Gly, 1.71; Phe, 0.88; Ala, 1.86; and Ile, 1.00. Cleavage with TFA−H$_2$O (19:1, 1 mL) at 25 °C for 2 h of an aliquot of the resin, followed by the same workup as for the H-Val-Tyr-Gly-Gly-Phe-Ala-OAllyl synthesis, gave the desired peptide thioester (64% cleavage yield), which was characterized by analytical HPLC−ESMS (t_R 23.6 min, 91% purity; condition A; ESMS *m*/*z* calcd for $C_{38}H_{53}N_7O_{10}S$ 799.9, found 800.2 $[M + H]^+$). The crude (unfractionated) material was checked by amino acid analysis (Val, 1.01; Tyr, 0.95; Gly, 2.07; Phe, 1.04; Ala, 1.94) and FABMS (*m*/*z* calcd for $C_{38}H_{53}N_7O_{10}S$ 799.9, found 800.3 $[M + H]^+$). The main byproducts detected and characterized by analytical HPLC−ESMS using condition A were H-Val-Tyr-Gly-Gly-Phe-Ala-OH (t_R 12.5 min, 0.6%; ESMS *m*/*z* calcd for $C_{30}H_{40}N_6O_8$ 612.7, found 613.0 $[M + H]^+$) and H-Val-Tyr-Gly-Gly-Phe-Ala-Ala-OH (t_R 13.2 min, 1.6%; ESMS *m*/*z* calcd for $C_{33}H_{45}N_7O_9$ 683.8, found 684.2 $[M + H]^+$).

Another aliquot of the resin was cleaved with reagent B at 25 °C for 2 h, followed by the same workup as described previously for this reagent. The desired peptide thioester (63% cleavage yield) was characterized by analytical HPLC−ESMS (t_R 23.6 min, 91% purity; condition A). The main byproducts were detected and characterized by analytical HPLC−ESMS using condition A (see Table 2 for product distribution).

Finally, another aliquot of the resin was cleaved with reagent R at 25 °C for 2 h, followed by the same workup as described previously for this reagent. The desired peptide thioester (69% cleavage yield) was characterized by analytical HPLC (t_R 23.6 min, 94% purity; condition A). The main byproducts were detected and characterized by analytical HPLC−ESMS using condition A (see Table 2 for product distribution).

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Supporting Information Available: Experimental procedures for glycine thioesters, and data and documentation for less satisfactory peptide thioester syntheses summarized in the main text. This material is available free of charge via the Internet at http://pubs.acs.org.

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