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ORIGINAL ARTICLE

Solid-phase route to Fmoc-protected cationic amino acid building blocks

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Abstract Diamino acids are commonly found in bioactive compounds, yet only few are commercially available as building blocks for solid-phase peptide synthesis. In the present work a convenient, inexpensive route to multiple-charged amino acid building blocks with varying degree of hydrophobicity was developed. A versatile solid-phase protocol leading to selectively protected amino alcohol intermediates was followed by oxidation to yield the desired di- or polycationic amino acid building blocks in gram-scale amounts. The synthetic sequence comprises loading of (*S*)-1-(*p*-nosyl)aziridine-2-methanol onto a freshly prepared trityl bromide resin, followed by ring opening with an appropriate primary amine, on-resin N^{β}-Boc protection of the resulting secondary amine, exchange of the N^{α}-protecting group, cleavage from the resin, and finally oxidation in solution to yield the target γ-aza substituted building blocks having an Fmoc/Boc protection scheme. This strategy facilitates incorporation of multiple positive charges into the building blocks provided that the corresponding partially protected di- or polyamines are available. An array of compounds covering a wide variety of γ-aza substituted analogs of simple neutral amino acids as well as analogs displaying high bulkiness or polycationic side chains was prepared. Two building blocks were incorporated into peptide sequences using microwave-assisted solid-phase peptide synthesis confirming their general utility.

Keywords Aziridine · Cationic amino acids · Hydrophobic amino acids · Peptides · Solid-phase synthesis

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Introduction

Traditionally, native peptides have been deemed less appealing as pharmaceuticals due to their inherent drawbacks: (1) short duration of action caused by low in vivo stability and fast renal clearance, (2) lack of oral bioavailability, (3) difficult and costly synthesis (Nestor 2009; Otvos 2008), or (4) inefficient recombinant expression. Nevertheless, in recent years, peptide-based drugs have attracted considerable attention as improved synthetic methods and medicinal chemistry advances have alleviated many of their shortcomings. Stability towards proteolysis may be achieved by e.g., incorporation of unnatural amino acids, backbone modification or N-terminal acylation (Bellmann-Sickert et al. 2011). Further, PEGylation and conjugation to fatty acids have proved beneficial for a prolonged action due to slow release from subcutaneous depots into circulation as well as improved clearance profiles (Bellmann-Sickert et al. 2011; Pollaro and Heinis 2010). Together with reduced production prices, this has led to a revived interest in synthetic peptides, which now account for a substantial fraction of compounds undergoing

clinical trials in many pharmaceutical companies (Haberl et al. 2009; Stevenson 2009). In the last decade, several peptide therapeutics have in fact been marketed towards blockbuster indications such as diabetes, cancer, autoimmune inflammatory and cardiovascular diseases, CNS-related conditions as well as infections (Bellmann-Sickert and Beck-Sickinger 2010; Bellmann-Sickert et al. 2011; Briand and Muller 2010; Jenssen 2009; Nestor 2009; Otvos 2008). Other continuously evolving areas concern drug delivery with peptide carriers, i.e., cell-penetrating peptides capable of targeting, interacting with and crossing extracellular as well as subcellular membranes (D'Souza et al. 2011; Fischer et al. 2005; Foerg and Merkle 2008; Foged and Nielsen 2008; Mae and Langel 2006; Vives et al. 2008; Wender et al. 2008; Won et al. 2011).

Unnatural amino acids may be applied as sequence modifications that confer enhanced protease resistance as well as improved pharmacological properties to a bioactive peptide. For this purpose, a plethora of amino acids displaying variations of side chain structure have been introduced as commercial building blocks for solid-phase peptide synthesis (SPPS). Development of novel readily accessible amino acids is essential for continuous expansion of the possible chemical space for peptide design. While a vast number of hydrophobic and bulky amino acids have been widely employed, unnatural diamino acids are scarcely represented in modified peptides, the homologs of lysine being the only exceptions. However, since rare, complex non-essential diamino acids are commonly found in biologically active natural products (Nestor 2009), they appear to constitute a relevant, but quite unexplored motif for optimization of peptide leads. Structural diversity in α,β-diamino acids has been introduced via enantioselective synthesis based on catalytic asymmetric reactions, chiral auxiliaries as well as on utilization of the chiral pool, e.g., extensive functional group manipulation of cheap natural amino acids (Viso et al. 2005; Wang et al. 2010). Thus, efficient methodologies leading to chiral 3-alkyl or 3-aryl substituted 2,3-diaminobutyric acids have been reported, but most routes suffer from incompatibility with protecting groups suitable for standard fluorenylmethyloxycarbonyl (Fmoc)-SPPS, which ideally requires an N^{α}-Fmoc and side-chain *N*-butyloxycarbonyl (Boc) protection scheme.

Instead of carefully establishing additional chiral centers, our current focus is on establishing a general route to cationic mimics of neutral amino acids via formal insertion of an azamethylene moiety into their side chains. It was envisioned that a convenient way to construct such building blocks involved the formal alkylation of a suitable L-2,3-diaminopropanoic acid (Dap) derivative or precursor. A few such partially or fully N-protected building blocks are commercially available (e.g., N^{α}-Fmoc-N^{β}-methyl-Dap-OH, N^{α}-Fmoc-N^{β}-benzyl-Dap-OH, N^{α}-Fmoc-N^{β}-(2-hydroxyethyl)-Dap-OH, and N^{α}-Fmoc-N^{β}-(isopropyl, Boc)-Lys-OH), but usually at high cost.

