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Solid phase synthesis of selective caspase-3 peptide inhibitors

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Abstract—A robust method for the solid phase synthesis of a series of selective caspase-3 peptide inhibitors is described. The inhibitors can be obtained after cleavage from the solid support without further purification.

The solid phase method of synthesis has dramatically increased the accessibility of synthetic peptides and small molecules. In particular, the extension of solid phase techniques to allow for the automated synthesis of peptides and small molecules has received widespread attention.

In connection with our previous work on caspase-1[1] and caspase-3,[2] we became interested in a novel and robust protocol for the solid phase production of caspase-3 peptide inhibitors. A key challenge in the synthetic effort was the identification of an appropriate protocol that would allow for the preparation of ketone-based inhibitors on solid support. In this letter, we describe an efficient route to caspase-3 selective inhibitors[3,4] by application of an amino Merrifield resin tethered via a semicarbazone Webb linker.[5]

Our synthetic strategy called for linking the P_1 aspartic acid derivative **1** to resin **2** through the ketone carbonyl functionality[6] (Fig. 1). This linkage would not only temporarily block the reactive carbonyl functionality but would also serve as an anchor for the rapid construction of peptide inhibitors via automation. The fully functionalized ketone inhibitor **3** could be released from resin **4** by acidic cleavage.

1. Linker synthesis and loading onto resin

Initially we followed the procedure given by Webb for the preparation of amino acid aldehyde semicarbazone resins (Fig. 2). This method uses the solution synthesis of the semicarbazone acid linker **5** followed by coupling of the acid to a MBHA resin[7] to produce **6**.

While this method gave us access to the intermediate carboxylic acids **5**, it often required tedious purification and gave variable yields due to the sensitivity of the substrate. Coupling of **5** to the commercially available MBHA resin proceeded in overall good efficiency. Final cleavage of **6** under acidic conditions (trifluoroacetic acid/water) gave the corresponding ketones together with small amounts of impurities as determined by NMR analysis. This procedure often required HPLC purification of the final compounds and was deemed unsuitable for rapid analogue synthesis. We therefore turned our attention to other resins and found the Merrifield resin (100–200 mesh, 2% DVB)[8] to be ideally suited for our purposes. Coupling of **5** to the amino Merrifield resin gave the insoluble support **6** with a quantitative coupling efficiency. This material had all the physical and chemical properties required for the automated synthesis of inhibitors. Final cleavage gave the corresponding ketones essentially pure *without the need for further purification*.

To further streamline the synthesis, we modified Webb's procedure in the following way (Fig. 3). Treatment of acid **7**[5] with the amino Merrifield resin in the presence of 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (EDCl) and 1-hydroxybenzotriazole (HOBT) in dichloromethane followed by removal of the Boc group with trifluoroacetic acid (TFA) in dichloromethane gave resin **8**. This resin is extremely robust, it can be prepared in kilogram quantity, and can be stored at room temperature for many years.

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Hydrazone formation with ketones derived from Fmoc protected aspartic acid **1** was most conveniently accomplished by stirring resin **8** with two to three equivalents of ketone in THF in the presence of acetic acid. This modification of the Webb procedure allowed for quantitative loading of ketones onto the resin as well as recovery of unreacted starting material. Again, the aspartic acid derived resin **6** is very robust, can be prepared on large scale (>100 g), and is stable at room temperature.[9]

1.1. Preparation of ketones

N-Fmoc-L-aspartic acid-β-*tert*-butyl ester was converted to the primary alcohol **9** according to the procedure of Chapman for the *N*-alloc protected aspartic acid.[10] The alcohol was oxidized to the aldehyde under Swern conditions,[11] and immediately treated with commercially available or freshly prepared Grignard reagent at −78 °C to afford the corresponding secondary alcohol in good yield.[12] The alcohol was purified and then oxidized to the corresponding ketones **1a–f** using the Dess–Martin[13] reagent (Fig. 4).

1.2. Preparation of tetrapeptide inhibitors

Hydrazone formation was performed as described above to give the insoluble support **6** (Fig. 5). Toward this end the 9050 PepSynthesizer from PerSeptive Biosystems was used (Millipore Corporation). The synthesis procedure given in the user's guide was followed for the preparation of the tetrapeptides on solid support. Iterative acylation reactions using *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU) and *N,N*-diisopropylethylamine (DIEA) as coupling reagents were successful, including sequential *N*-α-Fmoc amino acid piperidine deblocking protocols to produce the uncapped tetrapeptide on solid support. The acetylation step was typically performed in a fritted reaction vessel using acetylimidazole in DMF. Final acidic hydrolysis with TFA:H_2O (9:1) cleaved the ketones from the Webb linker bound resin along with any acid-labile side chain protecting groups. Evaporation of the solvent, followed by trituration with diethylether for 30 min and filtration of the solid produced the desired compounds in pure form *without the need for further purification*.

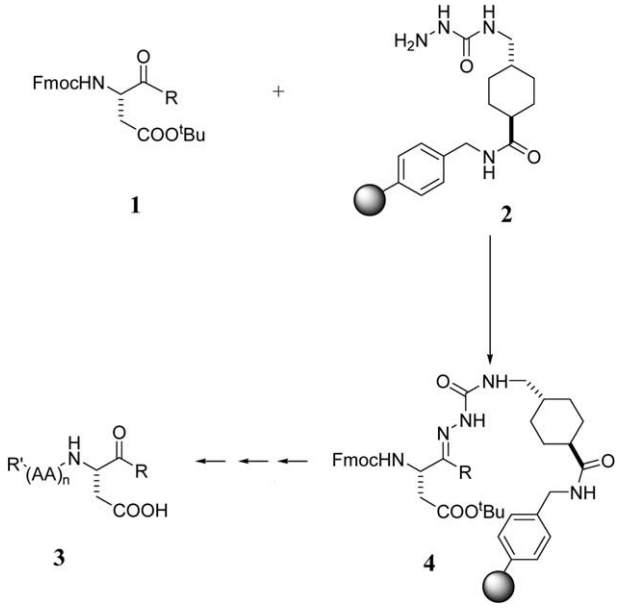


Figure 1.

