D-Leu-*L*-Phe-containing dipeptide inhibitors of α-chymotrypsin – the role of the *N*- and *C*-termini in enzyme affinity

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Abstract

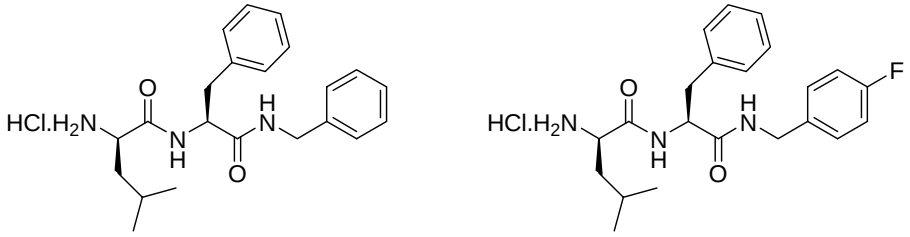
Three new compounds (**3-5**) based on a dipeptide non-covalent inhibitor (**1**) of α-chymotrypsin have been synthesised and assayed against the serine protease α-chymotrypsin. The stability of the compounds to α-chymotrypsin catalysed hydrolysis has been measured. All three compounds were inactive with a retained stability to enzyme catalysed hydrolysis.

Keywords: Chymotrypsin, enzyme inhibitors, peptidomimetics, azobenzene

Introduction

Serine proteases are an important group of enzymes that are involved in physiological processes implicated in a number of diseases.[1] As such, inhibitors of these enzymes have potential therapeutic use. For example, inhibitors of the chymotrypsin-like activity of the 20S proteasome have recently attracted attention as a new class of chemotherapy agents.[2] However, most potent inhibitors of serine proteases contain a highly electrophilic group such as a trifluoromethylketone, boronic acid or α-ketoester[1, 3] and as such lack good drug-like properties. It is thus of importance to develop potent, selective inhibitors of serine proteases that do not contain these reactive groups.

Dipeptide *D*-Leu-*L*-Phe-benzylamine **1** (Figure 1) has been identified as one such non-covalent inhibitor of α-chymotrypsin.[4] This compound inhibits α-chymotrypsin activity with a reported inhibition constant, K_i, of 3.6 μM, and exhibits high stability to α-chymotrypsin catalysed hydrolysis. It has been proposed that the activity of **1** results from its preference to adopt an unusual conformation involving hydrophobic-hydrophobic interactions between the *D*-Leu and *L*-Phe side chains. The crystal structure of the enzyme-inhibitor complex of the fluorinated derivative **2** reveals that the *D*-Leu and *L*-Phe side chains do in fact both occupy the large hydrophobic S_2 subsite, with the benzylamine group fitting into the narrow hydrophobic S_1 subsite (see notation of Schechter and Berger[5]).[6]



1 **2**

Figure 1. Compounds **1** and **2**, noncovalent dipeptide inhibitors of α-chymotrypsin.

Here we investigate the effect of substituting the primary amine group of **1** with a short peptide (or peptidomimetic) chain, where a similar modification has been reported to increase the activity of related boronic acid inhibitors of chymotrypsin.[7] As such, we report the synthesis, assay and stability studies of two derivatives of **1** extended at the *N*-terminus (compounds **3** and **4**), and compound **5**, in which the *C*-terminal phenyl group of the benzylamine moiety is replaced by the large and extended azobenzene group (Figure 2). Compounds **3** and **4** were designed to investigate the importance of the primary amine group in binding to the enzyme active site. An inspection of the crystal structure of **2** with chymotrypsin shows that the amine group appears to form a hydrogen bond to the Gly-216 carbonyl group. This hydrogen bonding interaction is likely to increase the affinity of the inhibitor for the active site. However, the amine group is exposed to the outside of the binding pocket, so it was of interest to investigate the importance of this group in binding. Compound **5** was designed to investigate whether or not a larger hydrophobic group of fixed and extended geometry is accommodated in the S_1 position, which is known to accommodate a naphthalene group.[8] Compound **5** exists as two interconvertable isomers (the *E* and *Z* isomers of the N=N bond) that are significantly different in structure and conformation. It is known that peptidomimetics containing the azobenzene group in the P_2 position are able to inhibit chymotrypsin as both the *E* and *Z* isomers,[9, 10] so it was of interest here to investigate the azobenzene group in the P_1 position. The binding of both *E* and *Z* isomers was investigated as a mixture to provide further information about inhibitor structure and enzyme binding of both. The binding of such a large group in the S_1 pocket might be expected to induce a change in the conformation of the enzyme active site in order to fit. Consequently, the enzyme binding affinity of compound **5** provides some information about the potential for induced fit binding of such compounds.

