Research Article

Journal of PeptideScience

Received: 8 November 2009 Revised: 11 January 2010 Accepted: 15 January 2010 Published online in Wiley Interscience: 1 March 2010

(www.interscience.com) DOI 10.1002/psc.1218

Novel method for the synthesis of urea backbone cyclic peptides using new Alloc-protected glycine building units

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Cyclization of bioactive peptides, utilizing functional groups serving as natural pharmacophors, is often accompanied with loss of activity. The backbone cyclization approach was developed to overcome this limitation and enhance pharmacological properties. Backbone cyclic peptides are prepared by the incorporation of special building units, capable of forming amide, disulfide and coordinative bonds. Urea bridge is often used for the preparation of cyclic peptides by connecting two amine functionalized side chains. Here we present urea backbone cyclization as an additional method for the preparation of backbone cyclic peptide libraries. A straightforward method for the synthesis of crystalline Fmoc-N^{α} [ω-amino(Alloc)-alkyl] glycine building units is presented. A set of urea backbone cyclic Glycogen Synthase Kinase 3 analogs was prepared and assessed for protein kinase B inhibition as anticancer leads. **Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: urea backbone cyclization; solid phase peptide synthesis; cyclic peptides; AGBU

Introduction

Linear peptides have a number of drawbacks as drug candidates, mainly the lack of important pharmacological properties such as metabolic stability and oral bioavailability. Many local and global modifications have been developed to improve the pharmacological properties of active peptides. Cyclization is one of the most common strategies used to prepare peptide-based drugs. Cyclization of natural bioactive peptides is not always feasible, either due to lack of suitable side chain functionalities for bond formation or because the functional groups are crucial for the bioactivity [1,2]. The backbone cyclization methodology [3] overcomes these limitations by conferring long-range conformational constraint upon the peptide backbone with minimal alterations to the native peptide sequence. Ring closure is achieved by artificial, functionalized alkyl spacers anchored to the peptide backbone that can either be covalently connected to another appropriate functionalized spacer, to a functional amino acid side chain on the peptide or to the *N*- or *C*-terminus of the peptide. Cyclization can thus be accomplished without changing the original sequence required for bioactivity. Restricting the conformational space of peptide structures by cyclization usually results in inactive peptides; thus, libraries should be screened to increase the probability of finding a backbone cyclic peptide that overlaps with the bioactive conformation of the original peptide [4–7]. Active backbone cyclic peptides discovered by library screening have been proven to possess enhanced selectivity [8], potency [9], bioavailability and metabolic stability compared to linear peptides [10,11].

Conformational libraries of backbone cyclic peptides can be prepared by varying four parameters: the mode of cyclization, ring position, ring size and ring chemistry. Ring chemistry is defined by the type of chemical functions that exist on the bridge, the chemistry of the bond used for cyclization and the position of these chemical entities on the bridge. Previous studies have shown that the chemical bond used for cyclization can affect the conformation and bioactivity of backbone cyclic peptides [12,13]. Extensive work has been dedicated to establishing new methods for the bridge bond formation of cyclic peptides. Novel approaches for bond formation such as metal cyclization [14,15] and metathesis mediated cyclization [16,17], in addition to amide [18] and disulfide bridge [19–24] have been used to synthesize new classes of biopharmaceutical cyclic peptides. Many of these methods have been adapted for the preparation of biologically relevant backbone cyclic peptides, e.g. amide [9], Azo [25], disulfide [8], metathesis [13,26,27] and metal ion complexation [25].

Urea bridge formation [28,29] was reported for the preparation of cyclic analogs of Enkephalin in solution and in SPPS [30,31] utilizing amino side chains (e.g. lysine, ornithine). In addition, we recently introduced a new procedure for the synthesis of urea macrocycles on a solid support [32]. This procedure enables straightforward formation of cyclic urea from two free amines anchored to the resin. Here we present urea backbone cyclization

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Abbreviations used: *EGTA, ethylene glycol tetraacetic acid; LTQ, linear trap quadrupole; IUPAC-IUB, International Union of Pure and Applied Chemistry-International Union of Biochemistry.*

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as a new bond formation method for the preparation of backbone cyclic peptides. In this method, the urea bridge is formed between two *N*-alkylated glycine derivatives bearing an amino moiety on the alkyl chain. The urea backbone cyclization approach is an alternative to standard amide backbone cyclization. It can be used to increase the diversity of backbone cyclic peptides by alteration of the bridge chemistry and the position of the urea bond in the bridge.

Improved procedures for the large-scale preparation of Fmoc-[*N*-(Alloc)ω-aminoalkyl]glycine building units [called herein alloc glycine buiding units (AGBU)] and their incorporation into peptide sequence using standard Fmoc SPPS protocols are also described.

Persistently activated protein kinase B (PKB/Akt) is associated with many human cancers, such as breast, colon, ovary, pancreas, head and neck, and prostate [33]. Therefore, PKB/Akt is considered an attractive candidate for targeted cancer therapy. The use of substrate mimetic peptides is an appealing strategy for the inhibition of protein kinases [34,35]. Recently, a series of peptides derived from the PKB/Akt substrate protein Glycogen Synthase Kinase 3 (GSK3) were synthesized and their interaction with PKB was studied [36], leading to the discovery of a selective PKB/Akt inhibitor, PTR6154 (Arg-Pro-Arg-Nva-Tyr-Dap-Hol). The urea backbone cyclization method was applied to the preparation of four cyclic peptides based on PTR6154. The compounds were screened by evaluating the *in vitro* inhibitory effect of the cyclic peptides on PKB/Akt function.

