A Photolabile Backbone Amide Linker for the Solid-Phase Synthesis of Cyclic Peptides and C-Terminal Thioesters

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Abstract

Introduction

A new backbone amide linker has been developed for the synthesis of cyclic and C-terminally modified peptides that permits photochemical detachment of the synthesized peptide from the solid support, thus avoiding problems associated with acid deprotection conditions.

Methods

An initial survey of known photolabile motifs for their ability to produce a linker-bound model dipeptide in high yield and their ability to undergo efficient photochemical detachment of the model dipeptide found that the 6-nitroveratryl (Nve) motif afforded the most efficient release of the dipeptide. The problematic acylation of Nve-bound amino esters was solved through the development of the 2-hydroxy-4-carboxy-6-nitrobenzyl (Hcnb) linker, which utilizes an O-to-N transacylation to afford efficient acylation of even sterically hindered, linker-bound amino esters. Two different approaches were carried out to avoid/minimize diketopiperazine formation. This methodology was then used to synthesize cyclic peptides and peptide thioesters.

Results

The Hcnb linker was found to afford high yields of amino acid loading, acylation, and photolytic cleavage of model tripeptides. Attachment of the Hcnb linker to the aminomethyl TG resin permitted the solid phase synthesis of representative cyclic peptides and C-terminal thioesters in high overall yield and purity.

Conclusion

Hcna is a new photolabile backbone amide linker that has been used to synthesize cyclic peptides and peptide thioesters. It was found that the linker efficiently released the synthesized peptides when UV light was shed on it. This linker is also stable to acids meaning global deprotections can be done on resin and can be washed to remove by-products, releasing only the peptide upon its photocleavage from the resin.

Introduction

The head-to-tail cyclization of peptides (Fusetani 1993; Humphrey 1997; Katsara 2006) is a common and important strategy, employed both in nature and in synthetic applications, for improving the bioavailability and conformational rigidity of polypeptides (Kessler 1982; Samanen 1991; Biron 2008). The lack of either an N- or C-terminus increases proteolytic stability while internal hydrogen bonding and a more compact structure improve the membrane permeability of cyclic peptides (White 2011; Dougherty 2019). These advantages are reflected in the prominence of cyclic peptides among peptidic therapeutic agents (Veber 1981; Darkin-Rattray 1996; Coulouarn 1998; Foister 2006), such as gramicidin S (Gause 1944) and cyclosporine A (Shevach 1985). The chemical synthesis of cyclic peptides, however, has been complicated by the traditional approach to solid-phase peptide synthesis using the C-terminal carboxylate to covalently attach the growing peptide to a polymeric support. The most straightforward approach involving detachment of a fully protected linear peptide precursor from the resin followed by macrocyclization in solution is complicated by solubility problems, oligomerization and C-terminal epimerization reactions. To avoid the problems associated with head-to-tail cyclization in solution, various approaches have been devised for on-resin cyclization of peptides, including Kaiser's Oxime resin (Mihara 1995), Richter's thioester linker (Richter 1994), Waldmann's hydrazide linker (Rosenbaum 2001), Kenner's sulfonamide linker (Kenner 1971) and side-chain attachment of the linear peptide to the resin (Krishnamoorthy 2006). An important general strategy for the on-resin, head-to-tail cyclization of peptides was introduced by the "backbone amide linker" (BAL) strategy of Albericio and Barany (Jensen 1998). In this approach, the nitrogen atom of an internal amide bond is used to tether the growing peptide to the polymeric resin, thereby liberating both N- and C-termini for modification. It is noteworthy that this strategy also addresses the more general problem of C-terminal modification of the resin-bound peptide. Since its introduction, the BAL strategy has been employed by a number of researchers and several modifications have been developed (Bourne 1999; Bourne 2001; Boas 2002; Brask 2003; Boas 2004; Pittelkow 2005; Jessing 2006; Liley 2006; Pittelkow 2006). Additional applications of the BAL strategy involve the synthesis of C-terminal modifications, such as trithioorthoesters (Guillaumie 2000; Kappel 2005), aldehydes (Mori 2004; Soural 2007; Springer 2008), or functionalized amides.

Another application of the C-terminal modification of peptides was revealed by the discovery of the native chemical ligation of peptide bonds by Kent, Muir and Dawson (Dawson 1994). Their use of peptide thioesters to couple with N-terminal cysteine residues has necessitated the

development of various methods for the solid-phase synthesis of peptide thioesters (Mende 2011; Li 2007). Since that landmark discovery, related processes such as Staudinger ligation (Nilsson 2000) have been developed that likewise depend on the availability of peptide thioesters. In response to this need, a number of creative approaches to the solid-phase synthesis of peptide thioesters have been developed, yet the availability of C-terminal modification in the BAL strategy remains one of the most straightforward approaches to the synthesis of peptide thioesters.

Although acidic deprotection conditions are widely used in peptide synthesis, there are situations involving acid-sensitive functionality that may require alternative deprotection conditions. For these cases, other methodologies have been explored and developed (Lloyd 1993; Guillier 2000; Bochet 2002; Heidler 2005; McAllister 2005; Scott 2006; Kang 2008). One of the most appealing ideas has been to use photochemical detachment from the resin as an extra dimension of protecting group orthogonality in solid phase peptide synthesis. Photolabile linkers (Gallop 1994; Liang 2016), in which long-wave UV radiation (300 ~ 360 nm) is used to detach an organic functional group – usually a carboxylic acid, a carbamate (a latent amine), an alcohol, or a phosphate – from a polymer or glass surface, provide an alternative to the classic acidic or basic cleavage protocols used in solid-phase organic synthesis. Ideally, photochemical deprotection should proceed with high functional group selectivity, low amounts of side reactions and involve no separation of product from reactants. Photochemical deprotection has also found favor with applications in the parallel synthesis of compound libraries (Sheehan 1973).

Herein we describe studies directed toward the development of a photolabile BAL linker (Fig. 1) to address the problems associated with the BAL strategy as a general method for the synthesis of cyclic and C-terminally modified peptides. Our studies began with a survey of several known photolabile motifs for their applicability to BAL linker design (Fig. 1). The candidate photolabile motifs studied were the *p*-oxyphenacyl (Op) (Bellof 1985), α-methyl-*p*-oxyphenacyl (Mop) (Sheehan 1965; Peach 1995), *p*-oxybenzoin (Obz) (Bergmark 1985), α,*o*-dimethyl phenacyl (Dmp) (Patchornick 1970) and 6-nitroveratryl (Nve). All of these photolabile groups undergo facile n to π* or π to π* excitation at 300–360 nm and release oxygen-based substituents with few side reactions. Appropriate synthetic precursors **1**–**5** were chosen for each of the motifs, as shown in Fig. 2.

Consequently, our preliminary survey focused on the Op, Mop, Obz, Dmp, and Nve motifs, their preparation and photolysis. Of specific interest were a) the ease of anchoring amino acids to the linker, b) the rate and efficiency of photocleavage, and c) the suppression of side reactions during photolytic deprotection. To assess these questions, the dipeptide Boc-Phe-Gly-OMe (**8**) was chosen as a model system. After the photolabile motif was chosen, effort was directed toward its attachment to a solid support compatible with the solvents and reagents common to peptide synthesis. Finally, the problems of diketopiperazine formation, photocleavage optimization, and maximization of cyclization yields were addressed.