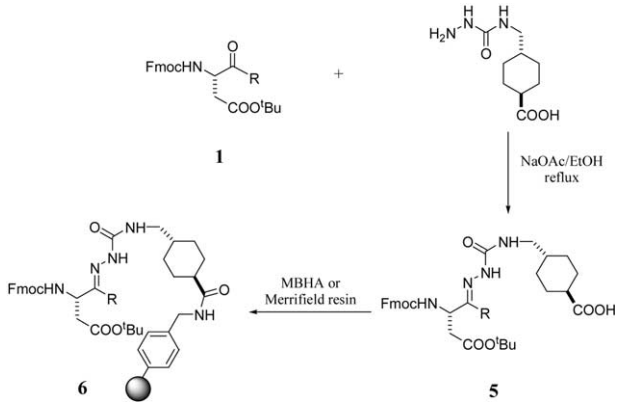


Figure 2.

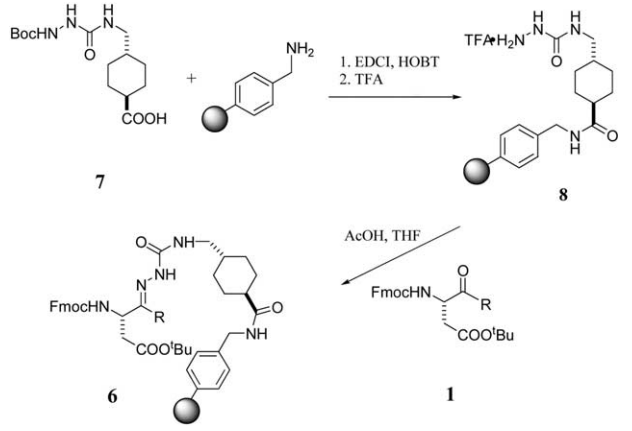
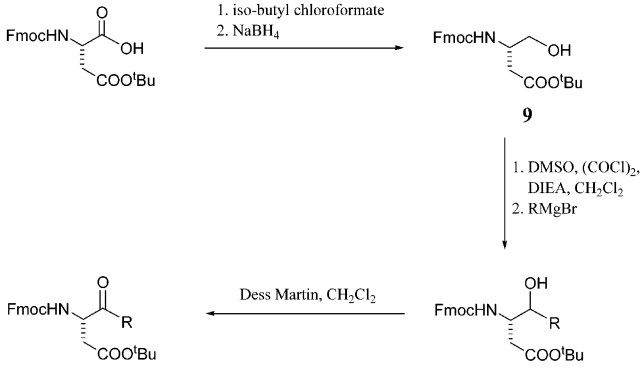


Figure 3.



1a: R= Me, 1b: R=nPr, 1c: R=Ph, 1d: R=$(CH_2)_3$Ph, 1e: R=$(CH_2)_5$Ph, 1f: R=$(CH_2)_3$Napth

Figure 4.

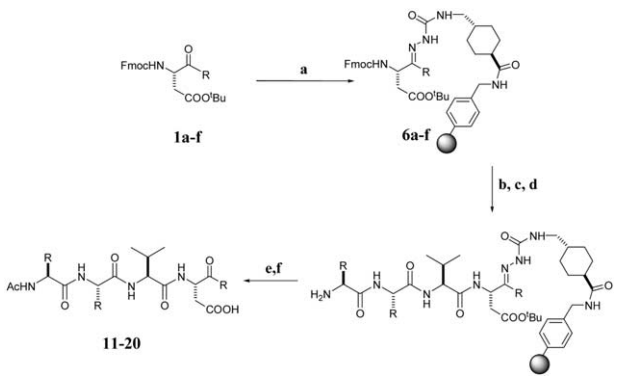


Figure 5. Reagents and conditions: (a) AcOH, THF, solid support **8**; (b) 20% piperidine in DMF, rt, 30 min; (c) Fmoc-(AA)n-OH, HATU, DIEA, DMF, rt, 2 h; (d) repeat steps (b), (c), (b) until P4 is attached; (e) acetylimidazole, DMF, 1 h; (f) TFA/H_2O (9:1), 30 min. Steps (b), (c), and (d) are performed on PepSynthesizer.

2. Results and discussion

Combinatorial substrate libraries developed by Chapman et al.[2b] established the optimum cleavage sequence for caspase-3 to be DEVD. Based on results gathered from work on caspase-1,[12] tetrapeptide aspartic acid ketone derivatives were synthesized. Inhibitors in this series were found to be considerably more potent against purified recombinant caspase-3 than the corresponding tetrapeptide aldehyde inhibitor Ac-DEVD-CHO (**10**) (Table 1).[14] While the phenylketone derivative **13** was equipotent to the aldehyde **1**, a 20- to 30-fold increase in potency was observed for the corresponding n-propyl (**12**) and methylketone (**11**) derivatives. The potency against caspase-3 could be further enhanced by the introduction of an aromatic ring on the aliphatic side chain. For example, the Ac-DEVD-phenyl propyl ketone **14**, the phenyl pentyl ketone **15**, and naphthyl propyl ketone **16** are subnanomolar caspase-3 inhibitors with IC_{50}'s of 0.8 nM, 0.8 nM, and 0.1 nM, respectively. This increase in potency is likely due to increased binding energy arising from hydrophobic interactions with the S1′prime binding site.[15]