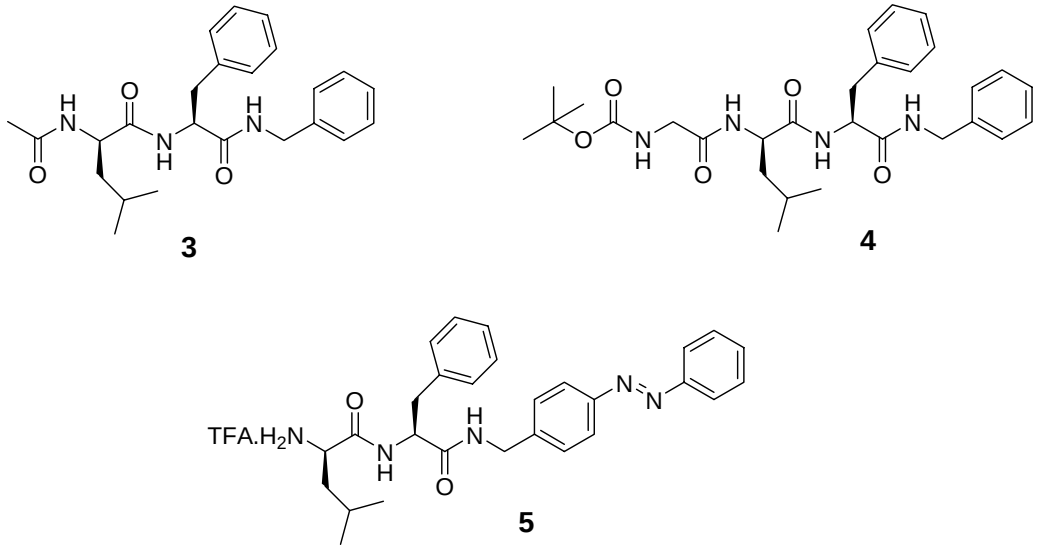
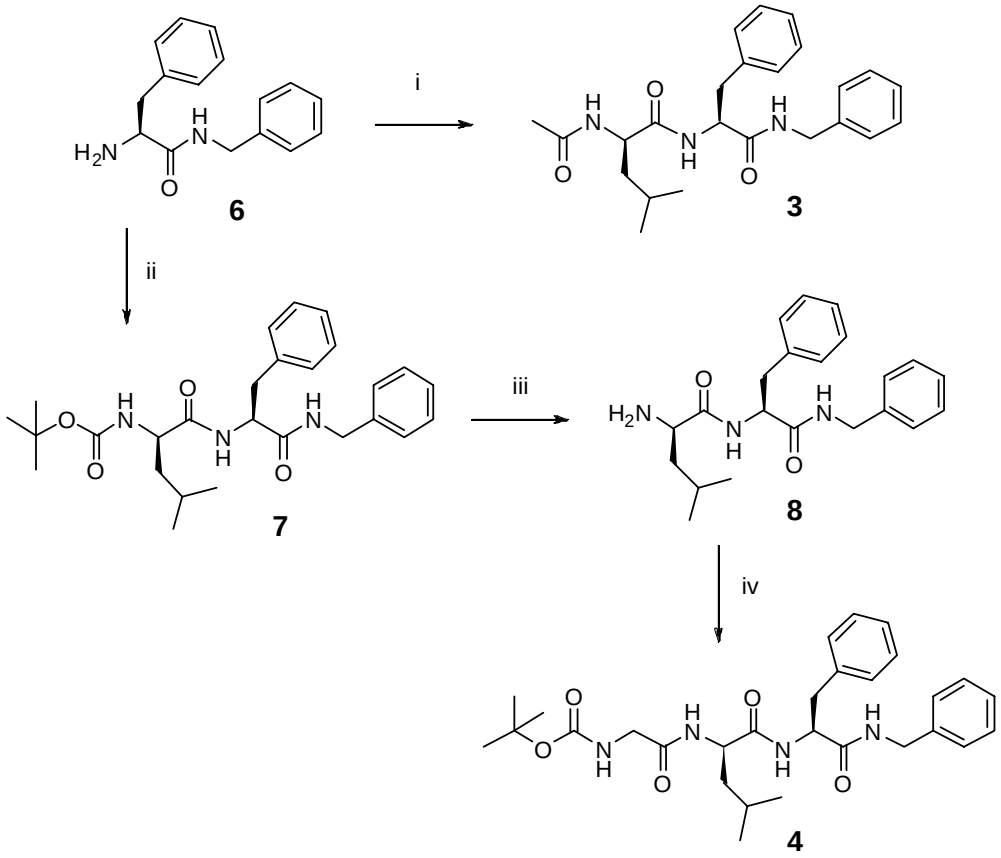