Protocols for reductive amination of Dap (Chhabra et al. 2002; Flouret et al. 1995) and lysine (Flouret et al. 1995; Huang et al. 2007; Schierholt and Lindhorst 2009; Stefanowicz et al. 2010) have been reported. Although this direct route is compatible with Fmoc protection, the starting Fmoc-Dap-OH is expensive or requires synthesis from asparagine via several steps (Akaji and Aimoto 2001). Alternatively, strategies based on readily accessible precursors of N-alkylated Dap derivatives may be employed. However, as nucleophilic conditions are involved, N^{α}-benzyloxycarbonyl (Z) protection is usually employed during the key step, e.g., ring opening of N^{α}-Z-serine-β-lactone constitutes a relatively efficient way of producing N^{α}-Z-protected intermediates (Cassidy and Poulter 1996; Kretsinger and Schneider 2003; Moura and Pinto 2007; Ratemi and Vederas 1994), albeit it involves subsequent protecting group manipulations to give the desired N^{α}-Fmoc-protected building blocks. Likewise, 4-nitrophenylsulfonyl (*p*-nosyl)-activated aziridines readily undergo ring opening with amines (Hu 2004; Olsen et al. 2007), and recently this has been exploited in solid-phase synthesis (SPS) (Ottesen et al. 2009). Attachment to the resin via a trityl linker (Crestey et al. 2008) proved favorable since ring opening of the resulting sterically congested activated chiral aziridine (i.e., resin **2** in Fig. 1) proceeded regioselectively (Ottesen et al. 2009), whereas solution-phase ring opening of *tert*-butyl *N*-(*o*-nosyl)-aziridine-2-carboxylate with benzylamine afforded the desired product and its regioisomer in a 3.4:1 ratio (Turner et al. 2001). The versatility of the chosen solid-phase synthesis strategy (Fig. 1) appears only limited by the diversity of primary amine nucleophiles, and by the ease of the on-resin protection of the resulting secondary amine (Scheme 1).

To investigate the scope of this protocol, several building blocks (Fig. 2) displaying di- and polyamino-functionalized side chains were selected as targets, e.g.,

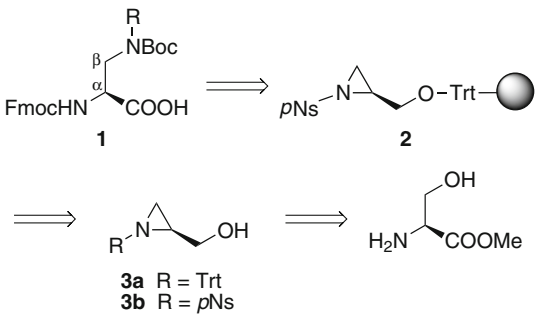
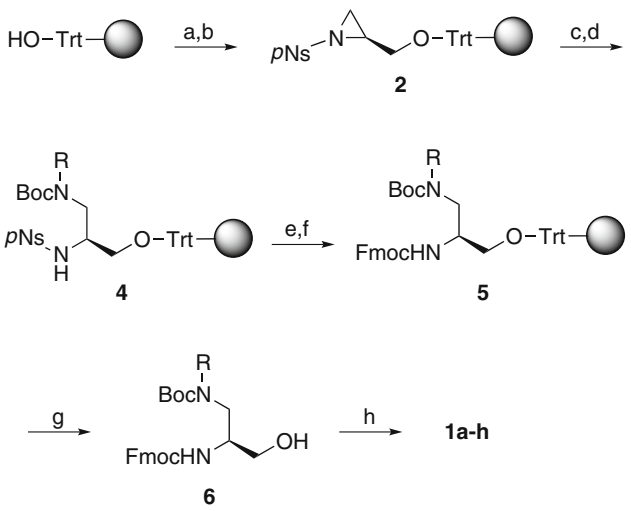


Fig. 1 Retro-synthesis of N$^{\beta}$-alkylated L-Dap building blocks from L-serine methyl ester



Scheme 1 Reagents and conditions: (*a*) AcBr (10 Eq), DCM, 30 min, rt; (*b*) (*S*)-1-(*p*-nosyl)aziridine-2-methanol (1.5 Eq, **3b**), DIPEA (3 Eq), DCM, 15 min, rt; (*c*) amine (R-NH_2) (4 Eq), DCE, 15 min, MW, 80°C; (*d*) Boc_2O (4 Eq), DCE, 30 min, MW, 120°C; (*e*) 2-mercaptoethanol (10 Eq), DBU (5 Eq), DMF, 2 h, rt; (*f*) Fmoc-OSu (5 Eq), DIPEA (5 Eq), THF, 16 h, rt; (*g*) TFA/DCM (2:98), 45 min, rt; (*h*) NaOCl (5 Eq), TEMPO (1 Eq), KBr (0.1 Eq), acetone/5% $NaHCO_3$ (aq), 1 h, 0°C

γ-aza-lysine derivative **1g** (Chhabra et al. 2002). Hence, synthesis of this series of N^{α}-Fmoc-protected amino acid building blocks for SPPS was undertaken using general conditions in the SPS approach. To confirm their synthetic utility, selected building blocks were to be incorporated into modified peptide sequences using SPPS.

Results and discussion

Solid-phase synthesis of cationic amino acid building blocks

An array of primary amines was chosen to meet our aim of designing amino acids that display a wide variety of cationic side chains comprising a few resembling those of essential amino acids except for the γ-aza substitution (Fig. 2). This strategy also facilitates incorporation of multiple positive charges provided that appropriate partially protected di- and polyamines are available. The selected array of building blocks covers a high diversity, as it comprises simple diamino acids (**1a**, **1b** and **1e**), high bulkiness (**1c**, **1d** and **1f**) as well as polycationic side chains (**1g** and **1h**).