Results and Discussion

Synthesis of Alloc Glycine Building Units

N^{α} (ω-functionalized alkylated) glycine derivatives bearing orthogonal protected amino moieties are used as building blocks for the synthesis of backbone cyclic peptides [12], peptoids [37–39], pseudopeptides [40], and in the synthesis of peptide nucleic acids (PNA) [41,42]. Glycine derivatives of the type Fmoc-N^{α} [ω-amino(Boc)-alkyl] glycine are commercially available but cannot be used for the synthesis of backbone cyclic peptides using standard SPPS protocols.

The allyloxycarbonyl group (Alloc) has been widely used for the protection of primary amines [43] and has proven to be orthogonal to the removal conditions of both Fmoc and Boc groups. We report a procedure for gram scale synthesis of a variety of AGBU, suitable for the preparation of backbone cyclic peptides using standard Fmoc SPPS.

AGBU (**3a–d**) (Scheme 1) were synthesized in three steps. The procedure was based on mono Alloc protection of diamines, followed by reductive alkylation in the presence of glyoxylic acid and *in situ* Fmoc protection.

Mono Alloc-protected diamines **1a–d** were obtained by treating allylchloroformate (Alloc-Cl) with a tenfold excess of the corresponding diamine (Scheme 1). Reductive alkylation of mono Alloc-protected diamines **1a–d** was performed by the simultaneous addition of 0.85 molar equivalent of $NaCNBH_3$ and glyoxylic acid to yield **2a–d**. The unprotected N^{α} [ω-amino(Alloc)-alkyl] glycine **2a–d** were not isolated, and the reaction mixture was treated with 0.75 molar equivalent of Fmoc-OSu to produce AGBU **3a–d**. We found that using sub-equivalent amounts of reagents (glyoxylic acid and Fmoc-OSu) significantly increased product purity and avoided tedious purification workup. The final products, **3a–d**, were obtained as sticky colorless pastes [over 80% purity as determined by high pressure liquid chromatography (HPLC)]. Handling of these oily AGBU was inconvenient for standard peptide synthesis.

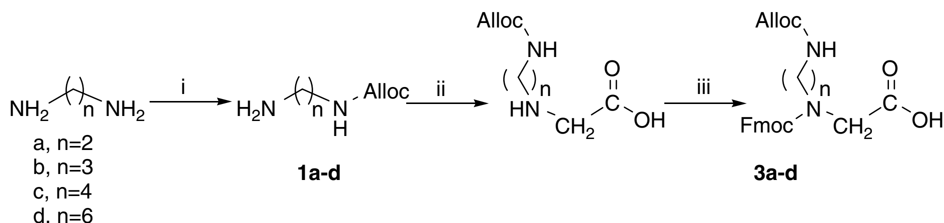
Crystallization protocols were developed to obtain **3a–d** as granular powders, in an attempt to increase the yield and purity of backbone cyclic peptides. Crude **3a–c** were recrystallized from boiling toluene, yielding sticky white pellets that gave white powder after extensive washing with petroleum ether. Crystallization of **3d** was achieved by slow addition of hexane to a toluene solution at room temperature. These protocols yielded **3a–d** as pure compounds (over 95% purity as determined by HPLC) that proved convenient for handling and storage. This procedure presents a general method for large-scale synthesis of amino-alkylated glycine derivatives using commercially available starting materials.

To demonstrate the usefulness of this procedure, we synthesized four backbone cyclic peptides by standard Fmoc SPPS protocols using the crystallized AGBU.

Design and Synthesis of the YM Analogs

In amide backbone cyclization, the cyclization can be performed either by connecting a dicarboxylic linker to the amino terminus of the peptide [9,10,12,25], which prevents peptide elongation beyond the point of cyclization, or by the use of an additional set of allyl ω-carboxy building units, appropriate for Fmoc SPPS protocols [44]. Urea backbone cyclization can overcome these limitations by incorporating two AGBU in the peptide sequence.

In order to mimic the inhibitory effect of PTR6154, four urea backbone cyclic analogs of *c*(YM3-m) were designed. The proline residue (cyclic *N*-alkylated amino acid) in PTR6154 was substituted by an AGBU (*N*-alkylated glycine moiety) that allowed both peptide elongation and cyclization. An additional AGBU was introduced at the carboxy terminus. All four compounds have the same sequence but differ in the bridge size. Previous studies indicated that the amino terminus plays a crucial role in PKB/Akt inhibition (Tal-Gan *et al*., in preparation), thus the amino terminus was protected throughout the cyclization step and the Fmoc protecting group was removed only in the final step of the peptide synthesis, before cleavage of the peptides from the resin.



Scheme 1. Synthesis of AGBU. Conditions: (i) Allylchloroformate; (ii) Glyoxylic acid, $NaCNBH_3$; (iii) Fmoc-OSu, Et_3N.

