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Synthesis of glutamic acid and glutamine peptides possessing a trifluoromethyl ketone group as SARS-CoV 3CL protease inhibitors

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Abstract—Trifluoromethyl-β-amino alcohol **11** [(4*S*)-*tert*-butyl 4-amino-6,6,6-trifluoro-5-hydroxyhexanoate] was synthesized in five steps starting from Cbz-L-Glu-OH **5** where the key step involved the introduction of the trifluoromethyl (CF_3) group to oxazolidinone **7**, resulting in the formation of silyl ether **8** [(4*S*,5*S*)-benzyl 4-(2-(*tert*-butoxycarbonyl)ethyl)-5-(trifluoromethyl)-5-(trimethylsilyloxy)oxazolidine-3-carboxylate]. Compound **11** was then converted into four tri- and tetra-glutamic acid and glutamine peptides (**1**–**4**) possessing a CF_3-ketone group that exhibited inhibitory activity against severe acute respiratory syndrome coronavirus protease (SARS-CoV $3CL^{pro}$).
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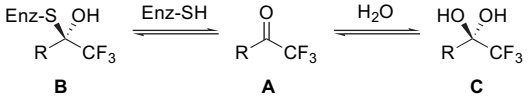
1. Introduction

In May 2003, two groups reported that a novel coronavirus (CoV) was the causative agent of severe acute respiratory syndrome (SARS).[1,2] CoV encodes a chymotrypsin-like protease ($3CL^{pro}$) that plays a pivotal role in the replication of the virus.[3] $3CL^{pro}$ is functionally analogous to the main picornavirus protease $3C^{pro}$ and both are cysteine proteases with a catalytic dyad (Cys-145 and His-41) in the active site, with Cys as the nucleophile and His as the general base.[4,5] Although a global SARS crisis was avoided in 2003 and the infection was contained, it is still a matter of necessity to find compounds that can inhibit SARS-CoV in case that the disease might re-emerge.

Compounds containing a trifluoromethyl ketone (CF_3-ketone) moiety form an important group of biologically useful fluorinated molecules[6] that can be used as protease inhibitors, as first described by Abeles et al.[7] The CF_3 group next to the carbonyl group thermodynamically stabilizes the hemi-ketal form relative to the ketone form, thus making the carbonyl prone to nucleophilic substitution by water, the active site Ser hydroxyl or Cys thiol group present in serine or cysteine proteases. Nucleophilic attack by the active site thiol in SARS-CoV $3CL^{pro}$ would convert the CF_3-ketone **A** to the tetrahedral adduct **B** (Scheme 1), which is believed to mimic the substrate–enzyme intermediate formed during substrate peptide-bond hydrolysis. Since adduct **B** is relatively stable, compound **A** would behave as a protease inhibitor,[8] suggesting that compounds containing a CF_3-ketone moiety may play an important role as $3CL^{pro}$ inhibitors. CF_3-ketone **A** also forms a relatively stable hydrate adduct **C** upon reacting with water. A unique and conservative recognition of the substrate's Gln residue at the P_1 site has been identified in the CoV cysteine protease family.[9] Therefore, a Gln-derived CF_3-ketone residue would contribute to the activity of SARS-CoV $3CL^{pro}$ inhibitors. Based on these considerations, a new synthetic method for forming Gln and Glu derivatives possessing a CF_3-ketone moiety was developed and this strategy was used in the synthesis of four peptides (compounds **1**–**4**).



B **A** **C**

Scheme 1. Trifluoromethyl ketone adducts.

Keywords: Trifluoromethyl ketone; Protease inhibitors; Severe acute respiratory syndrome coronavirus protease (SARS-CoV $3CL^{pro}$).
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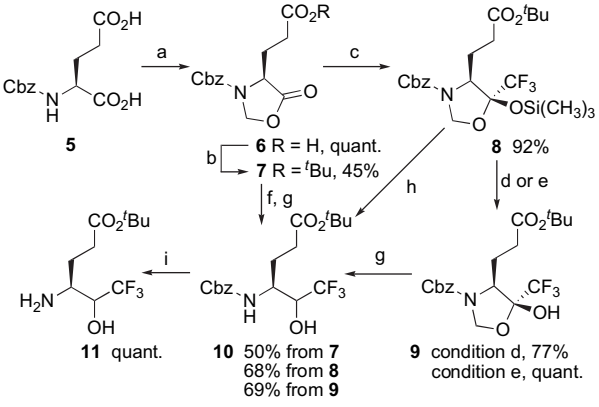
2. Results and discussion

2.1. Synthesis of trifluoromethyl-β-amino alcohol 11

The target compounds were envisioned being synthesized in two parts, viz, the peptide part and β-amino alcohol **11** containing the CF_3 unit. These two parts would then be coupled together and further elaborated to the desired target compounds. The synthesis of the key compound **11** started with oxazolidinone acid **6** prepared from Cbz-L-Glu-OH (**5**) under conditions described by Moore et al.[10] The resulting acid **6** was then converted to *tert*-butyl ester **7** (45%) that was expediently converted to silyl ether **8** (92% yield) (Scheme 2), which was isolated as a single diastereomer as determined by ^1H and ^{13}C NMR analyses, by utilizing a literature method.[11,12] The depicted stereochemistry for compound **8** is based on literature precedence for a very similar compound in which the addition of the CF_3 anion is *anti* to the side chain.[11] Product **8** was then readily desilylated upon treatment with tetrabutylammonium fluoride (TBAF) giving alcohol **9** in 77% yield.



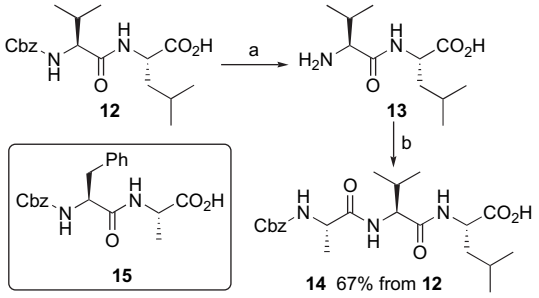
Scheme 2. Synthesis of β-amino alcohol **11**. Reagents and conditions: (a) paraformaldehyde, *p*-TsOH·H_2O, toluene, reflux, 1.67 h; (b) *t*-BuOH, EDC·HCl, DMAP, Et_3N, THF, rt, 16 h; (c) CsF, $CF_3Si(CH_3)_3$, THF, amb. temp, sonication, 2 h; (d) TBAF, THF, 0 °C–rt, 0.5 h; (e) MeOH/water (9:1), rt, 3 h; (f) CsF, $CF_3Si(CH_3)_3$, THF, sonication, amb. temp, 2 h then water, sonication, amb. temp, 0.5 h; (g) $NaBH_4$, MeOH, rt, 21 h; (h) $NaBH_4$, MeOH, rt, 16 h; (i) H_2, Pd/C (10%), MeOH, rt, 16 h.

We observed that compound **8** was partly converted to the desilylated product **9** when exposed to air. The cause of the partial protio-desilylation might be due to the moisture-sensitive nature of compound **8**. In the patent literature, there is one report of desilylation occurring upon stirring similar compounds in methanol,[13] most likely caused by water present in the methanol. For our substrate, we found that this method only proceeded when the reaction was carried out on a small scale (20 mg or less). However, by adding water to the methanol [methanol/water (9:1 v/v)], substrate **8** could be fully converted to compound **9** after 3 h stirring at room temperature (Scheme 2).