Thus, to obtain N^{α}-Fmoc-protected amino acid building blocks with Boc protection of the remaining amino group(s), an 8-step protocol was developed (Scheme 1). The sequence involves simple activation of an inexpensive trityl alcohol resin, loading of an activated aziridine alcohol followed by four efficient on-resin transformations,

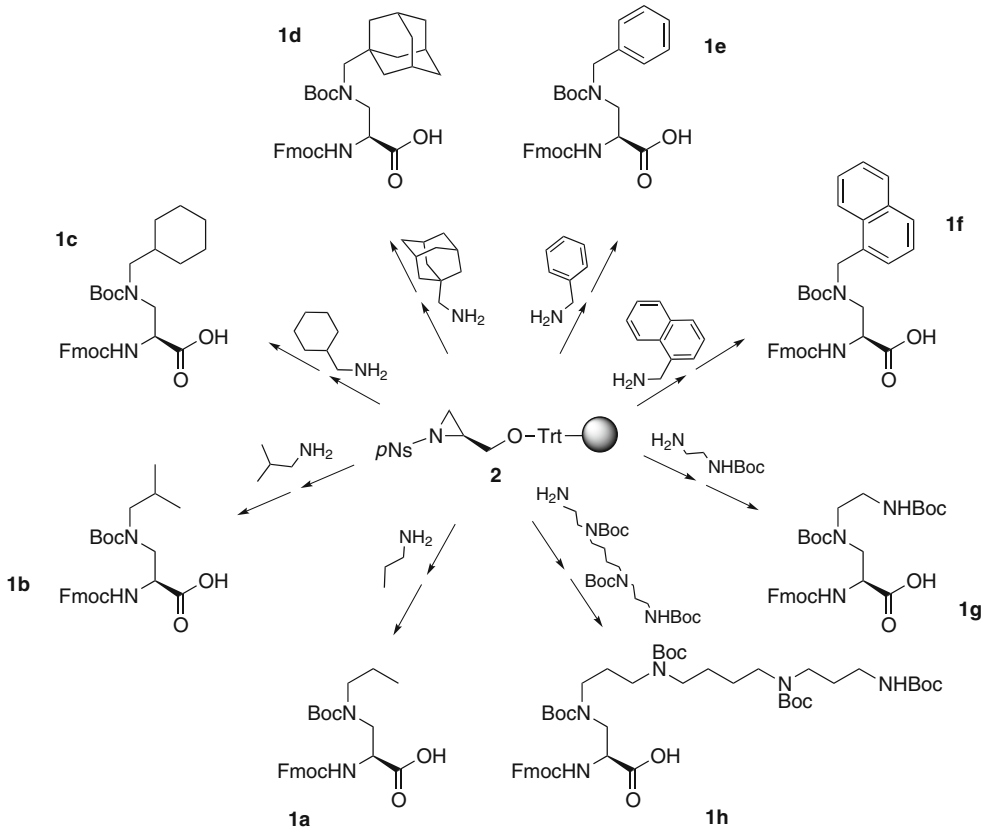


Fig. 2 Array of N^{β}-substituted Dap building blocks for SPPS displaying high diversity: varying degree of hydrophobicity (i.e., **1a**, **1b**, and **1e**), high bulkiness (i.e., **1c**, **1d**, and **1f**), or multiple charges (i.e., **1g** and **1h**)

thus minimizing loss of product as well as time spent on purification since only simple washing procedures were required between each step. Finally, the desired crude building blocks were obtained after cleavage under mild conditions and a subsequent oxidation of the corresponding alcohol.

(*S*)-1-Tritylaziridine-2-methanol (**3a**; Fig. 1) (Baldwin et al. 1993; McKeever and Pattenden 2003; Utsunomiya et al. 1993) was prepared from L-serine methyl ester on a 100-g scale (~tenfold up-scaling cf. literature procedure) resulting in an overall yield of 63% via esterification to the methyl ester followed by N-tritylation, O-mesylation, and ring closure to give the corresponding aziridine methyl ester, which then was reduced. All conversions required only purification by simple non-chromatographic work-up or crystallization (Baldwin et al. 1993; Ottesen et al. 2009). The key intermediate (*S*)-1-(*p*-nosyl)aziridine-2-methanol (**3b**) was in turn obtained from **3a** on a large scale (>60 mmol) that together with a slightly modified procedure resulted in an improved yield (75%). Then, **3b** was loaded onto freshly prepared polystyrene trityl bromide resin (Crestey et al. 2008) to give resin-bound aziridine **2**, which was subjected to microwave-assisted nucleophilic ring opening with primary amines. The resulting secondary amine intermediate was readily Boc-protected with Boc_2O under microwave-assisted conditions to give resin **4** (Scheme 1). Removal of the nosyl group with 2-mercaptoethanol/DBU and subsequent Fmoc protection with Fmoc-OSu/DIPEA gave resin **5** from which protected amino alcohol **6** was cleaved under mild conditions (2% TFA in DCM) compatible with its side chain Boc protection. Finally, oxidation of **6** with NaOCl/TEMPO/KBr in a heterogeneous mixture of acetone and 5% aqueous $NaHCO_3$ yielded the final building blocks **1a–h**.

Using the appropriate primary amines as nucleophiles in the sequence depicted in Scheme 1, the eight building blocks were obtained in moderate to high overall yields (33–77%; Table 1). The differences in the overall yields appear not to be correlated to steric hindrance of the primary amines. However, when multiple Boc protecting groups are present (e.g., in **1g** and **1h**) an alternative work-up procedure should be used to avoid a diminished yield due to partial loss of Boc groups (otherwise only 34% yield was obtained for **1g**). Yet, the combination of a high-loading resin and a building block of considerable size (i.e., as in the synthesis of **1h**) may also contribute to a significant lowering of the yield.