Material and Methods

Synthesis of Dmp

1-(2,5-dimethyl-4-alkylphenyl)-propanone (4b) To a mixture of propionyl chloride (0.51 mL, 5.88 mmol) and $AlCl_3$ (780 mg, 5.88 mmol) in CH_2Cl_2 (25 mL) was added a solution of 4-(2,5-dimethylphenyl)butanoic acid (**4a**, 377 mg, 1.96 mmol) in CH_2Cl_2 (5 ml) at 0°C. The reaction mixture was stirred for 1 h at the same temperature and poured into cold HCl solution (40 g ice and 10 mL of conc. HCl). The organic material was extracted by addition of CH_2Cl_2 (25 mL) and the aqueous layer was extracted with another portion of CH_2Cl_2 (50 mL). The combined organic layer was washed with brine (50 mL), dried with anhydrous $MgSO_4$, passed through 3 cm silica gel pad, and evaporated under reduced pressure. The crude product was dissolved in oxalyl chloride (3 mL) and stirred overnight. After removal extra oxalyl chloride by evaporation under reduced pressure, the organic material was dissolved in CH_2Cl_2 (30 mL). To this solution, benzyl amine (0.257 mL, 2.35 mmol) and DIEA (1.7 mL, 9.8 mmol) were added and stirred 3 h. The organic layer was washed with brine (30 mL), dried with anhydrous $MgSO_4$, evaporated under reduced pressure and purified by flash column chromatography using 33% EtOAc in Hexanes to give **4b** as a pale-yellow solid.

1-(2,5-dimethyl-4-alkylphenyl)-1-hydroxypropan-2-one oxime (4c) To a solution of **4b** (0.51g, 1.50 mmol) and isoamyl nitrite (0.24 mL, 1.80 mmol) in MeOH (10 mL) at 45°C was added dropwise to concentrated HC1 (0.25 mL) and the resulting mixture was stirred for 3 h at the same temperature. After cooling to room temperature, the organic material was partitioned into EtOAc (40 mL) and H_2O (40 mL). The organic layer was washed with brine (40 mL), dried with anhydrous $MgSO_4$, passed through 3cm silica gel pad, and evaporated under reduced pressure. The residue was dissolved in MeOH (15 mL) and $NaBH_4$ (50 mg) was added under ice bath. After stirring for 30 min, the organic material was partitioned into EtOAc (40 mL) and HCl solution (0.1 M, 30 mL). The organic layer was washed with saturated $NaHCO_3$ (30 mL) and with brine (30 mL), dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The residue was purified by flash column chromatography using 66% EtOAc in hexanes to give **4c** as a white solid.

1-(2,5-dimethyl-4-alkylphenyl)-1-hydroxypropan-2-one (4) To a solution of **4c** (0.30 g, 0.81 mmol) in DMF (2 mL) was added glyoxylic acid (60% water solution, 5 mL). After stirring for 5 h, the organic material was partitioned into EtOAc (40 mL) and H_2O (40 mL). The organic layer was washed with brine (2 X 30 mL), dried with anhydrous $MgSO_4$, evaporated under reduced pressure, and the residue was purified by flash column chromatography using 50% EtOAc in hexanes to give **4** as a pale-yellow oil.

Alkylation of amino ester with photolabile linker 1–5 (6a-e)

H-[Op]-Gly-OMe (6a). To a solution of **1**, (150 mg, 0.66 mmol) in anhydrous CH_3CN (5 mL) were added GlyOMe·HCl (248 mg, 1.98 mmol), and DIEA (0.52 mL, 2.97 mmol). The reaction mixture was stirred overnight at room temperature, evaporated under reduced pressure and partitioned into CH_2Cl_2 (20 mL) and H_2O (20 mL). The organic layer was washed with brine (20 mL), dried with anhydrous $MgSO_4$, evaporated under reduced pressure, and purified by flash column chromatography using 33% EtOAc in hexanes to give **6a** as a pale-yellow oil.

H-[Mop]-Gly-OMe (6b). To a solution of **2**, (200 mg, 0.63 mmol) in anhydrous CH_3CN (8 mL) were added GlyOMe·HCl (237 mg, 1.89 mmol), DIEA (0.49 mL, 2.84 mmol) and tetrabutylammonium iodide (25 mg). The reaction mixture was refluxed for overnight, cooled to room temperature, evaporated under reduced pressure, and partitioned into CH_2Cl_2 (20 mL) and H_2O (20 mL). The organic layer was washed with brine (20 mL), dried with anhydrous $MgSO_4$, evaporated under reduced pressure, and purified by flash column chromatography using 33% EtOAc in hexanes to give **6b** as a colorless oil.

H-[Obz]-Gly-OMe (6c). To a solution of **3**, (123 mg, 0.39 mmol) in anhydrous MeOH (20 mL) was added GlyOMe·HCl (242 mg, 1.93 mmol). The reaction mixture was refluxed for 24 h, cooled to room temperature, evaporated under reduced pressure, and partitioned into CH_2Cl_2 (20 mL) and H_2O (20 mL). The organic layer was washed with brine (20 mL), dried with anhydrous $MgSO_4$, evaporated under reduced pressure, and purified by flash column chromatography using 33% EtOAc in hexanes to give **6c** as a pale-yellow oil.

H-[Dmp]-Gly-OMe (6d). To a solution of **4**, (200 mg, 0.57 mmol) in anhydrous toluene (20 mL) was added GlyOMe·HCl (215 mg, 1.71 mmol). The reaction mixture was refluxed for 5 h, cooled to room temperature, and partitioned into EtOAc (20 mL) and H_2O (40 mL). The organic layer was washed with brine (40 mL), dried with anhydrous $MgSO_4$, evaporated under reduced pressure, and purified by flash column chromatography using 33% EtOAc in hexanes to give **6d** as a colorless oil.

H-[Nve]-Gly-OMe (6e). To a solution of **5** (515 mg, 2.44 mmol) in MeOH (20 mL), were added GlyOMe·HCl (459 mg, 3.66 mmol), sodium bicarbonate (348 mg, 4.15 mmol). The reaction mixture was stirred for 4 h, cooled to 0°C. After portionwise addition of $NaBH_4$ (6.0 mmol, 227 mg), the mixture was stirred for 30 min and poured into HCl solution (0.1N, 50 mL). The pH of water layer was adjusted to 4 ~ 6 by dropwise addition of NaOH (1 M), and the organic material was extracted with CH_2Cl_2 (50 mL). The aqueous layer was extracted with another portion of CH_2Cl_2 (50 mL). The combined organic layer was dried with anhydrous $MgSO_4$, evaporated under reduced pressure and purified by flash column chromatography using 50% EtOAc in hexanes to give **6e** as a colorless oil.