Compound **7** was also converted to the corresponding alcohol **9** (72% yield) in a one-pot reaction by adding small amount of water to the reaction mixture of intermediate **8** followed by sonication for an additional half hour. Finally, the desired alcohol **10** was obtained by treating compound **9** with $NaBH_4$ in methanol at room temperature. This gave target compound **10** as a ca. 4.5:1 mixture of diastereomers, as determined by ^1H and ^{13}C NMR analyses, in 69% yield. Among the different synthetic routes tried, treating a methanol solution of silyl ether **8** with $NaBH_4$ seems to be an efficient route to synthesize alcohol **10**. Under these conditions, we obtained the desired compound **10** in 68% yield (Scheme 2). Finally, the protecting group within substrate **10** could be easily cleaved off by hydrogenation over Pd/C (10%) affording alcohol **11** in quantitative yield.

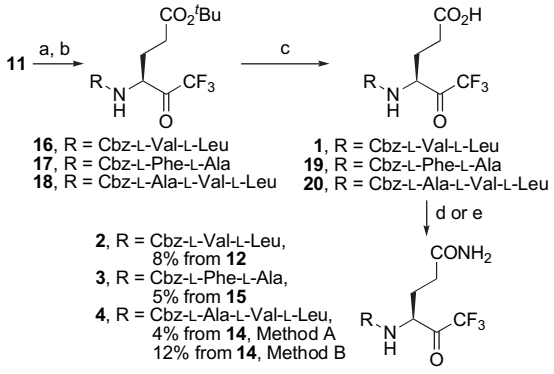
2.2. Synthesis of glutamic acid and glutamine peptides with a CF_3-ketone unit

With compound **11** prepared, focus could now shift toward the synthesis of the acid component coupling partners, namely peptides **12**, **14**, and **15**. Protected dipeptides **12** and **15** could be prepared following literature procedures[14,15] while tripeptide **14** could be prepared from dipeptide **12** as outlined in Scheme 3. The Cbz group within compound **12** could be removed using standard hydrogenation conditions, thus giving dipeptide **13** that was used directly in the next step. Coupling compound **13** with Cbz-L-Ala-OSu[16] afforded dipeptide **14** in 67% yield over the two steps.



Scheme 3. Synthesis of tripeptide **14** from dipeptide **12**. Reagents and conditions: (a) H_2, Pd/C (10%), MeOH/water/AcOH (9.5:5:1), rt, 2 h; (b) Cbz-L-Ala-OSu,[16] Et_3N, DMF, 0 °C–rt, 16 h.

Coupling of peptide **12** with β-amino alcohol **11** gave the expected amide (Scheme 4) that was used directly in the next step affording ketone **16**. Peptides **14** and **15** were subjected to the exactly same reaction sequences giving ketones **17** and **18**.



Scheme 4. The final steps toward the target compounds. Reagents and conditions: (a) peptide (**12**, **14** or **15**), HOBt, EDC·HCl, DMF, 0 °C–rt, 21 h; (b) Dess–Martin periodinane, CH_2Cl_2, rt, 19 h; (c) TFA, CH_2Cl_2, rt, 16 h; (d) HOBt, EDC·HCl, ammonia solution (28% aq solution), DMF, 16 h (Method A); (e) Boc_2O, NH_4HCO_3, pyridine, 1,4-dioxane, rt, 23 h (Method B).

Treating compound **16** with trifluoroacetic acid (TFA) resulted in clean removal of the *tert*-butyl group forming tripeptide **1**. Examination of the ^{13}C NMR spectrum did reveal that inhibitor **1** exists predominantly as the hydrate form in $CDCl_3$ (containing one drop of DMSO-d_6). ^{19}F NMR analysis of the same sample not only showed that the hydrate form was the dominant tautomer in the sample but that the two other possible tautomeric forms of tripeptide **1** were also present in small amount.[17] The equilibrium between the different tautomeric forms of this compound might shift depending on solvent. Due to the small amount of compound available, it was decided to study this in more detail by using a simpler model compound (vide infra).

Compounds **17** and **18** were subjected to the exactly same reaction conditions as ester **16** affording peptides **19** and **20**. The remaining crude tripeptide **1** and peptides **19** and **20** were subjected directly to the coupling conditions outlined in Scheme 4 (Method A), thus giving products **2**–**4** in 8, 5, and 4% yield over the four steps, respectively, after HPLC purification. The low chemical yield for the target compounds is a result of the last reaction sequence that seems to be very inefficient giving rise to many side products. In an effort to improve the yield for the last step, compound **4** was prepared by a mixed anhydride strategy using a slightly modified literature procedure (Method B).[18] By such means, we were able to improve the overall yield of inhibitor **4**, from peptide **14**, from 4 to 12%.

^{19}F NMR studies of the three glutamine peptides showed that compounds **2** and **4** only existed in the cyclic form while tripeptide **3** was a ca. 3.3:1 mixture of the cyclic and keto forms in $CDCl_3$.[19] Recently, similar observations were reported for glutamine fluoromethyl ketones by Cai et al.[20] Previously, there have also been reports that glutaminal compounds mostly exist as the hemiaminal in organic solvent.[21,22]

2.3. Synthesis of model Glu-CF_3 compounds

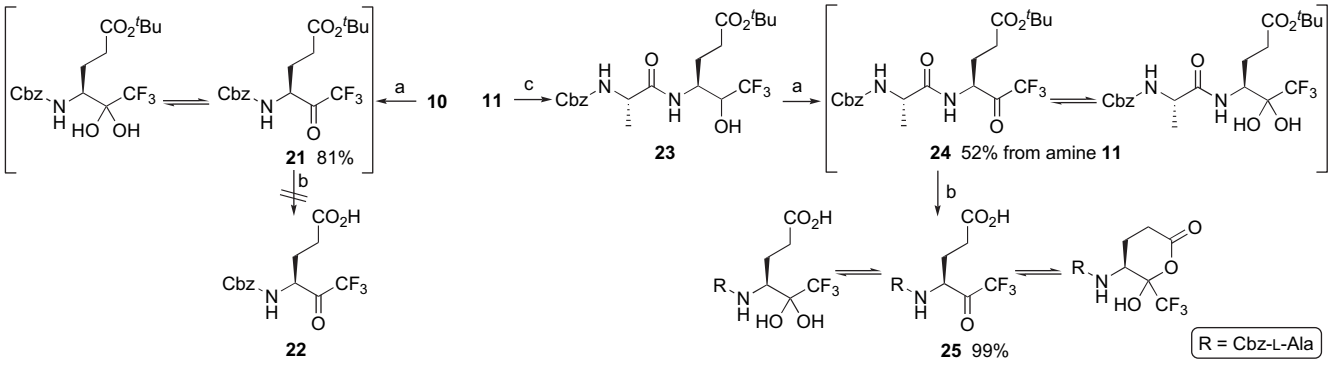
As previously noted, target peptide **1** was predominantly present in the hydrate form in $CDCl_3$. However, as alluded to in the previous section, this might differ depending on the solvent used for the NMR studies. Therefore, we decided to synthesize acid **22**, which is a much simpler molecule than the real system but, nevertheless, thought to be a good model for this study. To this end, alcohol **10** was converted to ketone **21** in 81% yield and as a ca. 2:1 mixture of the keto and hydrate forms as evident from ^{19}F and ^{13}C NMR analyses (Scheme 5). Attempts to convert compound **21** to the free acid **22** only resulted in the formation of decomposition products.