Figure 2. Compounds **3-5**, derivatives of **1**.

Results/Discussion

Compounds **3-5** were synthesised using solution peptide coupling methods, as shown in Scheme 1 (compounds **3** and **4**) and Scheme 2 (compound **5**). Compounds **3** and **4** were prepared by sequential coupling and deprotection steps, starting from the C-terminal amino acid, *L*-Phe, followed by coupling *D*-Leu then finally the *N*-terminal component. This method is analogous to that reported for **1**, where H-Phe-benzylamine was coupled with Boc-*D*-Leu-OH, followed by acid catalysed Boc cleavage. In detail, compound **3** was synthesised by the reaction of **6**[11, 12] with acetyl-*D*-Leu-OH using the HATU coupling reagent in good yield (72%). Compound **4** was synthesised by coupling of **6** with Boc-*D*-Leu-OH and HATU to obtain compound **7** in good yield (76%), followed by Boc cleavage of **7** to give the dipeptide **8**, the free amine equivalent of **1**, in moderate yield (65%). Compound **8** was then coupled to Boc-Gly-OH using HATU coupling reagent to obtain **4** in moderate (55%) yield.

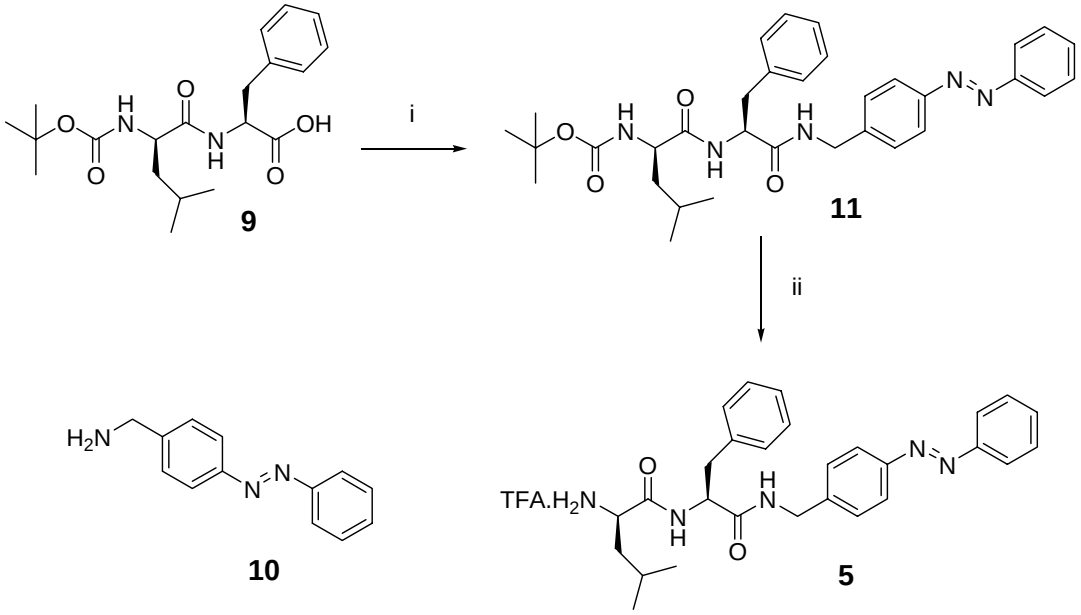


Reagents and conditions: (i) Ac-*D*-Leu-OH, HATU, DIEA, DMF, 72% (ii) Boc-*D*-Leu-OH, HATU, DIEA, DMF, 76% (iii) TFA, DCM, 65% (iv) Boc-Gly-OH, HATU, DIEA, DMF, 55%

Scheme 1. Synthesis of compounds **3** and **4**.

Compound **5** was prepared by a slightly different route. In this synthesis the *N*-terminal components were coupled first and the *C*-terminal component (the azobenzene group) was introduced in the final step. This route was employed due to the ready availability of key starting material **9**.[13] In addition, this synthetic route allows the reaction of compound **10** in the final step. This is advantageous since this compound is the most expensive component of the synthesis. In detail, compound **9** was coupled to azobenzene **10**[14] using HATU coupling reagent, followed by TFA cleavage of the Boc group to obtain **5** in good yield (66% over two steps).

