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Exploiting differences in caspase-2 and -3 S_2 subsites for selectivity: Structure-based design, solid-phase synthesis and in vitro activity of novel substrate-based caspase-2 inhibitors

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ABSTRACT

Several caspases have been implicated in the pathogenesis of Huntington's disease (HD); however, existing caspase inhibitors lack the selectivity required to investigate the specific involvement of individual caspases in the neuronal cell death associated with HD. In order to explore the potential role played by caspase-2, the potent but non-selective canonical Ac-VDVAD-CHO caspase-2 inhibitor **1** was rationally modified at the P_2 residue in an attempt to decrease its activity against caspase-3. With the aid of structural information on the caspase-2, and -3 active sites and molecular modeling, a 3-(*S*)-substituted-L-proline along with four additional scaffold variants were selected as P_2 elements for their predicted ability to clash sterically with a residue of the caspase-3 S_2 pocket. These elements were then incorporated by solid-phase synthesis into pentapeptide aldehydes **33a**–**v**. Proline-based compound **33h** bearing a bulky 3-(*S*)-substituent displayed advantageous characteristics in biochemical and cellular assays with 20- to 60-fold increased selectivity for caspase-2 and ~200-fold decreased caspase-3 potency compared to the reference inhibitor **1**. Further optimization of this prototype compound may lead to the discovery of valuable pharmacological tools for the study of caspase-2 mediated cell death, particularly as it relates to HD.

1. Introduction

The human caspases (Csp) are a family of intracellular cysteine aspartyl-specific proteases with at least 12 members that play essential roles in inflammation, apoptosis and a variety of other physiological and pathological processes.[1] The promise of pro-inflammatory Csp-1 as a drug target for rheumatoid arthritis has encouraged intensive research efforts culminating with two Csp-1 inhibitors reaching clinical stage evaluation.[2] Additionally, in apoptotic-driven indications, especially neurodegenerative diseases, efficacy in proof-of-concept in vivo experiments has resulted in an increased focus on several members of the family.[3] Consequently, caspases represent a key drug target family of particular interest to our research organization whose mission is to improve the understanding of Huntington's disease (HD) as it relates to expediting the development of therapies.

HD is a fatal genetic disorder characterized pathologically by the selective loss of neurons, predominantly in the striatum, and clinically by uncontrollable movements, progressive cognitive deficit, and psychiatric disturbance.[4] The underlying genetic deficit of this disease is a CAG trinucleotide repeat expansion encoding an abnormally long polyglutamine tract in the huntingtin (HTT) protein. Early reports suggested that polyglutamine expansion in HTT mediates apoptosis through caspase activation, in particular Csp-1, -3, -8 and -9,[5] while more recently Csp-2 and -7 activation has been reported in HD post-mortem tissue.[6] Moreover, as many as five caspases (Csp-1, -2, -3, -6 and -7)[6,7] have been implicated in mutant HTT (mHTT) cleavage resulting in the production of potentially more toxic polyglutamine-expanded HTT fragments.[8] In testing this toxic fragment hypothesis, YAC mice expressing Csp-6 cleavage-resistant mHTT did not develop striatal neurodegeneration.[9] Thus, whether it is through the induction of apoptosis or through the production of toxic

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HTT fragments and their downstream consequences, there is sufficient in vitro and in vivo experimental evidence suggesting a role for caspase inhibition in HD to warrant further investigation.

Further substantiating these findings is the efficacy of broad-spectrum peptide-based caspase inhibitors in preventing HTT cleavage and reducing toxicity in vitro[7b,10] and in HD animal models.[11] However, the lack of selective caspase inhibitors has hampered further progress in understanding the specific involvement of individual caspases in mHTT-induced apoptosis or mHTT cleavage; consequently, we set out to develop these selective inhibitors. Selective Csp-1,[12] Csp-3[12a,13] and Csp-7[13a] inhibitors have been disclosed; however, for Csp-2 and Csp-6, the two leading caspase targets for HD, there are no reports to our knowledge of selective inhibitors. In order to be able to rapidly test our inhibitor design strategy, we decided to focus our effort initially on the development of Csp-2 inhibitors with reduced activity for Csp-3.

We report here the successful application of structure-based design to the development of potent inhibitors of Csp-2 with improved selectivity. We discuss the rationale for targeting the S_2 pocket and choosing proline-based templates as the P_2 element. Synthesis of these key P_2 scaffolds and their incorporation into pentapeptide aldehydes via solid-phase chemistry are presented along with a detailed analysis of the biochemical and cellular selectivity of these new inhibitors.

2. Inhibitor design

Ideally, non-peptide small-molecule inhibitors would represent the most desirable starting point to achieve our goal of developing a selective and cell permeable Csp-2 inhibitor. Unfortunately, almost twenty years after the cloning of the first caspase,[14a] the identification of non-covalent small-molecule caspase inhibitors remains the 'Holy Grail' in the field because of the extreme challenges in transforming an electrophilic warhead-based tetra- or pentapeptide with an aspartic acid into a non-peptide mimetic. Not surprisingly, screening campaigns have had very limited success with only a few reports of validated hits[15] including isatins.[13] However, we recently disclosed unsuccessful attempts to prepare potent Csp-2 isatin inhibitors and to reproduce the activity of several published non-peptidic Csp-3 and -6 inhibitors identified by screening.[16]

In the peptide class, potent inhibitors have been derived from substrate sequences obtained by positional scanning combinatorial libraries (PSCL) or peptide sequence variants employed in the seminal work of the Thornberry and Talanian groups.[14b,17] It is sobering to note that over a decade later, the vast majority of the numerous substrate-derived caspase inhibitors including the commercial ones lack sufficient specificity to inhibit individual caspase pathways. In particular for Csp-2, the canonical inhibitor Ac-VDVAD-CHO (**1**, Table 1) is more potent against Csp-3 which casts some doubt about the validity of the data generated with this reagent when employed in a cellular context as a 'selective' Csp-2 inhibitor. Other groups have already expressed the same specificity concerns regarding existing reagents and stressed the urgent need for more selective inhibitors in the caspase field.[18] In particular, selectivity against Csp-3 is critically important because of the high concentration of active Csp-3 relative to other caspases in almost all tissues and of its promiscuous nature.[18c] Furthermore, the neuroprotective effects of a Csp-3 inhibitor has already been reported in a HD model.[11b] For these reasons, it was important to develop a Csp-2 tool with no or little Csp-3 activity in order to be able to specifically evaluate the role of Casp-2 in HD.

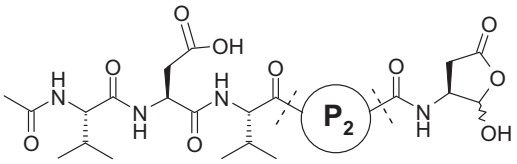
Due to the absence of small-molecule leads, a substrate-based approach represented, in our opinion, the best option for the rapid development of selective and cell permeable Csp-2 inhibitors. Relatively recently, a PSCL approach was used to attempt to address this issue of cross reactivity of peptide-based caspase inhibitors.[19] Unfortunately, despite the use of both natural and unnatural amino acids, the selectivity against Csp-3 of the described Csp-8 and Csp-9 inhibitors remained insufficient. In this context, we favored a rational design approach based on structural information of the targeted caspases of interest. At the time of this work, crystal structures were available for Csp-2[20] and Csp-3[21] but not for Csp-6,[22] therefore we decided to target Csp-2 with the goal to design potent inhibitor with >100-fold selectivity against Csp-3.

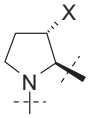
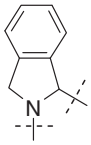
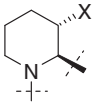
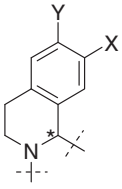
Our design strategy commenced with a detailed comparative structural analysis of the S_1 through S_5 Csp-2 and Csp-3 subsites (Table 2) searching for residue differences in the active site cleft from which selectivity against Csp-3 could be derived. The S_4 pocket was examined first as this subsite is the most divergent in the caspase family and it had been successfully exploited to develop potent Csp-1 tetrapeptidic inhibitors with >100-fold selectivity against Csp-3.[12] In the case of Csp-2, the triad Asn-Trp-Tyr of the S_4 subsite is unfortunately almost completely conserved in Csp-3 (Asn-Trp-Phe) which is reflected in the nearly exclusive preference for an Asp in S_4 for both caspases.[14b,17] Our analysis continued with the key recognition S_1 pocket which was quickly discounted because S_1 is completely conserved.[23] Similarly, Csp-2 and -3 share structurally equivalent hydrophobic solvent exposed S_5 pockets[24] which are not amenable to our selectivity design. The same conclusion was also reached for the S_3 pockets which in Csp-2 and -3 are both flat polar surfaces providing no opportunity to drive selectivity.[14b,25]

Finally, important differences in terms of shape and size were observed in the S_2 subsites of Csp-2 and -3. The most distinguishable residues are Ala-228 in Csp-2 which is replaced in Csp-3 by Tyr-204 and Phe-279 in Csp-2 which is moved by about 3 Å inside the pocket in Csp-3 (Phe-256). In part as a result of this amino acid change, the bowl-shaped lipophilic S_2 subsite of Csp-3 is changed into a groove, with a narrow opening perpendicular to the active-site cleft (Fig. 1A and B). Thus, these S_2 subsite differences represented the best opportunity on the unprime side for the design of selective Csp-2 inhibitors as we hypothesized that a bulky P_2 accommodated in Csp-2 would be likely to clash sterically with Tyr-204 of Csp-3. Molecular modeling also predicted limited mobility for Tyr-204 which further reinforced our idea. The second stage of the design was to 'engineer' a vector from the peptidic backbone to provide an adequate trajectory for the selected bulky P_2 to clash with Tyr-204. Modeling experiments indicated that direct substitution on the peptidic backbone α-carbon often led to the large P_2 side chains to point away from the Tyr-204 into solvent as a result of movement at the P_1–P_2 junction. This led us to consider L-proline analogues since we envisioned that a rigid template could lock a substituent in the required trajectory and reduce the possibility of movement from the peptidic backbone (Fig. 1C). Another attractive feature of L-proline is that it has been frequently used as a key building block in peptidomimetic design including for Csp-1.[2b,26]

To our knowledge, there is no report of P_2 proline Csp-2 inhibitors; however, there are P_2 Pro-containing inhibitors for Csp-3,[27] -7[28] and the caspase-like granzyme B.[29] Crystal co-complexes of these three proteins with P_2 proline substrate-like inhibitors are also available.[27–29] Examination of these three co-structures revealed that a common feature is the presence of a Phe residue nestled against the P_2 proline. These three P_2 Phe residues overlay well with Phe-279 of Csp-2 which supports the notion that a proline residue should also be well tolerated as a P_2 in Csp-2. Furthermore, PSCL results corroborated our structure-based hypothesis that

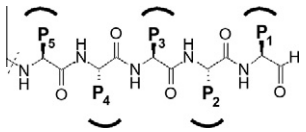
Table 1
Biological activity of pentapeptide aldehydes



	P_2		Enzyme assay Casp-2 IC_{50} (nM)	Enzyme assay Casp-3 IC_{50} (nM)	Casp-3/ Casp-2 ratio	Cell assay Casp-2 IC_{50} (nM)	Cell assay[a] Casp-3 IC_{50} (nM)	Casp-2 cell/ enzyme ratio
1	(*S*)-Ala		46	15	0.3	1334	346	29
2	(*S*)-*O*-*t*Bu-serine		41	29	0.7	1690	437	41
33a		X = H	41	12	0.3	635	92	15
33b		X = OCH_3	108	59	0.5	1940	965	18
33c		X = $OCH(CH_3)_2$	246	5533	22	12,600	>100,000	51
33d		X = $OCH(CH_2CH_3)CH_3$	413	5125	12	>50,000	>25,000	>121
33e		X = $OCH_2CH(CH_3)_2$	453	9300	20	32,000	nd	71
33f		X = *O*-cyclopentyl	772	22,500	29	35,667	nd	46
33g		X = *O*-(2-pyrimidyl)	219	9920	45	17,400	>100,000	79
33h		X = $CH_2C(CH_3)_3$	50	2925	60	2360	78,350	65
33i		X = phenyl	441	10,400	24	50,000	nd	113
33j		X = 4-CH_3-phenyl	1600	2765	1.7	>65,000	>50,000	>41
33k		X = cyclohexyl	136	4075	30	13,607	42,160	100
33l			150	289	2	781	nd	5
33m		X = H	86	26	0.3	1120	24	13
33n		X = $OCH(CH_3)_2$	52,800	81,500	1.5	>100,000	>100,000	>2
33o		X = H, Y = H, * = *S*	22	233	11	2260	nd	103
33p		X = CH_3, Y = H, * = *R*/*S*	60	1410	25	5533	>35,000	92
33q		X = H, Y = CH_3, * = *R*/*S*	50	3000	60	6670	>60,000	130
33r		X = OCH_3, Y = OCH_3, * = *R*/*S*	2190	7970	4	>100,000	>100,000	>45
33s		X = H, Y = OH, * = *R*/*S*	1980	58,600	30	12,823	nd	6
33t		X = OH, Y = H, * = *R*/*S*	459	23,000	50	23,567	nd	50
33u		X = H, Y = Br, * = *R*/*S*	163	907	6	7470	nd	46
33v		X = Br, Y = H, * = *R*/*S*	573	1075	1.8	3110	39,170	5

[a] nd, not determined.

Table 2
Generalized structure of pentapeptide aldehyde inhibitors: (P_5–P_4–P_3–P_2–P_1–CHO) and key residues of S_1–S_5 subsites in Csp-2 and Csp-3



Csp-2	Tyr-273	Asn-232	Ser-55	Ala-228	Arg-54
	Ala-274	Trp-238	Thr-233	Met-230	Arg-231
	Pro-275	Tyr-273	Arg-231	Phe-279	Gln-153
Csp-3	Phe-250	Asn-208	Ser-65	Tyr-204	Arg-64
	Ser-251	Trp-214	Ser-209	Trp-206	Arg-207
	Phe-252	Phe-250	Arg-207	Phe-256	Gln-161
Subsite	S_5	S_4	S_3	S_2	S_1

proline would be accepted in the S_2 subsite of Csp-2.[14b] The final step in the design was the identification of the position, orientation and size of the substituent on the proline ring. Additional modeling experiments indicated that from the 3-position large alkyl or alkoxy groups (isobutyl, isopropoxy) in the (*S*)-configuration would have the required trajectory and size to clash with Tyr-204 of Csp-3 and would be tolerated in the S_2 groove of Csp-2. Finally, taking a variable degree of synthetic accessibility into account, we designed a set of analogs based on five different proline-based motifs as P_2 scaffolds (i)–(v) (Fig. 1D) which would assess the design principles described above.

3. Chemistry

3.1. P_2 Amino acid synthesis

The synthetic routes to the P_2 amino acids are outlined in Schemes 1–4. The synthesis of 3-oxyproline analogues **6a–f** is described in Scheme 1. Reaction of *N*-Fmoc-3-(*S*)-hydroxy-L-proline[30] **3** with methanol under acidic conditions afforded the methyl ester **4** which was then alkylated using a series of alkyl iodides in a Ag_2O mediated etherification to give compounds **5a–e**; final acid mediated hydrolysis of the ester afforded compounds **6a–e**. The 2-pyrimidyl analogue **6f** was obtained via a S_NAr reaction of *N*-Boc-3-(*S*)-hydroxy-L-proline **7** with 2-chloropyrimidine and subsequent protective group interconversion. Synthesis of the 3-(*S*)-alkyl and aryl proline analogues **15a–d** were performed using the

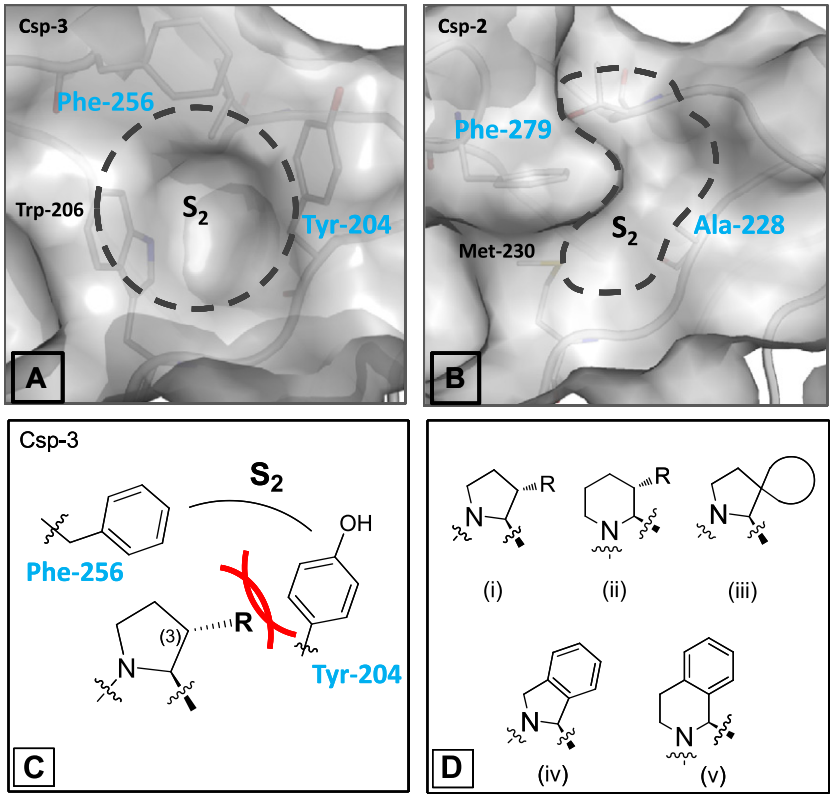
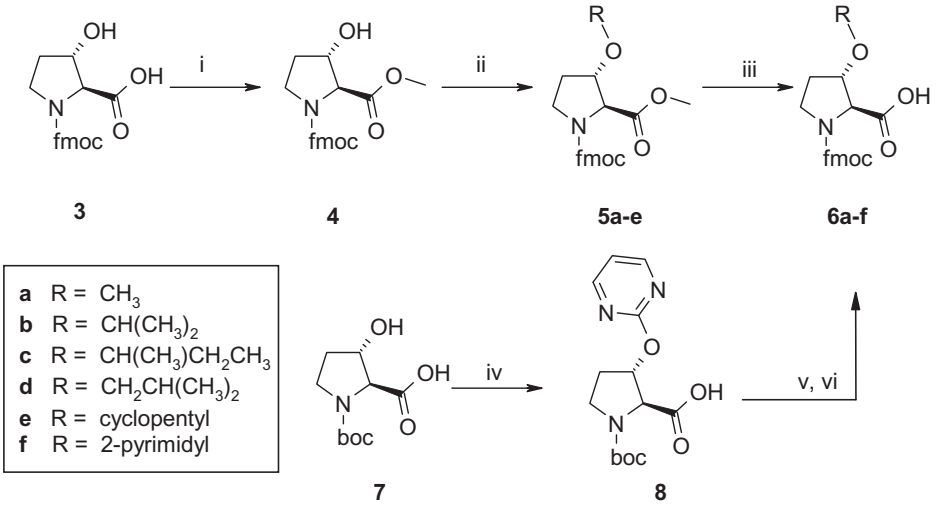