The lack of stability for our desired model compound forced us to use a slightly more complex acid for these studies. Compound **25** was synthesized in a three-step process, as outlined in Scheme 5, by first coupling Cbz-L-Ala-OH with amine **11**. This gave the desired alcohol **23**, which was directly oxidized to ketone **24** (52% yield over the two steps). From the ^{13}C and ^{19}F NMR analyses of this ketone, it became evident that the ketone exists as a ca. 7:3 mixture of the hydrate and keto forms. The rather unstable ketone **24** was then deprotected giving dipeptide **25** in almost quantitative yield in ca. 90% purity as determined by HPLC analysis. The ^{13}C NMR spectrum suggested that compound **25** exists mostly as the cyclic hemiacetal in $CDCl_3$ (resonance shifts from >170 to 75 ppm). This was also the case when the ^{13}C NMR spectrum was obtained for the same sample in CD_3OD.

NMR studies of model compounds **21**, **24**, and **25** in the predominant keto, hydrate, and hemiacetal forms, respectively, supported our assignment of compound **1** as existing mainly in the hydrate form in $CDCl_3$. This evidence was derived from the ^{19}F and ^{13}C NMR spectra of ketones **21** and **24** that were both present as a mixture of the keto and hydrate forms.[23] The work with the model compound also suggests that the form these acids appear in solution is highly solvent- and concentration-dependent.

2.4. Inhibitory activity of synthesized compounds

The inhibitory activity of the target compounds against SARS-CoV 3CL^{pro} was tested using a fluorescence-based peptide cleavage assay (Table 1).[23] We originally thought that the glutamine peptides (compounds **2**–**4**) would be the more potent inhibitors in these assays. However, the glutamate-possessing inhibitor **1** was the most potent of the group. The conformation that these compounds exist in during their interaction with the active site of SARS-CoV 3CL^{pro} is believed to contribute to binding affinity. Cai and co-workers found that their Gln fluoromethyl ketones exhibited low activity in their assays, a fact which they explained by referring to that their inhibitors predominantly exist in the cyclic form as evident from NMR studies.[20] Indeed, the cyclic form is



Scheme 5. Attempted synthesis of model compound **22** and synthesis of model compound **25**. Reagents and conditions: (a) Dess–Martin periodinane, CH_2Cl_2, rt, 16 h; (b) TFA, CH_2Cl_2, rt, 16 h; (c) HOBt, EDC·HCl, Cbz-L-Ala-OH, DMF, rt, 21 h.

Table 1. Inhibitory activity of peptides against the SARS-CoV 3CL^{pro}

Compound	Structure	K_i (μM)
1	Cbz-Val-Leu-Glu-CF_3	116.1±13.6
2	Cbz-Val-Leu-Gln-CF_3	>1000
3	Cbz-Phe-Ala-Gln-CF_3	844.4±120.3
4	Cbz-Ala-Val-Leu-Gln-CF_3	134.5±31.6

not expected to interact effectively with the active site of SARS-CoV.[20] The Gln compounds synthesized in our study were also found to be mainly in the cyclic form which may explain the low biological activity for these compounds. However, the Glu inhibitor **1** was found to mainly exist in the hydrate form, which is a form that most likely will interact more effectively with the active site.

3. Conclusion

A simple five-step procedure for the synthesis of β-amino alcohol **11** containing a CF_3 group was developed. This alcohol was further elaborated into four tri- and tetra-Glu and Gln peptides. Compounds **1** and **4** were found to be moderate SARS-CoV 3CL^{pro} inhibitors. Current work is focused on the co-crystallization of compounds **1** and **4** with SARS-CoV 3CL^{pro} in an attempt to elucidate their mode of action.

4. Experimental

4.1. General procedures

Melting points were measured on a Yanagimoto micro hot-stage apparatus and are uncorrected. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on either a JEOL JNM-AL300 spectrometer operating at 300 MHz for proton and 75 MHz for carbon, or a Varian UNITY INOVA 400NB spectrometer operating at 400 MHz for proton and 101 MHz for carbon. Chemical shifts were recorded as δ values in parts per million (ppm) downfield from tetramethylsilane (TMS). Fluorine (^{19}F) NMR spectra were recorded on a Varian UNITY INOVA 400 spectrometer operating at 376 MHz for fluorine. Fluorine NMR spectra were referenced externally to C_6F_6 at 0.00 ppm. Low-resolution mass spectra (ESI) were recorded on a Finnigan SSQ-7000 spectrophotometer. Low- and high-resolution mass spectra (FAB) were recorded on a JEOL JMS-SX102A spectrometer equipped with JMA-DA7000 data system. Low- and high-resolution mass spectra (CI) were recorded on a JEOL JMS-GCmate. Optical rotations were measured with a Horiba High-speed Accurate Polarimeter SEPA-300 at the sodium-D line (589 nm) at the concentrations (c, g 100 mL^{-1}). The measurements were carried out between 22 and 28 °C in a cell with path length (l) of 0.5 dm. Specific rotations $[\alpha]_D$ are given in 10^{-1} deg cm^2 g^{-1}. Preparative HPLC was carried out on a C18 reverse phase column (20×250 mm; YMC Pack ODS SH343-5) with a binary solvent system (a linear gradient of CH_3CN and aq TFA (0.1%) at a flow rate of 5.0 mL min^{-1}), detected at 230 nm. Analytical HPLC was performed using a C18 reverse phase column (4.6×150 mm; YMC Pack ODS AM302) with a binary solvent system (a linear gradient of CH_3CN and aq TFA (0.1%) at a flow rate of 0.9 mL min^{-1}), detected at 230 nm. The t_R given for the target compounds are obtained from analytical HPLC. Solvents used for HPLC were of HPLC grade and all other chemicals were of analytical grade or better.

4.1.1. (*S*)-3-[3-(Benzyloxycarbonyl)-5-oxooxazolidin-4-yl]propanoic acid 6.[10] This compound was synthesized according to the procedure in Ref. 10. $[\alpha]_D^{26}$ +80.5 (c 3.9, MeOH) {lit.[24] $[\alpha]_D^{25}$ +73 (c 2.35, MeOH)}.