Incorporation of cationic hydrophobic amino acids into peptides

Membrane-interacting peptides generally contain numerous basic residues as a high number of positive charges appear to be correlated with high uptake efficiency in human cells (Patel et al. 2007; Stewart et al. 2008). Recently, designed 8-meric peptides were reported to be capable of efficiently transporting a fluorescent cargo even across the highly negative membranes of mitochondria. The uptake of such mitochondria-penetrating peptides (MPPs) was found to depend distinctly on the ratio and distribution of hydrophobic and basic residues (Kelley et al. 2011; Yousif et al. 2009). Hence, we chose the overall most promising peptide sequence $[FrF_xK]_2$ (F = L-Phenylalanine = Phe, r = D-Arginine, F_x = L-Cyclohexylalanine = Cha, K = L-Lysine) reported from those studies as the template for preliminary model sequences incorporating our amino acids displaying similar hydrophobic side chains as well as conferring additional positive charge to the peptide. Several residues (e.g., Phe and Arg) constitute sites for possible replacement with the present synthetic cationic amino acids.

Thus, in order to prove the synthetic utility of the present amino acid building blocks, two of these (**1e** and **1g**) were incorporated into 12-meric analogs (Table 2) of the above selected peptide sequence using Fmoc-based SPPS. By performing the syntheses on an automated microwave-assisted peptide synthesizer, their compatibility with such conditions was also shown. The sequences were prepared as C-terminal amides using a Rink amide resin, while DIC/HOAt was employed as coupling system. As the applicability of cationic peptides is often investigated by cellular uptake studies using detection by fluorescence, the

Table 1 The outcome of target Dap building block synthesis

Compound	N^{β}-alkyl substituent (R)	Scale (mmol)	Overall yield (%)
1a	Propyl	5.7	54
1b	Isobutyl	5.7	77
1c	Cyclohexylmethyl	4.5	76
1d	Adamant-1-ylmethyl	5.6	64
1e	Benzyl	4.6	63
1f	Naphth-1-ylmethyl	4.5	67
1g	*N*-Boc-(2-aminoethyl)	11.0	71
1h	Boc_3(spermine)	5.6	33

Table 2 Modified 12-mer peptides based on a sequence exhibiting mitochondrial penetration

Peptide	Sequence	Charge	Residues	MW
7	TAMRA-**1e**RF_xKFR**1e**RF_xKFR-NH_2	+9	12	2,264.9
8	TAMRA-F**1g**F_xKFRF**1g**F_xKFR-NH_2	+9	12	2,153.7

N-terminal required labelling with a fluorescent cargo. Polarity and charge of fluorescence probes may confer significantly changed overall properties to the labelled peptides, however, only a negatively charged probe (e.g., carboxyfluorescein) is expected to impede the uptake efficiency of the peptides (Yousif et al. 2009). Thus, the positively charged 5(6)-carboxytetramethylrhodamine (TAMRA) was conjugated manually to the N-terminal upon SPPS of the sequences (Table 2). After purification by HPLC, the two peptides were obtained in purities above 95%.

Conclusion

The main advantage of the present protocol is that it allows for a fast convenient gram-scale parallel solid-phase synthesis of arrays of cationic amino acid building blocks suitably protected for applications in peptide modification using standard SPPS methods. Thus, we have obtained eight different N^{α}-Fmoc-protected cationic amino acid building blocks using the serine-derived (*S*)-1-(*p*-nosyl)aziridine-2-methanol as the common key intermediate that was readily obtained in multi-gram scale from an inexpensive starting material and cheap chemicals.

It is expected that the currently improved access to this relatively unexplored type of amino acid building blocks may promote further developments in drug-related peptide research, as incorporation in different classes of membrane-interacting peptides, e.g., cell-penetrating peptides or antimicrobial peptides may indeed prove beneficial due to their cationic-hydrophobic character. Such unnatural amino acids may also be introduced as a means of enhancing stability of peptides towards proteolytic degradation. Incorporation of N^{β}-alkylated Dap residues in peptides may also generate surface patches that allow for strong polar interactions that are able to interfere with hydrophobic protein–protein interactions. Finally, N^{β}-alkylated Dap derivatives and analogs displaying di- and polycationic side chains constitute unique building blocks for combinatorial chemistry, and for construction of dendrimers with a higher degree of amine functionalization than e.g., traditional lysine dendrimers.

Experimental

General

Starting materials were purchased from commercial suppliers and used without further purification. Trityl alcohol polystyrene resin (loading: 1.75 mmol/g) was obtained from IRIS Biotech. Rink amide AM resin (0.71 mmol/g) was from NovaBiochem. DMF dried over 4 Å molecular sieves ($H_2O < 0.01\%$) was from Fluka, and anhydrous CH_2Cl_2 was distilled from P_2O_5 and kept over 4 Å molecular sieves. Water for reversed-phase HPLC was filtered through a 0.22 μm membrane filter (Millipak40, Millipore). Other solvents were analytical or HPLC grade and were used as received. Microwave-assisted ring opening of **2** in the syntheses of **1a–h** was carried out in an initiator single-mode microwave cavity producing controlled irradiation at 2.45 GHz (Biotage AB, Uppsala). The reactions were run in sealed vessels (10–20 mL), and magnetic stirring was used. Variable power was employed to reach the temperature desired (80 and 120°C, respectively) (within 1–2 min) and then to maintain it during the period of time programmed. Temperature was monitored by an IR sensor focused on a point on the reactor vial; the sensor was calibrated to an internal solution reaction temperature by the manufacturer. Preparation of resin-bound peptides was performed on a CEM Liberty microwave peptide synthesizer, and the final introduction of TAMRA was performed manually at rt. Analytical DAD–HPLC was performed using a Phenomenex Luna C18(2), 3 μm, 4.6 × 150 mm column eluted at a rate of 0.8 mL/min. Injection volumes were 20 μL of a 1 mg/mL solution, and separations were performed at 40°C. Solvent mixtures A (H_2O–MeCN–TFA 95:5:0.1) and B (MeCN–H_2O–TFA 95:5:0.1) were used. A linear gradient 0–80% B during 5 min followed by a linear rise to 100% B during 25 min was used for elution. Building blocks were detected with UV at $\lambda = 269$ nm (detection of the Fmoc group). Preparative HPLC separations of peptides were carried out on an XBridge™ Prep C_{18}, 5 μm, OBD™ 50 × 250 mm column. Solvent A: H_2O; Solvent B: MeCN; Solvent C: 1% TFA in H_2O were used in a gradient rising linearly from 30 to 50% of B during 40 min with 10% C constantly at a flow rate of 60 mL/min. The fractions of interest were analyzed by LCMS. Fractions of >95% purity were pooled and lyophilized. Vacuum liquid chromatography (VLC) was performed using Merck silica gel 60 (particle size 0.015–0.040 mm). ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, using CD_3OD as solvent. Multiplicities of ^{1}H NMR signals are reported as follows: s, singlet; bs, broad singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublet of doublets; t, triplet; m, multiplet. All target amino acid building blocks were characterized by HRMS performed on an Agilent Technologies 1200 Series LC/MSD-TOF instrument using a C18 column (5→95% MeCN in H_2O, 15 min), while HRMS of peptides was performed using a Bruker MicrOTOF-Q II Quadropol MS Detector in positive mode. Optical rotations were measured with a Perkin–Elmer model 241 polarimeter.