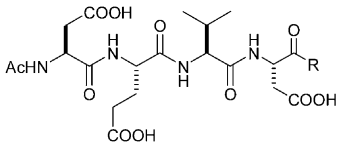
Several Ac-DEVD-ketone inhibitors were also assessed in a whole cell assay using NT-2 neuronal precursor cells[16] (Table 2). The aldehyde inhibitor **10** is essentially inactive while the ketone inhibitors revealed only weak inhibition. We presume this to be indicative of the tetrapeptide's poor cell permeability and therefore decided to replace the carboxylates in the P_3 and P_4 position with neutral groups.

Replacement of the P_3 glutamic acid by alanine or a methylsulfone equivalent furnished inhibitors **18** and **17**. Despite a loss in intrinsic potency (IC_{50} = 1.4 nM for **18**, 1.7 nM for **17**) a moderate increase in whole cell activity was observed (IC_{50} = 5 μM for **18**, 10 μM for **17**). Efforts have also been made to increase the permeability of **18** by esterifying the P_4 aspartic acid residue. Although the methyl ester **19** is 13-fold less potent than **18**, the two compounds were found to be approximately equipotent in the whole cell assay, suggesting that the methyl ester **19** is more cell permeable. Interestingly, incubation studies of **19** with cellular extracts from the NT-2 whole cell assay indicated that the ester moiety is not cleaved to the corresponding acid, suggesting the ester's enhanced cell permeability.[17] Molecular modeling suggested that in P_4 the sulfone **20** would retain the same hydrogen bonding interactions as the parent aspartic acid residue.[2d] This inhibitor was synthesized and was found to be equipotent to **18** against caspase-3 (IC_{50} = 1.9 nM) and demonstrated equivalent whole cell potency (IC_{50} = 12 μM).

In summary, a solid phase method for the synthesis of potent caspase-3 inhibitors was developed which allowed for the expeditious preparation of tetrapeptide derivatives *without the need for HPLC purification*. Neutralizing the acidic moieties in P_3 and P_4 (glutamic acid and aspartic acid) gave inhibitors with improved whole cell potency. The potency of compounds such as **18–20** make them ideal tools for studying caspase-3 activation in cell based systems.

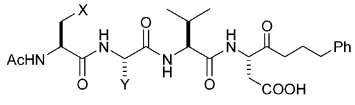
The solid phase synthetic sequence using a modified Webb linker/Merrifield resin combination developed here, enabled us to rapidly and efficiently prepare libraries of ketone-based caspase-3 inhibitors with improved selectivity and potency profiles and is the subject of separate communications.[18,19] So far, we prepared more than 2000 compounds for biological screening. The target inhibitors were produced in excellent

Table 1. In vitro caspase inhibition results



Compd	R	rh-caspase IC_{50} (nM)				NT2 cells IC_{50} (μM)
		1	3	7	8	
10	H	292	44	716	286	> 100
11	Me	3775	1.3	12	185	n.d.
12	*n*-Pr	4575	1.9	14	140	n.d.
13	Ph	1395	46	363	656	n.d.
14	$(CH_2)_3Ph$	307	0.8	7.7	16	30
15	$(CH_2)_5Ph$	24	0.8	7.2	9.6	n.d.
16	$(CH_2)_3$-naphth-1-yl	14	0.1	1.2	1.9	26

Table 2. In vitro caspase-3 inhibition results



Compd	X	Y	rh-caspase-3 IC_{50} (nM)	NT2 cells IC_{50} (μM)
14	COOH	$(CH_2)_2COOH$	0.8	30
17	COOH	$(CH_2)_2SO_2Me$	1.7	10
18	COOH	Me	1.4	5
19	COOMe	Me	18	10
20	SO_2Me	Me	1.9	12

(>90%) purity for in vitro screening. Structural integrity and purity was confirmed by LC-MS and NMR analysis.

In conclusion, the modified Webb linker compliments existing approaches for the preparation of ketone-based inhibitors. It is a truly 'traceless' linker and it has significant potential to speed up solid phase synthesis since there is no need for HPLC purification of the final compounds after cleavage from solid support.

3. Experimental

3.1. *tert*-Butyl(3S)-3-[(9H-9-fluorenylmethoxy)carbonyl]-amino-4-hydroxybutanoate (9)

This compound is commercially available but can also be prepared easily: To a solution of *N*-Fmoc-L-aspartic acid β-tert-butyl ester (19.0 g, 46.2 mmol) in 300 mL of tetrahydrofuran (THF) at −78 °C was added *N*-methyl morpholine (NMM, 5.9 mL, 53.3 mmol) followed by isobutyl chloroformate (IBCF, 6.9 mL, 53.3 mmol). After 10 min this mixture was warmed to 0 °C for 40 min and then recooled to −78 °C. A suspension of sodium borohydride (3.85 g, 102 mmol) in 25 mL of methanol was added and the mixture was stirred at −78 °C for 2 h. The reaction was quenched into 400 mL saturated aqueous ammonium chloride and extracted with ethyl acetate (4×100 mL). The combined organic layers were washed with brine and dried over anhydrous magnesium sulfate, filtered and concentrated. The residue was purified on silica gel (50% ethyl acetate/hexane) to give **9**: ^{1}H NMR (400 MHz, CD_3COCD_3) δ 7.85 (d, 2H, *J* = 7.30 Hz), 7.67 (d, 2H, *J* = 7.37 Hz), 7.40 (t, 2H, *J* = 7.30 Hz), 7.30 (t, 2H, *J* = 7.30 Hz), 6.32 (brd, 1H), 4.40–4.15 (m, 3H), 4.10–3.98 (m, 1H), 3.92 (t, 1H), 3.65–3.48 (m, 2H), 2.60 (dd, 1H, *J* = 6.24, 16.80 Hz), 2.41 (dd, 1H, *J* = 6.30, 16.91 Hz), 1.40 (5, 9H).