4.1.2. (*S*)-*tert*-Butyl-3-[3-benzyloxycarbonyl-5-oxooxazolidin-4-yl]propanoate 7. DMAP (428 mg, 3.50 mmol) and EDC·HCl (1.83 g, 9.56 mmol) were added to a stirred solution of oxazolidinone acid **6** (2.26 g, 7.78 mmol) and *t*-BuOH (2.2 mL, 23.0 mmol) in THF (80 mL) at room temperature. The reaction mixture was then stirred for 5 min before triethylamine (1.1 mL, 7.89 mmol) was added dropwise. The reaction mixture was then stirred for 16 h before being diluted with EtOAc (100 mL) and washed with citric acid (2×50 mL of a 5% aq solution), $NaHCO_3$ (2×50 mL of a 5% aq solution) and brine (2×50 mL) before being dried (Na_2SO_4). Filtration and concentration under reduced pressure gave a light-yellow oil, which was subjected to flash chromatography (silica, hexane→hexane/EtOAc 9:1→4:1 gradient eluent). Concentration of the relevant fractions (R_f 0.3 in hexane/EtOAc 4:1) gave the title compound **7**[25] (1.22 g, 45%) as a clear, colorless oil: $[\alpha]_D^{25}$ +63.2 (c 3.86, EtOH) {lit.[25] $[\alpha]_D^{22}$ +27.9 (c 1.58, EtOH)}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41–7.30 (m, 5H), 5.54 (br s, 1H), 5.22 (d, J=4.8 Hz, 1H), 5.19 (s, 2H), 4.37 (t, J=5.3 Hz, 1H), 2.37–2.11 (m, 4H), 1.43 (s, 9H); MS (ESI+) m/z 372 (M$^+$+Na, 100%).

4.1.3. (4*S*,5*S*)-Benzyl 4-[2-(*tert*-butoxycarbonyl)ethyl]-5-trifluoromethyl-5-(trimethylsilyloxy)oxazolidine-3-carboxylate 8. Cesium fluoride (87.8 mg, 0.58 mmol) and (trifluoromethyl)trimethylsilane (0.73 mL, 4.94 mmol) were added to a solution of oxazolidinone **7** (986.0 mg, 3.98 mmol) in dry THF (20 mL) maintained under an argon atmosphere. The reaction mixture was then sonicated for 2 h at ambient temperature before being diluted with EtOAc (40 mL). The resulting solution was washed with water (1×20 mL) and brine (1×20 mL) before being dried ($MgSO_4$). Filtration and concentration under reduced pressure gave the title compound **8** (1.80 g, 92%) as a clear, yellow oil, which was >95% pure (as judged by ^{1}H NMR analysis): $[\alpha]_D^{28}$ +37.8 (c 1.26, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36–7.27 (m, 5H), 5.41–5.29 (m, 1H), 5.14 (s, 2H), 4.83 (br s, 1H), 4.37 (br s, 1H), 2.32 (app. br s, 2H), 1.94 (6, J=7.0 Hz, 1H), 1.79 (app. br s, 1H), 1.41 (s, 9H), 0.20 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.0, 153.9, 135.7, 128.5, 128.2, 127.9, 122.2 (q, J_{C-F}=287.3 Hz), 102.2 (br), 80.3, 77.8, 67.8, 59.1, 31.7, 28.0, 23.9, 1.0; MS (CI+) m/z 492 (M$^+$+H, 1%), 436 (10), 401 (4), 392 (6), 334 (7),107 (7), 91 (100), 57 (47); HRMS (CI+): calcd for $C_{22}H_{33}NO_6F_3Si$ (M$^+$+H) 492.2029, found 492.2030.

4.1.4. (4*S*,5*R*)-Benzyl 4-[2-(*tert*-butoxycarbonyl)ethyl]-5-trifluoromethyl-5-hydroxyoxazolidine-3-carboxylate 9. Method A: TBAF (0.11 mL of a 1 M solution in THF, 0.11 mmol) was added dropwise to a stirred solution of compound **8** (44.5 mg, 0.091 mmol) in THF (2.0 mL) at 0 °C. The reaction mixture was then allowed to heat to room temperature and stirred for 0.5 h before being diluted with

EtOAc (15 mL). The organic phase was washed with water (2×10 mL) and brine (1×10 mL) before being dried ($MgSO_4$). Filtration and concentration under reduced pressure gave a yellow oil, which was subjected to flash chromatography (silica, hexane/EtOAc 4:1 eluent). Evaporation of the relevant fractions (R_f 0.2) gave the title alcohol **9** (29.3 mg, 77%) as a clear, yellow oil: $[\alpha]_D^{26}$ +41.0 (*c* 0.97, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.30 (m, 5H), 5.34 (br s, 1H), 5.17 (s, 2H), 4.88 (d, *J*=4.8 Hz, 1H), 4.40 (t, *J*=6.7 Hz, 1H), 2.40 (t, *J*=7.1 Hz, 2H), 2.14–1.97 (m, 2H), 1.43 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.5, 153.9, 135.6, 128.6, 128.4, 128.0, 122.4 (q, J_{C-F}=286.1 Hz), 101.1 (q, J_{C-F}=33.2 Hz), 81.4, 77.9, 67.9, 58.3, 31.2, 28.0, 22.8; MS (CI+) *m*/*z* 420 (M^++H, 2%), 364 (15), 91 (100); HRMS (CI+): calcd for $C_{19}H_{25}NO_6F_3$ (M^++H) 420.1634, found 420.1633. Method B: A solution of silyl ether **8** (129.3 mg, 0.26 mmol) in MeOH (4.5 mL) and water (0.5 mL) was stirred at room temperature for 3 h. The reaction mixture was then concentrated under reduced pressure to give the title alcohol **9** (110.2 mg, quant.), which was identical, in all respects, with the material obtained by Method A. The product was >95% pure (as judged by ^{1}H NMR analysis).

4.1.5. One-pot synthesis of compound 9 from compound 7. Method C: Cesium fluoride (37.9 mg, 0.25 mmol) and (trifluoromethyl)trimethylsilane (0.31 mL, 2.02 mmol) were added to a solution of oxazolidinone **7** (427.0 mg, 1.72 mmol) in dry THF (9.0 mL) maintained under an argon atmosphere. The reaction mixture was then sonicated for 2 h at ambient temperature before water (0.30 mL) was added and the reaction mixture was sonicated for an additional 0.5 h. The reaction mixture was then diluted with EtOAc (30 mL) and washed with water (1×10 mL) and brine (1×10 mL) before being dried ($MgSO_4$). Filtration and concentration under reduced pressure gave the title alcohol **9** (520.8 mg, 72%), which was identical, in all respects, with the material obtained via the stepwise method.