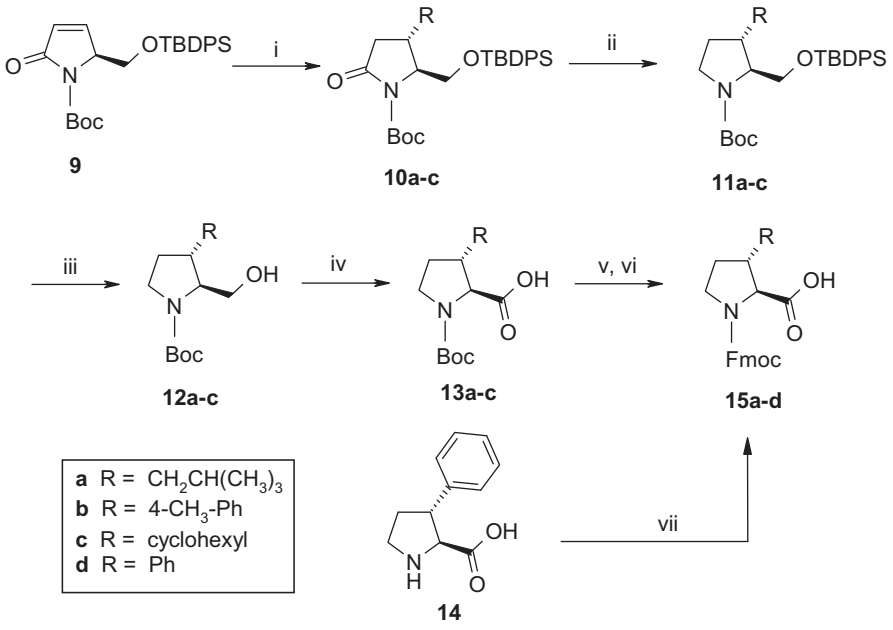
Figure 1. Shape of S_2 binding subsites with surface colored in grey for (A) Csp-3 (PDB code 2DKO[21b]) and (B) Csp-2 (PDB code 1PYO[20]). (C) Putative steric clash of 3(*S*)-proline substituent (-**R**) in S_2 pocket of Csp-3. (D) Selected scaffold variants (i)–(v). Parts (A) and (B) were generated using PyMOL.[39]



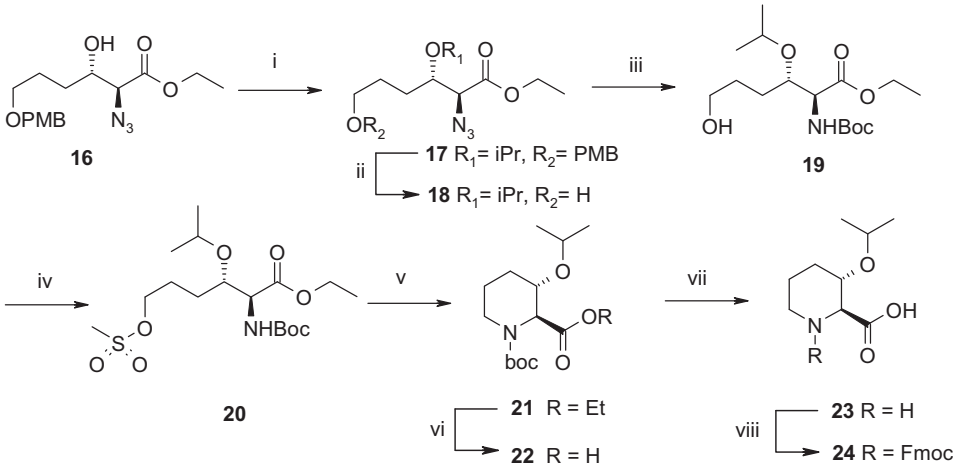
Scheme 1. Synthetic procedure for 3-oxyproline amino acid analogues **6a–f**. Reagents and conditions: (i) 4N HCl in dioxane, MeOH, rt, 24 h, 68%; (ii) RI, Ag_2O, DCE, rt or reflux, 24 h, 3–63%; (iii) 6N HCl (aq), dioxane, reflux, 15 h, 42–95%; (iv) 2-chloropyrimidine, KO*t*Bu, *t*BuOH, 1 h, 35%; (v) 4 N HCl in dioxane, rt, 1 h; (vi) $NaHCO_3$, FmocCl, dioxane, water, 0 °C to rt, 48 h, 65%.

chemistry described in Scheme 2. Compound **9** was synthesized following the procedure of Herdeis and Hubmann[31] and subsequent Michael addition of organocuprates afforded 3-(*S*)-substituted pyrrolidinones **10a–c** in good yield. Reduction of the pyrrolidine amide using borane–dimethylsulfide complex generated 3-(*S*)-substituted pyrrolidines **11a–c** and TBAF deprotection of the silyl protecting group gave hydroxymethylpyrrolidines **12a–c** which were subjected to Jones oxidation to give acids **13a–c**. Switching of the carbamate protecting group from Boc to Fmoc was performed using standard deprotection and protection conditions to give Fmoc-3-(*S*)-substituted prolines **15a–c**. Compound **15d** was synthesized from the commercially available 3-(*R*)-phenyl-L-proline **14** via Fmoc protection.

Scheme 3 describes the synthesis of *N*-Fmoc-3-(*S*)-isopropoxypipecolic acid analogue **24**. The PMB protected hydroxyl azide **16** was synthesized following the procedure described by Kumar et al.[32] and alkylation of this compound was successfully performed using isopropyl iodide and Ag_2O to give compound **17**. Deprotection of the PMB group was performed using DDQ resulting in hydroxy azide **18** which was converted to **19** using a one pot



Scheme 2. Synthetic procedure for Fmoc-3-(*S*)-alkyl proline **15a–d**. Reagents and conditions: (i) RLiCu or RMgBrCu, TMSCl, Et_2O, −78 °C to rt, 2 h, 70–80%; (ii) BH_3·DMS, THF, reflux, 1 h, 82–100%; (iii) TBAF, THF, rt, 24 h, 65–91%; (iv) Jones reagent, acetone, rt, 5 h, 61–100%; (v) TFA/DCM (1:1), 0 °C, 1 h; (vi) FmocCl, $NaHCO_3$, dioxane, rt, 18 h, 55–100%; (vii) FmocCl, $NaHCO_3$, dioxane, water, 0 °C to rt, 18 h, 78%.



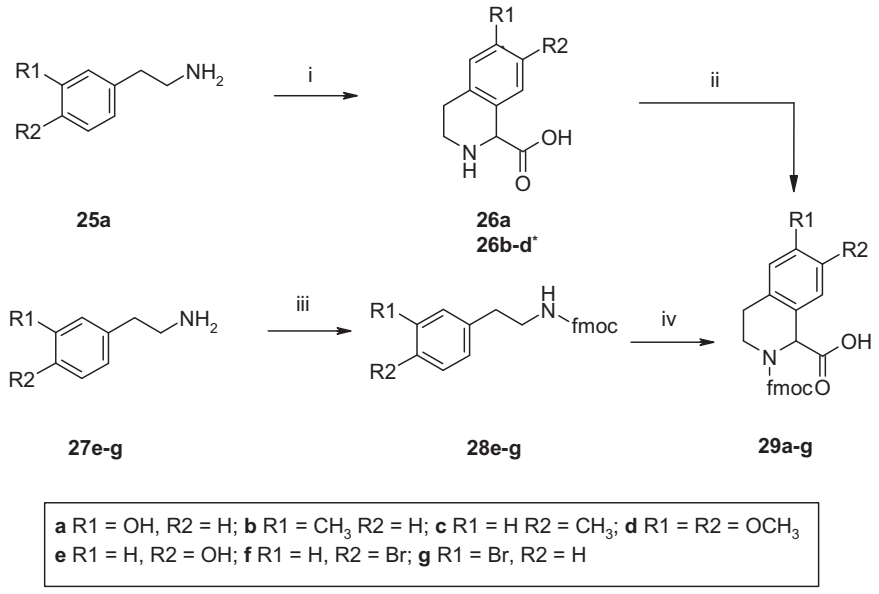
Scheme 3. Synthetic procedure for 3-isopropoxypiperidine amino acid analogue **24**. Reagents and conditions: (i) ^{i}PrI, Ag_2O, DCE, 70 °C, 17 h, 18%; (ii) DDQ, DCM, H_2O, rt, 1 h 30, 73%; (iii) H_2, 10%Pd/C, Boc_2O, EtOAc, rt, 15 h, 80%; (iv) MsCl, TEA, DCM, 0 °C to rt, 2 h, 76%; (v) NaH, DMF, 0 °C to rt, 4 h, 59%; (vi) LiOH, EtOH/H_2O, rt, 3 h, 50%; (vii) TFA, DCM, 0 °C, 2 h, 90%; (viii) FmocCl, dioxane, 0 °C to rt, 16 h, 50%.

reduction and N-protection strategy. Mesylation of **19** with methanesulfonyl chloride afforded **20** which was successfully cyclized using NaH to give the pipecolic ester **21**. Transformation of **21** into the Fmoc pipecolic acid **24** was performed following standard hydrolysis and Boc deprotection/Fmoc protection strategies.

Scheme 4 describes the synthesis of *N*-Fmoc-6-, 7-mono substituted and 6,7-disubstitued tetrahydroisoquinoline-1-carboxylic acids **29a–g**. The *N*-Fmoc-6-hydroxy tetrahydroisoquinoline-1-carboxylic acid **29a** was prepared by reaction of the commercially available 3-(2-amino-ethyl)-phenol **25a** with glyoxylic acid followed by Fmoc-protection. Compounds **29b–d** were prepared by Fmoc protection of the commercially available tetrahydroisoquinoline-1-carboxylic acids **26b–d**. Fmoc protection of the substituted phenethylamines **27e–g** followed by a Pictet–Spengler cyclisation using glyoxylic acid afforded compounds **29e–g**.

3.2. Solid-phase synthesis

Synthesized Fmoc amino acids **6a–f**, **15a–d**, **24** and **29a–g** and commercially available, Fmoc amino acids ((*S*)-Fmoc-*O*-*tert*-butyl-serine, (*S*)-Fmoc-proline, Fmoc-2,3-dihydro-1*H*-isoindole-1-carboxylic acid, (*S*)-Fmoc-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid, (*S*)-Fmoc pipecolic acid) were loaded onto aspartic acid functionalized semi-carbazide resin **30** as described by Grimm et al.[33] (Scheme 5). Fmoc deprotection of resins **31** using 20% piperidine in DMF followed by coupling of the resin immobilized P_2 amino acids with AcVD(O*t*Bu)V-OH (synthesized using classic linear



Scheme 4. Synthetic procedure for Fmoc-tetrahydroisoquinoline-1-carboxylic acid analogues **29a–g**. * denotes commercial compounds. Reagents and conditions: (i) Glyoxylic acid, TEA, EtOH, 5 °C to rt, 1 h, 70%; (ii) FmocCl, $NaHCO_3$, dioxane, water, 0 °C to rt, 18 h, 33–83%; (iii) Fmoc, DIPEA, DCM, 0 °C to rt, 20 h, 23–55%; (iv) Glyoxylic acid, H_2SO_4, AcOH, rt, 24 h, 21–36%.

peptide elongation)[34] using HATU gave resin bound peptides **32**. The final peptide aldehydes **2** and **33a–v** were obtained using a one step acid mediated deprotection and cleavage from resin using TFA/water.

4. Results and discussion

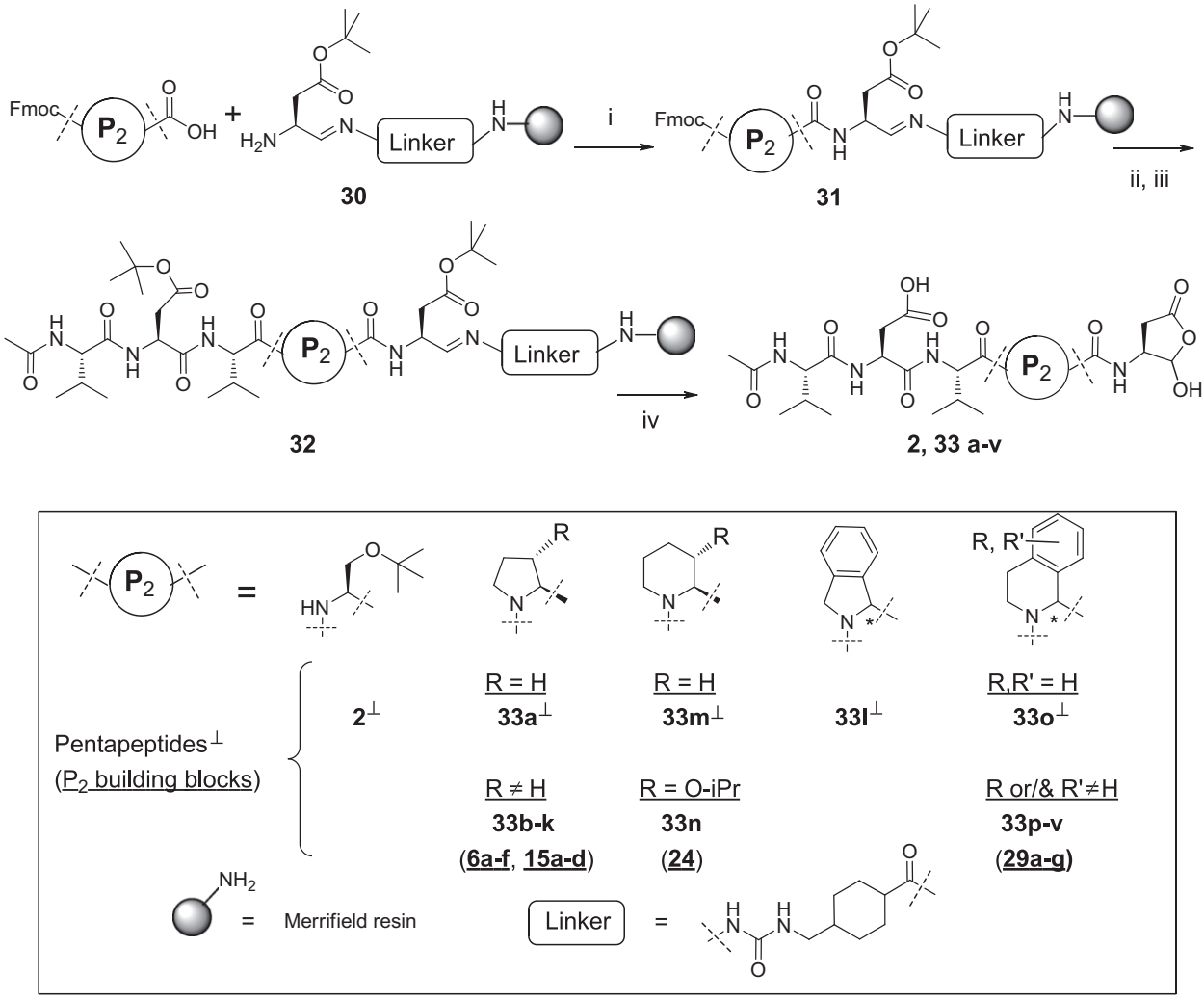
4.1. Unsubstituted P_2 scaffolds

We set out to synthesize first the unsubstituted compounds corresponding to the five scaffolds (i–v) represented in Figure 1D. It was gratifying to see our P_2 proline hypothesis proven correct as analog Ac-VDVPD-CHO **33a** (Table 1) had the same activity in Casp-2 as the reference inhibitor Ac-VDVAD-CHO **1**. As expected, the homologous piperidine analog **33m** also had similar activity to proline **33a**, and hence, both scaffolds (i) and (ii) were validated for the next step of substitution at the 3-position. According to molecular docking, the isoindole **33l** and isoquinoline **33o** presented a reduced steric clash with the Tyr-204 of Csp-3 when compared to the large 3(*S*)-substituent on the proline, however these two analogs were easily accessible and prepared to test the robustness of the docking model. It was interesting to observe that, unlike the proline **33a** and piperidine **33m**, these homologous bicyclic systems had different activity profile: isoquinoline **33o** showed a 10-fold selectivity against Csp-3 whilst maintaining high Casp-2 activity but the isoindoline **33l** had no selectivity against Csp-3 and showed a decrease in Csp-2 and Csp-3 activity compared to the proline **33a**. The resolution of the modeling was insufficient to explain this difference in activity. Finally, we found the 3-spiro series (iii) to be chemically intractable, probably due to steric hindrance caused by the 2-position substituent on the proline; however, having already identified three scaffolds (i), (ii) and (v) which were tolerated in the S_2 pocket of Csp-2, we elected to deprioritize scaffold (iii).