Acylation of 6a-e using symmetric anhydrides (7a-e). The symmetric anhydride of Boc-Phe was prepared *in situ* by mixing Boc-Phe (860 mg, 4 mmol) with EDCI (0.422 g, 2.2 mmol) in CH_2Cl_2 (10 ml, 0.2 M) for 5 min. To the solution of symmetric anhydride was added **6a-e** (1 mmol), and the reaction mixture was stirred for 24 h at room temperature. After dilution with CH_2Cl_2 (50 ml), the organic layer was washed with brine (50 mL), dried with anhydrous $MgSO_4$, and evaporated under reduced pressure. The residue was purified by flash column chromatography to obtain an Hcnb-linked dipeptide (**7a-e**).

Synthesis of Hcna

Methyl (E)-4-(2-(dimethylamino)vinyl)-3,5-dinitrobenzoate (13). To a stirred mixture of 4-Methyl-3,5-dinitrobenzoic acid (**12**) (2.0 g, 8.8 mmol) in anhydrous toluene (29 mL) was added DMF dimethyl acetal (3.2 mL, 24 mmol) dropwise at ambient temperature. The resulting mixture was heated to reflux in an oil bath and became dark red in color. After 16 h, the reaction was cooled to room temperature and the solvent removed under reduced pressure by co-evaporation with MeOH. The crude residue was recrystallized from hot ethanol to afford enamine **13** as a lustrous green solid which became red upon grinding.

Methyl 4-formyl-3,5-dinitrobenzoate (14). To a mixture of **13** (1.08 g, 3.66 mmol) and $RuCl_3$ solution (0.1 M in H_2O, 1.2 mL, 0.13 mmol) in CH_3CN-H_2O (6:1, 37 mL) was added $NaIO_4$ (2.0 g, 9.1 mmol) in portions at rt. The color quickly dissipated, and solids formed. The reaction continued until TLC analysis showed consumption of starting material (typically 10–30 min). After stirring for 30 min, the solids were removed by vacuum filtration and washed with CH_2Cl_2. The filtrate was partitioned between H_2O and CH_2Cl_2 and the aqueous layer was extracted twice with CH_2Cl_2. The combined organic extracts were washed with brine and sat. aq. $Na_2S_2O_4$, dried over Na_2SO_4, and

concentrated under reduced pressure. The crude residue was purified by flash column chromatography (30% EA/Hex) to afford **14** as an off-white solid.

Methyl 4-formyl-3-hydroxy-5-nitrobenzoate (15). A solution of **14** (678 mg, 2.67 mmol) in anhydrous DMF (5.1 mL) was added dropwise to a mixture of acetaldoxime [mixture of isomers] (325 µL, 5.33 mmol) and K_2CO_3 (811 mg, 5.87 mmol) in anhydrous DMF (5.1 mL) dropwise at rt. The reaction mixture immediately turned bright red purple upon mixing, then brownish green after stirring overnight. After 21 h, H_2O (10 mL) was added to the reaction and the resulting solution extracted 5x with Et_2O. The aqueous layer was then acidified to pH ~ 1–2 with concentrated HCl and extracted 4x with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude residue was purified by flash chromatography (25% EA/Hex) to afford **15** as a bright yellow solid.

4-Formyl-3-hydroxy-5-nitrobenzoic acid (16). Solid $Ba(OH)_2$•8 H_2O (1.97 g, 6.24 mmol) was added in portions to a solution of **15** (468 mg, 2.08 mmol) in MeOH (23 mL) at rt. Following reaction completion (TLC), the mixture was concentrated under reduced pressure and the crude residue was partitioned between EtOAc and H_2O. The aqueous layer was extracted twice with EtOAc, acidified to pH ~ 1–2 with concentrated HCl and extracted with EtOAc (3x). The combined organic extracts were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford **16** (379 mg, 86%) as a brown solid.

General procedure for solution-phase reductive amination (17a-c). HCl-AA-OtBu (0.666 mmol) was added to a solution of **15** (200.0 mg, 0.666 mmol) and DIEA (244 µL, 1.40 mmol) in CH_3CN (9.5 mL). The reaction was stirred at rt for 45 min. Volatiles were removed and the residue was dissolved in MeOH (3.3 m). $NaBH_4$ (75.6 mg, 2.00 mmol) was added, and the reaction was stirred for 15 min. Volatiles were removed and the residue was purified by flash column chromatography (20–45% EtOAc/Hex) to yield **17a-d.**

General procedure for solution-phase acylation (18a-c). A mixture of Fmoc-AA-OH (0.29 mmol) and DIPC (22.5 µL, 0.14 mmol) was preactivated in dry CH_2Cl_2: DMF (1:1) (0.6 mL) for 5 min. **17a-c** (0.13 mmol) as a solution in CH_2Cl_2 (2.1 mL) was added and the reaction was stirred for 2 h. The volatiles were removed and then dissolved in methanol (1.0 mL) and a solution of ammonium acetate (80.9mg, 1.05 mmol) in water (0.3 mL) was added and the reaction was stirred overnight. Volatiles were removed and the residues were washed with water and extracted with ethyl acetate three times, concentrated, and then purified by flash column chromatography (30–40% EA/Hex).

Solid Phase Peptide Synthesis

Preparation of Hcna-loaded resin (22a-b). Fmoc-Sieber Amide PS Resin (0.6 mmol/g) or TentaGel S NH_2 (0.26 mmol/g) was swelled in DMF for 15 min, drained, and treated with piperidine-DMF (1:4, 1 x 2 mL, 30 min). The mixture was drained and washed with standard wash protocol [DMF (3 x 1 mL), CH_2Cl_2 (3 x 1 mL), MeOH (3 x 1 mL), CH_2Cl_2 (3 x 1 mL), then DMF (3 x 1 mL)] to afford a positive Kaiser ninhydrin test. To a mixture of *N*-Fmoc-6-aminohexanoic acid (4 eq) and HATU (3.9 eq) in DMF (0.03 M) was added DIEA (8 eq) and this mixture was added to the resin (pre-washed with DMF) after a 5 min pre-activation period. After 2 h, the mixture was drained and washed with the standard wash protocol to afford a negative Kaiser ninhydrin test. The resin was then agitated with piperidine-DMF (1:4, 1 x 2 mL, 30 min). The mixture was drained, and the resin washed with standard wash protocol to afford a positive Kaiser ninhydrin test. To a mixture of Fmoc-Pro-OH (4 eq), HATU (3.9 eq) and HOAt (4 eq) in DMF (0.03 M) was added DIEA (8 eq) and this mixture was added to the resin (pre-washed with DMF) after a 5 min pre-activation period. After 2 h, the mixture was drained and washed with the standard wash protocol to afford a negative Kaiser ninhydrin test. The resin was then treated with piperidine-DMF (1:4, 1 x 2 mL, 30 min). The mixture was drained, and resin washed with standard wash protocol to afford a positive chloranil test. To a mixture of **16** (3 eq) and PyBOP in DMF (0.03 M) was added DIEA (6 eq) and this mixture was added to the resin (pre-washed with DMF) after a 5 min pre-activation period. After 2 h, the mixture was drained and washed with the standard wash protocol to afford a negative chloranil test.

General procedure for solid-phase reductive amination. The prepared resin **22a** or **22b** was swelled in DMF for 15 min then treated with a mixture of H-AA-ODMA or HCl-AA-O*t*Bu (10 eq) and AcOH (10 eq) in CH_2Cl_2 (0.03 M). After 3 h, the mixture was drained and washed briefly with standard wash protocol then THF-MeOH (2:1). The resin was then taken up in THF-MeOH (2:1, 0.03 M) and treated with solid $NaBH_4$ (5 eq). After 2 h, the mixture was drained and washed with H_2O, standard wash protocol, then piperidine-DMF (1:4, 5 min). The resin was washed once more with standard wash protocol to afford a positive chloranil test.