(*S*)-[1-(4-Nitrophenylsulfonyl)aziridin-2-yl]methanol (**3b**)

(*S*)-1-tritylaziridin-2-methanol (**3a**; 20 g, 63.4 mmol) dissolved in $CHCl_3$–MeOH (3:1; 40 mL) was added dropwise to TFA (47 mL, 634 mmol) at 0°C. The resulting mixture was stirred at 0°C for 1 h. Toluene (100 mL) and MeOH (100 mL) were added to the reaction mixture, which then was concentrated in vacuo (3×) to remove TFA. The residue was dissolved in EtOAc (100 mL), which was extracted with water (2 × 100 mL). The combined aqueous phases were treated with $NaHCO_3$ (~20 g) to pH ~8 and then added EtOAc (100 mL). Then 4-nitrobenzene-1-sulfonyl chloride (13.35 g, 60.2 mmol) in EtOAc (100 mL) was added dropwise to the two-phase mixture, which was stirred for 1 h at rt. The phases were separated, and the aqueous phase was diluted with water (100 mL) and extracted with additional EtOAc (3 × 100 mL). The combined organic phases were washed with brine (200 mL), dried (Na_2SO_4), filtered and concentrated in vacuo to yield the crude as an orange oil, which was purified by VLC (∅8 cm; stepwise gradient ending with 3:2:0.01 Heptane–EtOAc–AcOH) to yield the desired product as a slightly yellowish solid. Yield 12.3 g (75%).

Large-scale loading of resin

AcBr (25.9 mL, 350 mmol) was carefully added to trityl alcohol resin (20.0 g, 1.75 mmol/g) pre-swollen in dry DCM (100 mL) at rt into a 300 mL manual reaction vessel with baffles/coarse glass frit/screw cap/Teflon stopcock mounted on a 180° variable-speed flask shaker. The reaction mixture was shaken at rt for 30 min and then washed with dry DCM (3 × 100 mL, each for 5 min) to remove excess AcBr. A solution of DIPEA (18.3 mL, 105 mmol) and **3b** (13.56 g, 52.5 mmol) in DCM (100 mL) was added to the Trt-Br resin. The resulting mixture was shaken for 15 min at rt. The resin was drained and washed (DMF, MeOH, DCM; each for 3 times). Upon draining, the resin was transferred to a round-bottomed flask and dried in vacuo to give resin-bound (*S*)-1-(*p*-nosyl)aziridine-2-methanol (**2**; 30.65 g, 1.14 mmol/g) (Crestey et al. 2008).

General procedure for SPS of amino acid building blocks (GP)

The appropriate amine (4 Eq) was added to resin-bound (*S*)-1-(*p*-nosyl)aziridine-2-methanol pre-swollen in dichloroethane (DCE) (6 mL/g resin) in one or more microwave reactors (each of 20 mL). The mixture was heated by microwave (MW) to 80°C for 15 min. The resin was transferred to a syringe (50 mL) fitted with a PP filter, and then washed and drained (DMF, MeOH, DCM; each 3 times). Then Boc_2O (4 Eq) in DCE (4 mL/g resin) was added to the resin in 20 mL MW reaction vials, and then the mixture was heated by MW to 120°C for 30 min. The resin was transferred to a 300 mL manual reaction vessel with baffles/coarse glass frit/screw cap/Teflon stopcock mounted on a 180° variable-speed flask shaker and washed and drained (DMF, MeOH, DCM; each 3 × 100 mL).

1,8-Diazabicycloundec-7-ene (DBU; 5 Eq) in dry DMF (3 mL/g resin) and 2-mercaptoethanol (10 Eq) in dry DMF (3 mL/g resin) were added to the resin which then was shaken at rt for 90 min. The resin was drained and treated once more with DBU (5 Eq) and 2-mercaptoethanol (10 Eq) in dry DMF for 30 min. The resin was washed and drained (DMF, MeOH, DCM; each 3 × 100 mL). 9-Fluorenylmethyl-succinimidyl carbonate (Fmoc-OSu) (5 Eq) in dry THF (12 mL/g resin) was added to the resin pre-swollen in dry THF (4 mL/g resin) with diisopropylethylamine (DIPEA) (5 Eq) added. The mixture was shaken at rt for 16 h. The resin was washed and drained (DMF, MeOH, DCM; each 3×). The product was cleaved off the resin by treatment with 2% TFA in DCM (10 mL/g resin) for 30 min and then repeated (10 mL/g resin) for 15 min. The resin was eluted with additional DCM (10 mL/g resin), and the pooled eluates were concentrated in vacuo with toluene to give the crude product as orange oil. The crude was suspended in a mixture of acetone (10 mL/mmol) and 5% aqueous $NaHCO_3$ (5 mL/mmol). Upon cooling to 0°C, KBr (0.1 Eq) and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO; 1 Eq) was added to the mixture. Then, an aqueous NaOCl solution (10–15%; 5 Eq) was added dropwise. The mixture was stirred vigorously at 0°C until TLC showed full conversion (approx. 1 h). Acetone was removed in vacuo, and the resulting aqueous phase was acidified with 10% citric acid to pH ~5–6, and then the product was extracted with EtOAc (3 × 25 mL/mmol). The combined organic phases were washed with brine (25 mL/mmol), dried (Na_2SO_4), filtered and concentrated in vacuo to yield the crude as a yellow oil, which was purified by VLC (∅8 cm; stepwise gradient ending with 3:2:0.01 Heptane–EtOAc–AcOH) to yield the desired building block as pale amorphous foam (34–76%). Purity was analyzed by reversed-phase analytical HPLC.