4.1.6. (4*S*)-*tert*-Butyl 4-(benzyloxycarbonyl)amino-6,6,6-trifluoro-5-hydroxyhexanoate 10. Sodium borohydride (0.76 g, 20.09 mmol) was added to a stirred solution of alcohol **9** (1.09 g, 2.60 mmol) in THF (70 mL) under an atmosphere of argon. The resulting reaction mixture was stirred for 23 h before being quenched by addition of water (10 mL). The water phase was then extracted with EtOAc (3×30 mL) and the combined organic fractions were dried ($MgSO_4$). Filtration and concentration under reduced pressure gave a light-yellow oil, which was subjected to flash chromatography (silica, hexane/EtOAc 3:1 eluent). Concentration of the relevant fractions (R_f 0.2) gave the title alcohol **10** (702.1 mg, 69%) as a clear, viscous, colorless oil and as a ca. 4.5:1 mixture of diastereomers (as judged by ^{1}H and ^{13}C NMR analyses): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33–7.23 (m, 5H), 5.58 (d, *J*=9.3 Hz, 0.17H), 5.54 (d, *J*=9.3 Hz, 0.83H), 5.10 (br s, 0.83H), 5.05 (s, 1.67H), 4.87 (br s, 0.17H), 4.62 (s, 0.33H), 4.09–3.82 (m, 2H), 2.29 (t, *J*=7.4 Hz, 2H), 1.97–1.74 (m, 2H), 1.41 (s, 7.4H), 1.40 (s, 1.6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.1, 173.0, 156.5 (9), 156.5 (5), 136.0, 135.9, 128.4, 128.3, 128.1, 128.0, 127.9, 127.7, 127.5, 126.9, 124.4 (q, J_{C-F}=283.2 Hz), 81.0 (4), 81.0 (1), 71.9 (q, J_{C-F}=29.4 Hz), 70.6 (q, J_{C-F}=30.1 Hz), 67.0, 66.9, 51.2, 49.6, 31.8, 31.6, 27.8, 27.2, 23.7; MS (CI+) *m*/*z* 392 (M^++H, 3%), 336 (27), 292 (12), 91 (100); HRMS (CI+): calcd for $C_{18}H_{25}NO_5F_3$ (M^++H) 392.1684, found 392.1689.

4.1.7. Synthesis of compound 10 from compound 8. Sodium borohydride (78.8 mg, 2.08 mmol) was added to a stirred solution of silyl ether **8** (89.0 mg, 0.18 mmol) in MeOH (5.0 mL) at room temperature. The reaction mixture was then stirred at room temperature for 16 h before being quenched by addition of water (5.0 mL). The water phase was extracted with EtOAc (3×15 mL) and the combined organic fractions were dried ($MgSO_4$). Filtration and concentration under reduced pressure gave a light-yellow oil, which was subjected to flash chromatography (silica, hexane/EtOAc 3:1 eluent). Concentration of the relevant fractions (R_f 0.2) gave the title alcohol **10** (48.3 mg, 68%) as a viscous colorless oil and as a ca. 4.5:1 mixture of diastereomers (as judged by ^{1}H and ^{13}C NMR analyses). The material obtained via this method was identical, in all respects, with the material obtained via the reduction of alcohol **9**.

4.1.8. (4*S*)-*tert*-Butyl 4-amino-6,6,6-trifluoro-5-hydroxyhexanoate 11. Amine **10** (204.3 mg, 0.52 mmol) and Pd/C (10%) (21.0 mg) were stirred vigorously for 16 h in MeOH (6.0 mL) under an atmosphere of H_2. The reaction mixture was then diluted with MeOH (10 mL) and filtered through a plug of Celite® and washed afterwards with MeOH (3×10 mL). Concentration of the filtrate under reduced pressure gave the title amine **11** (134.3 mg, quant.) as a white solid: mp 93–95 °C and as a ca. 4.5:1 mixture of diastereomers (as judged by ^{1}H and ^{13}C NMR analyses): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.50–4.20 (br s, 3H), 4.10–3.85 (m, 1H), 3.54 (app. br s, 0.18H), 3.39 (app. br s, 0.82H), 2.55–2.29 (m, 2H), 2.17–1.85 (m, 2H), 1.45 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.7, 172.2, 125.3 (q, J_{C-F}=284.0 Hz), 125.2 (q, J_{C-F}=283.4 Hz), 81.0, 80.8, 71.4 (q, J_{C-F}=28.6 Hz), 70.2 (q, J_{C-F}=29.4 Hz), 51.6, 48.3, 32.3, 32.0, 29.7, 28.0 (2), 27.9 (9); MS (CI+) *m*/*z* 258 (M^++H, 12%), 242 (8), 202 (36), 102 (100); HRMS (CI+): calcd for $C_{10}H_{19}NO_3F_3$ (M^++H) 258.1317, found 258.1319. Anal. Calcd for $C_{10}H_{18}F_3NO_3\cdot 0.5H_2O$: C, 45.11; H, 7.19; N, 5.26. Found: C, 45.27; H, 6.79; N, 5.44.

4.1.9. *N*-Benzyloxycarbonyl-L-valyl-L-leucine 12.[14] This compound was synthesized according to the procedure in Ref. 14: mp 134–136 °C (lit.[4] mp 135–137 °C); $[\alpha]_D^{25}$ −20.5 (*c* 0.47, CH_2Cl_2) {lit.[4] $[\alpha]_D^{20}$ −24.0 (*c* 0.49, CH_2Cl_2)}. Anal. Calcd for $C_{19}H_{28}N_2O_5$: C, 62.62; H, 7.74; N, 7.69. Found: C, 62.82; H, 7.94; N, 7.87.

4.1.10. *N*-Benzyloxycarbonyl-L-alanyl-L-valyl-L-leucine 14. Protected dipeptide **12** (1.00 g, 2.74 mmol) and Pd/C (10%) (100.0 mg) were stirred vigorously in a mixture of MeOH (9.5 mL), water (5.0 mL), and acetic acid (1.0 mL) under an atmosphere of H_2 for 2 h. The reaction mixture was then filtered through a plug of Celite® and washed afterwards with MeOH (3×10 mL). Concentration under reduced pressure gave peptide **13**[26] (500.0 mg), which was used directly in the next step without further purification. A solution of *N*-hydroxysuccinimide ester of Cbz-L-Ala-OH[16] (694.0 mg, 2.17 mmol) in DMF (5.0 mL) was added dropwise to a solution of amine **13** (500.0 mg) and triethylamine (0.606 mL, 4.34 mmol) in DMF (10 mL) maintained at 0 °C

over the course of 30 min. The reaction mixture was then allowed to heat to room temperature and stirred for 16 h before being concentrated under reduced pressure. The resulting substrate was dissolved in EtOAc (40 mL) and washed with citric acid (2×20 mL of a 5% aq solution) and brine (1×20 mL) before being dried (Na_2SO_4). Filtration and concentration under reduced pressure gave a light-yellow solid, which was recrystallized from hexane/EtOAc to give the title protected peptide **14**[27] (800.0 mg, 67% over the two steps) as a white solid: mp 194–195 °C; $[\alpha]_D^{25}$ −52.4 (*c* 0.71, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38–7.30 (m, 5H), 6.99 (br d, *J*=7.3 Hz, 1H), 6.65 (d, *J*=7.3 Hz, 1H), 5.47 (br d, *J*=6.6 Hz, 1H), 5.11 (s, 2H), 4.56–4.49 (m, 1H), 4.26 (app. t, *J*=7.9 Hz, 2H), 3.49–3.40 (m, 1H), 2.17–1.54 (m, 3H), 1.3 (d, *J*=7.0 Hz, 3H), 0.95–0.88 (m, 12H) (one signal due to OH in COOH could not be discerned); ^{13}C NMR (75 MHz, $CDCl_3$+one drop of DMSO-d_6) δ 174.0, 172.3, 170.5, 155.6, 136.1, 128.1, 127.7, 66.3, 58.0, 50.4, 40.8, 33.6, 30.5, 22.4, 21.5, 18.9, 17.6 (one signal obscured or overlapping); MS (FAB+) *m*/*z* 436 (M+H, 7%), 305 (8), 222 (7), 91 (100); HRMS (FAB+): calcd for $C_{22}H_{40}N_3O_6$ (M^++H) 436.2448, found 236.2451. Anal. Calcd for $C_{22}H_{33}N_3O_6$: C, 60.67; H, 7.64; N, 9.65. Found: C, 60.89; H, 7.91; N, 9.88.