3.1.1. *tert*-Butyl(3*S*)-3-[(9H-9-fluorenylmethoxy) carbonyl]amino-4-oxo-7-phenylheptanoate (1d). Step 1: Oxalyl chloride (960 μL, 11 mmol) was added to a solution of DMSO (852 μL, 12 mmol) in 50 mL CH_2Cl_2 at −78 °C. The resulting mixture was stirred at −78 °C for 30 min and *tert*-butyl (3S)-3-[(9H-9-fluorenylmethoxy)-carbonyl]amino-4-hydroxybutanoate (3.98 g, 10 mmol) in CH_2Cl_2 (15 mL) was added dropwise. The mixture was stirred at −78 °C for 1 h, then *i*-Pr_2NEt (5.20 mL, 30 mmol) was added dropwise. The resulting mixture was stirred at −78 °C for 50 min and at 0 °C for 25 min. The mixture was recooled to −78 °C and phenylpropylmagnesium bromide (1.0M in Et_2O, 30 mL) was added dropwise. The mixture was stirred at −78 °C for 2 h and at 0 °C for 75 min. Saturated aqueous NH_4Cl (100 mL) was added and the two layers were separated. The aqueous layer was further extracted with Et_2O (4×100 mL). The combined organic layers were washed with 5% aq HCl, 5% aq.$NaHCO_3$, brine, and dried over magnesium sulfate. The solvent was removed in vacuo and the residue was chromatographed over silica gel (15% EtOAc/toluene) to provide the secondary alcohol (4.48 g, 87%).

Step 2: The secondary alcohol (1.5 g., 2.90 mmol) was dissolved in CH_2Cl_2 (20 mL) and Dess–Martin reagent (1.23 g, 2.90 mmol) was added. The resulting mixture was stirred for 2 h at room temperature and then filtered through a block of silica gel (15% EtOAc/toluene) to provide **1d** (1.23 g, 83%): ^{1}H NMR (400 MHz, CD_3COCD_3) δ 7.85 (d, 2H, *J* = 7.59 Hz), 7.68 (d, 2H, *J* = 7.44 Hz), 7.40 (t, 2H, *J* = 7.47 Hz), 7.31 (t, 2H, *J* = 7.86 Hz), 7.25–7.08 (m, 5H), 6.85 (brd, 1H), 4.55–4.35 (m, 3H), 4.22 (t, 1H, *J* = 6.95 Hz), 2.80 (dd, 1H, *J* = 7.1, 16.1 Hz), 2.70–2.50 (m, 5H), 1.88 (q, 2H), 1.40 (s, 9H); MS (+APCI) *m*/*z* 412 $(M+H)^+$.

3.1.2. *tert*-Butyl(3*S*)-3-[(9H-9-fluorenylmethoxy)carbonyl]amino-4-oxopentanoate (1a). Following the procedure outlined for the preparation of **1d**, substituting phenylpropylmagnesium bromide for methylmagnesium bromide, **1a** was obtained: ^{1}H NMR (400 MHz, CD_3COCD_3) δ 7.85 (d, 2H, *J* = 7.60 Hz), 7.68 (d, 2H, *J* = 7.45 Hz), 7.41 (t, 2H, *J* = 7.47 Hz), 7.32 (t, 2H, *J* = 7.80 Hz), 6.85 (brd, 1H), 4.55–4.35 (m, 3H), 4.26 (t, 1H, *J* = 6.95 Hz), 2.80 (dd, 1H, *J* = 7.1, 16.1 Hz), 2.65 (dd, 1H), 2.18 (s, 3H), 1.41 (s, 9H); MS (+APCI) *m*/*z* 309 $(M+H)^+$.

3.1.3. *tert*-Butyl(3*S*)-3-[(9H-9-fluorenylmethoxy)carbonyl]amino-4-oxoheptanoate (1b). Following the procedure outlined for the preparation of **1d**, substituting phenylpropylmagnesium bromide for *n*-propylmagnesium bromide, **1b** was obtained: ^{1}H NMR (400 MHz, CD_3COCD_3) δ 7.84 (d, 2H, *J* = 7.5 Hz), 7.68 (d, 2H, *J* = 7.5 Hz), 7.40 (t, 2H, *J* = 7.4 Hz), 7.31 (t, 2H, *J* = 7.4 Hz), 6.83 (d, 1H, *J* = 8.3 Hz), 4.36–4.55 (m, 3H), 4.23 (t, 1H, *J* = 6.9 Hz), 2.82 (dd, 1H, *J* = 6.9, 22.1 Hz), 2.48–2.67 (m, 3H), 1.51–1.61 (m, 2H), 1.41 (s, 9H), 0.88 (t, 3H, *J* = 7.4 Hz); MS (+APCI) *m*/*z* 337 $(M+H)^+$.