*N^{α}-Fmoc-N^{β}-(Propyl, Boc)-L-Dap-OH (**1a**)*

Preparation according to GP using propan-1-amine (1.85 mL, 22.6 mmol). Yield: 1.44 g (54%) as pale foam. Purity: 99%. $[\alpha]_D^{20} = -16.8°$ ($c = 0.53$, MeOH); ^{1}H NMR (CD_3OD, 300 MHz): δ 7.76 (d, $J = 7.5$ Hz, 2H), 7.63 (bd, $J = 7.0$ Hz, 2H), 7.36 (t, $J = 7.5$ Hz, 2H), 7.28 (t, $J = 7.5$ Hz, 2H), 4.50 (dd, $J = 5.0$, 8.0 Hz, 1H), 4.33 (m, 2H), 4.17 (t, $J = 6.5$ Hz, 1H), 3.63–3.79 (m, 1H), 3.30–3.54 (m, 1H; hidden in part under the solvent peak),

3.04–3.30 (m, 2H), 1.53 (m, 2H), 1.44 (s, 9H), 0.83 (t, $J = 7.5$ Hz, 3H). ^{13}C NMR (CD_3OD, 75 MHz): δ 173.7, 158.2, 156.9/157.7, 144.9/145.0 (2C), 142.4 (2C), 128.6 (2C), 128.0 (2C), 126.1 (2C), 120.8 (2C), 81.5/81.2, 68.0, 54.6, 50.8/51.1, 49.7, 48.3, 28.7 (3C), 22.1/22.8, 11.5. HRMS: (*m/z*): $[M + Na]^+$ calcd for $C_{26}H_{32}N_2O_6$, 491.2152; found, 491.2158; $\Delta M = 1.2$ ppm.

*N^{α}-Fmoc-N^{β}-(Isobutyl, Boc)-L-Dap-OH (**1b**)*

Preparation according to GP using 2-methylpropan-1-amine (2.25 mL, 22.6 mmol). Yield: 2.10 g (77%) as pale foam. Purity: 98%. $[\alpha]_D^{20} = -18.9°$ (*c* 0.53, MeOH); ^{1}H NMR (CD_3OD, 300 MHz): δ 7.77 (d, $J = 7.5$ Hz, 2H), 7.64 (d, $J = 7.5$ Hz, 2H), 7.36 (t, $J = 7.5$ Hz, 2H), 7.28 (t, $J = 7.5$ Hz, 2H), 4.55 (dd, $J = 5.0$, 9.0 Hz, 1H), 4.33 (m, 2H), 4.18 (t, $J = 7.0$ Hz, 1H), 3.65–3.82 (m, 1H), 3.28–3.53 (m, 1H; hidden in part under the solvent peak), 3.06–3.22 (m, 1H), 2.91–3.04 (m, 1H), 1.90 (n, $J = 7.0$ Hz, 1H), 1.45* (s, 9H), 1.43 (s, 9H), 0.85 (d, $J = 6.5$ Hz, 6H), 0.84* (d, $J = 6.5$ Hz, 6H), * = Minor rotamer. ^{13}C NMR (CD_3OD, 75 MHz): δ 173.7, 158.2, 157.0/157.8, 145.0/145.2 (2C), 142.4 (2C), 128.6 (2C), 128.0 (2C), 126.1 (2C), 120.8 (2C), 81.5/81.3, 68.0, 56.3/56.7, 54.4/54.2, 50.1/49.7, 48.3, 29.0/28.4, 28.7 (3C), 20.5 (2C). HRMS: (*m/z*): $[M + Na]^+$ calcd for $C_{27}H_{34}N_2O_6$, 505.2309; found, 505.2329; $\Delta M = 3.9$ ppm.

*N^{α}-Fmoc-N^{β}-(Cyclohexylmethyl, Boc)-L-Dap-OH (**1c**)*

Preparation according to GP using cyclohexylmethanamine (2.33 mL, 17.9 mmol). Yield: 1.77 g (76%) as pale foam. Purity: 99%. $[\alpha]_D^{20} = -15.9°$ (*c* 0.52, MeOH); ^{1}H NMR (CD_3OD, 300 MHz): δ 7.77 (d, $J = 7.5$ Hz, 2H), 7.64 (d, $J = 7.5$ Hz, 2H), 7.37 (t, $J = 7.5$ Hz, 2H), 7.28 (t, $J = 7.5$ Hz, 2H), 4.54 (dd, $J = 5.0$, 9.0 Hz, 1H), 4.33 (m, 2H), 4.18 (t, $J = 7.0$ Hz, 1H), 3.64–3.81 (m, 1H), 3.26–3.53 (m, 1H; hidden in part under the solvent peak), 3.07–3.24 (m, 1H), 2.92–3.06 (m, 1H), 1.54–1.75 (m, 6H), 1.44 (s, 9H), 1.43* (s, 9H), 1.11–1.31 (m, 3H), 0.81–0.98 (m, 3H), * = Minor rotamer. ^{13}C NMR (CD_3OD, 75 MHz): δ 173.7, 158.2, 157.0/157.8, 145.0/145.0 (2C), 142.4 (2C), 128.6 (2C), 128.0 (2C), 126.1 (2C), 120.8 (2C), 81.5/81.3, 68.0, 55.2/55.5, 54.4/54.3, 50.2/49.7, 48.3, 37.9/38.6, 32.0, 31.9/31.8, 28.7 (3C), 27.6, 27.0 (2C). HRMS: (*m/z*): $[M + Na]^+$ calcd for $C_{30}H_{38}N_2O_6$, 545.2622; found, 545.2647; $\Delta M = 4.5$ ppm.