The syntheses presented here are simple, effective methods to both *C*- and *N*-terminally substituted derivatives of **1**. Using intermediates **8** and **9**, a range of other derivatives of **1** that are additionally substituted at the *N*- and *C*-termini for similar studies can potentially be synthesised using the simple approaches presented here.



Reagents and conditions: (i) **10**, HATU, DIEA, DMF, 72% (ii) TFA, DCM, 92%

Scheme 2. Synthesis of compound **5**.

Compounds **3-5** were assayed for inhibition against α-chymotrypsin by a spectrophotometric assay. The stock solution of compound **5** in acetonitrile was left for 24 h in ambient lighting before the assay, so that it would reach an equilibrium photostationary state of both *E* and *Z* azobenzene isomers (83% *E*, 17% *Z*, determined by HPLC). All compounds **3-5** were found to be inactive up to their solubility limits (25-91 μM), as shown in Table 1. Intermediate **8** was assayed in order to verify the assay procedure, and to confirm previously reported results.[4] This compound is essentially identical to the reported inhibitor **1**, simply existing as a free amine instead of a hydrochloride salt. A K_i value of 4 μM was obtained, which compares favourably to the reported value of 3.6 μM for compound **1**.[4]

Table 1. K_i inhibition constants for assay of compounds **3**-**5** and **8** against α-chymotrypsin

Compound	K_i (μM)
3	>60
4	>91
5	>25
8	4

The lack of inhibitory activity of compounds **3** and **4**, as compared to compound **1**, suggests that the free amine of **1** is in fact critical for enzyme binding, probably due to hydrogen bonding

between the amine group and the Gly-216 residue of the active site. However, it is also possible that there is simply insufficient space for an amide to fit into the active site in this position.