4.2. (*S*)-Substituted proline and piperidine P_2 scaffolds

The next step in the proline series was the introduction of the 3(*S*)-substituent designed to clash with Tyr-204 of Csp-3. We chose to introduce alkoxy groups because functionalization of commercially available 3-(*S*)-hydroxy-L-proline would allow rapid access to a range of analogs to study the influence of substituent size on selectivity. We started to probe the steric requirements with two analogs: the methoxy derivatives **33b** which, as predicted by modeling, showed no improvement in selectivity because the 3-(*S*)-OMe was too small to clash with Tyr-204 and the 3-(*S*)-isopropoxy analogs **33c** which had ~20-fold selectivity against Csp-3. Encouraged by this result, compounds **33d–f** bearing larger substituents were prepared in an attempt to further improve this selectivity ratio. Unfortunately, we obtained only modest improvements (Table 1) with the *O*-(2-pyrimidyl) derivatives **33g** having the best selectivity (45-fold) and the activity in Csp-2 for this compound decreased in the triple-digit nanomolar range as in **33c**. A crystal structure of **33c** bound to Csp-2 was obtained and it revealed that the 3-*i*Pro substituent is in a pseudo axial orientation (Fig. 2). On the basis of this structural information and docking studies, we decided to introduce the 3-*i*Pro substituent on the piperidine scaffold (ii) because this substituent would then be locked in the putative bioactive axial conformation and would have a favorable trajectory to clash with the Tyr-204 of Csp-3. As intended, compound **33n** had only residual Csp-3 activity but very disappointingly Csp-2 activity was also almost totally abolished. This result was surprising because molecular modeling had predicted the axial conformation to be the lowest energy conformation for **33n** while the equatorial diastereoisomer (3*R*) could not be docked in Csp-2. Therefore, for this analogue, other factors as yet unknown must be exerting an effect.

In a further attempt to achieve higher selectivity against Csp-3 in the proline series, it was sought to increase the steric bulk in the vicinity of the 3-(*S*)-hydroxy by introducing a *tert*-butyl group. A *tert*-butoxy was however not compatible with the final deprotection step of the solid-phase route and instead the neopentyl analog was prepared **33h**. This compound offered no significant improvement in selectivity (60-fold) compared to our previously prepared analogs, however unlike in the 3-(*S*)-alkoxyproline series, the dougle-digit nanomolar Csp-2 activity was maintained. The oxygen to carbon replacement could explain this difference because a carbon atom in the 3-position is likely to make a better contact with Phe-279 than an oxygen in the same position. Interestingly, the flexible (*S*)-*O*-(*tert*-butyl)-serine P_2 analog **2** corresponding to **33h** is a



Scheme 5. General synthetic procedure for solid phase synthesis of pentapeptides **2**, **33a–v**. * denotes (2*R*/*S*)-configuration except for **33o** (2*S*), ⊥ denotes commercial Fmoc protected P_2 building block. Reagents and conditions: (i) HATU, DIPEA, DMF, rt, 18 h, (ii) 20% piperidine in DMF, rt, 1 h; (iii) AcValAsp(OtBu)Val-OH, HATU, DMF, rt, 18 h; (iv) trifluoroacetic acid/water (9:1), rt, 1 h.

double-digit nanomolar inhibitor of both Csp-2 and Csp-3. This absence of selectivity against Csp-3 of the flexible analog of **33h** is a vindication of our choice of rigid templates. Finally, three analogs **33i–k** with a ring directly attached to the 3-proline position were prepared, however they did not display better selectivity than the lead compound **33h**.

4.3. Isoquinoline P_2 scaffold

In this series, attempts were made to improve on the 10-fold selectivity observed with the unsubstituted isoquinoline **33o**. The 6- and 7-positions of the isoquinoline nucleus were selected based on modeling considerations, that is, the possibility of creating a contact with the upper part of the S_2 pocket of Csp-2 (6-, 7-OH analogs **33s–t**) and the introduction of a bulky group in order to try to take advantage of the different steric requirements of Csp-2 and Csp-3 (6-, 7-Me and 6-, 7-Br; **33p–q**, **u–v**). The results for these six analogues are summarized in Table 1 and showed that the 6-Me analog **33q** had coincidently the same profile has our best 3(*S*)-substituted analog **33h** (60-fold selectivity and 50nM in Csp-2). The other five analogs had lower selectivity and weaker activity in both Csp-2 and Csp-3. The two bulky 6- and 7-bromo substituents **33u–v** were too large but the improved selectivity of both the 6-Me **33p** (60-fold) and 7-Me **33q** (25-fold) led us to prepare the easily accessible 6,7-diOMe **33r** which unfortunately suffered a ~40-fold loss of Csp-2 activity compared to the Me analogs. Similarly, the 6- and 7-OH analogs **33s–t** lost Csp-2 activity which indicated that creating a substantial interaction with the upper S_2 pocket of Csp-2 from these two positions will be difficult. Taken together, these results suggest that despite the encouraging profile of 6-Me analog **33q**, it will probably be more challenging to improve the selectivity and potency in Csp-2 in the isoquinoline series than in the 3(*S*)-substituted proline series which remains the most promising series with the neopentyl analog **33h** as the lead compound.

4.4. Cellular activity and future direction

Csp-2 and Csp-3 cell activities and cell-to-enzyme (C/E) potency ratio in Csp-2 are reported in Table 1. With the exception of VDVPD-CHO **33a** and isoindoline **33l** which have respectively an IC_{50} of 630 nM and 780 nM in the Csp-2 cell assay, all other compounds display micromolar activity with C/E ratios ranging from 5 to 130. In the 3-(*S*)-substituted proline series, the C/E ratio generally increases with the size of the 3-substituent and the most Csp-2 selective analogs **33h** had a C/E of 65. In the isoquinoline series, the Csp-2 C/E ratio does not correlate with size as the bromo analog **33v** had surprisingly one of the best C/E ratios (C/E = 5) whereas the unsubstituted

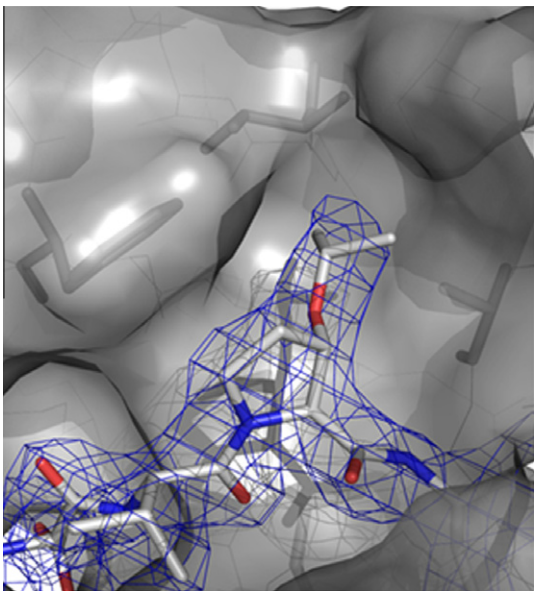


Figure 2. X-ray co-crystal structure at 2.6 Å of compound **33c** bound to Csp-2 (PDB code 3RJM). The surface of Csp-2 active site is colored in grey and compound **33c** by atom type. The $F_o - F_c$ composite omit electron density map contoured at 2.5σ is displayed in blue mesh around **33c**. Figure generated by PyMOL.[39]

isoquinoline **33o** had a C/E of 100. The most Csp-2 selective isoquinoline analog **33q** also had a poor C/E of 130. In the end, the compound that emerged from this effort with the most interesting profile is 3(*S*)-neopentyl-proline **33h** which has a number of attractive characteristics compared to the reference inhibitor Ac-VDVAD-CHO (**1**). Compounds **33h** and **1** are equipotent both in Csp-2 biochemical and cell assays with IC_{50} values of respectively ~50 nM & ~2 μM. However, in the biochemical assays, **33h** has a 60-fold selectivity against Csp-3 and is 200-fold less potent in Csp-3 than **1** (~3 μM vs 15 nM, Table 1). In the cell assays, compound **33h** is 20-fold selective for Casp-2 and also very weakly active in Csp-3 (78 μM) whereas compound **1** is quite potent (346 nM). All together, these data make **33h** a better tool compound than other VDVAD-based inhibitors including compound **1** and demonstrates the validity of the structured-based approach. These results are particularly encouraging for an exploratory study and we expect that additional progress can be made in the 3(*S*)-substituted proline series. The current limitation with this series comes from the fact that the neopentyl group in **33h** does not improve Csp-2 activity compared to the unsubstituted proline **33a**. Based on the analysis of the S_2 pocket of Csp-2, we hypothesize that it should be possible to identify an alternative bulky substituent capable of not only clashing sterically with a residue of the Csp-3 S_2 pocket but also engaging in interactions with residue(s) of the S_2 pocket of Csp-2. These new interactions should increase Csp-2 potency which could allow deletion of the P_5 group. The resulting tetrapeptide would be expected to be potent with an improved C/E ratio compared to **33h**, thus making it a valuable pharmacological tool compound.

5. Conclusion

The paucity of selective caspase inhibitors is an obstacle to elucidating the precise role that caspases may play in HD pathogenesis. We demonstrate here that a structure-based design strategy is effective for the identification of pentapeptide-based Csp-2 inhibitors with a superior Csp-3 selectivity profile in enzyme and cellular assays compared to existing VDVAD-based reagents. The proline scaffold introduced as the P_2 element along with the bulkiness of its 3-(*S*)-substituent have proven to be crucial features to improve selectivity against Csp-3. Replacement of the neopentyl group in the most selective inhibitor **33h** by an alternative large substituent, from which additional Csp-2 potency could be derived may ultimately produce the pharmacological tool compound needed to uncover the precise role of Csp-2 in particular pathways or disease mechanisms.

6. Experimental

6.1. Biochemical assays for Csp-2 and Csp-3

6.1.1. Materials

Coumarin-120-based Csp-2, and -3 substrates Ac-VDVAD-AMC (AMC: 7-amino-4-methyl-coumarin, Coumarin-120) and Ac-DEVD-AMC were purchased from PeptaNova GmbH (Sandhausen, Germany) or Peptide Institute (Osaka, Japan). Csp-2 (170–452aa containing large and small subunits) and Csp-3 (1–277aa containing full length pro-enzyme) fused with a C- terminal histidine-tag were cloned into a pET-23b vector (Novagen). The plasmids were transformed into E. Coli BL21 (DE3) pLysS cell and expression was carried out by overnight induction with 0.2 mM IPTG at 16 °C. The protein was purified in two steps using Ni-NTA affinity and anion exchange chromatography. 2-(*N*-Morpholino)ethanesulfonic acid (MES), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), ethylenediamine-tetra acetic acid (EDTA), dimethyl sulfoxide (DMSO), Pluronic F127 (PF127), and glutathione (GSH) were ordered from SIGMA-Aldrich (St. Louis, MO, USA). 4-(2-Hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES) was obtained from Merck (Darmstadt, Germany). Substrate-based Csp-2 (Ac-VDVAD-CHO, **1**) and Csp-3 (Ac-DEVD-CHO) inhibitors were purchased from BACHEM (Bubendorf, Switzerland).

6.1.2. Fluorescence-based enzyme inhibition assays

The assay is based on the cleavage of a fluorogenic substrate comprised of the recognition sequence of the individual caspase (see above) and coumarin-120. Cleavage of the substrate converts the quenched fluorophore into a fluorescent product. All assays were performed in a 384 well format (Matrix 384 well plate, black, polystyrene; cat.-no. 4318; Thermo Fisher Scientific, Hudson, NH, USA) in a total volume of 20 μl per well. Standard reaction conditions for Csp-2 consisted of 50 mM MES (pH 6.5), 150 mM NaCl, 1.5% sucrose, 5 mM GSH and 0.03% Pluronic F127. Standard reaction conditions for Csp-3 consisted of 50 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM GSH and 0.03% Pluronic F127. The chosen concentration of fluorogenic substrates corresponded to the respective K_M-values (see below for details). The peptidic inhibitors Ac-VDVAD-CHO (Csp-2) and Ac-DEVD-CHO (Csp-3) were used as reference inhibitors on each individual assay plate. The activity measured for the reference inhibitors was as expected from the literature.[14b,24a,35] Reference inhibitors and test compounds were dissolved in DMSO, threefold serial dilutions with eleven concentrations were prepared in 100% DMSO at 100-fold final concentration and 0.2 μl of the compound dilutions added to each well containing 10 μl of freshly prepared solutions of either 20 nM Csp-2 or 0.6nM Csp-3 in the respective reaction buffers using a CyBi®—Well vario manipulator (CyBio AG, Jena, Germany). Plates were incubated at 37 °C for 5 min before the enzymatic reaction was started by adding 10 μl of the substrate solution (twofold of final concentration in the individual reaction buffer) resulting in 20 μM Ac-VDVAD-AMC for Csp-2 and 10 μM Ac-DEVD-AMC for Csp-3, respectively. The final concentration of DMSO was 1%. Plates were incubated for 20 min at 37 °C and reaction was stopped by addition of 3 μl of 5 M aqueous acetic acid solution followed by a short centrifugation step to remove air-bubbles. Fluorescence was quantified using a Tecan Safire 2 microplate fluorometer (Tecan Austria GmbH, Grödig, Austria) with the following

wavelength settings: excitation at 380 nm and emission at 440 nm for coumarin-120, respectively.

6.2. Cellular assays for Csp-2 and Csp-3

Determination of cellular potency of the compounds was performed using HEK293 T17 cells that were transiently transfected with the full-length cDNAs of Csp-2 or Csp-3. The cDNAs used were human Myc-DDK tagged Csp-2 in pCMV6 (RC200669, Origene, Rockville, USA) and full-length cDNA of Csp-3 in pcDNA 3.1 with in-frame fusion and FLAG tag at the C-terminus. Cells were grown at 8% CO_2 and 37 °C in culture medium consisting of DMEM (cat.-no. D5796, Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal calf serum and 1% (v/v) penicillin/streptomycin-solution (cat.-no. P11-013, PAA Laboratories, Cölbe, Germany).

6.2.1. Csp-2 cellular assay

The assay was performed in poly-L-lysine coated 96-well plates (CellCoat, cat.-no. 655,936, Greiner bio-one, Frickenhausen, Germany). For one 96-well plate 20 ml of cell suspension was prepared as follows: 1,100,000 cells/ml in culture medium in a total volume of 18 ml were harvested. A solution containing 20 μg plasmid mixture (2 μg Csp-2 and 18 μg empty vector pcDNA3.1neo) and 60 μl Fugene HD (FuGENE® HD Transfection Reagent, Roche, Switzerland) in OptiMEM (cat.-no. 31,985, Invitrogen, Darmstadt, Germany) was prepared, vortexed and kept at room temperature for 15 min. Cells were then transfected by adding transfection solution to cells. Cells were then seeded into the 96-well plate at a density of 20,000 cells/well (200 μl of transfection suspension). After 4 h of incubation at 37 °C and 8% CO_2 in a humidified incubator the standard inhibitor Ac-VDVAD-CHO (final concentration 10 μM) was added and cells were incubated another 20 h before culture medium and inhibitor were replaced. 48 h after transfection the assay was performed by removing the medium and replacing it with 40 μl of phenol red-free medium (DMEM High Glucose, cat.-no. E15-870, PAA Laboratories) per well. Compounds were diluted from a DMSO stock solution using the phenol red-free medium to yield a concentration 10-fold higher than final test concentration in 10% DMSO. Five microliters of this compound dilution was subsequently added per well and after incubation for 2 h 5 μl of Ac-VDVAD-AMC substrate (Peptide Institute, Osaka, Japan) were added to yield a final concentration of 200 μM. Fluorescence was measured every hour for 5 h to monitor the reaction using a Tecan Safire 2 plate-reader and settings for the AMC substrate as described above. Typically, the fluorescence values determined after 5 h were used to calculate the IC_{50} values.

6.2.2. Csp-3 cellular assay

The assay was performed in poly-L-lysine coated 384 well plates (CellCoat, cat.-no. 78,1936, Greiner bio-one, Frickenhausen, Germany). For one 384 well plate 20 ml of cell suspension was prepared entirely as described above and using 2 μg of plasmid encoding Csp-3 instead of Csp-2. Transfected cells were seeded into the 384 well plate at a density of 10,000 cells/well (50 μl of transfection suspension). 48 h after transfection, the assay was performed by removing the medium and replacing it with 40 μl of phenol red-free medium per well. Compounds were diluted and added to cells entirely as described above and reaction started by addition of 5 μl of Ac-DEVD-AMC substrate (Peptide Institute Osaka, Japan) at a final concentration of 200 μM. Readout was again performed as described above.

6.3. Data analysis

Data from the various biochemical and cellular assays were corrected, normalized and concentration–response relationships determined with the A+-software using a four parameter Hill equation. Normalized percent activity was calculated using the median values of the controls containing 1% DMSO (no inhibition) and 1 μM of the peptide inhibitors Ac-VDVAD-CHO (Csp-2) and Ac-DEVD-CHO (Csp-3) (full inhibition). Final IC_{50} values are the average of at least two independent experiments in triplicates.