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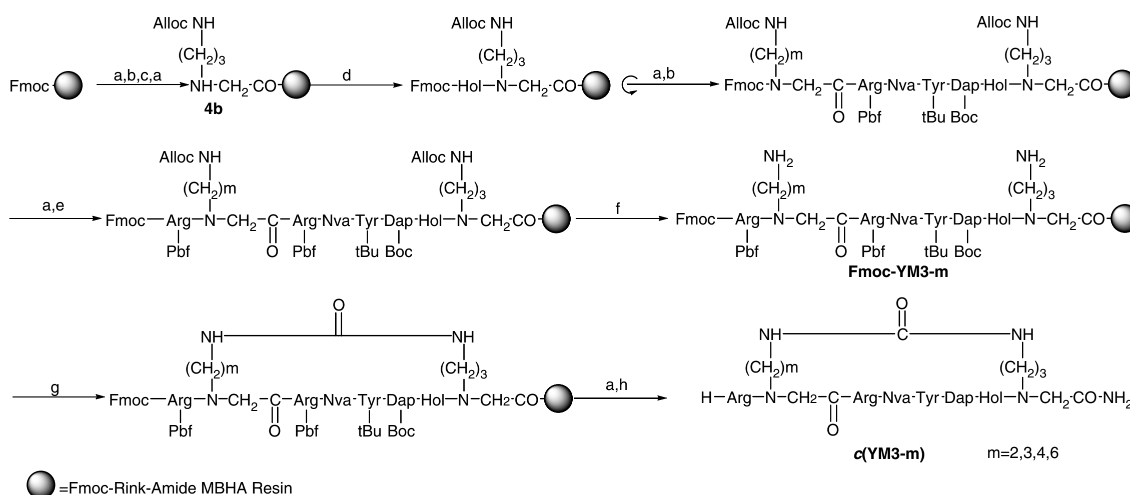

Scheme 2. Synthesis of the *c*(YM3-m) compounds. Nomenclature, in the general name, *c*(YM3-m), m stands for the number of methylenes in the alkyl chain of the AGBU at position 2 and *c* represents a cyclic peptide while the absence of c indicates a pre-cyclic peptide. Reagents and conditions: a, 20% piperidine; b, Fmoc-AA-OH, HBTU, HOBt, DIPEA; c, Ac_2O,DIPEA; d, Fmoc-Hol-OH, HATU, HOAt, DIPEA; e, Fmoc-Arg(Pbf)-OH, HATU, HOAt, DIPEA; f, $(PPh_3)_4Pd(0)$, *N*-methylmorpholine, AcOH; g, BTC, DIPEA; h, TFA, Triisopropylsilane, TDW.

Generally, coupling to a secondary amine by standard HBTU mediated procedures results in incomplete attachment. Previously, effective coupling of Fmoc amino acids to secondary amines was achieved by the use of bis(trichloromethyl) carbonate, (triphosgene, BTC) to form the Fmoc amino acid chlorides [45]. To ensure reaction completion, elevated temperatures and long reaction times were also used [9,25,45].

For the synthesis of the YM analogs, standard Fmoc protocols on solid support [44] were used as shown in Scheme 2. All coupling steps were executed using HBTU as the standard coupling reagent, except coupling of Fmoc amino acids to the secondary amine of AGBU, which was performed using HATU. HATU is more reactive than HBTU and allows coupling in highly hindered peptide sequences [46]. We managed to reduce the excess of reagents and HATU to 1.5 equivalents without reducing the coupling yields. To ensure complete conversion, longer coupling periods were used. After the attachment of Fmoc-Arg(Pbf), the resin was divided into four portions, and different AGBU (**3a–d**) were introduced to form four peptides. Simultaneous removal of the orthogonal Alloc protecting groups from both AGBU yielded the desired pre-cyclic peptidyl resin with two *N*-alkylated omega amines. Optimization of the cyclization step forming urea bridge was studied on a model peptide and was implemented for all four peptides.

Backbone Urea Cyclization

The N^{α} Fmoc-protected pre-cyclic peptide, Fmoc-YM3-3, was selected as a model for optimization of the urea cyclization conditions. The efficiency of several carbonyl donating reagents, namely phosgene, BTC and dipentafluorophenyl carbonate (PFPC), for urea cyclization was compared. In addition, the effect of the consecutive base addition approach previously described [32] on urea backbone cyclization was evaluated. The reaction progress was monitored using HPLC, and product formation was confirmed using mass spectrometry. Three batches of 50 mg resin, loaded with Fmoc-YM3-3, were swelled in dichloromethane and were introduced with each of the above reagents. Samples from all three batches were collected prior to the addition of the base. Small portions of peptidyl resin were cleaved and samples were analyzed. We observed a major difference between PFPC and the two phosgene derivatives. PFPC converted the pre-cyclic peptide to urea cyclic peptide almost completely before the addition of base, while phosgene and BTC led only to partial conversion before the addition of the base (Figure 1(A–C)). This observation reaffirms the assumption that the first step in the mechanism of phosgene and BTC mediated urea cyclization involves the formation of *N*-alkylated omega ammonium chloride salt. A second portion of peptidyl resin was sampled 1 h after adding the base. HPLC indicated that the base addition contributed to the complete conversion to the urea cyclic form only in the BTC and phosgene mediated procedures (Figure 1(D–F)). There was a decrease in product purity in the PFPC mediated urea cyclization compared to BTC or phosgene mediated procedure. We decided to use the BTC urea cyclization procedure as BTC is easier to handle than phosgene. For *c*(YM3-m), more than 80% conversion to the urea form was obtained for all peptides (Table 1). Urea cyclic peptides were cleaved after Fmoc removal and purified using HPLC. Analytical HPLC and high resolution mass spectrometry were used to characterize the isolated compounds (Figure 2).