*N^{α}-Fmoc-N^{β}-(Adamant-1-ylmethyl, Boc)-L-Dap-OH (**1d**)*

Preparation according to GP using 1-adamantylmethanamine (2.00 g, 12.1 mmol). Yield: 2.04 g (64%) as pale foam. Purity: 99%. $[\alpha]_D^{20} = -21.0°$ (*c* 0.53, MeOH); ^{1}H NMR (CD_3OD, 300 MHz): δ 7.76 (d, $J = 7.5$ Hz, 2H), 7.64 (d, $J = 7.5$ Hz, 2H), 7.37 (t, $J = 7.5$ Hz, 2H), 7.28 (t, $J = 7.5$ Hz, 2H), 4.54–4.69 (m, 2H), 4.23–4.40 (m, 2H), 4.18 (t, $J = 7.0$ Hz, 1H), 3.74–3.86 (m, 1H), 3.34–3.50 (m, 1H), 3.10 (m, 1H), 2.83 (m, 1H), 1.92 (bs, 3H), 1.66 (m, 6H), 1.50 (bm, 6H), 1.46* (s, 9H), 1.42 (s, 9H), * = Minor rotamer. ^{13}C NMR (CD_3OD, 75 MHz): δ 173.8/173.7, 158.2, 157.3/158.0, 145.0 (2C), 142.4 (2C), 128.6 (2C), 128.0 (2C), 126.1 (2C), 120.8 (2C), 81.4, 68.0, 60.9/61.2, 54.3/53.6, 51.9/52.3, 48.3, 42.0/42.3 (3C), 38.0 (3C), 37.2/37.0, 29.8 (3C), 28.7/28.7 (3C). HRMS: (*m/z*): $[M + Na]^+$ calcd for $C_{34}H_{42}N_2O_6$, 597.2935; found, 597.2912; $\Delta M = 3.8$ ppm.

*N^{α}-Fmoc-N^{β}-(Benzyl, Boc)-L-Dap-OH (**1e**)*

Preparation according to GP using benzylamine (2.00 mL, 18.2 mmol). Yield: 1.47 g (63%) as pale foam. Purity: 97%. $[\alpha]_D^{20} = -13.4°$ (*c* 0.52, MeOH); ^{1}H NMR (CD_3OD, 300 MHz): δ 7.76 (d, $J = 7.5$ Hz, 2H), 7.64 (d, $J = 7.0$ Hz, 2H), 7.16–7.40 (m, 9H), 4.53 (dd, $J = 5.0$, 9.0 Hz, 1H), 4.42–4.63 (m, 1H), 4.24–4.40 (m, 3H), 4.17 (t, $J = 7.0$ Hz, 1H), 3.69 (m, 1H), 3.41 (m, 1H; hidden in part under the solvent peak), 1.48* (s, 9H), 1.38 (s, 9H), * = Minor rotamer. ^{13}C NMR (CD_3OD, 75 MHz): δ 173.5, 158.2, 157.2/157.6, 144.9/145.0 (2C), 142.4 (2C), 138.8/139.3, 129.5 (2C), 128.6 (2C), 128.6, 128.4, 128.2, 128.0 (2C), 126.1 (2C), 120.8 (2C), 82.0/81.8, 68.0, 54.3, 51.6/52.6, 48.8, 48.3, 28.6 (3C). HRMS: (*m/z*): $[M + Na]^+$ calcd for $C_{30}H_{32}N_2O_6$, 539.2152; found, 539.2168; $\Delta M = 2.9$ ppm.

*N^{α}-Fmoc-N^{β}-(Naphth-1-ylmethyl, Boc)-L-Dap-OH (**1f**)*

Preparation according to GP using naphthalen-1-ylmethanamine (2.63 mL, 17.9 mmol). Yield: 1.70 g (67%) as pale foam. Purity: 98%. $[\alpha]_D^{20} = -5.2°$ (*c* 1.01, MeOH); ^{1}H NMR (CD_3OD, 300 MHz): δ 7.16–7.80 (m, 15H), 4.80–5.10 (m, 2H), 4.54 (m, 1H), 4.31 (m, 2H), 4.17 (t, $J = 6.5$ Hz, 1H), 3.22–3.70 (m, 2H; hidden in part under the solvent peak), 1.49 (s, 9H), 1.40* (s, 9H), * = Minor rotamer. ^{13}C NMR (CD_3OD, 75 MHz): δ 173.6, 158.1, 156.9/157.5, 145.0/145.0 (2C), 142.4 (2C), 135.2, 133.6/133.9, 132.6/132.4, 129.7/129.8, 129.0/129.2, 128.6 (2C), 128.0 (2C), 127.1, 126.7, 126.2, 126.1 (2C), 125.8, 124.2/123.8, 120.8 (2C), 82.0, 67.9/68.3, 54.1, 50.4, 48.3, 48.1–48.6, 28.7 (3C). HRMS: (*m/z*): $[M + Na]^+$ calcd for $C_{34}H_{34}N_2O_6$, 589.2309; found, 589.2326; $\Delta M = 2.8$ ppm.