General procedure for loading of second amino acid residue. A flame-dried flask was charged with the 2nd Amino Acid residue (4 eq) and dry CH_2Cl_2 (0.05 M), to which was added DIPC (2 eq) dropwise at rt. The mixture was stirred at rt for 12 min and the resulting solution was solubilized by addition of DMF (0.05 M). This solution was added directly to prepared resin **23a** or **23b** and the mixture was agitated for 2 h, followed by the standard wash protocol to afford a slightly positive chloranil test. This procedure was repeated once more to afford resin-bound dipeptide (and a negative chloranil test).

General procedure for coupling of third amino acid residue. A solution of 1-octanthiol (10 eq), DIEA (5 eq), and DMF (0.03 M) was added to the prepared resin **24a** or **24b** and the mixture was agitated for 1 h, followed by the standard wash protocol. Next, a mixture of TIPS-OTf (10 eq), 2,6-Lutidine (10 eq), and DMF (0.03 M) was added to the resin and the mixture was agitated for 4 h, followed by the standard wash protocol. A flame-dried flask was charged with an Fmoc-amino acid (4 eq) and dry CH_2Cl_2 (0.05 M), to which was added DIPC (2 eq) dropwise at rt. The mixture was stirred at rt for 12 min and the resulting mixture was diluted with DMF (0.05 M).

The resin was agitated with a solution of DBU-1-Octanthiol-DMF (2:2:96, 1 x 2 mL, 3 min), washed with the standard wash protocol, and the pre-activated amino acid solution was added, taking care to minimize the time between deprotection and coupling. The mixture was agitated for 2 h, followed by standard wash protocol to afford a negative chloranil test. The resin was agitated with piperidine-DMF (1:4, 1 x 2 mL, 30 min). The mixture was drained, and the resin washed with standard wash protocol to afford a positive Kaiser ninhydrin test.

Fmoc half-cycle method. A solution of 1-octanthiol (10 eq), DIEA (5 eq), and DMF (0.03 M) was added to the prepared resin **24a** or **24b** and the mixture was agitated for 1 h, followed by the standard wash protocol. Next, a mixture of TIPS-OTf (10 eq), 2,6-Lutidine (10 eq), and DMF (0.03 M) was added to the resin and the mixture was agitated for 4 h, followed by the standard wash protocol. The C-terminal allyl protecting group was removed using $Pd(PPh_3)_4$ (0.25 eq), $PhSiH_3$ (25 eq) and DCM (3 mL) and agitating the reaction mixture for 2 hours after which the following wash was done: DCM (X 3), DMF (X 3), 0.03 M sodium N,N-diethyldithiocarbamate in DMF (X 3), and DMF (X 3).

H-AA_n-OAllyl•CF_3COOH (10 eq), HATU (10 eq), HOAt (10 eq) and DIEA (20 eq) in DMF (3 mL) was mixed and added to the reaction vessel and agitated for 4 hours. The resin was washed using standard wash protocol.

General procedure for peptide cyclization (condition A). After complete synthesis of the desired Fmoc or Boc-protected peptide, the C-terminal DMA protecting group was removed using $Pd(PPh_3)_4$ (1 eq) and phenylsilane (20 eq) in CH_2Cl_2 under N_2 for 2 h, after which the resin was drained and washed with DMF (3 x 1 min), sodium *N,N*-diethyldithiocarbamate (0.03 M in DMF, 3 x 1 min), and CH_2Cl_2 (3 x 1 min). The resin was agitated with piperidine-DMF (1:4, 1 x 2 mL, 30 min). The mixture was drained, and the resin washed with standard wash protocol to afford a positive Kaiser ninhydrin test. A mixture of PyAop (5 eq), HOAt (5 eq), and DIEA (10 eq) in DMF (0.03 M) was added directly to the resin. After 2 h, the mixture was drained and washed with the standard wash protocol to afford a negative Kaiser ninhydrin test.

General procedure for peptide cyclization (condition B). After complete synthesis of the desired Fmoc or Boc-protected peptide, the C-terminal DMA protecting group was removed using $Pd(PPh_3)_4$ (1 eq) and phenylsilane (20 eq) in CH_2Cl_2 under N_2 for 2 h, after which the resin was drained and washed with DMF (3 x 1 min), sodium *N,N*-diethyldithiocarbamate (0.03 M in DMF, 3 x 1 min), and CH_2Cl_2 (3 x 1 min). The resin was agitated with piperidine-DMF (1:4, 1 x 2 mL, 30 min). The mixture was drained, and the resin washed with standard wash protocol to afford a positive Kaiser ninhydrin test. A mixture of PyAOP (3 eq), 2,4,6-collidine (6 eq), and DIEA (6 eq) in 9:1 CH_2Cl_2-DMF (0.03 M) was added directly to the resin. After 2 h, the mixture was drained and washed with the standard wash protocol to afford a negative Kaiser ninhydrin test.

General procedure for thioester synthesis. After synthesis of the desired Boc-protected peptide, the C-terminal DMA protecting group was removed using $Pd(PPh_3)_4$ (1 eq) and phenylsilane (20 eq) in CH_2Cl_2 under N_2 for 2 h, after which the resin was drained and washed with DMF (3 x 1 min), sodium *N,N*-diethyldithiocarbamate (0.03 M in DMF, 3 x 1 min), and CH_2Cl_2 (3 x 1 min). A solution of 1-octanthiol (10 eq), DIEA (10 eq), and DMF (0.03 M) was added to the resin and the mixture was agitated for 1 h, followed by the standard wash protocol. Next, a mixture of TIPS-OTf (10 eq), 2,6-Lutidine (10 eq), and DMF (0.03 M) were added to the resin and the mixture was agitated for 4 h, followed by the standard wash protocol. A solution of HATU (5 eq) and DIEA (20 eq) in DMF (0.03 M) was added to the resin and agitated for a 5 min period. Without draining, thiophenol (10 eq) was added to the mixture. After 2 h, the mixture was drained and washed with the standard wash protocol to afford a negative Kaiser ninhydrin test

General Procedure for Sieber Amide cleavage. An aliquot (~ 1 mg) of resin-bound peptide was treated with 200 µL TFA/H_2O/CH_2Cl_2 (2:1:97) and the mixture agitated for 5 min at rt. Following this, volatiles were removed by N_2 stream. The resulting residue was dissolved in 200 µL MeOH-H_2O (1:1), filtered and analyzed by UPLC-MS. Note: Product / DKP ratio was determined by integrating 214 and 254 nm peaks and are reported as relative percentages.

General Procedure for photocleavage. The resin-bound peptide was suspended in a 5 mL solution of 1:9 MeOH:CH_2Cl_2 in a fused quartz tube and agitated under 365 nm UV light for 1 h. The solvent was filtered to remove the resin and removed under reduced pressure. The resulting residue was dissolved in MeOH-H_2O (1:1), filtered and analyzed by UPLC-MS and HPLC.