3.1.4. *tert*-Butyl(3*S*)-3-[(9H-9-fluorenylmethoxy)carbonyl]amino-4-oxo-4-phenylbutanoate (1c). Following the procedure outlined for the preparation of **1d**, substituting phenylpropylmagnesium bromide for phenylmagnesium bromide **1c** was obtained: ^{1}H NMR (400 MHz, CD_3COCD_3) δ 8.05 (d, 2H, *J* = 7.6 Hz), 7.83 (d, 2H, *J* = 7.6 Hz), 7.61-7.65 (m, 3H), 7.53 (t, 2H, *J* = 7.8 Hz), 7.38 (d, 1H, *J* = 7.5 Hz), 7.27 (dt, 2H, *J* = 0.9, 7.5 Hz), 7.05 (brd, 1H, *J* = 8.7 Hz), 5.55 (brq, 1H, *J* = 8.6 Hz), 4.32–4.34 (m, 2H), 4.19 (t, 1H, *J* = 7.0 Hz), 2.95 (dd, 1H, *J* = 7.1, 16.1 Hz), 2.66 (dd, 1H, *J* = 6.3, 16.1 Hz), 1.38 (s, 9H); MS (+APCI) *m*/*z* 371 $(M+H)^+$.

3.1.5. *tert*-Butyl(3*S*)-3-[(9H-9-fluorenylmethoxy)carbonyl]amino-4-oxo-9-phenylnonanoate (1e). Following the procedure outlined for the preparation of **1d**, substituting phenylpropylmagnesium bromide for phenylpentylmagnesium bromide, **1e** was obtained: ^{1}H NMR (400 MHz, CD_3COCD_3) δ 7.85 (d, 2H, *J* = 7.6 Hz), 7.68 (d, 2H, *J* = 7.4 Hz), 7.40 (t, 2H, *J* = 7.5 Hz), 7.31 (t, 2H, *J* = 7.4 Hz), 7.11–7.27 (5H, m), 6.83 (brd, 1H, *J* = 8.4 Hz), 4.35–4.51 (m, 3H), 4.24 (t, 1H, *J* = 6.8 Hz), 2.80 (dd, 1H, *J* = 6.0, 9.0 Hz), 2.46–2.65 (m, 5H), 1.55–1.64 (m, 4H), 1.28–1.41 (m, 2H), 1.41 (s, 9H); MS (+APCI) *m*/*z* 441 $(M+H)^+$.

3.1.6. *tert*-Butyl(3*S*)-3-[(9H-9-fluorenylmethoxy)carbonyl] amino-7-(1-naphthyl)-4-oxoheptanoate (1f). Following the procedure outlined for the preparation of **1d**, substituting phenylpropylmagnesium bromide for 1-naphthylpropylmagnesium bromide, **1f** was obtained: ^{1}H NMR (400 MHz, CD_3COCD_3) δ 8.16 (d, 1H, J = 8.4 Hz), 7.84–7.88 (m, 3H), 7.68–7.73 (m, 3H), 7.29–7.53 (m, 8H), 6.87 (d, 1H, J = 8.4 Hz), 4.51–4.57 (m, 1H), 4.37–4.47 (m, 2H), 4.23 (t, 1H, J = 6.8 Hz), 3.05–3.09 (m, 2H), 2.64–2.87 (m, 4H), 1.96–2.03 (m, 2H), 1.41 (s, 9H); MS (+APCI) m/z 463 $(M+H)^+$.

3.2. Synthesis of resin 8

A 12 l three-necked round bottom flask with overhead stirrer and reflux condenser was charged with chloromethyl Merrifield resin (100–200 mesh), 2% DVB, 0.8 mmol/g, Novabiochem cat. no. 01-64-0104 (1.0 kg, 0.8 mol), potassium phthalimide (0.52 kg, 2.8 mol), and DMF (7 l). The mixture was heated at 50 °C for 20 h and then stirred at room temperature for another 20 h. The resin was filtered, washed with DMF (4×2 l), MeOH (4×2 l), H_2O (4×2 l), MeOH (4×2 l), and dried at room temperature.

The solid was suspended in EtOH (8 l) in a 12 l three-necked round bottom flask and hydrazine hydrate (0.16 kg, 3.2 mol) was added. The mixture was heated at 80 °C for 20 h. Water (2 l) was added after cooling to room temperature, the resin was filtered and washed with H_2O (4×2 l), 5% aq KOH (4×2 l), H_2O (4×2 l), EtOH (4×2 l), and dried at 50 °C under vacuum for 18 h to afford 0.96 kg of amine resin.

To the amine resin (0.96 kg, loading 0.8 mmol/g) in DMF (15 l) was added 1-(*t*-butoxycarbonyl)-semicarbazidyl-*trans*-4-methyl cyclohexane carboxylic acid[5] (292 g, 0.924 mol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (193 g, 1.01 mol), and 1-hydroxybenzotriazole hydrate (154 g, 1.01 mol). The resulting mixture was stirred at room temperature under nitrogen atmosphere for 48 h. The resin was then filtered, washed with DMF (2×2 l), H_2O (3×2 l), THF (2×2 l), EtOAc (2×2 l), CH_2Cl_2 (1×2 l), and dried at 50 °C under vacuum for 18 h to afford 1.25 kg.

The resin (1.2 kg) was suspended in a mixture of CH_2Cl_2 (8 l) and TFA (1.5 l) and stirred at room temperature for 4 h. The resin was then filtered, washed with CH_2Cl_2 (2×2 l), THF (2×2 l), MeOH (2×2 l), CH_2Cl_2 (2 l), and allowed to dry for 24 h to give 1.19 kg of resin **8**.