PKB/Akt Model

All backbone cyclic peptides were screened for inhibition of PKB/Akt in a cell-free radioactive assay, and compared with PTR6154 (Table 1). *c*(YM3-4) had poor anti PKB/Akt activity while the other peptides preserved anti PKB/Akt potency. *c*(YM3-2) and *c*(YM3-3) inhibited PKB/Akt activity in the same manner as the parent peptide (0.92 and 0.59 μM, respectively). *c*(YM3-6) significantly improved the inhibitory effect compared to the parent peptide (0.16 μM). The concept of conformational screening was demonstrated in this study of four peptides, which differ by gradual increase of ring size.

Methods

The following abbreviations were used throughout the text. The abbreviations for amino acids are according to the IUPAC-IUB Commission of Biochemical Nomenclature, http://www.chem.qmul.

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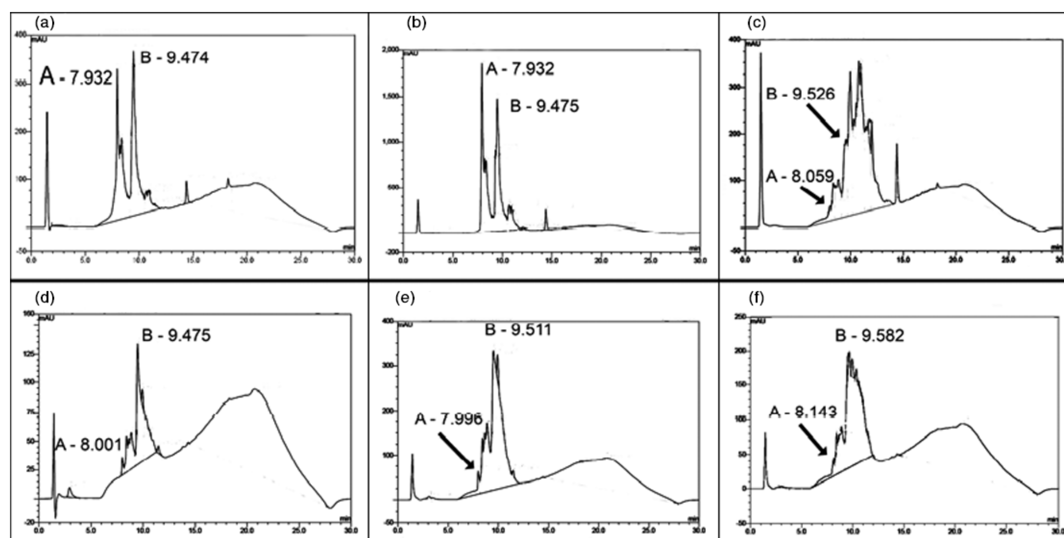

Figure 1. HPLC monitoring of urea cyclization optimization for YM3-3: (a) BTC 2 h (b) Phosgene 2 h (c) PFPC 2 h (d) BTC + DIPEA 1 h (e) Phosgene + DIPEA 1 h (f) PFPC + DIPEA 1 h. Peak A represents the pre-cyclic peptide. Peak B represents urea backbone cyclic peptide.

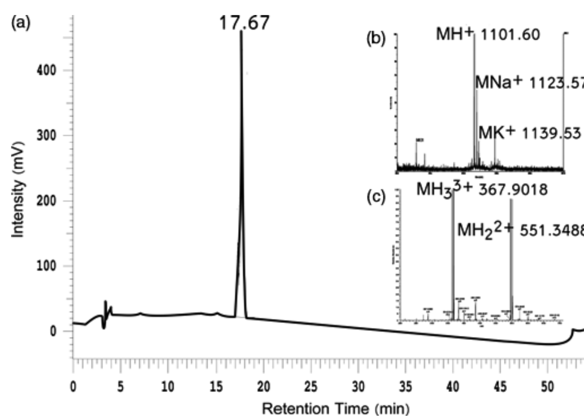

Figure 2. Characterization of *c*(YM3-6): (a) analytical HPLC; (b) MALDI-TOF MS: MH^+, MNa^+, MK^+ observed; (c) HRMS: MH_2^{2+}, MH_3^{3+} observed.

ac.uk/iupac/AminoAcid/. ACN, acetonitrile; Alloc, allyloxycarbonyl; Boc, *t*-butoxycarbonyl; BTC, bis(trichloromethyl)carbonate; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ESI, electrospray ionization; EtOAc, ethyl acetate; Fmoc, 9-fluorenyl methyloxycarbonyl; Fmoc-OSu, 9-fluorenylmethyloxycarbonyl-*N*-hydroxysuccinimide; HATU, [2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]; HBTU, [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]; HOAt, 1-hydroxy-7-aza-benzotriazole; HOBt,1-hydroxybenzotriazole; HRMS, high resolution mass spectrometry; MALDI, matrix assisted laser desorption ionization; MBHA, methylbenzhydrylamine; NMR, nuclear magnetic resonance; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PFPC, pentafluorophenyl carbonate; RP-HPLC, reverse phase high pressure liquid chromatography; SPPS, solid phase peptide synthesis; tBu, *tert*-butyl; TDW, tri-distilled water; TFA, trifluoroacetic acid; TLC, thin layer chromatography; TOF, time of flight.