Results and Discussion

Synthesis of linker-anchored dipeptides

Using the precursor molecules **1–5** (Fig. 2), each linker was covalently attached to glycine methyl ester to afford an anchored glycine residue (**6a**-**e**), which was then acylated with Boc-*L*-phenylalanine to afford an anchored dipeptide **7a**-**e** (Table 1). Each anchored dipeptide was photolyzed at 300 nm to afford Boc-Phe-Gly-OMe (**8**).

The linker precursors **1–5** were either commercially available or prepared using literature procedures (Stowell 1996; Vishwanathan 2006). Linkers **1** and **2** were anchored to methyl glycinate via nucleophilic displacement, whereas **3** and **4** employed the Amadori rearrangement (Amadori 1925; Isbell 1958) of an a-hydroxyketone, and **5** was attached to methyl glycinate through reductive amination. In all cases, acylation with the symmetric anhydride of Boc-*L*-Phe was found to afford the highest yield of anchored dipeptide. A survey of conditions for photolytic cleavage of the dipeptide found that photoexcitation at 300 nm in either methanol or chloroform, depending on the motif, afforded the fastest and cleanest reactions, but only the Dmp (**7d**) and Nve-linked dipeptides showed acceptable photolytic reactivity. Problems arose, however, when amino acids larger than glycine were anchored to Dmp and Nve. Both the initial attachment and subsequent acylation of those anchored amino acids proved to be quite problematic and afforded anchored dipeptides in modest yields at best.

In summary, Op, Mop, and Obz failed to release the dipeptide product efficiently, regardless of solvent and wavelength used, and so were discarded as linker candidates. Both the Nve and Dmp motifs displayed acceptable photolytic product release with the appropriate choices of solvent and irradiation wavelength; however, in both cases the modest yields for amino acid anchoring and acylation were insufficient for our needs, necessitating further modification to improve both steps of the reaction sequence.

Development of the second-generation photolabile linker

On the basis of both acylation and photocleavage yields the *o*-nitrobenzyl motif of the Nve linker was chosen as the basic skeleton for a second-generation linker, and the transacylation motif of the 6-hydroxy-4-methoxybenzyl (Hmb) (Sheppard 1993) and 6-hydroxynitrobenzyl (Hnb) (Smythe 1999) auxiliaries was incorporated into the linker design to improve acylation efficiency. The acid-labile Hmb auxiliary was devised by Sheppard for the protection of peptide amide backbones during the synthesis of ‘difficult’ peptide sequences. The related, photolabile Hnb auxiliary was developed by Smythe for the covalent modification of ‘difficult’ peptide sequences to facilitate their cyclization. Both the Hmb and Hnb auxiliaries employ an *ortho*-hydroxyl group to acylate bulky amino acids via esterification and subsequent O-to-N transacylation.

It was reasoned that the addition of an *o*-hydroxy substituent to the *o*-nitrobenzyl skeleton would assist both the acylation of the first amino acid and later photolytic cleavage. Attachment of a *para*-carboxylate to the *o*-nitrobenzyl skeleton provides a tether for covalent attachment of the linker to a spacer or resin and affords additional electron withdrawal to assist both the transacylation and photolysis reactions. Thus, the 2-hydroxy-4-carboxy-6-nitrobenzyl (Hcnb) linker emerged as a second-generation candidate for a photolabile BAL linker, as exemplified by the Hcnb-glycine adduct **9** (Fig. 3), derived from reductive amination of the aldehyde precursor 2-hydroxy-4-carboxy-6-nitrobenzaldehyde (Hcna) with glycine methyl ester. Treatment of **9** with a symmetric anhydride (Fig. 3) can result in the formation of the desired dipeptide **11** either through direct N-acylation or via initial acylation of the less hindered phenol followed by subsequent O-to-N acyl transfer. This alternate pathway was envisaged as a means to overcome the limitations of the Nve motif when attached to sterically hindered amino acids.

Synthesis of Hcna linker

4-formyl-3-hydroxy-5-nitrobenzoic acid (**16**) was prepared from commercially available 4-Methyl-3,5-dinitrobenzoic acid (**12**) (Fig. 4). A condensation reaction between **12** and DMF-DMA afforded the previously reported (Starosotnikov 2005) enamine **13** in 72% yield. Ruthenium (III) chloride-catalyzed oxidative cleavage of the enamine using sodium metaperiodate generated the aldehyde **14** in 61% yield. Unilateral nucleophilic aromatic substitution of a single nitro- group using acetaldoxime to install the *ortho*-phenol group afforded ester-protected linker precursor **15** in 68% yield, which was used for solution-phase studies. Finally, hydrolysis of the methyl ester group using barium hydroxide afforded the free carboxylic acid **16** in 90% yield.

Solution-phase synthesis of the dipeptides using Hcnb

We envisioned linking the peptide to the linker via a reductive amination between the primary amine of the first amino acid and the aldehyde precursor, in similar fashion to the loading protocol for the original BAL Linker. The resulting secondary amine can then be acylated with the next amino acid in sequence, forming a tertiary amide linkage between the peptide and linker. The presence of the *ortho*-phenol in Hcnb is essential for complete acylation of the secondary amine, especially when utilizing amino acids with bulky side chains. Finally, photolysis in the short-wave UV range should cause a clean release of the dipeptide from the linker.

A series of dipeptides was synthesized in solution on the methyl ester-protected Hcna linker precursor **15** (Table 2). In solution, addition of the desired C-terminally protected amino acid to the aldehyde handle in the presence of DIEA in CH_3CN forms the imine intermediate. Reduction of this intermediate by sodium borohydride in methanol yields the Hcnb-linked amino acids **17a-c**. Acylation of the secondary amine with a pre-formed symmetric anhydride of the second amino acid resulted in acylation to give Hcnb-linked dipeptides, even in the case of bulky side-chains such as Thr. Photolysis at 365 nm in MeOH releases the desired dipeptides from the linker to give **19a-c.** Due to rotomers present in peptides attached to Hcnb, yields were reported over 2 steps.

Preparation of the resin-bound Hcna linker

The free acid form of the Hcna linker, **16** (Fig. 4), was covalently anchored to aminomethyl-functionalized Tentagel resin **21a**, or Sieber TG resin **21b** via acylation of a resin-bound amine. A 6-aminohexanoic acid (Ahx) spacer was employed with both solid supports to improve reactivity. Additionally, a proline residue was introduced to reduce cross-linking between the amine and the aldehyde of the Hcna linker. In both cases, acylation of resin-bound amines was accomplished by coupling **16** to the free amine using either PyBOP or DCC (Fig. 5). Subsequent reactions of **22a** were monitored by photolytic cleavage from the linker and analysis of the crude product, whereas reactions of **22b** were monitored by cleavage using dilute acid and analysis of the crude product. Because of the time required for photolysis of Hcnb-linked peptides (1–2 h), reaction conditions were optimized using **22b** and then applied to **22a**.

Loading the dipeptide by reductive amination followed by acylation

The first residue was anchored to the resin-bound Hcna linker via a 2-step reductive amination under acidic conditions. (Fig. 6). Quantitative acylation of the resin-bound amine was accomplished using a symmetric anhydride of the second residue (4 eq, 2 x 2h) as determined by LC-MS analysis.