3.3. Synthesis of resins 6a–f

General procedure for loading ketones **1a–f** to resin **8**: A suspension of ketone **1d** (8.83 g, 21.4 mmol) and resin **8** (8.91 g, 7.13 mmol/g) in THF (70 mL) and AcOH (0.2 mL, 0.2 g, 3.4 mmol) was shaken on an orbital shaker for 20 h. The resin was filtered and was washed with THF (2×25 mL), CH_2Cl_2 (2×25 mL), EtOAc (2×25 mL), and Et_2O (2×25 mL). Drying under high vacuum afforded resin **6d** (11.4 g).

3.4. Preparation of peptides on solid support via automation

The PerSeptive 9050 Plus PepSynthesizer (Millipore Corporation, 34 Maple Street, Milford, MA 01757, User's Guide 9050 Plus OM 1.0 was used for the preparation of the peptides on solid support.

3.4.1. (3*S*)-3-((2*S*)-2-[((2*S*)-2-[(2*S*)-2-(Acetylamino)-3-carboxypropanoyl]amino-4-carboxybutanoyl)amino]-3-methylbutanoylamino)-4-oxo-7-phenylheptanoic acid (14). Using the synthesis procedure given in the user's guide the tetrapeptide was prepared with the following reagents: 290 mg of resin **6d** (0.7 meq/g loading), Fmoc-L-Val-OH (1.65 g), Fmoc-L-Glu (O*t*Bu)-OH (1.83 g), and Fmoc-L-Asp (OtBu)-OH (1.80 g); *O*-(7-azabenzotriazol-1-yl)*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIEA) were used as coupling reagents instead of TBTU and HOBt as described in the user's guide.

Acetylation: The uncapped tetrapeptide on solid support was transferred into a fritted reaction vessel and treated with acetylimidazole (550 mg, 5 mmol) in 5 mL DMF. The mixture is agitated for 1.5 h, filtered and then washed with DMF (3×), THF (3×), CH_2Cl_2 (4×) (2 mL each).

Cleavage from solid support: The above polymer was treated with TFA:H_2O (9:1, 2 mL) and agitated for 30 min. The solution was filtered, the solid support washed with TFA (2×0.5 mL) and the filtrate was evaporated. Trituration from Et_2O gave 58 mg of the tetrapeptide derivative **14** as a colorless solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.32 (d, 1H, J = 7.20 Hz), 7.90 (d, 1H, J = 8.08 Hz), 7.30–7.10 (m, 5H), 4.67 (t, 1H, J = 6.60 Hz), 4.62 (t, 1H, J = 6.23 Hz), 4.40–4.32 (m, 1H), 4.13 (t, 1H, J = 7.60 Hz), 2.90–2.80 (m, 2H), 2.78–2.35 (m, 8H), 2.20–2.05 (m, 2H), 2.02–1.90 (m, 1H), 1.98 (s, 3H), 1.89–1.79 (m, 2H), 0.94 (d, 6H, J = 6.71 Hz); MS (+APCI) m/z 621 $(M+H)^+$.

3.4.2. (4*S*)-4-[(2*S*)-2-(Acetylamino)-3-carboxypropanoyl]-amino-5-[(1*S*)-1-([(1*S*)-1-(carboxymethyl)2-oxopropyl]-aminocarbonyl)-2-methylpropyl amino-5-oxopentanoic acid (11). Following the procedure for the preparation of **14**, using resin **6a**, Fmoc-L-Val-OH (1.65 g), Fmoc-L-Glu (OtBu)-OH (1.83 g), and Fmoc-L-Asp (O*t*Bu)-OH (1.80 g), title compound **11** was obtained as colorless solid (59 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 8.33 (d, 1H, J = 6.95 Hz), 7.92 (d, 1H, J = 7.90 Hz), 4.67 (t, 1H, J = 6.70 Hz), 4.60 (t, 1H, J = 6.20 Hz), 4.40–4.33 (m, 1H), 4.14 (t, 1H, J = 7.70 Hz), 2.91–2.81 (m, 2H), 2.78–2.65 (m, 2H), 2.50–2.34 (m, 2H), 2.20–2.08 (m, 2H), 2.17 (s, 3H), 2.01–1.89 (m, 1H), 1.99 (s, 3H), 0.96 (d, 6H, J = 6.79 Hz); MS (+APCI) m/z 517 $(M+H)^+$.

3.4.3. (3*S*)-3-((2*S*)-2-[((2*S*)-2-[(2*S*)-2-(Acetylamino)-3-carboxypropanoyl]amino-4-carboxybutanoyl) amino]-3-methylbutanoylamino)-4-oxoheptanoic acid (12). Following the procedure for the preparation of **14**, using resin **6b**, Fmoc-L-Val-OH (1.65 g), Fmoc-L-Glu (O*t*Bu)-OH

(1.83 g), and Fmoc-L-Asp (OtBu)-OH (1.80 g), title compound **12** was obtained as colorless solid (54 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 4.68 (t, 1H, $J=6.7$ Hz), 4.63 (t, 1H, $J=6.3$ Hz), 4.37 (dd, 1H, $J=5.0$, 9.1 Hz), 4.14 (d, 1H, $J=7.2$ Hz),), 2.86 (dd, 1H, $J=6.4$, 16.9 Hz), 2.85 (dd, 1H, $J=6.2$, 16.9 Hz), 2.72 (dd, 1H, $J=7.0$, 17.0 Hz), 2.68 (dd, 1H,$J=6.4$, 18.2 Hz), 2.34–2.54 (m, 4H), 2.08–2.16 (m, 2H), 1.99 (s, 3H), 1.90–1.99 (m, 1H), 1.51–1.58 (m, 2H), 0.96 (d, 6H, $J=6.7$ Hz), 0.89 (d, 3H, $J=7.4$ Hz); MS (-APCI) m/z 543 $(M-H)^-$.