Chemistry – General

All starting materials were purchased from commercial sources and were used without further purification. Routine ^1H NMR and ^{13}C NMR spectra were acquired on Bruker 300, Bruker 400 or Bruker 500 MHz spectrometer. Chemical shifts are reported downfield, relative to internal solvent peaks. Coupling constants *J* are reported in Hertz. HRMS spectra were recorded on nanospray ionization LTQ orbitrap. MALDI-TOF spectra were recorded on a PerSeptive Biosystems MALDI-TOF MS, using α-cyano-4-hydroxycinnamic acid as matrix. TLC plates used were aluminum sheets silica gel 60 F_{254}. Column chromatography was performed on silica gel 60 (230–400 mesh).

All analytical HPLC were recorded at 220 nm at a flow of 1 ml/min on (A) RP-18 column (5 µ 75 × 4.6 mm), eluents A (0.1% TFA in TDW) and B (0.1% TFA in ACN) were used in a linear gradient (95% A → 5% A in 12 min) or (B) RP-18 column (5 µ 250 × 4.6 mm, 110 Å), eluents A (0.05% TFA in TDW) and B (ACN) were used in a linear gradient (95% A → 5% A in 35 min). Preparative HPLC were recorded at 220 nm on RP-18 column (10 µ 250 × 10 mm, 110 Å), eluents A (0.05% TFA in TDW) and B (ACN) were used in a linear gradient of (C) (85% A → 65% A in 25 min) at a flow rate of 5 ml/min.

General Procedure for the Preparation of Carbamates 1a–d

A solution of 1 mol alkylene diamine dissolved in 500 ml chloroform was stirred and cooled to 0 °C. A solution of 0.1 mol allylchloroformate in 250 ml chloroform was added dropwise

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Table 1. Characterization and IC_{50} of the *c*(YM3-m) analogs

Peptide name	m	Ring size	Calculated mass $[MH_2^{2+}]$	Observed mass $[MH_2^{2+}]/2$	Percentage urea conversion (HPLC)	Percentage purity (HPLC)	IC_{50} (μM) (95% confidence)
PTR6154							0.94 (0.78–1.14)
c(YM3-2)	2	27	1046.6450	523.3220	79	>95	0.92 (0.58–1.45)
c(YM3-3)	3	28	1060.6607	530.3303	86	>95	0.59 (0.48–0.73)
c(YM3-4)	4	29	1074.6763	537.3373	>95	>95	4.0 (2.8–5.7)
c(YM3-6)	6	31	1102.7076	551.3488	90	>95	0.16 (0.03–0.77)

Observed mass was determined by HRMS. Purity was determined by analytical HPLC method B. Urea conversion was determined by preparative HPLC method C. IC_{50} of PKB inhibition was determined according to radioactive kinase assay as described [48]. IC_{50} values and 95% confidence range (parenthesis) were determined using Graphpad Prism 5.

over 3 h and the reaction mixture was stirred overnight at room temperature. For **1a–c**, the organic layer was washed with water until no trace of starting material was apparent in the TLC (32 : 8:1 CH_2Cl_2–MeOH–AcOH), dried over Na_2SO_4 and solvent was evaporated to yield colorless oil. For **1d**, the crude was extracted with aqueous HCl 1 M solution followed by the addition of NaOH 4 M until pH >8. The solution was extracted with EtOAc, dried over Na_2SO_4 and evaporated to yield a clear oil of **1d**. The resulting crude **1a–d** proved pure enough for further use. When higher purity is needed, the products can be purified using flash chromatography (20% MeOH in CH_2Cl_2).

Allyl 2-aminoethylcarbamate 1a

Yield: 51%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.60–1.75 (br, 2H), 2.59–2.94 (t, J = 5.6, 2H), 2.99–3.38 (q, J = 5.8, 2H), 4.36–4.66 (d, J = 5.1, 2H), 5.02–5.36 (m, 2H), 5.35–5.68 (br, 1H) 5.73–6.03 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 41.4, 43.2, 65.4, 117.5, 132.8, 156.5.

Allyl 3-aminopropylcarbamate 2a

Yield: 58%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.55–1.63 (m, 2H), 1.64–1.69 (br, 2H), 2.68–2.79 (t, J = 6.6, 2H), 3.16–3.29 (m, 2H), 4.44–4.58 (d, J = 5.1, 2H), 5.10–5.29 (m, 2H), 5.36–5.58 (br, 1H), 5.79–5.94 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 32.7, 38.9, 39.5, 65.2, 117.3, 132.9, 156.3.

Allyl 4-aminobutylcarbamate 1c

Yield: 80%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40–1.80 (br, 6H), 2.55–2.72 (t, J = 6.6, 2H), 3.0–3.2 (q, J = 5.6, 2H), 4.48–4.53 (d, J = 5.4, 2H), 5.1–5.3 (m, 2H), 5.7–5.8 (br, 1H), 5.8–6.0 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 27.2, 30.4, 40.7, 41.5, 65.2, 117.3, 132.9, 156.2.