6.4. Structural biology

Csp-2 used for crystallography was produced by co-expression of the P12 subunit (amino acids 345–452) with a C-terminal hexa-histidine affinity tag, and the untagged P19 subunit (amino acids 167–333) in E. coli overnight at 16 °C. The soluble complex was purified by Ni affinity chromatography followed by heparin-sepharose chromatography. The protein was dialyzed into 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% sucrose, 8 mM DTT and concentrated to 2 mg/ml for crystallization. Complexes of Csp-2 and **33c** were prepared by incubating Csp-2 with a 5× molar excess of **33c** at 4 °C. Crystallization trials were carried out using sitting drop vapor diffusion at 16 °C. Crystals grew from 20% PEG3000, NH_4Citrate, pH 5.5. Crystals display a symmetry of $P2_12_12_1$ with cell dimensions $a = 63.77$ Å, $b = 97.38$ Å, $c = 97.98$ Å. Atomic coordinates for the Csp-2 complex with **33c** have been deposited in the Protein Data Bank under PDB ID code 3RJM and statistics for the X-ray data sets are shown in the Supplementary data.

6.5. Computational chemistry—Docking of peptides using low-mode MD simulations

The crystal structures of Csp-2 and Csp-3 used in the in silico docking experiments were obtained from the Brookhaven protein data bank (PDB codes: 1PYO[20] and 2DKO respectively[21b]). These structures were prepared for docking by removing all ligands and water molecules followed by adding hydrogen atoms using the Protonate-3D tool within MOE.[36] Tetrapeptides were utilized for docking purposes rather than pentapeptides to conserve the computation time for the docking of these peptides. It has been shown that the P_1, P_3 and P_4 groups were sufficient to stabilize and correctly orient the P_2 group in the S_2 pocket and that there is no benefit to using the pentapeptides for docking when considering the P_2 substituent orientation (unpublished results). Low-mode MD simulations were chosen as a method to dock the tetrapeptides due to their ability to effectively sample the conformational space of both the ligand and the side chains of the protein residues involved in the binding.[37]

The tetrapeptides were manually placed in the binding pocket of Csp-2. The starting position and conformation of the tetrapeptides for the docking were chosen by optimizing the overlap with the Ac-LDESD peptide in Csp-2 crystal structure (1PYO). A covalent bond was set between the sulphur atom of the catalytic Cys-155 and the reactive carbon atom of the P_1 aldehyde of the tetrapeptide. In order to take into account the protein flexibility in the S_2 pocket, the side chains of Cys-155, Ala-228, Thr-160, Met-230 and Phe-279 were left unconstrained in the calculations. The geometry of the ligand–protein complex was then optimized within MOE using the MMFF94x force-field.[38] Covalent docking was carried out using the low-mode MD simulations tool under default settings as implemented in MOE, using the MMFF94x force-field. All the binding hypotheses output were individually analyzed and the most representative poses among the ones with the lowest energy were chosen. The model was initially validated by comparing the binding hypotheses generated for Ac-DESD with the crystal structure of Ac-LDESD in Csp-2 (1PYO).

The docking of the tetrapeptides in Csp-3 was completed following the same protocol as described above except that the P4 group of the tetrapeptide was constrained. This was necessary as

the P_4 group had a tendency to exit the subsite S_4, thus destabilizing the low-mode MD simulation. A covalent bond was set between the sulphur atom of the catalytic Cys-163 of Csp-3 and the reactive carbon atom of the P_1 aldehyde of the tetrapeptide. The side chains of Cys-163, Leu-168, Tyr-204, Trp-206 and Phe-256 were left unconstrained in the calculations. The model was validated by comparing the binding hypotheses generated for Ac-DEVD with the crystal structure of Ac-DEVD in Csp-3 (2DKO).

6.6. Chemistry

6.6.1. Material and general methods

All reagents including (*S*)-Fmoc-*O*-*tert*-butyl-serine, (*S*)-Fmoc-proline, Fmoc-2,3-dihydro-1*H*-isoindole-1-carboxylic acid, (*S*)-Fmoc-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid, (*S*)-Fmoc pipecolic acid were used as supplied from commercial sources and all solvents were HPLC grade.

^{1}H NMR spectra were recorded on a Bruker DRX 500 MHz spectrometer or Bruker DPX 250 MHz spectrometer in deuterated solvents. Chemical shifts (δ) are in parts per million. Thin-layer chromatography (TLC) analysis was performed with Kieselgel 60 F_{254} (Merck) plates and visualized using UV light.

Analytical HPLC-MS was performed on Shimadzu LCMS-2010EV systems using reverse phase Atlantis dC18 columns (3 μm, 2.1 × 50 mm), gradient 5–100% B (A = water/0.1% formic acid, B = acetonitrile/0.1% formic acid) over 3 min, injection volume 3 μl, flow = 1.0 ml/min. UV spectra were recorded at 215 nm using a Waters 2788 dual wavelength UV detector. Mass spectra were obtained over the range *m*/*z* 150–850 at a sampling rate of 2 scans per second using Waters LCT or analytical HPLC-MS on Shimadzu LCMS-2010EV systems using reverse phase Water Atlantis dC18 columns (3 μm, 2.1 × 100 mm), gradient 5–100% B (A = water/0.1% formic acid, B = acetonitrile/0.1% formic acid) over 7 min, injection volume 3 μl, flow = 0.6 ml/min. UV spectra were recorded at 215 nm using a Waters 2996 photo diode array. Data were integrated and reported using Shimadzu psiport software.

Preparative-HPLC for intermediate compounds was performed using a Gilson 215 Liquid Handler and Gilson 321 Pump, utilising a reverse phase Waters Sunfire C18 column (5 μm, 19 mm × 100 mm), gradient 5–100% B (A = water, B = acetonitrile) over 18 min, injection volume 1000 μl, flow = 20 ml/min. UV spectra were recorded at 215 nm using a Gilson 151 UV/vis Detector system. Data was processed using Gilson Unipoint V5.11 software. Preparative-HPLC for final compounds (**2**, **33a–v**) was performed using a Gilson 215 Liquid Handler and 2 × Gilson 306 Pumps with Gilson 805 Manometric Module, utilising a Waters Atlantis T3 OBD column (5 μm, 19 mm × 100 mm), gradient 0–100% B (A = water, B = methanol) over 18 min, injection volume 1000 μl, flow = 20 ml/min. UV spectra were recorded at 215 nm using a Gilson 119 UV/vis Detector system. Data was processed using Gilson Unipoint V5.11 software.

High Resolution Mass Spectra (HRMS) were collected using a Waters Micromass LCT Premier orthogonal acceleration Time-of-Flight Mass Spectrometer 4 GHz TDC with LockSpray™ under electrospray positive detection and operating MassLynx v4.1 SCN 704 software. All compounds prepared for biochemical screening displayed purities determined by HPLC as ⩾95% unless otherwise stated.

The following abbreviations were used in the text: $CDCl_3$ (deuterated chloroform), D_2O (deuterium oxide), DCE (1,2-dichloroethane), DCM (dichloromethane), DDQ (2,3-dichloro-5,6-dicyanobenzoquinone), DIPEA (*N*,*N*-diisopropylethylamine), DMF (dimethylformamide), EDCI (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), Et_2O (diethyl ether), EtOAc (ethyl acetate), EtOH (ethanol), FmocCl (9-fluorenylmethyl chloroformate), HATU (2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HOBt (1-hydroxybenzotriazole), MeOH (methanol), $MgSO_4$ (magnesium sulfate), $NaHCO_3$ (sodium bicarbonate), Na_2SO_4 (sodium sulfate), TBME (*tert*-butyl methyl ether), THF (tetrahydrofuran).

6.6.2. Synthetic procedures

6.6.2.1. (2*S*,3*S*)-3-Hydroxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester 2-methyl ester (4). A 4 N hydrochloric acid solution in dioxane (50 mL) was added to a stirred solution of (2*S*,3*S*)-3-hydroxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester **3** (19.97 g, 56.51 mmol) in MeOH (150 mL). The reaction mixture was stirred at room temperature for 24 h, then concentrated under vaccum and the resulting oil was purified by flash column chromatography (heptane/EtOAc 1:0 to 1:1) to provide the title compound (14.2 g, 68% yield) as a white solid. MS (ES+) *m*/*z* (M+1) 368.0. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.77 (t, *J* = 6.78 Hz, 2H) 7.60–7.66 (m, 1H) 7.56 (t, *J* = 7.41 Hz, 1H) 7.37–7.44 (m, 2H) 7.29–7.36 (m, 2H) 4.34–4.55 (m, 3.6H) 4.26–4.33 (m, 0.5H) 4.25 (s, 0.4H) 4.19 (t, *J* = 6.62 Hz, 0.5H) 3.65–3.81 (m, 5H) 1.90–2.28 (m, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.09, 171.06, 155.31, 154.81, 144.10, 143.93, 143.85, 141.39, 127.84, 127.82, 127.77, 127.19, 127.15, 125.30, 125.23, 125.06, 125.03, 120.10, 120.06, 75.34, 74.18, 68.31, 67.88, 67.82, 67.63, 52.68, 52.64, 47.33, 47.26, 45.00, 44.69, 32.74, 31.99. Mixture of rotamers. HPLC purity 85%.

6.6.2.2. (2*S*,3*S*)-3-Methoxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester 2-methyl ester (5a). Silver oxide (4.48 g, 16.33 mmol) and methyl iodide (1.02 mL, 2.32 g, 16.33 mmol) were added to a solution of (2*S*,3*S*)-3-hydroxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester 2-methyl ester **4** (2.00 g, 5.44 mmol) in DCE (20 mL) at room temperature under nitrogen. The reaction mixture was stirred in the dark at room temperature for 24 h, then the whole was filtered through Keiselguhr and the filtrate was concentrated under vacuum to provide a crude residue which was purified by flash column chromatography (heptane/EtOAc 1:0 to 4:1) to afford the title compound (1.30 g, 63% yield) as a colorless oil. MS (ES+) *m*/*z* (M+23) 404.4. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.77 (dd, *J* = 7.4, 4.6 Hz, 2H) 7.63 (dd, *J* = 11.5, 7.6 Hz, 1H) 7.56 (t, *J* = 8.0 Hz, 1H) 7.38–7.44 (m, 2H) 7.29–7.36 (m, 2H) 4.53 (s, 0.5H) 4.33–4.49 (m, 2.5H) 4.26–4.31 (m, 0.5H) 4.18–4.23 (m, 0.5H) 3.94–3.99 (m, 1H) 3.75–3.81 (m, 2H) 3.58–3.74 (m, 3H) 3.40 (d, *J* = 17.50 Hz, 3H) 2.00–2.19 (m, 2H). Mixture of rotamers. HPLC purity 100%.

6.6.2.3. (2*S*,3*S*)-3-Isopropoxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester 2-methyl ester (5b). Silver oxide (7.88 g, 34.00 mmol) and isopropyl iodide (27.76 mL, 34.70 g, 204.14 mmol) were added to a solution of (2*S*,3*S*)-3-hydroxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester 2-methyl ester **4** (2.50 g, 6.80 mmol) in DCE (50 mL) at room temperature under nitrogen. The reaction mixture was stirred in the dark at reflux for 15 h, then cooled to room temperature and filtered through Keiselguhr. The filtrate was concentrated under vacuum to provide a crude residue which was purified successively by flash column chromatography (heptane/EtOAc 1:0 to 7:3) and preparative HPLC to provide the title compound (0.077 g, 3% yield) as a colorless oil. MS (ES+) *m*/*z* (M+1) 410.0. ^{1}H NMR (250 MHz, $CDCl_3$) δ ppm 7.77 (dd, *J* = 7.2, 2.5 Hz, 2H) 7.52–7.68 (m, 2H) 7.27–7.46 (m, 4H) 4.12–4.49 (m, 5H) 3.61–3.83 (m, 6H) 1.92–2.22 (m, 2H) 1.20 (dd, *J* = 6.2, 2.3 Hz, 6H). Mixture of rotamers. HPLC purity 100%.

6.6.2.4. (2*S*,3*S*)-3-*sec*-Butoxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester 2-methyl ester (5c). The title compound was prepared following the procedure used for compound **5b**. The crude residue was purified by preparative HPLC to provide the title compound (0.204 g, 9% yield) as a viscous

colorless oil. MS (ES+) *m*/*z* (M+23) 446.2. ^{1}H NMR (250 MHz, $CDCl_3$) δ ppm 7.74–7.81 (m, 2H) 7.52–7.67 (m, 2H) 7.29–7.45 (m, 4H) 4.12–4.46 (m, 5H) 3.66–3.80 (m, 5H) 3.40–3.56 (m, 1H) 1.95–2.17 (m, 2H) 1.46–1.55 (m, 2H) 1.12–1.22 (m, 3H) 0.84–0.98 (m, 3H). Mixture of rotamers. HPLC purity 89%.

6.6.2.5. (2*S*,3*S*)-3-Isobutoxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester 2-methyl ester (5d). The title compound was prepared following the procedure used for compound **5b**. The crude residue was purified successively by flash column chromatography and preparative HPLC to provide the title compound (0.555 g, 19% yield) as a colorless oil. MS (ES+) *m*/*z* (M+1) 424.1. ^{1}H NMR (250 MHz, $CDCl_3$) δ ppm 7.77 (m, 2H) 7.50–7.68 (m, 2H) 7.27–7.46 (m, 4H) 4.15–4.53 (m, 4H) 3.98–4.08 (m, 1H) 3.57–3.86 (m, 5H) 3.17–3.38 (m, 2H) 1.95–2.17 (m, 2H) 1.76–1.95 (m, 1H) 0.92 (dd, *J* = 6.6, 2.1 Hz, 6H). Mixture of rotamers. HPLC purity 100%.

6.6.2.6. (2*S*,3*S*)-3-Cyclopentyloxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester 2-methyl ester (5e). The title compound was prepared following the procedure used for compound **5b**. The crude residue was purified successively by flash column chromatography and preparative HPLC to provide the title compound (0.161 g, 5% yield) as a colorless oil. MS (ES+) *m*/*z* (M+1) 436.4. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.77 (t, *J* = 6.5 Hz, 2H) 7.63 (dd, *J* = 12.6, 7.4 Hz, 1H) 7.56 (t, *J* = 8.3 Hz, 1H) 7.41 (q, *J* = 6.7 Hz, 2H) 7.29–7.36 (m, 2H) 4.26–4.50 (m, 3.6H) 4.21 (t, *J* = 6.7 Hz, 0.4H) 3.98–4.14 (m, 2H) 3.59–3.81 (m, 5H) 1.94–2.17 (m, 2H) 1.50–1.85 (m, 8H). Mixture of rotamers. HPLC purity 98%.

6.6.2.7. (2*S*,3*S*)-3-Methoxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester (6a). A 6 N hydrochloric acid aqueous solution (5 mL) was added to a stirred solution of (2*S*,3*S*)-3-methoxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester 2-methyl ester **5a** (1.30 g, 3.40 mmol) in dioxane (5 mL) at room temperature. The reaction mixture was stirred at reflux for 18 h. The mixture was cooled to room temperature, dioxane was removed under vacuum and the aqueous residue was extracted with EtOAc (3 × 30 mL). The organic extracts were combined, dried over Na_2SO_4, filtered and concentrated under vacuum. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 7:3) to provide the title compound (0.70 g, 56% yield) as a white solid. MS (ES+) *m*/*z* (M+1) 368.0. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.70–7.82 (m, 2H) 7.52–7.64 (m, 2H) 7.35–7.45 (m, 2H) 7.28–7.35 (m, 2H) 4.24–4.54 (m, 4H) 4.14–4.22 (m, 1H) 4.00 (br s, 0.3H) 3.76–3.81 (m, 0.3H) 3.57–3.72 (m, 2.4H) 3.35–3.43 (m, 3H) 1.99–2.16 (m, 2H). Mixture of rotamers. HPLC purity 100%.

6.6.2.8. (2*S*,3*S*)-3-Isopropoxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester (6b). The title compound was prepared following the procedure used for compound **6a**. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 8:2) to provide the title compound (0.148 g, 95% yield) as a colorless oil. MS (ES+) *m*/*z* (M+1) 396.1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.69–7.80 (m, 2H) 7.52–7.65 (m, 2H) 7.25–7.45 (m, 4H) 4.15–4.51 (m, 5H) 3.61–3.81 (m, 4H) 1.91–2.17 (m, 2H) 1.20 (d, *J* = 6.0 Hz, 6H). Mixture of rotamers. HPLC purity 99%.

6.6.2.9. (2*S*,3*S*)-3-*sec*-Butoxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester (6c). The title compound was prepared following the procedure used for compound **6a**. The crude residue was purified by preparative HPLC to afford the title compound (0.173 g, 50% yield) as a colorless glass. MS (ES+) *m*/*z* (M+1) 410.0. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.78 (d, *J* = 7.6 Hz, 1.3H) 7.73 (d, *J* = 7.5 Hz, 0.7H) 7.61 (t, *J* = 8.1 Hz, 1.3H) 7.55 (t, *J* = 7.0 Hz, 0.7H) 7.26–7.44 (m, 4H) 5.06 (br s, 1H) 4.16–4.50 (m, 5H) 3.59–3.74 (m, 2H) 3.39–3.53 (m, 1H) 1.92–2.19 (m, 2H) 1.41–1.59 (m, 2H) 1.13–1.19 (m, 3H) 0.84–0.94 (m, 3H). Mixture of rotamers. HPLC purity 100%.

6.6.2.10. (2*S*,3*S*)-3-Isobutoxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester (6d). The title compound was prepared following the procedure used for compound **6a**. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 1:1) to afford the title compound (0.403 g, 42% yield) as a colorless oil. MS (ES+) *m*/*z* (M+1) 410.1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.72–7.81 (m, 2H) 7.52–7.64 (m, 2H) 7.28–7.44 (m, 4H) 4.04–4.51 (m, 6H) 3.58–3.72 (m, 2H) 3.17–3.33 (m, 2H) 1.98–2.15 (m, 2H) 1.80–1.91 (m, 1H) 0.87–0.94 (m, 6H). Mixture of rotamers. HPLC purity 100%.