The fact that the mixture of both *E* and *Z* isomers of **5** was inactive implies that neither isomer binds to α-chymotrypsin at the assay concentrations used. The decreased inhibitory activity of (*E*)- and (*Z*)-**5** compared to **1** suggests that an azobenzene group is too large to fit into the S_1 subsite. It can also be inferred that the active site is insufficiently flexible to allow induced fit binding of the azobenzene group into the S_1 pocket.

The previously reported inhibitors **1** and **2** also exhibit high stability to chymotrypsin catalysed hydrolysis, presumably due to the presence of a *D*-amino acid (*D*-Leu) in the P_2 position, since *D*-amino acids often increase the stability of peptides. Compounds **3-5** contain this same core (*D*-Leu, *L*-Phe) as **1** and **2**, and so would be expected to show similar stability, despite having additional substitution at the *C* and *N* termini which prevents binding in the unusual inhibitory conformation observed for **2**. The stability of these compounds to chymotrypsin hydrolysis was thus investigated by incubation of a solution of each with α-chymotrypsin for 24 h, with HPLC analysis of aliquots to monitor hydrolysis. Hydrolysis was not observed in any case, confirming that the *D*-Leu-*L*-Phe sequence does provide stability to chymotrypsin hydrolysis.

Conclusions

Three derivatives of a non-covalent inhibitor of the serine protease α-chymotrypsin have been synthesised and assayed against the enzyme. All three compounds, including both *E* and *Z* isomers of **5**, were found to be inactive up to their solubility limits. This decrease in activity, compared to reported compound **1**, confirms the importance of the *N*-terminal amine of **1** to enzyme binding and shows that a very large group is not tolerated at the *C*-terminus. The stability of the new compounds to enzyme catalysed hydrolysis was also studied by HPLC, and all were found to be stable. This suggests that the *D*-Leu-*L*-Phe core provides stability to compounds of this type.

Experimental Section

General Procedures. NMR spectra were obtained on a Varian INOVA spectrometer, operating at 500 MHz for ^{1}H NMR and at 126 MHz for ^{13}C NMR, or on a Varian UNITY 300 spectrometer, operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. Electrospray ionisation mass spectra were detected on a Micromass LCT TOF mass spectrometer operating in electrospray mode with 50% acetonitrile/H_2O as solvent. Dry DMF was purchased from Acros, dry DCM was distilled from CaH_2. EtOAc was distilled before use. HPLC grade acetonitrile was purchased from BDH. All other commercial reagents were used as received.

Enzyme assays

Buffer solution (Tris): Tris(hydroxymethyl)aminomethane (1.21 g), $CaCl_2.6H_2O$ (0.44 g) and Triton X-100 (0.05 g) were dissolved in Milli-Q deionised water (75 mL), adjusted to pH 7.8 with NaOH solution (1 M) and made up to 100 mL with Milli-Q water.

Substrate solution: N-Succinyl-$(Ala)_2$-Pro-Phe-4-nitroanilide (21 mg) was dissolved in Tris buffer solution (10 mL) by ultrasonication. The solution was stored at -18 ^oC for up to two weeks. The concentration of the solution was determined at the start of each day from its UV spectrum (ε_{315} = 14000 L mol^{-1} cm^{-1})

Enzyme solution: A stock solution was prepared from α-chymotrypsin (15 mg) in HCl solution (10 mL, pH 3, made up by dilution of conc. HCl with Milli-Q water). The stock solution was stored at -18 ^oC for up to 1 month. Each day an enzyme solution was prepared: Stock solution (200 μL) and Triton X-100 (25 mg) were made up to 50 mL with Milli-Q water.