Allyl 6-aminohexylcarbamate 1d

Yield: 75%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.18–1.36 (m, 4H), 1.37–1.53 (m, 4H), 1.70–1.97 (br, 2H), 2.54–2.78 (t, J = 7.0, 2H), 3.03–3.22 (m, 2H), 4.39–4.62 (m, 2H,), 4.65–4.85 (br, 1H), 4.9–5.1 (br, 1H), 5.1–5.34 (m, 2H), 5.78–6.00 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 26.45, 26.50, 29.9, 33.3, 40.9, 42.0, 65.6, 117.8, 133.3, 156.5.

General Procedure for the Preparation of *N*-Fmoc-[*N*-(Alloc) *ω*-alkyl]glycine 3a–d

A stirred solution of 0.1 mol of **1a–d** and 0.085 mol $NaCNBH_3$ dissolved in 200 ml MeOH was cooled to 0 °C. A measured quantity of 0.085 mol of glyoxylic acid monohydrate (0.85 equiv) was added in portions and the reaction was stirred overnight at room temperature. Solvent was evaporated to yield an oily residue of **2a–d**. Crude **2a–d** was dissolved in 260 ml water followed by the addition of 0.17 mol of Et_3N and set to stir at room temperature until the solution became clear. A solution of 0.075 mol Fmoc-OSu (0.75 equiv in relation to **1a–d**) in 400 ml ACN was added to the reaction mixture. The reaction was stirred for 4 h while the pH was kept alkaline by adding Et_3N if necessary. The reaction mixture was washed with petroleum ether (3 × 250 ml) and ether: petroleum ether 7 : 3 (3 × 250 ml). The aqueous layer was cooled and acidified to pH 3–4 with HCl 2 M, and extracted with EtOAc (4 × 250 ml). The organic layer was washed with HCl 1 M (2 × 150 ml), saturated $KHSO_4$ (2 × 150 ml) and dried over Na_2SO_4. Solvent was evaporated to yield colorless oil that later solidified upon cooling to give a sticky white paste. Products **3a–c** were recrystallized from refluxing toluene to yield a white paste, which was collected and washed extensively with cold petroleum ether to give a white powder. Product **3d** was recrystallized from a mixture of toluene and *n*-hexane. The mixture was left to crystallize overnight at −20 °C and the crystals were collected and washed extensively with cold petroleum ether to give white powder.

N-Fmoc-[*N*-(Alloc)-aminoethyl]glycine 3a

Yield: 80%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.9–3.1 (br, 1H), 3.15–3.25 (br, 1H), 3.25–3.35 (br, 1H), 3.25–3.60 (br, 1H), 3.8–3.9 (s, 1H), 3.9–4.0 (s, 1H), 4.1–4.3 (m, 1H), 4.4–4.7 (br, 4H), 5.0–5.3 (m, 2H), 5.7–5.9 (m, 1H), 7.27–7.46 (m, 2H), 7.5–7.6 (m, 2H), 7.65–7.80 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 46.49 (46.55), 47.2 (47.7), 48.7 (49.0), 64.2 (64.25), 67.2, 116.78 (116.85), 120.1, 125.1, 127.10 (127.15), 127.6, 133.6, 140.5 (140.7), 143.6 (143.7), 155.4 (155.6), 155.9, 171.0 (171.2). ES–MS mass calculated for $C_{23}H_{24}N_2O_6$ 425.16 (MH^+). Found 425.27. HRMS (NSI-orbitrap) calculated for $C_{23}H_{24}N_2NaO_6$ 447.1532 (MNa^+). Found 447.1528. HPLC purity >99% room temperature 12.58.

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N-Fmoc-(*N*-(Alloc)3-aminopropyl)glycine 3b

Yield: 74%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.35–1.45 (br, 1H), 1.55–1.75 (br, 1H), 2.85–2.95 (br, 1H), 2.95–3.11 (br, 1H), 3.11–3.25 (br, 1H), 3.25–3.45 (br, 1H), 3.8–3.9 (s, 1H), 3.9–4.0 (s, 1H), 4.0–4.2 (m, 1H), 4.3–4.8 (br, 4H), 5.1–5.4 (br, 2H), 5.8–6.1 (m, 1H), 7.27–7.45 (br, 4H), 7.45–7.6 (m, 2H), 7.7–7.8 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.8 (28.1), 37.7 (37.8), 45.6 (45.9), 46.57 (46.61), 48.4 (48.8), 64.1, 66.6, 67.0, 115.8, 119.97 (120.02), 124.79 (125.03), 127.1, 133.7, 140.6 (140.7), 143.6 (143.8), 155.34 (155.48), 155.8, 170.9 (171.2). HRMS (NSI-orbitrap) calculated for $C_{24}H_{26}N_2O_6$ 439.1791 (MH^+). Found 439.1868. HPLC purity >99% room temperature 12.70.