6.6.2.11. (2*S*,3*S*)-3-Cyclopentyloxy-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester (6e). The title compound was prepared following the procedure used for compound **6a**. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 8:2) to afford the title compound (0.085 g, 54% yield) as a colorless oil. MS (ES+) *m*/*z* (M+1) 422.0. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.70–7.82 (m, 2H) 7.52–7.65 (m, 2H) 7.25–7.45 (m, 4H) 4.35–4.52 (m, 2.5H) 4.25–4.34 (m, 1.5H) 4.13–4.22 (m, 0.7H) 3.97–4.09 (m, 1H) 3.59–3.72 (m, 2.3H) 1.93–2.15 (m, 2H) 1.49–1.84 (m, 7H) 1.23–1.36 (m, 2H). Mixture of rotamers. HPLC purity 99%.

6.6.2.12. (2*S*,3*S*)-3-(Pyrimidin-2-yloxy)-pyrrolidine-1,2-dicarboxylic acid 1-*tert*-butyl ester (8). Potassium *tert*-butoxide (1.81 g, 16.23 mmol) was added to a stirred solution of (2*S*,3*S*)-3-hydroxy-pyrrolidine-1,2-dicarboxylic acid 1-*tert*-butyl ester **7** (0.50 g, 2.16 mmol) in *tert*-butanol (20 mL) at room temperature and the mixture was stirred for 15 min. 2-Chloropyrimidine (1.24 g, 10.82 mmol) was added at room temperature and the reaction mixture was stirred for 1 hour. The mixture was quenched with a 10% w/w aqueous citric acid solution (~2 mL), and partitioned between EtOAc (100 mL) and brine (100 mL). The layers were separated and the aqueous phase was extracted with EtOAc (3 × 100 mL). The organic extracts were combined, washed with brine (1 × 50 mL), dried over $MgSO_4$, filtered and concentrated under vacuum. The resulting crude residue was purified by flash column chromatography (DCM/MeOH 1:0 to 4:1) to provide the title compound (0.237 g, 35% yield) as a yellow gum. MS (ES+) *m*/*z* (M+23) 332.1. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 13.07 (br s, 1H), 8.65 (d, *J* = 4.7 Hz, 2H), 7.20 (t, *J* = 4.8 Hz, 1H), 5.55 (d, *J* = 4.3 Hz, 0.6H), 5.51 (d, *J* = 4.0 Hz, 0.4H), 4.25 (s, 0.4H), 4.20 (s, 0.6H), 3.50–3.60 (m, 1H), 3.36–3.49 (m, 1H), 1.98–2.28 (m, 2H), 1.41 (s, 4H), 1.34 (s, 5H). Mixture of rotamers. HPLC purity 100%.

6.6.2.13. (2*S*,3*S*)-3-(Pyrimidin-2-yloxy)-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester (6f). (2*S*,3*S*)-3-(Pyrimidin-2-yloxy)-pyrrolidine-1,2-dicarboxylic acid 1-*tert*-butyl ester **8** (0.220 g, 0.71 mol) was dissolved in a 4 N hydrochloric acid solution in dioxane (4 mL) and the whole was stirred at room temperature for 1 hour before being concentrated to dryness under vacuum. The resulting orange solid was dissolved in dioxane (5 mL) and a solution of $NaHCO_3$ (0.226 mg, 2.13 mmol) in water (2 mL) was added, the mixture was cooled to 0 °C and a solution of FmocCl (0.202 g, 0.78 mmol) in dioxane (1 mL) was added drop wise. The mixture was stirred at 0 °C for 30 min and room temperature for a further 48 h. Dioxane was removed under vacuum and the resulting aqueous residue was diluted with water (5 mL), washed with TBME (3 × 3 mL), acidified to pH 2 using a 2 N

hydrochloric acid aqueous solution and extracted with EtOAc (3 × 5 mL). The organic extracts were combined, dried over $MgSO_4$, filtered and concentrated under vacuum to provide the title compound (0.201 g, 65% yield) as a colorless glass. MS (ES+) *m*/*z* (M+1) 432.0. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 8.67 (dd, *J* = 16.4, 4.7 Hz, 2H) 7.82–7.94 (m, 2H) 7.68 (dd, *J* = 7.2, 2.0 Hz, 1H) 7.57 (d, *J* = 7.6 Hz, 1H) 7.32–7.5 (m, 3H) 7.19–7.31 (m, 2H) 5.55–5.66 (m, 1H) 4.50 (s, 0.4H) 4.23–4.37 (m, 2.6H) 4.14–4.23 (m, 1H) 3.52–3.73 (m, 3H) 2.14–2.31 (m, 2H). Mixture of rotamers. HPLC purity 97%.

6.6.2.14. (2*S*,3*S*)-2-(*tert*-Butyl-diphenyl-silanyloxymethyl)-3-(2,2-dimethyl-propyl)-5-oxo-pyrrolidine-1-carboxylic acid *tert*-butyl ester (10a). A solution of neopentylmagnesium bromide (0.5 M in THF) (57.57 mL, 28.78 mmol) was added to a stirred solution of (methylsulfanyl)methane–bromocopper (1:1) (0.592 g, 2.88 mmol) in THF (5 mL) at −35 °C and the mixture was stirred at this temperature for 20 min. The mixture was cooled to −78 °C and a solution of (*S*)-2-(*tert*-butyl-diphenyl-silanyloxymethyl)-5-oxo-2,5-dihydro-pyrrole-1-carboxylic acid *tert*-butyl ester **9** (1.30 g, 2.88 mmol) in THF (5 mL) and chlorotrimethylsilane (0.73 mL, 0.625 mg, 5.76 mmol) was added. The reaction mixture was slowly warmed to room temperature and stirred for 2 h before being cooled to 0 °C, quenched with a saturated ammonium chloride aqueous solution (3 mL) and warmed to room temperature. The mixture was extracted with EtOAc (3 × 5 mL), the organic extracts were combined, washed with a saturated ammonium chloride aqueous solution (3 × 3 mL), dried over Na_2SO_4, filtered and concentrated under vacuum. The resulting residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 3:1) to afford the title compound (1.05 g, 70% yield) as a colorless oil. MS (ES+) *m*/*z* (M+23) 546.4. ^{1}H NMR (250 MHz, $CDCl_3$) δ ppm 7.54–7.71 (m, 4H) 7.30–7.51 (m, 6H) 3.76–3.97 (m, 2H) 3.58–3.77 (m, 1H) 2.93 (dd, *J* = 17.6, 8.9 Hz, 1H) 2.28–2.51 (m, 1H) 2.19 (dd, *J* = 17.6, 2.7 Hz, 1H) 1.44 (s, 9H) 1.27 (s, 2H) 1.06 (s, 9H) 0.93 (s, 9H). HPLC purity 74%.

6.6.2.15. (2*S*,3*R*)-2-(*tert*-Butyl-diphenyl-silanyloxymethyl)-5-oxo-3-*p*-tolyl-pyrrolidine-1-carboxylic acid *tert*-butyl ester (10b). The title compound was prepared according to the procedure used for compound **10a**. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 98.5:1.5) to afford the title compound (0.971 g, 68% yield) as a white gum. MS (ES+) *m*/*z* (M+23) 566.0. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.60–7.70 (m, 4H) 7.34–7.49 (m, 6H) 7.14 (d, *J* = 7.9 Hz, 2H) 7.06 (d, *J* = 8.1 Hz, 2H) 4.09 (dt, *J* = 4.2, 2.1 Hz, 1H) 3.97 (dd, *J* = 10.5, 4.3 Hz, 1H) 3.81 (dd, *J* = 10.5, 2.3 Hz, 1H) 3.40–3.54 (m, 1H) 3.19 (dd, *J* = 18.0, 9.5 Hz, 1H) 2.57 (dd, *J* = 17.8, 2.8 Hz, 1H) 2.34 (s, 3H) 1.42 (s, 9H) 1.09 (s, 9H). HPLC purity 91%.

6.6.2.16. (2*S*,3*R*)-2-(*tert*-Butyl-diphenyl-silanyloxymethyl)-3-cyclohexyl-5-oxo-pyrrolidine-1-carboxylic acid *tert*-butyl ester (10c). The title compound was prepared according to the procedure used for compound **10a**. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 95:5) to afford the title compound (0.693 g, 80% yield) as a pale yellow oil. (ES+) *m*/*z* (M+23) 558.0. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.56–7.68 (m, 4H) 7.32–7.51 (m, 6H) 3.94–4.01 (m, 1H) 3.89 (dd, *J* = 10.3, 4.3 Hz, 1H) 3.65 (dd, *J* = 10.3, 2.3 Hz, 1H) 2.82 (dd, *J* = 18.0, 9.8 Hz, 1H) 2.31 (dd, *J* = 18.0, 2.0 Hz, 1H) 2.05–2.19 (m, 1H) 1.76 (dd, *J* = 12.6, 2.2 Hz, 2H) 1.57–1.72 (m, 2H) 1.46 (s, 9H) 1.11–1.38 (m, 5H) 1.05 (s, 9H) 0.86–1.03 (m, 2H). HPLC purity 95%.

6.6.2.17. (2*S*,3*S*)-2-(*tert*-Butyl-diphenyl-silanyloxymethyl)-3-(2,2-dimethyl-propyl)-pyrrolidine-1-carboxylic acid *tert*-butyl ester (11a). A solution of borane-dimethyl sulfide (2 M in THF) (2.00 mL, 4.00 mmol) was added to a stirred solution of (2*S*,3*S*)-2-(*tert*-butyl-diphenyl-silanyloxymethyl)-3-(2,2-dimethyl-propyl)-5-oxo-pyrrolidine-1-carboxylic acid *tert*-butyl ester **10a** (1.05 g, 2.00 mmol) in THF (5 mL) at room temperature. The resulting solution was stirred at reflux for 1 hour, then cooled to room temperature, quenched with MeOH (20 mL) and concentrated under vacuum. The resulting white residue was dissolved in water (50 mL) and the solution was extracted with EtOAc (3 × 50 mL); the organic extracts were combined, dried, filtered over $MgSO_4$, filtered and concentrated under vacuum. The title compound (1.02 g, 88% yield) was obtained as a white gum and used in the subsequent step without any further purification. MS (ES+) *m*/*z* (M+23) 532.2. ^{1}H NMR (250 MHz, $CDCl_3$) δ ppm 7.54–7.79 (m, 4H) 7.30–7.51 (m, 6H) 3.40–4.05 (m, 4H) 3.26 (dt, *J* = 10.7, 7.0 Hz, 1H) 2.54 (br s, 1H) 1.97–2.16 (m, 3H) 1.61–1.76 (m, 1H) 1.22–1.44 (m, 9H) 1.06 (s, 9H) 0.81–1.01 (m, 9H). HPLC purity 72%.

6.6.2.18. (2*S*,3*R*)-2-(*tert*-Butyl-diphenyl-silanyloxymethyl)-3-*p*-tolyl-pyrrolidine-1-carboxylic acid *tert*-butyl ester (11b). The title compound was prepared according to the procedure used for compound **11a**. The crude residue was not purified and the title compound (82% yield) was obtained as a colorless oil. (ES+) *m*/*z* (M+23) 552.1. HPLC purity 84%.

6.6.2.19. (2*S*,3*R*)-2-(*tert*-Butyl-diphenyl-silanyloxymethyl)-3-cyclohexyl-pyrrolidine-1-carboxylic acid *tert*-butyl ester (11c). The title compound was prepared according to the procedure used for compound **11a**. The crude residue was not purified and the title compound (100% yield) was obtained as a colorless oil. (ES+) *m*/*z* (M+23) 544.2. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.58–7.70 (m, 4H) 7.31–7.49 (m, 6H) 3.43–4.04 (m, 4H) 3.27 (dt, *J* = 10.6, 7.4 Hz, 1H) 2.08–2.26 (m, 1H) 1.86–1.99 (m, 1H) 1.59–1.80 (m, 6H) 1.33–1.50 (m, 9H) 1.12–1.24 (m, 3H) 1.05 (s, 9H) 0.84–1.01 (m, 3H). HPLC purity 92%,

6.6.2.20. (2*S*,3*S*)-3-(2,2-Dimethyl-propyl)-2-hydroxymethyl-pyrrolidine-1-carboxylic acid *tert*-butyl ester (12a). A 1 M solution of tetrabutyl ammonium fluoride in THF (3.54 mL, 3.54 mmol) was added to a stirred solution of (2*S*,3*S*)-2-(*tert*-butyl-diphenyl-silanyloxymethyl)-3-(2,2-dimethyl-propyl)-pyrrolidine-1-carboxylic acid *tert*-butyl ester **11a** (0.723 g, 1.42 mmol) in THF (15 mL) at room temperature. The reaction mixture was stirred at room temperature for 24 h before being concentrated under vacuum. The resulting residue was dissolved in EtOAc (10 mL) and the solution was washed with a saturated ammonium chloride aqueous solution (2 × 10 mL) and water (2 × 10 mL), dried over $MgSO_4$, filtered and concentrated under vacuum. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 9:1) to afford the title compound (0.251 g, 65% yield) as a colorless crystalline solid. MS (ES+) *m*/*z* (M+23) 294.1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 5.22 (d, *J* = 8.9 Hz, 1H) 3.71 (d, *J* = 9.8 Hz, 1H) 3.49–3.64 (m, 2H) 3.44 (t, *J* = 7.9 Hz, 1H) 3.21 (d, *J* = 10.5, 6.5 Hz, 1H) 1.98–2.11 (m, 1H) 1.62–1.77 (m, 1H) 1.48 (s, 11H) 1.28 (dd, *J* = 14.1, 8.6 Hz, 1H) 0.92 (s, 9H). HPLC purity 100%.

6.6.2.21. (2*S*,3*R*)-2-Hydroxymethyl-3-*p*-tolyl-pyrrolidine-1-carboxylic acid *tert*-butyl ester (12b). The title compound was prepared according to the procedure used for compound **12a**. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 4:1) to afford the title compound (91% yield) as a colorless oil. (ES+) *m*/*z* (M+23) 314.1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.14 (s, 4H) 5.03 (d, *J* = 7.9 Hz, 1H) 3.67–4.01 (m, 3H) 3.58–3.65 (m, 1H) 3.35 (d, *J* = 10.5, 6.5 Hz, 1H) 2.77–2.96 (m, 1H) 2.34 (s, 3H) 2.07–2.20 (m, 1H) 1.95 (m, *J* = 12.3, 10.3, 10.3, 8.1 Hz, 1H) 1.51 (s, 9H). HPLC purity 96%.

6.6.2.22. (2*S*,3*R*)-3-Cyclohexyl-2-hydroxymethyl-pyrrolidine-1-carboxylic acid *tert*-butyl ester (12c). The title compound was prepared according to the procedure used for compound **12a**. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 9:1) to afford the title compound (66% yield) as a colorless oil. (ES+) *m*/*z* (M+23) 306.1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 4.82 (d, *J* = 7.3 Hz, 1H) 3.44–3.86 (m, 4H) 3.06–3.24 (m, 1H) 1.51–1.97 (m, 9H) 1.48 (s, 8H) 0.84–1.37 (m, 6H). HPLC purity 75%.

6.6.2.23. (2*S*,3*S*)-3-(2,2-Dimethyl-propyl)-pyrrolidine-1,2-dicarboxylic acid 1-*tert*-butyl ester (13a). A solution of chromium trioxide (0.180 mg, 1.801 mmol) in sulfuric acid (98 μl, 1.84 mmol) and water (0.5 ml) was added to a stirred solution of (2*S*,3*S*)-3-(2,2-dimethyl-propyl)-2-hydroxymethyl-pyrrolidine-1-carboxylic acid *tert*-butyl ester **12a** (0.251 g, 0.901 mmol) in acetone (5 mL) at room temperature. The reaction mixture was stirred at room temperature for 5 h before being quenched with a saturated ammonium chloride aqueous solution (10 mL). The mixture was extracted with EtOAc (3 × 10 mL) and the combined organics were washed with water (2 × 20 mL) and extracted with a 1 M sodium hydroxide aqueous solution (3 × 40 mL). The aqueous extracts were combined, acidified to pH 4 with 1 M hydrochloric acid aqueous solution and extracted with EtOAc (3 × 40 mL). These organic extracts were combined, dried over $MgSO_4$, filtered, and concentrated to afford the title compound (0.187 g, 73% yield) as a colorless solid. MS (ES+) *m*/*z* (M+23) 308.0. HPLC purity 88%.

6.6.2.24. (2*S*,3*R*)-3-*p*-Tolyl-pyrrolidine-1,2-dicarboxylic acid 1-*tert*-butyl ester (13b). The title compound was prepared according to the procedure used for compound **13a**. The crude residue was not purified and the title compound (61% yield) was obtained as a white solid. (ES−) *m*/*z* (M−1) 304.2. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.05–7.21 (m, 4H) 4.18–4.53 (m, 1H) 3.71–3.82 (m, 1H) 3.54–3.70 (m, 1H) 3.42–3.54 (m, 1H) 2.25–2.37 (m, 4H) 1.96–2.09 (m, 1H) 1.37–1.58 (m, 9H). HPLC purity 96%.

6.6.2.25. (2*S*,3*R*)-3-Cyclohexyl-2-hydroxymethyl-pyrrolidine-1-carboxylic acid *tert*-butyl ester (13c). The title compound was prepared according to the procedure used for compound **13a**. The crude residue was not purified and the title compound (100% yield) was obtained as a colorless oil. (ES+) *m*/*z* (M+23) 320.1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 3.96–4.17 (m, 1H) 3.27–3.64 (m, 2H) 2.13–2.43 (m, 1H) 1.93–2.03 (m, 1H) 1.61–1.87 (m, 6H) 1.37–1.54 (m, 9H) 0.90–1.31 (m, 6H). HPLC purity 85%.