*N^{α}-Fmoc-γ-aza-L-Lys(Boc_2)-OH (**1g**)*

Preparation using *tert*-butyl 2-aminoethylcarbamate (5.00 g, 31.2 mmol) according to GP until cleavage, upon

which the resin was drained into a DCM solution (50 mL) with an equivalent amount (relative to TFA) of DIPEA added. The organic phase was washed (1 × sat. $NaHCO_3$, 2 × H_2O, each 25 mL). The crude was concentrated in vacuo before the final oxidation step. Yield: 4.42 g (71%) as pale foam. Purity: 97%. $[\alpha]_D^{20} = -13.5°$ (*c* 0.53, MeOH); ^1H NMR (CD_3OD, 300 MHz): δ 7.77 (d, $J = 7.5$ Hz, 2H), 7.64 (d, $J = 7.0$ Hz, 2H), 7.37 (t, $J = 7.0$ Hz, 2H), 7.29 (t, $J = 7.5$ Hz, 2H), 4.54 (dd, $J = 4.5$, 9.0 Hz, 1H), 4.33 (m, 2H), 4.19 (t, $J = 6.5$ Hz, 1H), 3.68–3.85 (m, 1H), 3.12–3.56 (m, 5H; hidden in part under the solvent peak), 1.45 (s, 9H), 1.41 (s, 9H). ^{13}C NMR (CD_3OD, 75 MHz): δ 173.8, 158.2, 157.5/157.0, 144.9/145.1 (2C), 142.4 (2C), 128.6 (2C), 128.0 (2C), 126.1 (2C), 120.8 (2C), 81.8/81.6, 80.0, 68.0/68.3, 54.5/54.6, 50.5/49.8, 48.9, 48.3, 28.8 (3C), 28.7 (3C). HRMS: (*m/z*): $[M + Na]^+$ calcd for $C_{30}H_{39}N_3O_8$, 592.2629; found, 592.2629; $\Delta M = 0.0$ ppm.

*N^{α}-Fmoc-N^{β}-(Spermine, Boc_4)-L-Dap-OH (**1h**)*

Preparation according to GP using N^1,N^5,N^{10}-tri-Boc-spermine (7.30 g, 14.5 mmol). Yield: 1.66 g (33%) as pale foam. Purity: 98%. $[\alpha]_D^{20} = -5.6°$ (*c* 1.01, MeOH); ^1H NMR (CD_3OD, 300 MHz): δ 7.79 (d, $J = 7.5$ Hz, 2H), 7.65 (d, $J = 7.5$ Hz, 2H), 7.39 (t, $J = 7.5$ Hz, 2H), 7.30 (t, $J = 7.5$ Hz, 2H), 4.50 (dd, $J = 5.0$, 8.5 Hz, 1H), 4.34 (m, 2H), 4.20 (t, $J = 7.0$ Hz, 1H), 3.65–3.82 (m, 1H), 3.28–3.56 (m, 1H; hidden in part under the solvent peak), 3.08–3.28 (m, 10H), 3.02 (t, $J = 6.5$ Hz, 2H), 1.59–1.83 (m, 4H), 1.38–1.56 (m, 4H), 1.46 (s, 9H), 1.44 (s, 18H), 1.42 (s, 9H). ^{13}C NMR (CD_3OD, 75 MHz): δ 173.6, 158.2, 156.7-157.4 (4C), 145.0/145.1 (2C), 142.4 (2C), 128.7 (2C), 128.0 (2C), 126.1 (2C), 120.8 (2C), 81.6/81.5, 80.8 (2C), 79.8, 68.0/68.8, 54.5, 48.3, 47.7, 46.1/46.9 (5C), 38.9, 30.2, 29.5, 28.8 (12C), 27.0, 26.7. HRMS: (*m/z*): $[M + Na]^+$ calcd for $C_{48}H_{73}N_5O_{12}$, 934.5148; found, 934.5189; $\Delta M = 4.3$ ppm.

Peptide synthesis

The peptides were synthesized using Fmoc-based building blocks on a CEM Liberty microwave peptide synthesizer (CEM Corporation) at a scale of 0.10 mmol. The sequences were synthesized using Rink amide AM resin (0.71 mmol/g, 200–400 mesh; NovaBiochem). All building blocks (3 Eq, 0.3 M) were coupled using *N,N*'-diisopropylcarbodiimide (3 Eq; 0.75 M; DIC) and HOAt (3 Eq; 0.3 M) in NMP for 5 min at 75°C. Fmoc deprotection was performed with 5% piperidine in NMP at 75°C for 3 min. The arginine and unnatural residues were introduced using double-coupling.

Fluorophore conjugation and purification

Upon deprotection the N-terminal of each of the complete oligomer sequences, they were conjugated manually in a 10 mL syringe fitted with a PP-filter to 5(6)-carboxytetramethylrhodamine (TAMRA; 3 Eq) with HOAt (3 Eq) and DIC (3 Eq) in NMP overnight at rt. The resin was washed (NMP, MeOH, DCM; each 3×). The conjugated sequences were finally deprotected and cleaved in a single step with TFA–H_2O–triisopropylsilane (95:2.5:2.5) for 3 h at rt. The peptides were precipitated in ice-cold Et_2O and after centrifugation (3,200 rpm for 30 s), the ether layer was decanted off. The pellet was re-solubilized in 250 mL 5% MeCN, 0.1% TFA in water and directly purified by preparative reversed-phase HPLC, monitoring conjugate elution at 214 nm. The fractions of interest were analyzed by LCMS. Fractions of >95% purity were pooled and lyophilized. HRMS: **7** (*m/z*): $[M + 5H]^{5+}$ calcd for $C_{117}H_{168}N_{31}O_{16}^+$, 453.6712; found, 453.6712; $\Delta M = 0.0$ ppm; **8** (*m/z*): $[M + 5H]^{5+}$ calcd for $C_{113}H_{160}N_{27}O_{16}^+$, 431.2568; found, 431.2576; $\Delta M = 1.8$ ppm.

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