4.1.11. *N*-Benzyloxycarbonyl-L-phenylalanyl-L-alanine 15.[15] This compound was synthesized according to the procedure in Ref. 15: mp 157–159 °C (lit.[15] mp 157–158 °C); $[\alpha]_D^{24}$ −8.1 (*c* 0.65, EtOH) {lit.[15] $[\alpha]_D^{25}$ −9.5 (*c* 1.0, EtOH)}.

4.1.12. General procedure for the synthesis of (*S*)-4-[*N*-(benzyloxycarbonyl)-L-valyl-L-leucyl]amino-6,6,6-trifluoro-5-oxohexanoic acid 1, (*S*)-4-[*N*-(benzyloxycarbonyl)-L-phenylalanyl-L-alanyl]amino-6,6,6-trifluoro-5-oxohexanoic acid 19, and (*S*)-4-[*N*-(benzyloxycarbonyl)-L-alanyl-L-valyl-L-leucyl]amino-6,6,6-trifluoro-5-oxohexanoic acid 20. Coupling: HOBt (59.6 mg, 0.39 mmol) and EDC·HCl (80.4 mg, 0.42 mmol) were added to a stirred solution of the relevant protected peptide (0.39 mmol) in DMF (6.0 mL) at 0 °C. The reaction mixture was then stirred for 15 min before amine **11** (100.0 mg, 0.39 mmol) dissolved in DMF (6.0 mL) was added dropwise. The reaction mixture was then allowed to heat to room temperature and stirred for 21 h before DMF was removed under reduced pressure. The resulting residue was diluted with EtOAc (30 mL) and washed with citric acid (2×10 mL of a 5% aq solution), $NaHCO_3$ (2×10 mL of a 5% aq solution), and brine (2×10 mL) before being dried (Na_2SO_4). Filtration and concentration under reduced pressure gave the desired compound in quantitative yield. The crude product, which contained small amounts of impurities, was used in the next step without further purification. All products had satisfactory low-resolution mass spectra. Oxidation: Dess–Martin periodinane (439 mg, 1.04 mmol) was added to a stirred solution of the relevant peptide from the previous step in CH_2Cl_2 (15.0 mL) at 0 °C. The resulting reaction mixture was then allowed to heat to room temperature and stirred for 19 h before being filtered through a plug of Celite® and washed afterwards with EtOAc (3×15 mL). Concentration under reduced pressure gave the desired compound as a yellow oil. The material was used in the next step without further purification. All products had satisfactory low-resolution mass spectra. Deprotection: TFA (0.115 mL, 1.55 mmol) was added dropwise to a stirred solution of the relevant compound from the previous step in CH_2Cl_2 (4.0 mL) at 0 °C. The reaction mixture was then allowed to heat to room temperature and stirred for 16 h before being concentrated under reduced pressure. The crude product was used directly in the next step without further purification except for a small amount of the crude peptide **1**, which was purified at this stage in order to provide a sample for biological assaying.

4.1.13. (*S*)-4-[*N*-(Benzyloxycarbonyl)-L-valyl-L-leucyl]-amino-6,6,6-trifluoro-5-oxohexanoic acid 1. Part of the resulting yellow oil was subjected to preparative HPLC purification in order to provide a sample for biological testing. Concentration of the relevant fractions (t_R 23.7 min) gave the title compound **1** (5.9 mg) as a white solid and as a ca. 6:1 mixture of the hydrate and keto forms (as judged by ^{19}F NMR analysis) and the hydrate form existed as a ca. 1:1 mixture of rotamers (as judged by ^{13}C NMR analysis). Trace amounts of the cyclic form of this compound could also be seen by ^{19}F NMR: mp 172–173 °C; $[\alpha]_D^{22}$ −15.6 (*c* 0.28, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$+one drop of DMSO-d_6) δ 7.40–7.20 (m, 6H), 6.00 (dd, *J*=8.3 and 24.3 Hz, 1H), 5.1 (app. dd, *J*=12.1 and 16.5 Hz, 2H), 4.48–4.44 (m, 1H), 4.22–4.13 (m, 1H), 4.01 (q, *J*=8.1 Hz, 1H), 3.80–2.70 (br s, 2H), 2.35 (app. s, 2H), 2.23–1.88 (m, 3H), 1.72–1.47 (m, 3H), 0.96–0.88 (m, 12H); ^{13}C NMR (101 MHz, $CDCl_3$+one drop of DMSO-d_6) δ 175.9, 175.8, 174.4, 173.8, 172.0, 156.9, 156.7, 136.0, 128.4, 128.2, 128.1, 94.0 (q, J_{C-F}=29.8 Hz), 67.2, 67.0, 60.9, 60.8, 53.8, 53.6, 52.0, 51.9, 30.7, 30.6, 30.4, 24.5 (4), 24.4 (7), 23.1, 22.9, 22.8, 21.5, 21.4, 19.1, 17.7 (signal due to CF_3 group carbon could not be discerned); ^{19}F NMR (376 MHz, $CDCl_3$+one drop of DMSO-d_6) δ −74.8 (cyclic), −76.4 (keto), −76.5 (keto), −81.8 (hydrate), −81.9 (hydrate), −82.1 (hydrate), −82.2 (hydrate). (The appearance of four signals for the hydrate form of this compound in ^{19}F NMR is probably due to partial racemization over time at the α position of this compound. The extent of racemization at the time ^{19}F NMR was measured was less than 10%.[28]) MS (FAB+) *m*/*z* 546 (M^++H, 5%), 502 (1), 412 (1), 347 (4), 91 (100); HRMS (FAB+): calcd for $C_{25}H_{35}N_3O_7F_3$ (M^++H) 546.2427, found 546.2421. Anal. Calcd for $C_{25}H_{34}F_3N_3O_7 \cdot 1/4CF_3COOH \cdot H_2O$: C, 51.73; H, 6.17; N, 7.10. Found: C, 51.79; H, 6.34; N, 7.13.

4.1.14. (*S*)-4-[*N*-(Benzyloxycarbonyl)-L-phenylalanyl-L-alanyl]amino-6,6,6-trifluoro-5-oxohexanoic acid 19. MS (ESI−) *m*/*z* 550 (M−H, 64%), 442 (100).

4.1.15. (*S*)-4-[*N*-(Benzyloxycarbonyl)-L-alanyl-L-valyl-L-leucyl]amino-6,6,6-trifluoro-5-oxohexanoic acid 20. MS (ESI−) *m*/*z* 615 (M−H, 100%), 507 (60).