6.6.2.26. (2*S*,3*S*)-3-(2,2-Dimethyl-propyl)-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester (15a). (2*S*,3*S*)-3-(2,2-dimethyl-propyl)-pyrrolidine-1,2-dicarboxylic acid 1-*tert*-butyl ester **13a** (0.187 g, 0.66 mmol) was dissolved in a mixture of DCM/trifluoroacetic acid (1:1) (2 mL) at 0 °C and stirred for 45 min at this temperature. The reaction mixture was concentrated under vacuum and the resulting residue was dissolved in dioxane (1.5 mL); a 10% w/w sodium carbonate aqueous solution (1 mL) and a solution of FmocCl (0.170 g, 0.66 mmol) in dioxane (2 mL) were added and the mixture was stirred at room temperature for 18 h. The reaction mixture was diluted with water (5 mL), acidified to pH 1 with 1 N hydrochloric acid aqueous solution and extracted with EtOAc (2 × 5 mL). The organic extracts were combined, dried over $MgSO_4$, filtered and concentrated under vacuum. The crude residue was purified by flash column chromatography (DCM/MeOH 1:0 to 98:2) to afford the title compound (0.168 g, 63% yield) as a yellow brittle foam. MS (ES+) *m*/*z* (M+23) 430.1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.69–7.83 (m, 2H) 7.50–7.65 (m, 2H) 7.27–7.45 (m, 4H) 4.46–4.55 (m, 1H) 4.33–4.44 (m, 1H) 4.12–4.33 (m, 1H) 3.76–4.08 (m, 1H) 3.55–3.69 (m, 1H) 3.51 (dt, *J* = 10.4, 7.0 Hz, 1H) 2.27–2.62 (m, 1H) 2.11–2.25 (m, 1H) 1.52–1.68 (m, 2H) 1.29 (dd, *J* = 14.0, 7.3 Hz, 1H) 0.88–0.99 (m, 9H). Mixture of rotamers. HPLC purity 93%.

6.6.2.27. (2*S*,3*R*)-3-*para*-Tolyl-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-yl methyl) ester (15b). The title compound was prepared according to the procedure used for compound **15a**. The crude residue was purified by flash column chromatography (DCM/MeOH 1:0 to 99:1) to afford the title compound (0.317 g) in quantitative yield as a pale pink gum. (ES+) *m*/*z* (M+23) 450.2. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.68–7.85 (m, 2H) 7.49–7.65 (m, 2H) 7.30–7.46 (m, 4H) 7.04–7.19 (m, 4H) 4.55 (dd, *J* = 10.7, 6.9 Hz,1H) 4.48 (dd, *J* = 11.1, 6.4 Hz, 1.3H) 4.22–4.32 (m, 0.6H) 4.17 (t, *J* = 5.8 Hz, 0.7H) 3.67–3.80 (m, 2H) 3.54–3.67 (m, 1H) 3.48 (q, *J* = 6.5 Hz, 0.4H) 2.26–2.41 (m, 4H) 1.89–2.12 (m, 1H). Mixture of rotamers. HPLC purity 100%.

6.6.2.28. (2*S*,3*R*)-3-Cyclohexyl-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester (15c). The title compound was prepared according to the procedure used for compound **15a**. The crude residue was purified by flash column chromatography (DCM/MeOH 1:0 to 97:3) to afford the title compound (0.214 g, 55% yield) as a colorless oil. (ES+) *m*/*z* (M+1) 420.2. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.68–7.82 (m, 2H) 7.51–7.64 (m, 2H) 7.35–7.46 (m, 2H) 7.28–7.35 (m, 2H) 4.44–4.54 (m, 1H) 4.33–4.44 (m, 1H) 4.04–4.31 (m, 2H) 3.53–3.68 (m, 1H) 3.47 (dt, *J* = 10.4, 7.4 Hz, 1H) 2.06 (s, 2H) 1.57–1.85 (m, 6H) 1.08–1.42 (m, 4H) 0.90–1.08 (m, 2H). HPLC purity 98%.

6.6.2.29. (2*S*,3*R*)-3-Phenyl-pyrrolidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester (15d). (2*S*,3*R*)-3-Phenyl-pyrrolidine-2-carboxylic acid **14** (0.096 g, 0.502 mmol) was suspended in dioxane (3 mL) and the suspension was cooled to 0 °C. A solution of $NaHCO_3$ (0.088 g, 1.054 mmol) in water (5 mL) was added to the mixture and the stirring was continued at 0 °C for 5 min. A solution of FmocCl (0.130 g, 0.502 mmol) in dioxane (2 mL) was added slowly and the reaction mixture was stirred for 18 h with gradual warming to room temperature. The reaction mixture was concentrated under vacuum to remove the dioxane; the resulting aqueous residue was diluted with water (10 mL), washed with TBME (2 × 5 mL), acidified to pH 2 using 2 N hydrochloric acid aqueous solution and extracted with EtOAc (3 × 10 mL). The organic extracts were combined, dried over Na_2SO_4, filtered and concentrated under vacuum. The crude material was purified by flash column chromatography (heptane/EtOAc 1:2 to 2:3) to provide the title compound (0.163 g, 78% yield) as a white solid. (ES+) *m*/*z* (M+1) 414.0. ^{1}H NMR (250 MHz, $CDCl_3$) δ ppm 7.15–7.85 (m, 13H) 4.42–4.60 (m, 2.4H) 4.26–4.36 (m, 1H) 4.08–4.22 (m, 0.6H) 3.41–3.75 (m, 3H) 2.28–2.49 (m, 1H) 1.97–2.15 (m, 1H) 1.22–1.32 (m, 0.7H) 0.85–0.93 (m, 0.3H). Mixture of rotamers. HPLC purity 100%.

6.6.2.30. (2*S*,3*S*)-2-Azido-3-isopropoxy-6-(4-methoxy-benzyloxy)-hexanoic acid ethyl ester (17). 2-iodopropane (16.5 mL, 28.1 g, 165.3 mmol) and silver oxide (38.3 g, 165.3 mmol) were added to a solution of (2*S*,3*S*)-2-azido-3-hydroxy-6-(4-methoxy-benzyloxy)-hexanoic acid ethyl ester **16** (4.60 g, 13.8 mmol) in 1,2-dichloroethane (190 mL) under nitrogen at room temperature. The mixture was heated at 70 °C for 17 h. The reaction mixture was cooled to room temperature, filtered over glass fiber paper and the filtrate was concentrated under vacuum. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 6:4) to afford the title compound (0.92 g, 18% yield) as a colorless oil. MS (ES+) *m*/*z* (M+23) 402.5. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.26 (d, *J* = 8.6 Hz, 2H) 6.88 (d, *J* = 8.6 Hz, 2H) 4.43 (s, 2H) 4.25 (q, *J* = 7.1 Hz, 2H) 4.11 (d, *J* = 4.4 Hz, 1H) 3.78–3.83 (m, 4H) 3.72 (dt, *J* = 12.2, 6.1 Hz, 1H) 3.40–3.50 (m, 2H) 1.59–1.82 (m,

3H) 1.49–1.57 (m, 1H) 1.29–1.33 (m, 3H) 1.16 (dd, *J* = 9.7, 6.1 Hz, 6H). HPLC purity 100%.

6.6.2.31. (2*S*,3*S*)-2-Azido-6-hydroxy-3-isopropoxy-hexanoic acid ethyl ester (18). To a solution of (2*S*,3*S*)-2-azido-3-isopropoxy-6-(4-methoxy-benzyloxy)-hexanoic acid ethyl ester **17** (0.92 g, 2.42 mmol) in DCM (20 mL) at 0 °C was added water (1.5 mL) and DDQ (0.82 g, 3.61 mmol) portion wise. The reaction mixture was stirred at 0 °C for 10 min and room temperature for a further 90 min. The reaction mixture was filtered through glass fiber paper and the residue was washed with DCM (4 × 5 mL). The filtrate phases were separated and the aqueous phase was extracted with DCM (1 × 10 mL). The organic extracts were combined, dried over $MgSO_4$, filtered and concentrated under vacuum. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 4:6) to afford the title compound (0.46 g, 73% yield) as a colorless oil. MS (ES+) *m*/*z* (M+23) 282.0. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 4.27 (q, *J* = 7.1 Hz, 2H) 4.15 (d, *J* = 4.7 Hz, 1H) 3.84 (dt, *J* = 8.0, 4.02 Hz, 1H) 3.76 (dt, *J* = 12.2, 6.0 Hz, 1H) 3.67 (m, 2H) 1.68–1.79 (m, 2H) 1.59–1.68 (m, 2H) 1.52–1.59 (m, 1H) 1.33 (t, *J* = 7.2 Hz, 3H) 1.19 (d, *J* = 6.1 Hz, 6H). HPLC purity 100%.

6.6.2.32. (2*S*,3*S*)-2-*tert*-butoxycarbonylamino-6-hydroxy-3-isopropoxy-hexanoic acid ethyl ester (19). Boc anhydride (0.584 g, 2.661 mmol) was added to a stirred solution of (2*S*,3*S*)-2-azido-6-hydroxy-3-isopropoxy-hexanoic acid ethyl ester **18** (0.460 g, 1.774 mmol) in EtOAc (20 mL). The solution was degassed using nitrogen and palladium on carbon (10% w/w, 50% wet with water) (0.156 mg, 0.701 mmol) was added. The reaction vessel was purged and put under hydrogen. The reaction mixture was stirred for 15 h at room temperature. The mixture was filtered through celite and the residue was washed with EtOAc (10 mL). The filtrate was concentrated under vacuum and the resulting residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 0:1) to afford the title compound (0.591 g, 80% yield) as a colorless oil. MS (ES+) *m*/*z* (M+23) 356.0. ^{1}H NMR (250 MHz, $CDCl_3$) δ ppm 5.20 (d, *J* = 9.0 Hz, 1H) 4.55 (dd, *J* = 8.7, 4.0 Hz, 1H) 4.22 (q, *J* = 7.1 Hz, 2H) 3.59–3.82 (m, 4H) 1.41–1.87 (m, 14H) 1.29 (t, *J* = 7.1 Hz, 3H) 1.14 (dd, *J* = 6.02, 2.4 Hz, 6H). HPLC purity 100%.

6.6.2.33. (2*S*,3*S*)-2-*tert*-butoxycarbonylamino-3-isopropoxy-6-methanesulfonyloxy-hexanoic acid ethyl ester (20). Triethylamine (0.39 mL, 0.28 g, 2.80 mmol) and mesyl chloride (0.13 mL, 0.19 g, 1.68 mmol) were added to a solution of (2*S*,3*S*)-2-*tert*-butoxycarbonylamino-6-hydroxy-3-isopropoxy-hexanoic acid ethyl ester **19** (0.46 g, 1.38 mmol) in DCM (26 mL) at 0 °C under nitrogen. The mixture was stirred at 0 °C for 1 hour and room temperature for 1 hour before being concentrated under vacuum. The resulting residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 2:1) to provide the title compound (0.47 g, 76% yield) as a colorless oil. MS (ES+) *m*/*z* (M+23) 434.1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 5.13–5.25 (m, 1H) 4.46–4.56 (m, 1H) 4.19–4.31 (m, 4H) 3.74 (s, 2H) 3.01 (s, 3H) 1.90–2.01 (m, 1H) 1.77–1.87 (m, 1H) 1.59–1.66 (m, 1H) 1.49–1.56 (m, 1H) 1.46 (s, 9H) 1.30 (t, *J* = 7.09 Hz, 3H) 1.14 (d, *J* = 5.99 Hz, 6H). HPLC purity 100%.

6.6.2.34. (2*S*,3*S*)-3-Isopropoxy-piperidine-1,2-dicarboxylic acid 1-*tert*-butyl ester 2-ethyl ester (21). Sodium hydride (60% in mineral oil) (0.049 g, 1.215 mmol) was added to a stirred solution of (2*S*,3*S*)-2-*tert*-butoxycarbonylamino-3-isopropoxy-6-methanesulfonyloxy-hexanoic acid ethyl ester **20** (0.500 g, 1.215 mmol) in DMF (7 mL) at 0 °C under nitrogen. The mixture was allowed to warm to room temperature over 4 h. The reaction mixture was diluted with water (10 mL), pH was adjusted to 6 using a 10% w/w citric acid aqueous solution and the mixture was extracted with EtOAc (3 × 50 mL). The organic extracts were combined, washed with brine (2 × 30 mL), dried over $MgSO_4$, filtered and concentrated under vacuum. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 9:1) to afford the title compound (0.225 g, 59% yield) as a colorless oil. MS (ES+) *m*/*z* (M+23) 338.4. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 4.71–5.03 (m, 1H) 4.20 (q, *J* = 7.1 Hz, 2H) 3.88–4.11 (m, 2H) 3.65–3.76 (m, 1H) 2.83–3.10 (m, 1H) 1.75–1.94 (m, 2H) 1.31–1.52 (m, 11H) 1.28 (t, *J* = 7.1 Hz, 3H) 1.16 (d, *J* = 6.0 Hz, 6H). Mixture of rotamers. HPLC purity 86%.

6.6.2.35. (2*S*,3*S*)-3-Isopropoxy-piperidine-1,2-dicarboxylic acid 1-*tert*-butyl ester (22). A solution of lithium hydroxide (0.431 g, 17.980 mmol) in water (2 mL) was added to a stirred solution of (2*S*,3*S*)-3-isopropoxy-piperidine-1,2-dicarboxylic acid 1-*tert*-butyl ester 2-ethyl ester **21** (0.567 g, 1.798 mmol) in THF (5 mL) at room temperature. The reaction mixture was stirred at room temperature for 3 h. THF was removed under vacuum and the resulting aqueous residue was diluted with water (5 mL), washed with TBME (2 × 5 mL), acidified to pH 4 using a 1 N hydrochloric acid aqueous solution, extracted with EtOAc (3 × 20 mL). The organic extracts were combined, washed with brine (1 × 10 mL), dried over $MgSO_4$, filtered and concentrated to provide the title compound (0.256 g, 50% yield) as a white solid. MS (ES+) *m*/*z* (M+23) 310.0. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 4.83–5.09 (m, 1H) 3.92–4.15 (m, 2H) 3.66–3.78 (m, 1H) 2.85–3.09 (m, 1H) 1.78–1.94 (m, 2H) 1.33–1.54 (m, 11H) 1.17 (d, *J* = 6.0 Hz, 6H). Mixture of rotamers. HPLC purity 100%.

6.6.2.36. (2*S*,3*S*)-3-Isopropoxy-piperidine-2-carboxylic acid trifluoroacetic acid salt (23). Trifluoro acetic acid (50% v/v solution in DCM) (2 mL) was added to a stirred solution of (2*S*,3*S*)-3-isopropoxy-piperidine-1,2-dicarboxylic acid 1-*tert*-butyl ester **22** (0.130 g, 0.452 mmol) in DCM (1 mL) at 0 °C. The reaction mixture was stirred for 2 h at 0 °C and concentrated under vacuum to produce the title compound (0.123 g, 90% yield) as a viscous pale yellow oil. MS (ES+) *m*/*z* (M+1) 188.0. ^{1}H NMR (250 MHz, MeOD) δ ppm 4.04–4.16 (m, 2H) 3.80 (dt, *J* = 12.2, 6.1 Hz, 1H) 3.25–3.41 (m, 1H) 3.07–3.21 (m, 1H) 1.93–2.16 (m, 1H) 1.62–1.93 (m, 3H) 1.20 (d, *J* = 7.3 Hz, 6H). HPLC purity 91%.

6.6.2.37. (2*S*,3*S*)-3-Isopropoxy-piperidine-1,2-dicarboxylic acid 1-(9*H*-fluoren-9-ylmethyl) ester (24). A sodium carbonate aqueous solution (10% w/w) (0.791 mL, 0.950 mmol) was added to a stirred solution of (2*S*,3*S*)-3-isopropoxy-piperidine-2-carboxylic acid trifluoroacetic acid salt **23** (0.123 mg, 0.408 mmol) in dioxane (1 mL) at room temperature and the solution was cooled to 0 °C. A solution of FmocCl (0.117 g, 0.452 mmol) in dioxane (1 mL) was added drop wise at 0 °C and the reaction mixture was allowed to warm to room temperature and stirred for 16 h. Dioxane was removed under reduced pressure and the resulting basic solution was diluted with water (3 mL), washed with TBME (2 × 2 mL) and acidified to pH 2 using 2 N hydrochloric acid aqueous solution. The acidic phase was extracted with EtOAc (3 × 5 mL), the organic extracts were combined, dried over $MgSO_4$, filtered and concentrated under vacuum. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 1:1) to provide the title compound (0.092 g, 50% yield) as a colorless glass. MS (ES+) *m*/*z* (M+23) 432.4. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.70–7.83 (m, 2H) 7.53–7.66 (m, 2H) 7.28–7.44 (m, 4H) 5.10 (br s, 0.6H) 4.72 (br s, 0.4H) 4.39–4.53 (m, 2H) 4.22–4.37 (m, 1H) 3.97–4.17 (m, 2H) 3.74 (dt, *J* = 11.9, 5.7 Hz, 0.6H) 3.45–3.55 (m, 0.4H) 3.14–3.24 (m, 0.6H) 2.96 (t, *J* = 12.4 Hz, 0.4H) 1.78–2.04 (m,

2H) 1.36–1.59 (m, 2H) 1.22–1.35 (m, 1H) 1.04–1.22 (m, 6H). HPLC purity 99%.