3.4.4. (4*S*)-4-[(2*S*)-2-(Acetylamino)-3-carboxypropanoyl]-amino-5-[(1*S*)-1-([(1*S*)-1-(carboxymethyl)-2-oxo-2-phenylethyl]amino carbonyl)-2-methylpropyl]amino-5-oxopentanoic acid (13). Following the procedure for the preparation of **14**, using resin **6c**, Fmoc-L-Val-OH (1.65 g), Fmoc-L-Glu (O*t*Bu)-OH (1.83 g), and Fmoc-L-Asp (O*t*Bu)-OH (1.80 g), title compound **13** was obtained as colorless solid (52 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 7.96 (d, 1H, $J=7.2$ Hz), 7.59 (t, 1H, $J=7.4$ Hz), 7.47 (t, 2H, $J=7.8$ Hz), 5.70 (dd, 1H, $J=5.9$, 7.7 Hz), 4.68 (t, 1H, $J=7.0$ Hz), 4.33 (dd, 1H, $J=5.1$, 9.1 Hz), 4.06 (d, 1H, $J=7.2$ Hz), 3.02 (dd, 1H, $J=7.8$, 16.7 Hz), 2.85 (dd, 1H, $J=6.1$, 17.0 Hz), 2.71 (dd, 1H, $J=7.2$, 17.0 Hz), 2.66 (dd, 1H, $J=5.8$, 16.7 Hz), 2.31–2.40 (m, 2H), 1.83–2.10 (m, 3H), 1.99 (s, 3H), 0.82 (d, 3H, $J=6.8$ Hz), 0.77 (d, 3H, $J=6.8$ Hz); MS (–APCI) m/z 577 $(M-H)^-$.

3.4.5. (3*S*)-3-((2*S*)-2-[((2*S*)-2-[(2*S*)-2-(Acetylamino)-3-carboxypropanoyl]amino - 4 - carboxybutanoyl)amino] - 3-methylbutanoylamino)-4-oxo-9-phenylnonanoic acid (15). Following the procedure for the preparation of **14**, using resin **6e**, Fmoc-L-Val-OH (1.65 g), Fmoc-L-Glu (O*t*Bu)-OH (1.83 g), and Fmoc-L-Asp (O*t*Bu)-OH (1.80 g), title compound **15** was obtained as colorless solid (61 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 7.86 (d, 1H, $J=8.4$ Hz), 7.09–7.24 (m, 5H), 4.62–4.69 m, 2H), 4.35 (dd, 1H, $J=4.8$, 8.8 Hz), 4.13–4.18 (m, 1H), 2.79–2.90 (m, 2H), 2.33–2.77 (m, 8H), 2.05–2.20 (m, 2H), 1.99 (s, 3H), 1.86–2.00 (m, 1H), 1.50–1.64 (m, 4H), 1.24–1.87 (m, 2H), 0.95 (d, 6H, $J=6.7$ Hz); MS (–APCI) m/z 647 $(M-H)^-$.

3.4.6. (3*S*)-3-((2*S*)-2-[((2*S*)-2-[(2*S*)-2-(Acetylamino)-3-carboxypropanoyl]amino - 4 - carboxybutanoyl)amino] - 3-methylbutanoylamino)-7-(1-naphthyl)-4-oxoheptanoic acid (16). Following the procedure for the preparation of **14**, using resin **6f**, Fmoc-L-Val-OH (1.65 g), Fmoc-L-Glu (OtBu)-OH (1.83 g), and Fmoc-L-Asp (OtBu)-OH (1.80 g), title compound **16** was obtained as colorless solid (60 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 8.12 (d, 1H, $J=8.1$ Hz), 7.86 (d, 1H, $J=8.1$ Hz), 7.82 (d, 1H, $J=7.7$ Hz), 7.68 (d, 1H, $J=8.1$ Hz), 7.31–7.50 (m, 4H), 4.60–4.70 (m, 2H), 4.35 (dd, 1H, $J=4.7$, 8.9 Hz), 4.14 (brt, 1H, $J=7.9$ Hz), 3.00–3.08 (m, 2H), 2.83–2.92 (m, 2H), 2.55–2.78 (m, 4H), 2.35–2.45 (m, 2H), 2.07–2.19 (m, 2H), 1.90-2.03 (m, 5H), 0.94 (d, 6H, $J=6.8$ Hz); MS (–APCI) m/z 670 $(M-H)^-$.

3.4.7. (3*S*)-3-[((2*S*)-2-[(2*S*)-2-[(2*S*)-2-(Acetylamino)-3-carboxypropanoyl]amino-4-(methylsulfonyl) butanoyl]-amino-3-methylbutanoyl)amino]-4-oxo-7-phenylheptanoic acid (17). Following the procedure for the preparation of **14**, using resin **6d**, Fmoc-L-Val-OH (1.65 g), Fmoc-L-Met(O_2)-OH (1.80 g), and Fmoc-L-Asp (O*t*Bu)-OH (1.80 g), title compound **17** was obtained as colorless solid (72 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 8.23 (d, 1H, $J=7.60$ Hz), 7.93 (d, 1H, $J=7.34$ Hz), 7.25–7.05 (m, 5H), 4.68–4.58 (m, 2H), 4.57–4.48 (m, 1H), 4.12 (t, 1H, $J=7.47$ Hz), 3.25–3.10 (m, 2H), 2.95 (s, 3H), 2.90–2.48 (m, 8H), 2.40–2.28 (m, 1H), 2.20–2.02 (m, 2H), 1.97 (s, 3H), 1.90–1.78 (m, 2H), 0.93 (d, 6H, $J=6.57$ Hz); MS (–APCI) m/z 653 $(M-H)^-$.