Loading of the third amino acid

One of the major challenges with the Backbone Amide Linker concept is the formation of a diketopiperazine byproduct upon deprotection of the Fmoc group at the dipeptide stage. The most common method for overcoming this problem is to employ a sterically hindered C-terminal protecting group such as *t*-butyl or 1,1-dimethylallyl (DMA) (Hostetler 2018), thereby minimizing the formation of this undesired byproduct. However, it was found that quantitative DKP formation was still obtained when using the Hcnb linker, regardless of the C-terminal protecting groups used. It was hypothesized that this results from Brønsted acid activation of the C-terminus by the nearby phenol proton, thereby catalyzing the formation of DKP. It was reasoned that this effect could be eliminated through removal of the phenol proton, thus avoiding Brønsted acid activation. The phenol group is required for efficient loading of the second amino acid residue, as well as for photocleavage of the final peptide, and so a method for temporary protection of the hydroxyl is required. Following the loading of the secondary amine, the phenol remains acylated with an excess of the second amino acid, until it is removed by the strongly nucleophilic piperidine upon deprotection of the N-terminal Fmoc group. We theorized that replacing piperidine with DBU, a relatively non-nucleophilic alternative for Fmoc deprotection, would leave the acylated phenol intact and minimize formation of the DKP byproduct. However, it was found that rapid, quantitative DKP formation was still obtained even when using DBU. Other methods were considered for blocking the phenol from interacting with the C-terminus, including silylation. However, due to the electronics of the ring, silylation of the phenol results in an exceedingly unstable silyl ether. Even attempts to observe the presence of a silyl group by LC-MS analysis were unsuccessful. The most successful protection conditions used TIPS-OTf and 2,6-Lutidine for TIPS protection of the phenol on the linker. To monitor the effectiveness of various silylation conditions, the dipeptide N-terminus was deprotected for 3 min using a cocktail of 2% v/v DBU and 2% v/v 1-Octanethiol in DMF, followed by immediate introduction of the third amino acid as a pre-formed symmetric anhydride. Various tripeptide sequences were tested to determine the efficacy of the TIPS group for reducing DKP formation (Table 3). In most cases, only trace amounts of DKP were observed by LC-MS, if any. The worst sequence tested was the Ala-Ala dipeptide, resulting in 12% DKP formation.

To completely avoid diketopiperazine formation, we followed the method developed by the Barany group, also known as the Fmoc-half cycle. In an Fmoc-half cycle, the first amino acid used in the reductive amination step is an allyl ester. This is followed by the acylation of the second amino acid. Instead of attaching the third amino acid through the N-terminus, the allyl group of the first amino acid is deprotected and the final amino acid is attached through the C-terminus by a peptide coupling reaction. In this case, the allyl ester was deprotected with catalytic $Pd(PPh_3)_4$ and excess $PhSiH_3$ and then coupled with another amino acid allyl ester through the C-terminus (Fig. 7). Then the rest of the peptide was built through the N-terminus by Fmoc SPPS until the peptide of desired length was built.

Also worth noting that we switched from *t*-Bu/DMA esters to allyl esters for two of the amino acids in the sequence. When the DMA group was deprotected, and the final amino acid is coupled with another DMA ester of amino acid, the 1-octanethiol (left from the Fmoc deprotection using excess diethylamine and 1-octanethiol) could also react and generate thioesters. Therefore, we switched to using allyl

esters which are synthesized on Boc-protected amino acids and then esterified with allyl bromide. Before the reductive amination and Fmoc half-cycle, the Boc group is deprotected with trifluoroacetic acid and neutralized with excess DIEA for the allyl ester to participate in reaction.

The choice of model cyclic peptides

Two previously synthesized cyclic peptides were chosen to model the head-to-tail cyclized peptides to examine the performance of the Hcnb linker on solid support. The cyclic decapeptide c-[Arg-(D)-Phe-Pro-Glu-Asp-Asn-Tyr-Glu-Ala-Ala, **28**] was synthesized to demonstrate the efficacy of the original Backbone Amide Linker, published by Albericio and Barany. The 14-residue cyclic peptide c-[Pro-Asp-Gly-Arg-Cys-Thr-Lys-Ser-Ile-Pro-Pro-Ile-Cys-Phe, **30**], also known as the Sunflower Trypsin Inhibitor, is a well-known natural product. The 14-mer cyclic Sunflower Trypsin Inhibitor poses some interesting synthetic challenges due to the high prevalence of β-branched amino acids and proline residues, which make selection of a cyclization point difficult. After several attempts, it was found that reductive amination of Phe onto the linker as the first residue, followed by cyclization at Pro yielded the best results. Cyclization and subsequent photocleavage from the resin yielded the final peptide in 90% purity and 80% cleavage yield. The synthesis of the cyclic decapeptide was accomplished through reductive amination of Ala as the first residue and the subsequent linear synthesis. Following cyclization and photocleavage from the resin, the peptide was obtained in 95% purity and 90% cleavage yield. The first peptide chosen was a short-chain analogue of the natural product somatostatin, cyclo[(D)-Trp-Lys-Gly-(β)-Ala-Phe]. The synthesis was carried out according to the procedures mentioned previously. The first residue H-Phe-ODMA was loaded onto the prepared resin **22a** via reductive amination. The subsequent linear synthesis was then carried out as previously discussed. The head-to-tail cyclization was accomplished on the resin using PyAOP. Photocleavage from the resin at 365 nm for 1 h yielded the final cyclic decapeptide **29** in 50% purity and 96% cleavage yield.

Synthesis of C-terminal thioesters

The synthesis of C-terminal thioesters is an area of interest for their use in native chemical ligation (NCL) reactions. The Hcnb linker was used to demonstrate the on-resin synthesis of thioesters in high purities. It was established by solution-phase studies that the presence of a free phenol on the linker was problematic for synthesis of C-terminal thioesters, and so the phenol was TIPS protected prior to optimizing the coupling conditions for thioester formation. Use of carbodiimides as a coupling reagent yielded low conversions (0–30%). However, switching to HATU as a coupling reagent and preactivating the carboxylic acid for 5 min prior to addition of the thiol yielded complete conversion to the desired thioester in 2 h. A series of pentapeptides were synthesized to examine the scope of C-terminal residue compatibility with thioester synthesis. It was found that β-branched amino acids yield poor conversion to the thioester. However, it was reported by Kent that these sterically hindered amino acids are not amenable to NCL due to the steric hindrance of the carbonyl required for the transthioesterification and subsequent acyl transfer. The linear sequences were synthesized according to the standard procedure outlined previously. The final amino acid in the sequence was coupled as an N-Boc-protected residue. The C-terminal DMA protecting group was removed using Pd(0) and phenylsilane. Following TIPS protection of the linker phenol, the C-terminus was pre-activated for 5 min with HATU and Hunig's base in DMF. Addition of thiophenol resulted in 95–100% conversion of carboxylic acid to thioester in 2 h. The thioester peptides were then globally deprotected using 5% H_2O in TFA for 1 h and cleaved from the resin using 365 nm UV irradiation for 1 h. Depending on the identity of the first amino acid residue, peptide thioesters were obtained in 75–99% purity and 66–92% cleavage yield (Table 3). No epimerization of the C-terminal residue was observed.