6.6.2.38. 6-Hydroxy-1,2,3,4-tetrahydro-isoquinoline-1-carboxylic acid (26a). Triethylamine (0.76 mL, 0.55 g, 5.47 mmol) was added to a stirred solution of 3-(2-aminoethyl)phenol hydrochloride (1:1) **25a** (1.00 g, 5.76 mmol) in EtOH (26 mL). The mixture was cooled to 5 °C and a solution of glyoxylic acid (0.53 g, 5.76 mmol) in EtOH (6 mL) was added drop wise. The resulting mixture was stirred for 1 hour with gradual warming to room temperature. The resulting solid was filtered and washed with EtOH to provide the title compound (0.78 g, 70% yield) as an off-white solid. (ES+) *m*/*z* (M+1) 193.9. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.35 (br s, 1H) 7.50 (d, *J* = 8.6 Hz, 1H) 6.60 (dd, *J* = 8.6, 2.0 Hz, 1H) 6.49 (d, *J* = 2.0 Hz, 1H) 4.33 (s, 1H) 3.42–3.47 (m, 1H) 3.27–3.34 (m, 1H) 3.06–3.14 (m, 1H) 2.81–2.89 (m, 1H) 2.69–2.78 (m, 1H).

6.6.2.39. [2-(3-Hydroxy-phenyl)-ethyl]-carbamic acid 9*H*-fluoren-9-ylmethyl ester (28e). A solution of FmocCl (4.3 g, 16.8 mmol) in DCM (20 mL) was added to a stirred solution of 3-(2-amino-ethyl)-phenol **27e** (2.0 g, 14.6 mmol) and DIPEA (7.2 mL, 5.6 g, 43.7 mmol) in DCM (30 mL) at 0 °C. After 15 min at 0 °C, the mixture was stirred for 20 h at room temperature. The reaction mixture was diluted with DCM (50 mL), washed successively with a saturated aqueous $NaHCO_3$ solution (1 × 20 mL) and brine (1 × 20 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated under vacuum. The crude residue was purified by flash column chromatography (DCM/MeOH 1:0 to 95:5) to provide the title compound (1.2 g, 23% yield) as a white solid. (ES+) *m*/*z* (M+23) 382.3. HPLC purity 95%.

6.6.2.40. [2-(4-Bromo-phenyl)-ethyl]-carbamic acid 9*H*-fluoren-9-ylmethyl ester (28f). The title compound was prepared according to the procedure used for compound **28e**. The crude residue was purified by trituration in TBME to provide the title compound (1.24 g, 50% yield) as a white powder. (ES+) *m*/*z* (M+23) 444.1/445.9. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.78 (d, *J* = 7.6 Hz, 2H) 7.57 (d, *J* = 7.4 Hz, 2H) 7.39–7.46 (m, 4H) 7.29–7.36 (m, 2H) 7.05 (d, *J* = 8.2 Hz, 1.7H) 6.90 (br s, 0.3H) 4.74 (d, *J* = 4.7 Hz, 1H) 4.55 (br s, 0.3H) 4.43 (d, *J* = 6.6 Hz, 1.7H) 4.19–4.26 (m, 1H) 3.43 (q, *J* = 6.4 Hz, 1.7H) 3.22 (br s, 0.3H) 2.78 (t, *J* = 6.7 Hz, 1.7H) 2.53 (br s, 0.3H). Mixture of rotamers. HPLC purity 100%.

6.6.2.41. [2-(3-Bromo-phenyl)-ethyl]-carbamic acid 9*H*-fluoren-9-ylmethyl ester (28g). The title compound was prepared according to the procedure used for compound **28e**. The crude residue was purified by trituration in Et_2O to provide the title compound (2.45 g, 55% yield) as a white powder. (ES+) *m*/*z* (M+23) 444.2/446.2. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.78 (d, *J* = 7.6 Hz, 2H) 7.58 (d, *J* = 7.4 Hz, 2H) 7.30–7.45 (m, 6H) 7.08–7.22 (m, 2H) 4.78 (br s, 1H) 4.57 (br s, 0.3H) 4.43 (d, *J* = 6.8 Hz, 1.7H) 4.20–4.28 (m, 1H) 3.45 (q, *J* = 6.5 Hz, 1.7H) 3.21 (br s, 0.3H) 2.80 (t, *J* = 6.7 Hz, 1.7H) 2.52 (br s, 0.3H). Mixture of rotamers. HPLC purity 100%.

6.6.2.42. 6-Hydroxy-3,4-dihydro-1*H*-isoquinoline-1,2-dicarboxylic acid 2-(9*H*-fluoren-9-ylmethyl) ester (29a). 6-Hydroxy-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid **26a** (0.50 g, 2.59 mmol) was suspended in dioxane (7 mL) and the suspension was cooled to 0 °C. A solution of $NaHCO_3$ (0.24 g, 2.85 mmol) in water (4 mL) was added to the mixture and the stirring was continued at 0 °C for 5 min. A solution of FmocCl (0.67 g, 2.59 mmol) in dioxane (3 mL) was added slowly and the reaction mixture was stirred for 18 h with gradual warming to room temperature. The reaction mixture was concentrated under vacuum to remove the dioxane, the resulting residue was dissolved in a mixture EtOAc (15 mL):2 N HCl (aq) (15 mL). The phases were separated and the aqueous phase was extracted with EtOAc (3 × 15 mL). The organic extracts were combined, dried over Na_2SO_4, filtered and concentrated under vacuum to provide a sticky off-white solid which was triturated in Et_2O to provide the title compound (0.90 g, 83% yield) as an off-white solid. (ES+) *m*/*z* (M+1) 438.1. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 9.50 (s, 0.4H) 9.49 (s, 0.6H) 7.83–7.95 (m, 2H) 7.66 (q, *J* = 6.7 Hz, 2H) 7.38–7.48 (m, 2H) 7.21–7.37 (m, 3H) 6.62–6.70 (m, 1H) 6.59 (s, 1H) 5.34 (s, 0.4H) 5.27 (s, 0.6H) 4.22–4.41 (m, 3H) 3.63–3.72 (m, 1H) 3.51–3.63 (m, 1H) 2.75–2.85 (m, 1H) 2.61–2.75 (m, 1H). Mixture of rotamers. HPLC purity 83%.

6.6.2.43. 6-Methyl-3,4-dihydro-1*H*-isoquinoline-1,2-dicarboxylic acid 2-(9*H*-fluoren-9-ylmethyl) ester (29b). The title compound was prepared according to the procedure used for compound **29a** The crude residue did not require purification and the title compound (0.454 g, 63% yield) was obtained as a white powder. (ES+) *m*/*z* (M+1) 414.0. ^{1}H NMR (250 MHz, $CDCl_3$) δ ppm 6.95–7.83 (m, 12H) 5.62 (s, 0.6H) 5.33 (s, 0.4H) 4.38–4.61 (m, 2H) 4.16–4.36 (m, 1H) 3.66–3.97 (m, 2H) 2.73–3.06 (m, 2H) 2.29–2.37 (m, 3H). Mixture of rotamers. HPLC purity 100%.

6.6.2.44. 7-Methyl-3,4-dihydro-1*H*-isoquinoline-1,2-dicarboxylic acid 2-(9*H*-fluoren-9-ylmethyl) ester (29c). The title compound was prepared according to the procedure used for compound **29a**. The crude residue did not require purification and the title compound (0.402 g, 55% yield) was obtained as a white powder. (ES+) *m*/*z* (M+1) 414.0. ^{1}H NMR (250 MHz, $CDCl_3$) δ ppm 7.01–7.83 (m, 12H) 5.61 (s, 0.6H) 5.32 (s, 0.4H) 4.36–4.63 (m, 2H) 4.07–4.35 (m, 1H) 3.65–3.98 (m, 2H) 2.84 (d, *J* = 6.4 Hz, 2H) 2.35 (s, 3H). Mixture of rotamers. HPLC purity 89%.

6.6.2.45. 6,7-Dimethoxy-3,4-dihydro-1*H*-isoquinoline-1,2-dicarboxylic acid 2-(9*H*-fluoren-9-ylmethyl) ester (29d). The title compound was prepared according to the procedure used for compound **29a**. The crude residue was purified by flash column chromatography (heptane/EtOAc 1:0 to 1:1) to afford the title compound (0.219 g, 33% yield) as an off-white powder. (ES+) *m*/*z* (M+23) 482.5. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.52–7.83 (m, 4H) 7.18–7.45 (m, 5H) 7.01 (s, 0.6H) 6.93 (s, 0.4H) 6.61–6.69 (m, 1H) 5.58 (s, 0.6H) 5.33 (s, 0.4H) 4.42–4.59 (m, 2H) 4.21–4.35 (m, 1H) 3.78–4.00 (m, 7.4H) 3.62–3.76 (m, 0.6H) 2.77–2.96 (m, 2H). Mixture of rotamers. HPLC purity 98%.

6.6.2.46. 7-Hydroxy-3,4-dihydro-1*H*-isoquinoline-1,2-dicarboxylic acid 2-(9*H*-fluoren-9-ylmethyl) ester (29e). Glyoxylic acid (0.28 g, 3.74 mmol) was added to a stirred solution of 9H-fluoren-9-ylmethyl *N*-[2-(4-bromophenyl)ethyl]carbamate **28e** (1.21 g, 3.37 mmol) in a $AcOH/H_2SO_4$ mixture (3:1, 30 mL) at room temperature. The reaction mixture was stirred at room temperature for 24 h, wherupon the reaction mixture was poured carefully onto ice/water (~100 mL). The resulting mixture was left to warm to room temperature and then extracted with DCM (3 × 50 mL); the organic extracts were combined, dried over Na_2SO_4, filtered and concentrated under vacuum. The crude residue was purified by flash column chromatography (DCM/MeOH 1:0 to 97.5:5) to provide the title compound (0.31 g, 21% yield) as a sticky foam. (ES+) *m*/*z* (M+1) 416.1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 6.65–7.79 (m, 13H) 5.57 (br s, 1H) 4.06–4.53 (m, 3H) 3.55–3.89 (m, 2H) 2.63–2.89 (m, 2H). HPLC purity 94%.

6.6.2.47. 7-Bromo-3,4-dihydro-1*H*-isoquinoline-1,2-dicarboxylic acid 2-(9*H*-fluoren-9-ylmethyl) ester (29f). The title compound was prepared according to the procedure used for compound **29e**. The crude residue was purified by flash column chromatography (DCM/MeOH 1:0 to 97.5:2.5) to provide a mixture of

the 6- and 8-bromo isomers which were separated by preparative HPLC and the title compound (0.506 g, 36% yield) was obtained as a sticky oil. (ES+) *m*/*z* (M+23) 500.1/501.8. ^{1}H NMR (250 MHz, $CDCl_3$) δ ppm 7.49–7.83 (m, 5H) 7.18–7.47 (m, 5H) 6.97–7.11 (m, 1H) 5.63 (s, 0.5H) 5.24 (s, 0.5H) 4.38–4.61 (m, 2H) 4.16–4.34 (m, 1H) 3.76–4.00 (m, 1.5H) 3.50–3.70 (m, 0.5H) 2.71–2.99 (m, 2H). Mixture of rotamers. HPLC purity 89%.

6.6.2.48. 6-Bromo-3,4-dihydro-1*H*-isoquinoline-1,2-dicarboxylic acid 2-(9*H*-fluoren-9-ylmethyl) ester (29g). The title compound was prepared according to the procedure used for compound **29e**. The crude residue was purified by flash column chromatography (DCM/MeOH 1:0 to 97.5:2.5) to provide a mixture of the 6- and 8-bromo isomers which were separated by preparative HPLC and the title compound (0.826 g, 30% yield) was obtained as a white solid.(ES+) *m*/*z* (M+23) 499.9/501.9. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.78 (d, *J* = 7.6 Hz, 1H) 7.70 (t, *J* = 8.2 Hz, 1H) 7.50–7.63 (m, 2H) 7.28–7.44 (m, 6H) 7.22 (dd, *J* = 12.9, 7.1 Hz, 1H) 5.61 (s, 0.6H) 5.26 (s, 0.4H) 4.42–4.61 (m, 2H) 4.29 (t, *J* = 6.9 Hz, 0.6H) 4.21 (t, *J* = 5.7 Hz, 0.4H) 3.85–3.93 (m, 0.6H) 3.73–3.85 (m, 1H) 3.64 (ddd, *J* = 12.8, 8.0, 4.9 Hz, 0.4H) 2.76–2.99 (m, 2H). Mixture of rotamers. HPLC purity 99%.

6.7. General method for synthesis of peptides

6.7.1. Stage 1—Loading of P_2 amino acid to Webb semi-carbazide resin (31)

Asp semi-carbazide Merrifield resin **30** (0.625 mmol/g, 1.0 equiv, typically 600–700 mg of resin) was added to a 20 ml fritted plastic tube followed by P_2 amino acid (2.0 equiv) and HATU (2.2 equiv). To this mixture was added DMF (10 mL) and DIPEA (5.0 equiv) and the mixture was agitated at room temperature for 20 h. The resin was filtered and washed with 3 × 10 mL aliquots of solvent in the following order: DMF, DCM, DMF, DCM, MeOH, DCM, MeOH and Et_2O to afford the functionalized resin which was dried to constant weight under vacuum. Ninhydrin and IR analysis of the resin was performed to confirm that there were no free amine sites on the resin.

6.7.2. Stage 2—Resin deprotection followed by loading of Fmoc Val-Asp-Val-OH onto resin (32)

To the stage 1 resin **31** in a 20 ml fritted plastic tube was added 10 ml of a 20% v/v solution of piperidine in DMF and the reaction mixture was agitated for 1 h at room temperature. The resin was then filtered and washed with DMF, DCM, DMF, DCM, MeOH, DCM, MeOH and Et_2O to afford the deprotected resin which was dried to constant weight under vacuum. Ninhydrin and IR analysis of the resin was performed to confirm that the coupling reaction was completely successful.

To the deprotected resin (1.0 equiv) in a 20 ml fritted plastic tube was added Ac-Val-Asp(O*t*Bu)-Val-OH and HATU (2.2 equiv) followed by DMF (10 mL) and DIPEA (5.0 equiv). This mixture was agitated at room temperature for 20 h. The resin was then filtered and washed with 3 × 10 mL aliquots of solvent in the following order: DMF, DCM, DMF, DCM, MeOH, DCM, MeOH and Et_2O to afford the functionalized resin which was dried to constant weight under vacuum. Ninhydrin and IR analysis of the resin was performed to confirm that there were no free amine sites on the resin.

6.7.3. Stage 3—Cleavage of final compound from resin (2, 33a–v)

To the stage 2 resin (**32**) in a 20 ml fritted plastic tube was added 5 ml of a solution of TFA/water (9:1). The resin was then agitated at room temperature for 90 min after which the resin was filtered, the resin was washed with two further aliquots of mixture TFA/water (9:1, 2 ml) and the filtrate was concentrated under vacuum to dryness to give the crude product which was purified using preparative HPLC (MeOH/water mobile phase) to afford the final pentapeptide aldehyde product.

6.7.3.1. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(*S*)-2-*tert*-butoxy-1-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-ethylcarbamoyl]-2-methyl-propyl}-succinamic acid (2). The title compound was prepared following the procedure used for peptide synthesis described above (use of (*S*)-*O*-*tert*-butyl serine in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (5 mg) as a white solid. MS (ES+) *m*/*z* (M+1) 616.2. ^{1}H NMR (500 MHz, D_2O) δ ppm 4.97–5.07 (m, 1H) 4.70–4.71 (m, 1H) 4.40–4.54 (m, 1H) 4.18–4.28 (m, 1H) 4.02–4.16 (m, 2H) 3.61–3.75 (m, 2H) 2.45–3.14 (m, 4H) 1.96–2.18 (m, 5H) 1.17 (s, 9H) 0.85–0.95 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{27}H_{45}N_5O_{11}$ $[M+H]^+$ *m*/*z* 616.3194, found *m*/*z* 616.3179. HPLC purity 100%.

6.7.3.2. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(*S*)-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33a). The title compound was prepared following the procedure used for peptide synthesis described above (use of Fmoc-(*S*)-proline in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (15 mg) as a white solid. MS (ES+) *m*/*z* (M+1) 570.1. ^{1}H NMR (500 MHz, D_2O) δ ppm 4.88–5.04 (m, 1H), 4.64 (dd, *J* = 7.7, 6.1 Hz, 1H), 4.24–4.45 (m, 2H), 4.08–4.24 (m, 1H), 3.89–4.04 (m, 1H), 3.30–3.87 (m, 2H), 2.32–3.15 (m, 4H), 2.32–3.15 (m, 4H), 2.09–2.31 (m, 1H), 1.65–2.09 (m, 8H), 0.66–0.97 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{25}H_{39}N_5O_{10}$ $[M+H]^+$ *m*/*z* 570.2775, found *m*/*z* 570.2776. HPLC purity 100%.

6.7.3.3. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(2*S*,3*S*)-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-3-methoxy-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33b). The title compound was prepared following the procedure used for peptide synthesis described above (use of **6a** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (12 mg) as an off-white solid. MS (ES+) *m*/*z* (M+1) 600.5. ^{1}H NMR (500 MHz, D_2O) δ ppm 4.95–5.07 (m, 1H) 4.69–4.71 (m, 1H) 3.47–4.53 (m, 7H) 3.32–3.44 (m, 3H) 2.45–3.10 (m, 4H) 1.89–2.27 (m, 7H) 0.74–1.02 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{26}H_{41}N_5O_{11}$ $[M+H]^+$ *m*/*z* 600.2881, found *m*/*z* 600.2900. HPLC purity 100%.

6.7.3.4. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(2*S*,3*S*)-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-3-isopropoxy-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33c). The title compound was prepared following the procedure used for peptide synthesis described above (use of **6b** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (13 mg) as a white powder. MS (ES+) *m*/*z* (M+1) 628.1. ^{1}H NMR (500 MHz, D_2O) δ ppm 4.96–5.09 (m, 1H) 4.69 (m, 1H) 4.16–4.52 (m, 4H) 3.52–4.10 (m, 4H) 2.43–3.16 (m, 4H) 1.90 -2.32 (m, 7H) 1.07–1.30 (m, 6H) 0.73–1.02 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{28}H_{45}N_5O_{11}$ $[M+H]^+$ *m*/*z* 628.3194, found *m*/*z* 628.3195. HPLC purity 100%.