3.4.8. (3*S*)-3-((2*S*)-2-[((2*S*)-2-[(2*S*)-2-(Acetylamino)-3-carboxypropanoyl]aminopropanoyl)amino]-3-methylbutanoylamino)-4-oxo-7-phenylheptanoic acid (18). Following the procedure for the preparation of **14**, using resin **6d**, Fmoc-L-Val-OH (1.65 g), Fmoc-L-Ala-OH(1.56 g), and Fmoc-L-Asp (O*t*Bu)-OH (1.80 g), title compound **18** was obtained as colorless solid (73 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 8.32 (d, 1H, $J=7.20$ Hz), 7.82 (d, 1H, $J=8.12$ Hz), 7.30–7.10 (m, 5H), 4.68 (t, 1H, $J=6.70$ Hz), 4.63 (t, 1H, $J=6.21$ Hz), 4.30–4.10 (m, 1H), 4.13 (brt, 1H, $J=7.62$ Hz), 2.91–2.80 (m, 2H), 2.75–2.45 (m, 6H), 2.20–2.05 (m, 1H), 1.97 (s, 3H), 1.90–1.78 (m, 2H), 1.37 (d, 3H, $J=7.20$ Hz), 0.94 (d, 6H, $J=6.80$ Hz); MS (+APCI) m/z 563 $(M+H)^+$.

3.4.9. (3*S*)-3-((2*S*)-2-[((2*S*)-2-[(2*S*)-2-(Acetylamino)-4-methoxy-4-oxobutanoyl]aminopropanoyl)amino]-3-methylbutanoylamino)-4-oxo-7-phenylheptanoic acid (19). Following the procedure for the preparation of **14**, using resin **6d**, Fmoc-L-Val-OH (1.65 g), Fmoc-L-Ala-OH (1.56 g), and Fmoc-L-Asp (OMe)-OH (1.70 g), title compound **19** was obtained as colorless solid (17 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 7.30–7.08 (m, 5H), 4.70 (t, 1H, $J=6.76$ Hz), 4.62 (t, 1H, $J=6.12$ Hz), 4.35–4.25 (m, 1H), 4.19–4.10 (m, 1H), 3.67 (s, 3H), 2.95–2.45 (m, 8H), 2.20–2.05 (m, 1H), 1.96 (s, 3H), 1.90–1.78 (m, 2H), 1.37 (d, 3H, $J=7.17$ Hz), 0.94 (d, 6H, $J=6.77$ Hz); MS (+APCI) m/z 577 $(M+H)^+$.

3.4.10. (3*S*)-3-((2*S*)-2-[((2*S*)-2-[(2*R*)-2-(Acetylamino)-3-(methylsulfonyl)propanoyl]aminopropanoyl)amino]-3-methylbutanoylamino)-4-oxo-7-phenylheptanoic acid (20). Following the procedure for the preparation of **14**, using resin **6d**, Fmoc-L-Val-OH (1.65 g), Fmoc-L-Ala-OH (1.56 g), Fmoc-L-Cys(SMe)-OH (1.56 g), the thiomethyl derivative was obtained as colorless solid (108 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 7.76 (d, 1H, $J=8.02$ Hz), 7.25–7.05 (m, 5H), 4.60 (t, 1H, $J=6.03$ Hz), 4.51 (t, 1H, $J=7.28$ Hz), 4.40–4.30 (m, 1H), 4.15 (t, 1H, $J=7.56$ Hz), 2.96–2.45 (m, 8H), 2.15–2.08 (m, 1H), 2.11 (s, 3H), 1.97 (s, 3H), 1.90–1.88 (m, 2H), 1.35 (d, 3H, $J=7.18$ Hz), 0.92 (d, 3H, $J=6.76$ Hz), 0.91 (d, 3H, $J=6.74$ Hz); MS (–APCI) m/z 563 $(M-H)^-$.

The thiomethyl derivative (20 mg, 0.035 mmol) in 1 mL MeOH at 0 °C was treated with OXONE® (22 mg, 0.035 mmol) in 1 mL water. The mixture was allowed to warm to room temperature over 0.5 h and then poured into 30 mL EtOAc. Water was added, the layers were separated, and the aqueous layer was extracted with EtOAc (2×5 mL). The combined organic layers were dried over $MgSO_4$, filtered and evaporated to give 6 mg of title

compound **20** as colorless solid: ^{1}H NMR (400 MHz, CD_3OD) δ 8.14 (d, 1H, $J = 7.81$ Hz), 7.75 (d, 1H, $J = 7.24$ Hz), 7.25–7.05 (m, 5H), 4.94 (t, 1H, $J = 6.59$ Hz), 4.62 (t, 1H, $J = 6.20$ Hz), 4.35–4.20 (m, 1H), 4.15–4.05 (m, 1H), 3.78–3.70 (m, 1H), 3.48–3.38 (m, 1H), 3.29 (s, 3H), 2.88–2.80 (m, 1H), 2.75–2.45 (m, 5H), 2.15–2.03 (m, 1H), 1.97 (s, 3H), 1.90–1.80 (m, 2H), 1.37 (d, 3H, $J = 7.26$ Hz), 0.93 (d, 3H, $J = 6.77$ Hz), 0.92 (d, 3H, $J = 6.74$ Hz); MS (–APCI) m/z 595 $(M-H)^-$.

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