4.1.16. General procedure for the synthesis of (*S*)-4-[*N*-(benzyloxycarbonyl)-L-valyl-L-leucyl]amino-6,6,6-trifluoro-5-oxohexanamide 2, (*S*)-4-[*N*-(benzyloxycarbonyl)-L-phenylalanyl-L-alanyl]amino-6,6,6-trifluoro-5-oxohexanamide 3, and (*S*)-4-[*N*-(benzyloxycarbonyl)-L-alanyl-L-valyl-L-leucyl]amino-6,6,6-trifluoro-5-oxohexanamide 4. Method A: HOBt (25.0 mg, 0.16 mmol) and EDC·HCl (31.0 mg, 0.16 mmol) were added to a stirred solution of the relevant peptide from the previous step in DMF (7.0 mL) at 0 °C. The resulting reaction mixture

was then stirred for 15 min before ammonia solution (31.0 μL of a 28% aq solution) was added dropwise. The reaction mixture was then allowed to heat to room temperature and stirred for 16 h before DMF was removed under reduced pressure. The residue thus obtained was then dissolved in EtOAc (20 mL) and washed with citric acid (2×10 mL of a 5% aq solution), $NaHCO_3$ (2×10 mL of a 5% aq solution), and brine (2×10 mL) before being dried (Na_2SO_4). Filtration and concentration under reduced pressure gave the crude product, which was purified by preparative HPLC. Concentration of the relevant fractions gave the desired compounds in the yields stated below.

4.1.17. (*S*)-4-[*N*-(Benzyloxycarbonyl)-L-valyl-L-leucyl]-amino-6,6,6-trifluoro-5-oxohexanamide 2. Concentration of the relevant fractions (t_R 24.2 min) gave the title compound **2** (16.9 mg, 8% from acid **12**) as a white solid and as a ca. 1:1 mixture of rotamers (as judged by ^1H, ^{13}C, and ^{19}F NMR analyses): mp 112–113 °C; $[\alpha]_D^{26}$ +32.0 (*c* 0.07, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.45 (br d, *J*=8.1 Hz, 0.5H), 7.39–7.31 (m, 5H), 7.08 (app. br d, *J*=8.1 Hz, 0.5H), 6.83 (br s, 0.5H), 6.52 (br s, 0.5H), 6.48–6.41 (m, 1H), 5.38 (app. br d, *J*=5.7 Hz, 0.5H), 5.30 (app. br d, *J*=5.7 Hz, 0.5H), 5.12 (s, 2H), 4.66 (app. br s, 0.5H), 4.47 (app. br s, 1H), 4.38 (app. br d, *J*=6.6 Hz, 0.5H), 3.93 (app. br s, 1H), 2.66–2.43 (m, 2H), 2.22–1.45 (m, 11H), 0.97–0.88 (m, 12H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.5, 172.1, 172.0, 171.8 (4), 171.8 (0), 171.7 (6), 157.0, 135.6, 128.7, 128.6, 128.5, 128.2, 128.1, 67.8, 67.6, 61.3, 61.2, 52.3, 47.0, 40.0, 39.6 (4), 39.6 (0), 30.3, 24.8, 24.7, 22.8, 22.7, 21.6, 19.2, 19.1, 17.9, 17.8 (signal due to CF_3 group carbon and signal due to the carbon adjacent to the CF_3 group could not be discerned); ^{19}F NMR (376 MHz, $CDCl_3$) δ −82.7 (cyclic), −83.3 (cyclic); MS (ESI+) *m/z* 567 (M^++Na, 100%), 545 (M^++H, 5); HRMS (FAB+): calcd for $C_{25}H_{36}N_4O_6F_3$ (M^++H) 545.2587, found 545.2591. Anal. Calcd for $C_{25}H_{35}F_3N_4O_6 \cdot 1/4CF_3COOH \cdot 1/4H_2O$: C, 53.03; H, 6.24; N, 9.70. Found: C, 53.26; H, 6.35; N, 9.79.

4.1.18. (*S*)-4-[*N*-(Benzyloxycarbonyl)-L-phenylalanyl-L-alanyl]amino-6,6,6-trifluoro-5-oxohexanamide 3. Concentration of the relevant fractions (t_R 21.9 min) gave the title compound **3** (8.0 mg, 5% from acid **15**) as an off-white solid and as a ca. 1:1 mixture of rotamers (as judged by ^{13}C and ^{19}F NMR analyses) and as a ca. 3.3:1 mixture of the cyclic and keto forms (as judged by ^{19}F NMR): mp 108–111 °C; $[\alpha]_D^{27}$ −6.8 (*c* 0.24, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.07 (m, 14.5H), 5.47 (br s, 0.5H), 5.08–4.94 (m, 2H), 4.64–4.22 (m, 3H), 3.19–2.85 (m, 2H), 2.44 (app. br s, 2H), 2.11–1.77 (m, 2H), 1.28 (app. br s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.3 (3), 172.2 (8), 172.0 (2), 171.9 (7), 156.7, 156.6, 156.5, 135.7, 129.1, 128.9, 128.8, 128.6, 128.5, 128.3, 128.2, 128.0, 127.4, 67.6, 67.4, 56.5, 56.3, 49.2, 46.5, 45.8, 37.9, 37.8, 37.6, 29.6, 28.8, 22.8, 17.8, 17.1 (signal due to CF_3 group carbon and signal due to the carbon adjacent to the CF_3 group could not be discerned); ^{19}F NMR (376 MHz, $CDCl_3$) δ −76.2 (keto), −82.9 (cyclic), −83.2 (cyclic); MS (FAB+) *m/z* 573 (M^++Na, 7%), 551 (M^++H, 5); HRMS (FAB+): calcd for $C_{26}H_{30}N_4O_6F_3$ (M^++H) 551.2117, found 551.2114.

4.1.19. (*S*)-4-[*N*-(Benzyloxycarbonyl)-L-alanyl-L-valyl-L-leucyl]amino-6,6,6-trifluoro-5-oxohexanamide 4. Concentration of the relevant fractions (t_R 25.0 min) gave the title compound **4** (8.5 mg, 4% from acid **14**) as a white solid and as a ca. 1:1 mixture of rotamers (as judged by ^{13}C and ^{19}F NMR analyses): mp 140–141 °C; $[\alpha]_D^{27}$ −4.5 (*c* 0.11, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.36 (m, 3H), 7.33–7.30 (m, 2H), 7.13 (app. br d, *J*=8.4 Hz, 0.8H), 7.06–6.96 (m, 1.2H), 6.62 (app. br d, *J*=4.4 Hz, 0.8H), 6.49 (app. br d, *J*=4.4 Hz, 0.2H), 6.20 (s, 0.8H), 6.10 (s, 0.2H), 5.28 (br s, 0.8H), 5.24 (br s, 0.2H), 5.13 (d, *J*=4.0 Hz, 2H), 4.69 (dt, *J*=3.6 Hz, 0.2H), 4.64–4.56 (m, 0.2H), 4.54 (dt, *J*=3.6 Hz, 0.8H), 4.50–4.43 (m, 0.8H), 4.16–4.01 (m, 2H), 3.70–3.30 (br s, 0.2H), 2.64–2.14 (m, 4H), 1.95–1.90 (m, 1H), 1.87–1.76 (m, 1H), 1.45 (d, *J*=7.1 Hz, 3H), 0.96 (d, *J*=6.6 Hz, 6H), 0.91–0.86 (m, 6H) (the signal for three protons were obscured by the signal for residual water in the sample); ^1H NMR (400 MHz, $CDCl_3$+one drop of DMSO-d_6) δ 7.38–7.31 (m, 5H), 7.07–6.99 (m, 3H), 6.81 (app. br s, 1H), 6.42–6.23 (m, 2H), 5.11 (s, 2H), 4.55–4.39 (m, 2H), 4.19–4.08 (m, 2H), 3.70–3.30 (br s, 1H), 2.56–2.41 (m, 2H), 1.94–1.86 (m, 1H), 1.77–1.53 (m, 4H), 1.39 (d, *J*=7.1 Hz, 3H), 0.98–0.89 (m, 12H); ^{19}F NMR (376 MHz, $CDCl_3$+one drop of DMSO-d_6) δ −83.2 (cyclic), −83.3 (cyclic); MS (ESI+) *m/z* 654 (M^++K, 35%), 638 (M^++Na, 100), 616 (M^++H, 68); HRMS (FAB+): calcd for $C_{28}H_{41}N_5O_7F_3Na$ (M^++Na) 638.2778, found 638.2783.