N-Fmoc-[*N*-(Alloc)4-aminobutyl]glycine 3c

Yield: 60%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.1–1.3 (br, 2H), 1.4–1.6 (br, 1H), 2.9–3.5 (br, 4H), 3.8–3.9 (s, 1H), 3.9–4.0 (s, 1H), 4.15–4.25 (m, 1H), 4.35–4.65 (br, 4H), 5.1–5.4 (m, 4H), 5.85–6.1 (m, 1H), 7.27–7.45 (m, 4H), 7.45–7.65 (m, 2H), 7.65–7.8 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 25.1 (25.5), 27.11 (27.23), 47.15 (47.26), 47.92 (48.22), 48.68 (49.08), 64.59, 66.97 (61.4), 117.28, 120.55, 125.20 (125.60), 127.50 (127.53), 128.01 (128.13), 134.34, 141.14 (141.36), 144.27 (144.46), 155.98, 156.39, 1s71.42 (171.70). HRMS (NSI-orbitrap) calculated for $C_{25}H_{28}N_2O_6$ 453.1947 (MH^+). Found 453.2025. HPLC purity >95% room temperature 12.88.

N-Fmoc-[*N*-(Alloc)6-aminohexyl]glycine 3d

Yield: 58%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.0–1.5 (br, 8H), 3.0–3.2 (m, 3H), 3.2–3.35 (m, 1H), 3.8–3.9 (s, 1H), 3.9–4.0 (s, 1H), 4.1–4.3 (m, 1H), 4.4–4.5 (m, 1H), 4.5–4.65 (br, 3H), 5.1–5.35 (m, 2H), 5.8–6.0 (m, 1H), 7.27–7.45 (m, 4H), 7.5–7.65 (m, 2H), 7.65–7.80 (m, 2H). ^{13}C NMR (125 MHz, CDCl3): δ 26.18 (26.26), 27.5, 28.0, 29.55 (29.71), 40.7 (40.8), 47.1, 48.3 (48.5), 48.9, 65.4, 67.3 (67.6), 117.40 (117.57), 119.82, 124.69 (124.84), 126.9, 127.54 (127.57), 132.82, 141.12 (141.26), 143.8, 155.97, 156.3 (156.5), 173.5 (173.6). HRMS (NSI-orbitrap) calculated for $C_{27}H_{32}N_2O_6$ 503.2158 (MNa^+). Found 503.2153. HPLC purity >95% room temperature 13.50.

General Methods for SPPS

Swelling: The resin was swelled for at least 2 h in CH_2Cl_2. Fmoc removal: The resin was treated with a solution of 20% piperidine in 1-methyl-2-pyrrolidinone (NMP) (2 × 20 min), and then washed with NMP (5 × 2 min). HBTU coupling: Fmoc protected amino acids (1.5 equiv) were dissolved in NMP. *N,N*-DIPEA (1.5 equiv) and 1-hydroxybenzotriazole (HOBt) were added and the mixture was cooled to 0 °C. [2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HBTU) (1.5 equiv) was added and the mixture was preactivated by mixing for 10 min, added to the resin, and shaken for 1 h. The resin was washed with NMP (3 × 2 min). Capping: The resin was treated with a solution of Ac_2O (10 equiv) and DIPEA (7.15 equiv) in dimethylformamide (DMF) for 20 min and washed with NMP (3 × 2 min). HATU coupling: Fmoc protected amino acids (1.5 equiv) were dissolved in NMP, DIPEA (1.5 equiv) and 1-hydroxy-7-aza-benzotriazole (HOAt) were added and the mixture was cooled to 0 °C. [2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU) (1.5 equiv) was added and the mixture was preactivated by mixing for 10 min, added to the resin, and shaken overnight at room temperature. The resin was washed with NMP (3 × 2 min). Alloc removal: The resin was washed with CH_2Cl_2 (2 × 2 min) and dried in vacuum. A solution of 5% AcOH and 2.5% *N*-methyl morpholine in CH_2Cl_2 was added under Ar stream and Tetrakis (triphenylphosphine) palladium (0) (0.7 equiv) was added. The reaction was left to stir in the dark for 2 h and then washed with 0.5% DIPEA in NMP (3 × 5 min), 0.5% sodium diethyldithiocarbamate trihydrate in NMP (5 × 2 min), NMP (2 × 2 min) and CH_2Cl_2 (2 × 2 min). Cleavage: The resin was washed with CH_2Cl_2 (2 × 2 min) and dried under vacuum. A solution of 2.5% TDW and 2.5% triisopropylsilane in trifluoroacetic acid (TFA) was added and the reaction proceeded for 3 h at room temperature. The solution was separated by filtration and the resin was rinsed with neat TFA. The TFA mixture was treated with a cooled solution of ether : hexane 1 : 1 and peptides were precipitated by centrifugation. Crude peptides were dissolved in acetonitrile : TDW 1 : 1 solution and lyophilized.

Urea cyclization BTC-mediated: A solution of BTC, bis(trichloromethyl) carbonate (0.33 equiv) in CH_2Cl_2 was added to the resin and left to stir. After 2 h, DIPEA (2 equiv) was added and the reaction was left to stir overnight at room temperature. The resin was washed with CH_2Cl_2 (2 × 2 min). Urea cyclization PFPC mediated: A solution of PFPC (1 equiv) in CH_2Cl_2 was added to the resin and left to stir. After 2 h, DIPEA (2 equiv) was added and the reaction was left to stir overnight at room temperature. The resin was washed with CH_2Cl_2 (2 × 2 min). Urea cyclization phosgene mediated: A solution of 20% phosgene in toluene (1 equiv phosgene relative to resin) was added to resin preswelled in CH_2Cl_2 and left to stir. After 2 h, DIPEA (2 equiv) was added and the reaction was left to stir overnight at room temperature. The resin was washed with CH_2Cl_2 (2 × 2 min).