Inhibition of α-chymotrypsin was determined with N-Succinyl-$(Ala)_2$-Pro-Phe-4-nitroanilide as the substrate by an assay procedure developed from the technique described by Geiger,[15] except that the order of addition of enzyme and substrate was inverted. Briefly, inhibitors were dissolved in acetonitrile at a series of dilutions ranging from 0.5-100 μM as appropriate for each compound. For each rate measurement, inhibitor solution (or acetonitrile blank, 50 μL), substrate solution (30, 60, 90, 120 or 150 μL) and buffer solution (as required to make up the final solution to 1050 μL) were mixed in a cuvette, and incubated for 5 min at 25 ^oC. Enzyme solution (30 μL) was added, and the absorbance at 405 nm was monitored for 5 min. An absorbance vs time plot was obtained, and the slope used to find the initial rate. Where enzyme inhibition was observed, a dixon plot of 1/rate vs inhibitor concentration was used to find the inhibition constant, K_i.

HPLC stability studies

50 μL aliquots of acetonitrile solutions of compounds **3-5** (1 mg mL^{-1}) were diluted with Tris buffer (970 μL, as used in the enzyme assays). Enzyme solution (30 μL, as used in the enzyme assays) was added, then a 150 μL aliquot of the resulting solution was analysed by HPLC (Prodigy C18, 250 x 4.6, 5 μm; solvents: A: water with 0.05% TFA, B: acetonitrile; 40 °C; 1 mL min^{-1}; detection at 210 nm; compounds were eluted with a gradient as follows: 0-1 min 20% solvent B, 1-10 min gradient 20-100% B, 10-12 min 100% B, 12-14 min gradient 100-20% B, 14-20 min 20% B). The mixture was incubated for 24 h at 15 ºC, then a further 150 μL aliquot was analysed by HPLC. To confirm the identity of the peaks observed in HPLC chromatographs, HPLC analyses were also carried out for solutions of compounds **3-5** in i) acetonitrile (1 mg mL^{-1}) and ii) buffer (50 μL of the acetonitrile solution diluted with 1000 μL Tris buffer).

Synthesis

(*R*)-2-Acetamido-*N*-((*S*)-1-(benzylamino)-1-oxo-3-phenylpropan-2-yl)-4-methylpentan-amide (3) To a solution of **6**[11, 12] (50 mg, 0.20 mmol), acetyl-*D*-leucine (34 mg, 1 eq) and HATU

(82 mg, 1.1 eq) in DMF (2 mL) was added DIEA (76 µL, 56 mg, 2.2 eq) and the resulting mixture was stirred for 16 h, then diluted with EtOAc (50 mL), washed with HCl (1 M, 50 mL), sat. $NaHCO_3$ (50 mL), brine (50 mL), dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography, eluting with DCM to remove leading impurities, then EtOAc to give **3** (59 mg, 72%) as a white solid: mp 194-197 ºC, $[\alpha]_D^{20}$ -22.7 (*c* = 0.30, MeOH), ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 0.69 (3H, d, *J* = 6.1 Hz), 0.75 (3H, d, *J* = 6.0 Hz), 1.02-1.21 (3H, m), 1.75 (3H, s), 2.75 (1H, dd, *J* = 13.7, 11.1 Hz), 3.14 (1H, dd, *J* = 13.6, 3.9 Hz), 4.12 (1H, q, *J* = 7.4 Hz), 4.30 (2H, dd, *J* = 5.8, 3.0 Hz), 4.42-4.51 (1H, m), 7.14-7.33 (10H, m), 8.04 (1H, d, *J* = 6.8 Hz), 8.41-8.51 (2H, m), ^{13}C NMR (75 MHz, $(CD_3)_2SO$) δ 22.3, 22.3, 22.5, 23.9, 37.2, 40.5, 42.2, 51.8, 54.5, 126.2, 126.8, 127.2, 128.0, 128.3, 129.2, 138.2, 139.2, 169.8, 171.0, 172.4, HRMS (ES) Calc. for $C_{24}H_{32}N_3O_3$ 410.2444, found 410.2454 (MH^+).