Table 4
Structures and Yields of Peptide Thioesters

Structure	Purity, %	Cleavage Yield, %
H-Phe-Lys-Ala-Ala-Leu-SPh (**31a**)	99	83
H-Ala-Glu-Phe-Leu-Phe-SPh (**31b**)	99	66
H-Ala-Lys-Phe-Leu-Glu-SPh (**31c**)	75	71
H-Phe-Glu-Ala-Leu-Ala-SPh (**31d**)	99	92

Conclusions

In a quest for a photolabile backbone amide linker for the solid-phase synthesis of cyclic and C-terminally modified peptides, a survey of various photolabile motifs was conducted, establishing the *o*-nitrobenzyl motif as the best candidate. Problems associated with many of the candidate motifs including the nitroveratryl linker led to the development of the 2-hydroxy-4-carboxy-6-nitrobenzyl (Hcnb**)** linker. A convenient and scalable synthesis of a benzaldehyde precursor (**16**) was developed, and general conditions were found for the anchoring of various amino esters to the linker and their acylation to form anchored dipeptides in good yield. Photolysis of the various Hcnb-linked dipeptides (**18a-c)** at 365 nm cleanly deprotected them in good yield in all cases. The linker can be attached to a wide variety of amino acids, can be

selectively removed in the presence of most commonly used protecting groups and tolerates treatment with acid, base and Pd^0. It was found that the Hcnb linker could be conveniently attached to an aminoethyl TG resin using a prolyl-6-aminohexanoic acid spacer, permitting its use in solid phase peptide synthesis. The ability of the Hcnb linker to facilitate on-resin, head-to-tail cyclization of peptides was demonstrated through the synthesis of three cyclic peptides. The utility of Hcnb to synthesize C-terminal thioesters was also demonstrated. We believe that this new photolabile linker should provide a valuable tool for the synthesis of cyclic and modified peptides, depsipeptides and peptidomimetics such as oligoureas (Boeijen 1999; Boeijen 2001).

Declarations

Competing Interests.

The authors declare that they have no competing interests, financial or non-financial, that are related to the work in this publication.

Author Contribution

K. K., M.N. and M.L. wrote the main manuscript text. SSK, M.N. and K.K. prepared the figures. All authors reviewed the manuscript.

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Tables

Tables 1 to 3 are available in the Supplementary Files section.