6.7.3.5. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(2*S*,3*S*)-3-se-butoxy-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33d). The title compound was prepared following the procedure used for peptide synthesis

described above (use of **6c** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (17 mg) as a yellow powder. MS (ES+) *m*/*z* (M+1) 642.3. ^{1}H NMR (500 MHz, D_2O) δ ppm 4.96–5.06 (m, 0.5H) 4.66–4.70 (m, 0.5H) 4.15–4.51 (m, 4H) 3.54–4.10 (m, 5H) 2.51–3.12 (m, 4H) 1.96–2.31 (m, 7H) 1.37–1.57 (m, 2H) 1.07–1.22 (m, 3H) 0.74–0.99 (m, 15H). Mixture of isomers. HRMS (ESI) calcd for $C_{29}H_{47}N_5O_{11}$ $[M+H]^+$ *m*/*z* 642.3350, found *m*/*z* 642.3347. HPLC purity 94%.

6.7.3.6. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(2*S*,3*S*)-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-3-isobutoxy-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33e). The title compound was prepared following the procedure used for peptide synthesis described above (use of **6d** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (7 mg) as a white powder. MS (ES+) *m*/*z* (M+1) 642.3. ^{1}H NMR (500 MHz, D_2O) δ ppm 4.95–5.08 (m, 1H) 4.68 (m, 1H) 4.06–4.54 (m, 5H) 3.66–4.01 (m, 2H) 3.25–3.44 (m, 2H) 2.43–3.12 (m, 4H) 1.94–2.31 (m, 7H) 1.79 (m, 1H) 0.80–1.02 (m, 18H). Mixture of isomers. HRMS (ESI) calcd for $C_{29}H_{47}N_5O_{11}$ $[M+H]^+$ *m*/*z* 642.3350, found *m*/*z* 642.3351. HPLC purity 95%.

6.7.3.7. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(2*S*,3*S*)-3-cyclopentyloxy-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33f). The title compound was prepared following the procedure used for peptide synthesis described above (use of **6e** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (7 mg) as a white powder. MS (ES+) *m*/*z* (M+1) 654.2. ^{1}H NMR (500 MHz, D_2O) δ ppm 4.92–5.09 (m, 1H) 4.67–4.70 (m, 1H) 3.53–4.46 (m, 8H) 2.46–3.12 (m, 4H) 1.96–2.27 (m, 7H) 1.43–1.87 (m, 8H) 0.74–1.00 (m, 12H). Mixture of isomers. HPLC purity 100%.

6.7.3.8. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(2*S*,3*S*)-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-3-(pyrimidin-2-yloxy)-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33g). The title compound was prepared following the procedure used for peptide synthesis described above (use of **6f** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (18 mg) as a white powder. MS (ES+) *m*/*z* (M+1) 664.2. ^{1}H NMR (500 MHz, D_2O) δ ppm 8.52–8.69 (m, 2H) 7.14–7.28 (m, 1H) 5.50 (m, 1H) 5.03–5.19 (m, 1H) 4.82–4.86 (m, 1H) 4.68 (m, 1H) 3.61–4.50 (m, 5H) 2.46–3.15 (m, 4H) 2.25–2.43 (m, 2H) 1.90–2.17 (m, 5H) 0.62–1.04 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{29}H_{41}N_7O_{11}$ $[M+H]^+$ *m*/*z* 664.2942, found *m*/*z* 664.2960. HPLC purity 100%.

6.7.3.9. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(2*S*,3*S*)-3-(2,2-dimethyl-propyl)-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33h). The title compound was prepared following the procedure used for peptide synthesis described above (use of **15a** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (41 mg) as a colorless oil. MS (ES+) *m*/*z* (M+1) 640.3. ^{1}H NMR (500 MHz, D_2O) δ ppm 5.05–5.11 (m, 1H) 4.70–4.72 (m, 1H) 4.37–4.44 (m, 1H) 4.15–4.35 (m, 1H) 4.08–4.13 (m, 1H) 3.96–4.06 (m, 1H) 3.73–3.86 (m, 1H) 3.59–3.69 (m, 1H) 2.53–2.98 (m, 4H) 2.31–2.42 (m, 1H) 2.17–2.18 (m, 1H) 2.06–2.09 (m, 2H) 2.05 (s, 3H) 1.63–1.76 (m, 1H) 1.49–1.61 (m, 1H) 1.29–1.43 (m, 1H) 0.85–0.99 (m, 21H). Mixture of isomers. HRMS (ESI) calcd for $C_{30}H_{49}N_5O_{10}$ $[M+H]^+$ *m*/*z* 640.3558, found *m*/*z* 640.3546. HPLC purity 100%.

6.7.3.10. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(2*S*,3*R*)-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-3-phenyl-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33i). The title compound was prepared following the procedure used for peptide synthesis described above (use of **15d** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (8 mg) as a white solid. MS (ES+) *m*/*z* (M+1) 646.2. ^{1}H NMR (500 MHz, D_2O) δ ppm 7.23–7.47 (m, 5H) 4.87–5.00 (m, 1H) 4.66–4.71 (m, 1H) 3.90–4.55 (m, 5H) 3.74–3.89 (m, 1H) 3.32–3.54 (m, 1H) 2.44–3.09 (m, 4H) 2.17–2.42 (m, 2H) 1.97–2.14 (m, 5H) 0.70–1.16 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{31}H_{43}N_5O_{10}$ $[M+H]^+$ *m*/*z* 646.3088, found *m*/*z* 646.3093. HPLC purity 99%.

6.7.3.11. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(2*S*,3*R*)-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-lcarbamoyl)-3-p-tolyl-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33j). The title compound was prepared following the procedure used for peptide synthesis described above (use of **15b** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (23 mg) as a white solid. (ES+) *m*/*z* (M+1) 660.1. ^{1}H NMR (500 MHz, D_2O) δ ppm 7.19–7.27 (m, 4H) 4.85–4.99 (m, 1H) 4.68–4.72 (m, 1H) 4.43 (d, J = 6.1 Hz, 1H) 4.03–4.30 (m, 4H) 3.68–3.84 (m, 1H) 3.23–3.43 (m, 1H) 2.43–3.07 (m, 4H) 2.15–2.38 (m, 5H) 1.98–2.13 (m, 5H) 0.85–1.04 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{32}H_{45}N_5O_{10}$ $[M+H]^+$ *m*/*z* 660.3245, found *m*/*z* 660.3251. HPLC purity 100%.

6.7.3.12. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(2*S*,3*R*)-3-cyclohexyl-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-pyrrolidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33k). The title compound was prepared following the procedure used for peptide synthesis described above (use of **15c** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (15 mg) as a white solid. (ES+) *m*/*z* (M+1) 652.5. ^{1}H NMR (500 MHz, D_2O) δ ppm 4.99–5.06 (m, 1H) 4.66–4.69 (m, 1H) 4.24–4.46 (m, 2H) 4.04–4.20 (m, 2H) 3.85–4.00 (m, 1H) 3.54–3.68 (m, 1H) 2.45–3.14 (m, 4H) 1.95–2.21 (m, 7H) 1.55–1.86 (m, 6H) 0.96–1.48 (m, 6H) 0.84–0.95 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{31}H_{49}N_5O_{10}$ $[M+H]^+$ *m*/*z* 652.3558, found *m*/*z* 652.3549. HPLC purity 100%.

6.7.3.13. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[1-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-1,3-dihydro-isoindole-2-carbonyl]-2-methyl-propyl}-succinamic acid (33l). The title compound was prepared following the procedure used for peptide synthesis described above (use of Fmoc-1,3-dihydro-isoindole-1,2-dicarboxylic acid in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (36 mg) as an off-white solid. (ES+) *m*/*z* (M+1) 618.0. ^{1}H NMR (500 MHz, D_2O) δ ppm 7.28–7.55 (m, 4H) 5.52–5.68 (m, 1H) 5.11–5.23 (m, 2H) 5.01–5.04 (m, 1H) 4.65–4.70 (m, 1H) 3.87–4.59 (m, 3H) 2.48–3.13 (m, 4H) 1.74–2.22 (m, 5H) 0.54–1.16 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{29}H_{39}N_5O_{10}$ $[M+H]^+$ *m*/*z* 618.2775, found *m*/*z* 618.2790. HPLC purity 100%.

6.7.3.14. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(*S*)-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-piperidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33m). The title compound was prepared following the procedure used for peptide synthesis described above (use of (*S*)-*N*-Fmoc-piperidine-2-carboxylic acid in stage 1 and Ac-Val-Asp (O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (9 mg) as a white solid. ES+) *m*/*z* (M+23) 606.1. ^{1}H NMR (500 MHz, D_2O) δ ppm 4.86–5.05 (m, 2H) 3.97–4.68 (m, 4H) 3.88–3.96 (m, 1H) 3.12–3.35 (m, 1H) 2.37–3.05 (m, 4H) 1.89–2.30 (m, 6H) 1.52–1.77 (m, 3H) 1.18–1.49 (m, 2H) 0.74–0.93 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{26}H_{41}N_5O_{10}$ $[M+H]^+$ *m*/*z* 584.2932, found *m*/*z* 584.2927. HPLC purity 100%.

6.7.3.15. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(*S*)-2-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-piperidine-1-carbonyl]-2-methyl-propyl}-succinamic acid (33n). The title compound was prepared following the procedure used for peptide synthesis described above (use of **24** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (38 mg) as a white solid. (ES+) *m*/*z* (M+1) 642.3. ^{1}H NMR (500 MHz, D_2O) δ ppm 5.06–5.45 (m, 1H) 4.92–5.04 (m, 1H) 4.50–4.69 (m, 2H) 3.93–4.37 (m, 4H) 3.36–3.82 (m, 2H) 2.44–3.09 (m, 4H) 1.97–2.16 (m, 5H) 1.44–1.87 (m, 4H) 0.72–1.24 (m, 18H). Mixture of isomers. HRMS (ESI) calcd for $C_{29}H_{47}N_5O_{11}$ $[M+H]^+$ *m*/*z* 642.3350, found *m*/*z* 642.3343. HPLC purity 96%.

6.7.3.16. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[(*S*)-1-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-3,4-dihydro-1H-isoquinoline-2-carbonyl]-2-methyl-propyl}-succinamic acid (33o). The title compound was prepared following the procedure used for peptide synthesis described above (use of (1*S*)-2-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (22 mg) as a white solid. (ES+) *m*/*z* (M+1) 632.3. ^{1}H NMR (500 MHz, D_2O) δ ppm 7.14–7.38 (m, 4H) 5.52–6.06 (m, 1H) 4.91–4.97 (m, 1H) 4.59–4.68 (m, 2H) 3.64–4.22 (m, 4H) 2.34–3.18 (m, 6H) 1.82–2.14 (m, 5H) 0.48–0.98 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{30}H_{41}N_5O_{10}$ $[M+H]^+$ *m*/*z* 632.2932, found *m*/*z* 632.2948. HPLC purity 100%.

6.7.3.17. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[1-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-7-methyl-3,4-dihydro-1*H*-isoquinoline-2-carbonyl]-2-methyl-propyl}-succinamic acid (33p). The title compound was prepared following the procedure used for peptide synthesis described above (use of **29c** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (28 mg) as a white solid. (ES+) *m*/*z* (M+23) 668.2. ^{1}H NMR (500 MHz, D_2O) δ ppm 7.09–7.26 (m, 3H) 5.53–6.06 (m, 1H) 4.95–5.10 (m, 1H) 4.42–4.66 (m, 2H) 3.70–4.33 (m, 4H) 2.47–3.14 (m, 6H) 2.28–2.38 (m, 3H) 2.01–2.21 (m, 5H) 0.61–1.06 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{31}H_{43}N_5O_{10}$ $[M+H]^+$ *m*/*z* 646.3088, found *m*/*z* 646.3073. HPLC purity 96%.

6.7.3.18. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[1-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-6-methyl-3,4-dihydro-1*H*-isoquinoline-2-carbonyl]-2-methyl-propyl}-succinamic acid (33q). The title compound was prepared following the procedure used for peptide synthesis described above (use of **29b** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (39 mg) as a white solid. (ES+) *m*/*z* (M+23) 668.2. ^{1}H NMR (500 MHz, D_2O) δ ppm 7.05–7.37 (m, 3H) 5.59–6.10 (m, 1H) 4.95–5.05 (m, 1H) 4.39–4.67 (m, 2H) 3.64–4.26 (m, 4H) 2.43–3.20 (m, 6H) 2.29 (m, 3H) 1.70–2.18 (m, 5H) 0.56–0.99 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{31}H_{43}N_5O_{10}$ $[M+H]^+$ *m*/*z* 646.3088, found *m*/*z* 646.3097. HPLC purity 97%.

6.7.3.19. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[1-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-6,7-dimethoxy-3,4-dihydro-1*H*-isoquinoline-2-carbonyl]-2-methyl-propyl}-succinamic acid (33r). The title compound was prepared following the procedure used for peptide synthesis described above (use of **29d** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (21 mg) as an off-white solid. (ES+) *m*/*z* (M+1) 692.1. ^{1}H NMR (500 MHz, D_2O) δ ppm 6.74–7.07 (m, 2H) 5.46–5.99 (m, 1H) 4.90–4.98 (m, 1H) 4.54–4.68 (m, 2H) 3.61–4.27 (m, 10H) 2.32–3.05 (m, 6H) 1.69–2.11 (m, 5H) 0.42–0.98 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{32}H_{45}N_5O_{12}$ $[M+H]^+$ *m*/*z* 692.3143, found *m*/*z* 692.3124. HPLC purity 100%.

6.7.3.20. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[6-hydroxy-1-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-3,4-dihydro-1*H*-isoquinoline-2-carbonyl]-2-methyl-propyl}-succinamic acid (33s). The title compound was prepared following the procedure used for peptide synthesis described above (use of **29a** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (7 mg) as an off-white solid. (ES+) *m*/*z* (M+23) 670.4. ^{1}H NMR (500 MHz, D_2O) δ ppm 7.18–7.33 (m, 1H) 6.69–6.88 (m, 2H) 5.43–6.08 (m, 1H) 4.91–5.07 (m, 1H) 4.33–4.70 (m, 2H) 3.24–4.23 (m, 4H) 2.35–3.15 (m, 6H) 1.89–2.21 (m, 5H) 0.55–1.00 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{30}H_{41}N_5O_{11}$ $[M+H]^+$ *m*/*z* 648.2881, found *m*/*z* 648.2886. HRMS (ESI) calcd for $C_{30}H_{41}N_5O_{11}$ $[M+H]^+$ *m*/*z* 648.2881, found *m*/*z* 648.2863. HPLC purity 95%.

6.7.3.21. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[7-hydroxy-1-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-3,4-dihydro-1*H*-isoquinoline-2-carbonyl]-2-methyl-propyl}-succinamic acid (33t). The title compound was prepared following the procedure used for peptide synthesis described above (use of **29e** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (15 mg) as an off-white solid. (ES+) *m*/*z* (M+1) 648.0. ^{1}H NMR (500 MHz, D_2O) δ ppm 7.09–7.19 (m, 1H) 6.73–6.94 (m, 2H) 5.55–6.07 (m, 1H) 4.93–5.08 (m, 1H) 4.42–4.68 (m, 2H) 3.29–4.24 (m, 4H) 2.44–3.11 (m, 6H) 1.82–2.21 (m, 5H) 0.55–1.04 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{30}H_{41}N_5O_{11}$ $[M+H]^+$ *m*/*z* 648.2881, found *m*/*z* 648.2886. HPLC purity 89%.

6.7.3.22. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[6-bromo-1-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-3,4-dihydro-1*H*-isoquinoline-2-carbonyl]-2-methyl-propyl}-succinamic acid (33u). The title compound was prepared following the procedure used for peptide synthesis described above (use of **29g** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (18 mg) as an off-white solid. (ES+) *m*/*z* (M+1) 709.9/711.9. ^{1}H NMR (500 MHz, D_2O) δ ppm 7.41–7.56 (m, 2H) 7.17–7.34 (m, 1H) 5.61–6.08 (m, 1H) 4.95–5.05 (m, 1H) 4.40–4.66 (m, 2H) 3.39–4.34 (m, 4H) 2.41–3.18 (m,

6H) 1.84–2.17 (m, 5H) 0.57–1.03 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{30}H_{40}BrN_5O_{10}$ $[M+H]^+$ m/z 710.2037, found m/z 710.2047. HPLC purity 97%.

6.7.3.23. (*S*)-3-((*S*)-2-Acetylamino-3-methyl-butyrylamino)-*N*-{(*S*)-1-[7-bromo-1-((*S*)-2-hydroxy-5-oxo-tetrahydro-furan-3-ylcarbamoyl)-3,4-dihydro-1*H*-isoquinoline-2-carbonyl]-2-methyl-propyl}-succinamic acid (33v). The title compound was prepared following the procedure used for peptide synthesis described above (use of **29f** in stage 1 and Ac-Val-Asp(O*t*Bu)-Val-OH in stage 2). The crude residue was purified by preparative HPLC to afford the title compound (2 mg) as an off-white solid. (ES+) m/z (M+23) 709.9/712.0. ^{1}H NMR (500 MHz, D_2O) δ ppm 7.43–7.74 (m, 2H) 7.12–7.31 (m, 1H) 5.71–6.12 (m, 1H) 4.93–5.01 (m, 1H) 3.42–4.69 (m, 6H) 2.29–3.18 (m, 6H) 1.93–2.24 (m, 5H) 0.54–1.08 (m, 12H). Mixture of isomers. HRMS (ESI) calcd for $C_{30}H_{40}BrN_5O_{10}$ $[M+H]^+$ m/z 710.2037, found m/z 710.2028. HPLC purity 100%.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.08.020.

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