***tert*-Butyl 2-((*R*)-1-((*S*)-1-(benzylamino)-1-oxo-3-phenylpropan-2-ylamino)-4-methyl-1-oxo pentan-2-ylamino)-2-oxoethylcarbamate (4)** To a solution of **8** (100 mg, 0.27 mmol), Boc-glycine (48 mg, 1 eq) and HATU (115 mg, 1.1 eq) in DMF (8 mL) was added DIEA (105 µL, 78 mg, 2.2 eq) and the resulting mixture was stirred for 16 h, then diluted with EtOAc (50 mL), washed with sat. $NaHCO_3$ (50 mL), brine (50 mL), dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography, eluting with 2:3 EtOAc/DCM, then by recrystallisation from EtOAc/petroleum ether to give **4** (78 mg, 55%) as a white solid: mp 174-176 ºC, $[\alpha]_D^{20}$ -5.9 (*c* = 0.32, MeOH), ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 0.72 (6H, dd, *J* = 16.5, 5.7 Hz), 1.12-1.18 (3H, m), 1.36 (9H, s), 2.74 (1H, dd, *J* = 13.5, 10.9 Hz), 3.10 (1H, dd, *J* = 13.5, 4.1 Hz), 3.51 (2H, d, *J* = 5.8 Hz), 4.16-4.34 (3H, m), 4.44-4.51 (1H, m), 6.90 (1H, t, *J* = 6.0 Hz), 7.15-7.26 (8H, m), 7.30 (2H, t, *J* = 7.4 Hz), 7.81 (1H, d, *J* = 7.3 Hz), 8.42-8.49 (2H, m) ^{13}C NMR (126 MHz, $(CD_3)_2CO$) δ 22.2, 22.7, 23.8, 28.2, 37.4, 40.9, 42.2, 43.1, 51.4, 54.4, 78.1, 126.2, 126.8, 127.3, 128.1, 128.3, 129.3, 138.1, 139.2, 155.8, 169.4, 171.0, 171.9, HRMS (ES) Calc. for $C_{29}H_{41}N_4O_5$ 525.3077, found 525.3072 (MH^+).

(*R*)-2-Amino-4-methyl-*N*-((*S*)-1-oxo-3-phenyl-1-(4-((*E*)-phenyldiazenyl)benzylamino) propan -2-yl)pentanamide trifluoroacetate salt (5) To a solution of **9**[13] (60 mg, 0.16 mmol), **10**[14] (34 mg, 1 eq) and HATU (67 mg, 1.1 eq) in DMF (3 mL) was added DIEA (61 µL, 45 mg, 2.2 eq) and the resulting mixture was stirred for 16 h then diluted with EtOAc (50 mL), washed with H_2O (50 mL x 2), sat $NaHCO_3$ (50 mL), brine (50 mL), dried over MgSO4 and concentrated. The crude material was purified by flash chromatography, eluting with 1 : 4 EtOAc/DCM to give **11** (65 mg, 72%) as an orange solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.82-0.87 (6H, m), 1.29-1.34 (11H, m), 1.45-1.53 (1H, m), 3.14-3.27 (2H, m), 3.88-3.95 (1H, m), 4.35 (1H, dd, *J* = 15.3, 5.2 Hz), 4.61 (1H, dd, *J* = 15.3, 6.3 Hz), 4.82-4.91 (2H, m), 6.14 (1H, d, *J* = 8.8 Hz), 7.12-7.36 (8H, m), 7.45-7.54 (3H, m), 7.84 (2H, d, *J* = 8.3 Hz), 7.91 (2H, d, *J* = 7.1 Hz). To a solution of **11** (65 mg, 0.11 mmol) in DCM (0.8 mL) was added TFA (0.2 mL) and the resulting mixture was stirred for 1 h, then diluted with DCM (50 mL), washed with HCl (1 M, 50 mL), sat $NaHCO_3$ (50 mL), brine (50 mL), dried over $MgSO_4$ and concentrated. The crude product was purified by recrystallisation from EtOAc/petroleum ether to give **5** (48 mg, 92%) as an orange solid: mp 161-164 ºC, $[\alpha]_D^{20}$ -8.3 (*c* = 0.29, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$) δ