Figures

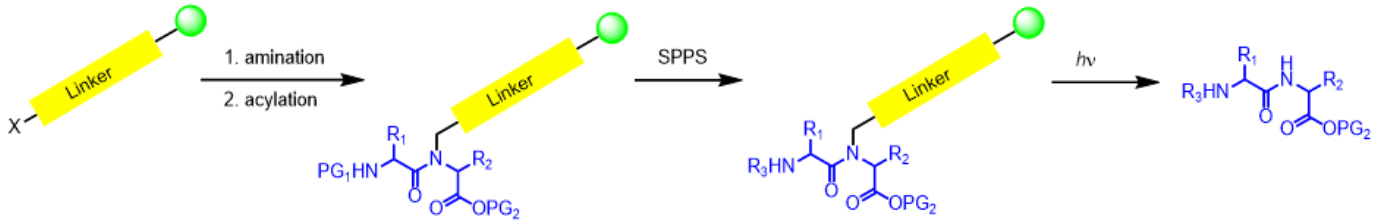

Figure 1

General concept of amino acid anchoring and photolytic deprotection

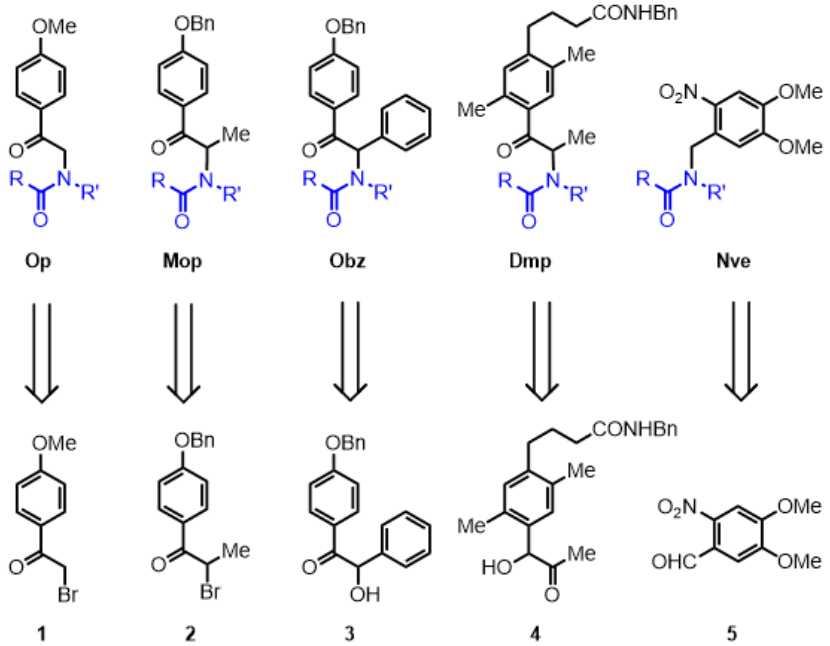

Figure 2

Candidate photolabile backbone amide linkers and their synthetic precursors

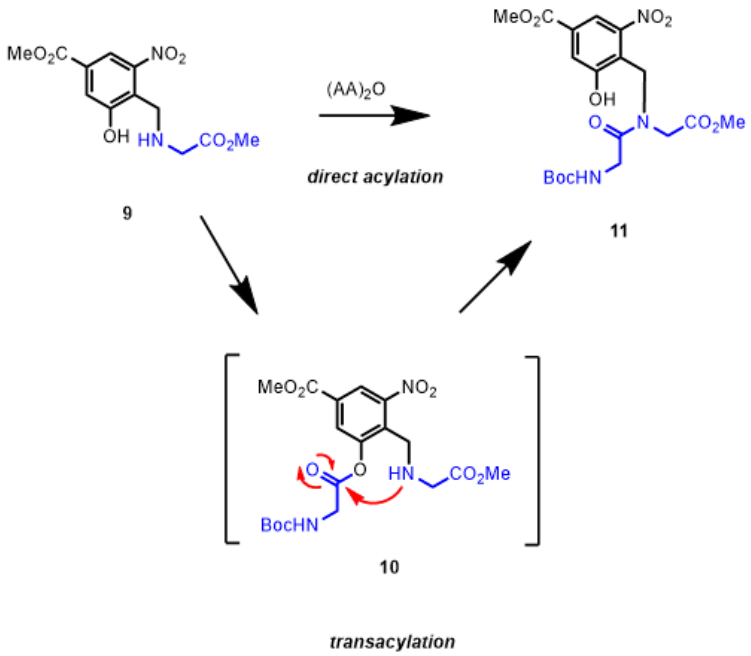

Figure 3

Transacylation motif in the Hcnb linker

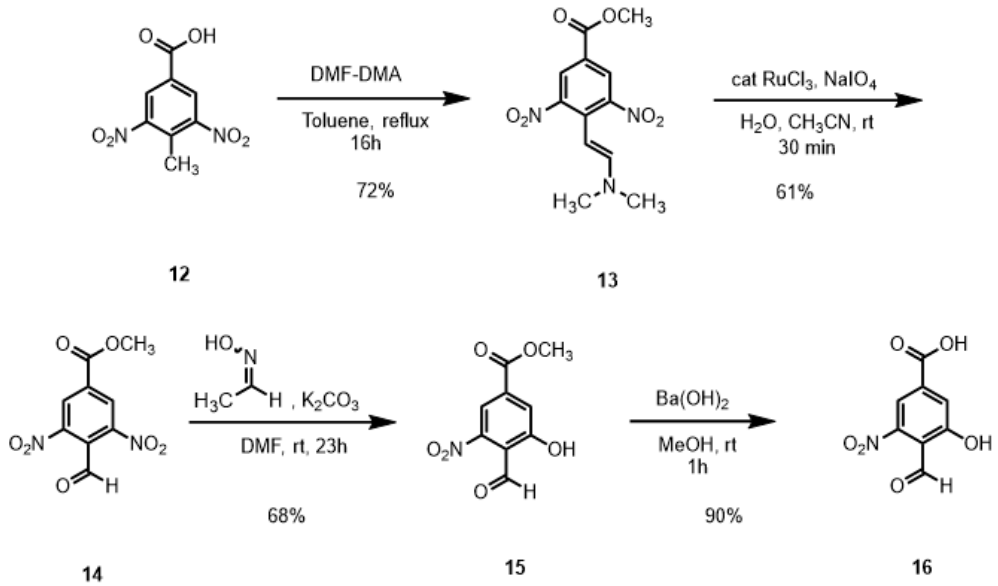

Figure 4

Preparation of Hnca linker **16**

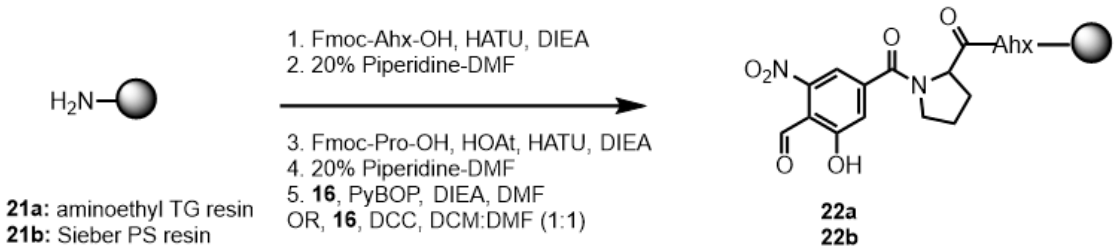

Figure 5

Preparation of resin-bound Hcna linkers **22a-b**

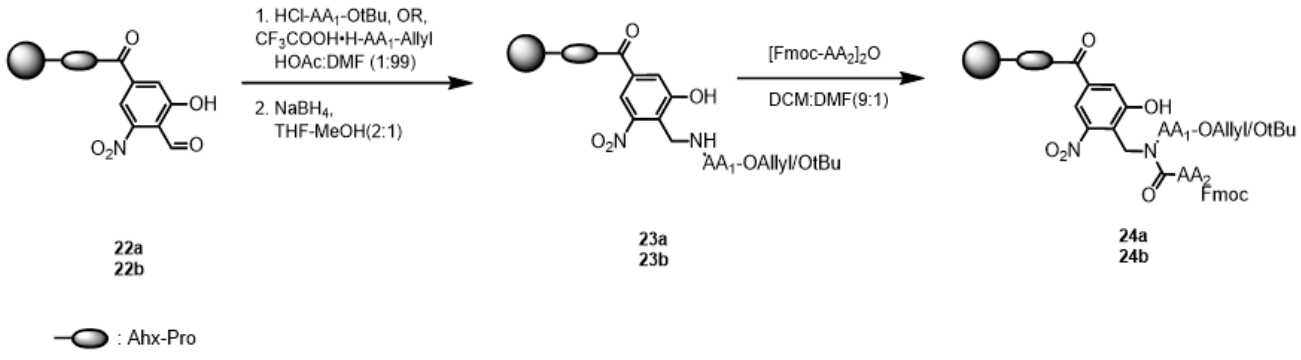

Figure 6

Addition of first and second amino acid residues to the Hcna linker

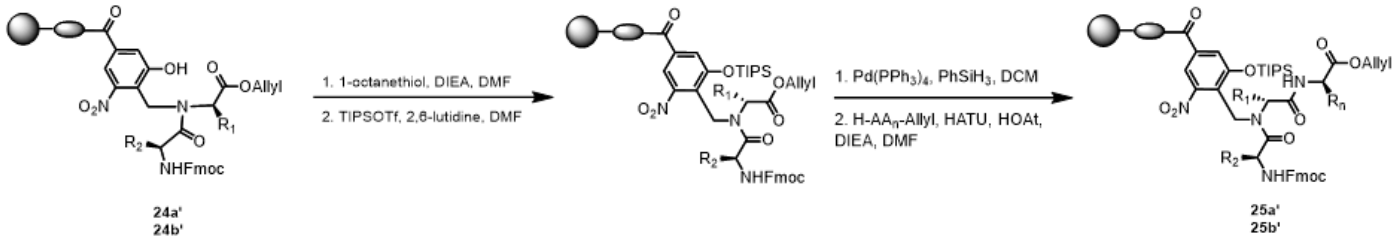

Figure 7

The Fmoc-half cycle to synthesize tripeptides and avoid diketopiperazine formation

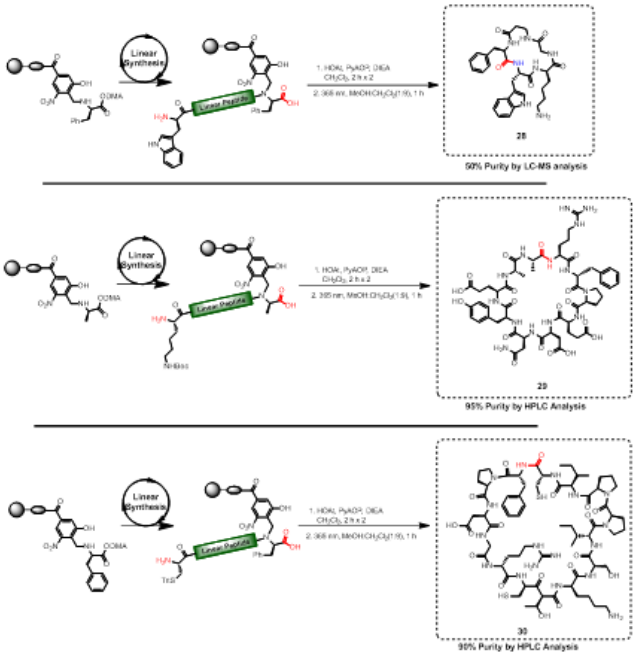

Figure 8

Cyclization of 5-, 10-, and 14- residue cyclic peptides

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformationAPhotolabileBackboneAmideLinkerfortheSolidPhaseSynthesisofCyclicPeptidesandCTerminalThioesters.docx
- Tables.docx