c(YM3-2) was prepared from 150 mg of Fmoc-Rink Amide MBHA resin. HPLC purity >95% room temperature (B) 16.48. MS (MALDI-TOF): calculated for $C_{46}H_{81}N_{18}O_{10}{}^{+}$ 1045.6378 (MH^+), found 1045.32. HRMS exact mass (ESI-orbitrap): calculated for $C_{46}H_{82}N_{18}O_{10}{}^{2+}$ 1046.6450, found ($MH_2{}^{2+}$) 523.3220; calculated for $C_{46}H_{83}N_{18}O_{10}{}^{3+}$ 1047.6523, found ($MH_3{}^{3+}$) 349.2176.

c(YM3-3) was prepared from 150 mg of Fmoc-Rink Amide MBHA resin. HPLC purity >95% room temperature (B) 16.39. MS (MALDI-TOF): calculated for $C_{47}H_{83}N_{18}O_{10}{}^{+}$ 1059.6534 (MH^+), found 1058.99. HRMS exact mass (ESI-orbitrap): calculated for $C_{47}H_{84}N_{18}O_{10}{}^{2+}$ 1060.6607, found ($MH_2{}^{2+}$) 530.3303; calculated for $C_{47}H_{85}N_{18}O_{10}{}^{3+}$ 1061.6680, found ($MH_3{}^{3+}$) 353.8897.

c(YM3-4) was prepared from 150 mg of Fmoc-Rink Amide MBHA resin. HPLC purity >95% room temperature (B) 16.29. MS (MALDI-TOF): calculated for $C_{48}H_{85}N_{18}O_{10}{}^{+}$ 1073.6691 (MH^+), found 1073.75. HRMS exact mass (ESI-orbitrap): calculated for $C_{48}H_{86}N_{18}O_{10}{}^{2+}$ 1074.6763, found ($MH_2{}^{2+}$) 537.3373; calculated for $C_{48}H_{87}N_{18}O_{10}{}^{3+}$ 1075.6836, found ($MH_3{}^{3+}$) 358.5612.

c(YM3-6) was prepared from 150 mg of Fmoc-Rink Amide MBHA resin. HPLC purity >95% room temperature (B) 17.67. MS (MALDI-TOF): calculated for $C_{50}H_{89}N_{18}O_{10}{}^{+}$ 1101.7004 (MH^+), found 1101.60. HRMS exact mass (ESI-orbitrap): calculated for $C_{50}H_{90}N_{18}O_{10}{}^{2+}$ 1102.7076, found ($MH_2{}^{2+}$) 551.3488; calculated for $C_{50}H_{91}N_{18}O_{10}{}^{3+}$ 1103.7149, found ($MH_3{}^{3+}$) 367.9018 (Figure 2).

PKB Assay

PKB kinase (HisΔPHPKBEEEFlag) was prepared as described by Klein *et al*. [47] except that for routine screening the enzyme was only partially purified in one step on Ni-NTA agarose (Qiagen) as described [47]. The radioactive kinase assay was as described by Reuveni *et al*. [48] except that the reaction mix comprised 50 mM Hepes pH 7.4, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol,

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10 mM magnesium acetate, 3-μM RPRTSSF peptide, 10 μM γ^{32}P-ATP (1 μCi/assay well), the inhibitory compound and 0.005 units HisΔPHPKBEEEFlag. A stock solution of each peptide was prepared and the concentration was determined by a UV spectrophotometer as described [49]. For initial screening, compounds were tested at three to four concentrations between 20 and 0.8 μM. Compounds that showed significant inhibition at 1 μM or less were retested and IC_{50} values and 95% confidence range were determined using Graphpad Prism 5 (Table 1). PTR6154 was included in every assay, as a standard.

Conclusions

There is a demand for chemical procedures that enable more extensive screening of backbone cyclic peptides. We established and optimized a new BTC mediated urea cyclization procedure that can be used for the formation of urea backbone cyclic peptides. This procedure allowed efficient connection of two relatively distant amines through a backbone cyclic urea bridge on solid support. The method was validated by synthesizing four compounds of PKB/Akt substrate mimetics. Urea cyclization was used to produce a backbone cyclic peptide that inhibited PKB/Akt in the nano-molar range *in vitro* and improved the activity of the parent peptide by almost tenfold. In addition, an improved and efficient procedure for the preparation of a set of crystalline orthogonally protected N^{α} (ω-functionalized alkylated) glycines was developed. These building units were later incorporated into the peptide using standard Fmoc SPPS protocols. Three reagents, phosgene, PFPC and BTC were assessed as the urea bridge formation agent. BTC, which is relatively easy to store and handle, was found to be an efficient reagent.

The methodology described here is general and is based on a small set of building blocks. This approach can be used to extend the diversity of backbone cyclic peptides and to increase the probability of finding potent backbone cyclic peptides against various targets.

The synthetic improvements presented here will facilitate the use of backbone cyclization by nonspecialist scientists, so that this methodology may become a powerful tool in peptide-based drug discovery research.

Acknowledgements

A.L. was supported by grants from the Prostate Cancer Foundation (PCF, USA) and The Goldhirsh Foundation (USA).

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