0.82-0.90 (6H, m), 1.17-1.42 (3H, m), 1.49-1.67 (2H, m), 3.09 (1H, dd, *J* = 13.7, 7.5 Hz), 3.18 (1H, dd, *J* = 13.7, 7.5 Hz), 3.28 (1H, dd, *J* = 9.2, 4.0 Hz), 4.37-4.46 (2H, m), 4.70 (1H, q, *J* = 7.5 Hz), 6.91 (1H, t, *J* = 5.8 Hz), 7.20-7.29 (7H, m), 7.44-7.53 (3H, m), 7.79-7.85 (3H, m), 7.88-7.91 (2H, m), ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.3, 23.5, 24.9, 37.9, 43.1, 44.0, 53.5, 54.5, 122.9, 123.2, 127.0, 128.2, 128.7, 129.2, 129.4, 131.1, 136.9, 141.1, 151.9, 152.7, 171.1, 176.4. HRMS (ES) Calc. for $C_{28}H_{34}N_5O_2$ 472.2713, found 472.2690 (M – TFA + H^+).

(*R*)-2-Amino-*N*-((*S*)-1-(benzylamino)-1-oxo-3-phenylpropan-2-yl)-4-methylpentanamide trifluoroacetate salt (8) To a solution of **6** (50 mg, 0.20 mmol), Boc-*D*-leucine (45 mg, 1 eq) and HATU (82 mg, 1.1 eq) in DMF (3 mL) was added DIEA (75 μL, 56 mg, 2.2 eq) and the resulting mixture was stirred for 16 h, then diluted with EtOAc (50 mL), washed with H_2O (50 mL), sat. $NaHCO_3$ (50 mL), brine (50 mL), dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography, eluting with 1:4 EtOAc/DCM to give **7** (70 mg, 76%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.81-0.88 (6H, m), 1.27-1.45 (11H, m), 1.54 (1H, dt, *J* = 13.5, 6.8 Hz), 3.12-3.22 (2H, m), 3.77-3.85 (1H, m), 4.27 (1H, dd, *J* = 14.8, 5.4 Hz), 4.50 (1H, dd, *J* = 14.8, 6.2 Hz), 4.74-4.84 (2H, m), 6.22 (1H, d, *J* = 7.4 Hz), 7.01 (1H, s), 7.14-7.30 (10H, m). To a solution of **7** (55 mg, 0.12 mmol) in DCM (0.8 mL) was added TFA (0.2 mL) and the resulting mixture was stirred for 1 h then concentrated. The residue was dissolved in H_2O (10 mL), washed with DCM (10 mL), basified by addition of sat. $NaHCO_3$ and extracted with DCM (10 mL × 3) to give **8** (37 mg, 85%) as a white solid: mp 130-132 ºC, $[\alpha]_D^{20}$ -20.9 (*c* = 0.44, MeOH), ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.84-0.90 (6H, m), 1.20-1.27 (1H, m), 1.50-1.64 (2H, m), 1.79 (2H, br s), 3.07 (1H, dd, *J* = 13.8, 7.6 Hz), 3.17 (1H, dd, *J* = 13.8, 7.6 Hz), 3.34 (1H, dd, *J* = 9.6, 4.0 Hz), 4.35 (2H, d, *J* = 5.8 Hz), 4.61 (1H, q, *J* = 7.6 Hz), 6.55 (1H, s), 7.09-7.13 (2H, m), 7.19-7.29 (8H, m), 7.84 (1H, d, *J* = 7.1 Hz), ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.4, 23.5, 24.9, 38.0, 43.5, 44.0, 53.5, 54.5, 127.0, 127.5, 127.6, 128.7, 128.7, 129.4, 137.0, 138.0, 171.0, 176.2. HRMS (ES) Calc. for $C_{22}H_{30}N_3O_2$ 368.2338, found 368.2328 (MH^+).

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