4.1.20. (*S*)-4-[*N*-(Benzyloxycarbonyl)-L-alanyl-L-valyl-L-leucyl]amino-6,6,6-trifluoro-5-oxohexanamide 4. Method B: Pyridine (0.133 mL, 1.64 mmol) was added dropwise to a stirred solution of peptide **20** (39.1 mg) and di-*tert*-butyl dicarbonate (23.6 mg, 0.18 mmol) in 1,4-dioxane (13 mL) under an argon atmosphere at room temperature. Ammonium bicarbonate (324 mg, 4.10 mmol) was then added to the resulting solution and the reaction mixture was stirred at room temperature for 23 h before being diluted with EtOAc (20 mL). The organic phase was washed with citric acid (1×10 mL of a 5% aq solution), $NaHCO_3$ (1×10 mL of a 5% aq solution), and brine (1×10 mL) before being dried (Na_2SO_4). Filtration and concentration under reduced pressure gave a light-yellow solid, which was purified by preparative HPLC. Concentration of the relevant fractions (t_R 25.4 min) gave the title compound **4** (8.4 mg, 12% from peptide **14**), which was identical, in all respects, with the material obtained via Method A.

4.1.21. (*S*)-*tert*-Butyl 4-(benzyloxycarbonyl)amino-6,6,6-trifluoro-5-oxohexanoate 21. Dess–Martin periodinane (220.0 mg, 0.52 mmol) was added to a stirred solution of alcohol **10** (91.8 mg, 0.24 mmol) in CH_2Cl_2 (5.0 mL) at room temperature. The reaction mixture was then stirred for 16 h before being filtered through a plug of Celite® and washed afterwards with EtOAc (3×5 mL). The filtrate was concentrated under reduced pressure to give a light-yellow crude product, which was purified by flash chromatography (silica, hexane/EtOAc/triethylamine 50:49.6:0.4). Concentration of the relevant fractions (R_f 0.56 in hexane/EtOAc 1:1) gave the title compound **21** (74.4 mg, 81%) as a clear oil and as a ca. 2:1 mixture of the keto and hydrated forms (as judged by ^{19}F and ^{13}C NMR analyses): $[\alpha]_D^{25}$ +3.6 (*c* 0.73, MeOH); ^1H NMR (300 MHz, $CDCl_3$) δ 7.38–7.31 (m, 5H), 5.57 (br d,

J=7.7 Hz, 0.5H), 5.46 (br d, J=7.7 Hz, 0.5H), 5.11 (s, 2H), 4.88–4.78 (m, 0.5H), 3.96–3.86 (m, 0.5H), 2.42–2.09 (m, 3H), 1.97–1.86 (m, 1H), 1.43 (s, 4.5H), 1.42 (s, 4.5H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 190.3 (q, J_{C-F}=34.7 Hz), 173.5, 171.8, 157.9, 155.8, 135.7, 128.6, 128.5 (0), 128.4 (8), 128.3, 128.2, 128.1, 128.0, 127.7, 127.6 (4), 127.5 (8), 127.0, 123.1 (q, J_{C-F}=288.6 Hz), 115.5 (q, J_{C-F}=292.3 Hz), 94.2 (q, J_{C-F}=30.5 Hz), 81.5, 81.3, 67.5, 67.4, 55.5, 55.0, 31.8, 30.9, 27.9, 25.3, 23.2; ^{19}F NMR (376 MHz, $CDCl_3$) δ −76.5 (keto), −82.3 (hydrate); MS (ESI−) m/z 388 (M−H, 70%), 280 (100).

4.1.22. (*S*)-*tert*-Butyl 4-[*N*-(benzyloxycarbonyl)-L-alanyl]amino-6,6,6-trifluoro-5-oxohexanoate 24. HOBt (31.8 mg, 0.21 mmol) and EDC·HCl (43.6 mg, 0.23 mmol) were added to a stirred solution of Cbz-L-Ala-OH (44.1 mg, 0.198 mmol) in DMF (3.0 mL) at 0 °C. The reaction mixture was then stirred for 15 min before amine **11** (49.0 mg, 0.19 mmol) dissolved in DMF (2.0 mL) was added dropwise. The reaction mixture was then allowed to heat to room temperature and stirred for 21 h before DMF was removed under reduced pressure. The residue thus obtained was dissolved in EtOAc (20 mL) and washed with citric acid (2×10 mL of a 5% aq solution), $NaHCO_3$ (2×10 mL of a 5% aq solution), and brine (2×10 mL) before being dried (Na_2SO_4). Filtration and concentration under reduced pressure gave the title compound **23** (66.8 mg) as a clear, yellow oil. The material was used directly in the next step without further purification: MS (FAB+) m/z 485 (M^++Na, 5%), 463 (M^++H, 10), 407 (32), 363 (18), 91 (100); HRMS (FAB+): calcd for $C_{21}H_{30}N_2O_6F_3$ 463.2056, found 463.2061.

Dess–Martin periodinane (138.8 mg, 0.33 mmol) was added to a stirred solution of alcohol **23** (66.8 mg) in CH_2Cl_2 (3.0 mL) at room temperature. The reaction mixture was then stirred for 16 h before being diluted with EtOAc (10 mL) and filtered through a plug of Celite® and washed with EtOAc (3×10 mL). Concentration under reduced pressure gave a light-yellow oil, which was subjected to flash chromatography (silica, hexane/EtOAc/Et_3N 50:49.8:0.2 eluent). Concentration of the relevant fractions (R_f 0.2 in hexane/EtOAc 1:1) gave the title compound **24** (45.7 mg, 52% over the two steps) as a clear, colorless oil and as a ca. 7:3 mixture of the hydrate and keto forms (as judged by ^{1}H and ^{19}F NMR analyses) and both tautomers exist as a ca. 1:1 mixture of rotamers (as judged by ^{1}H, ^{13}C, and ^{19}F NMR analyses): $[\alpha]_D^{24}$ −9.1 (c 2.02, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56–7.27 (m, 5H), 7.14 (app. br d, J=6.6 Hz, 0.3H), 7.03 (app. d, J=8.4 Hz, 0.3H), 6.08–5.42 (br m, 1.7H), 5.12–5.05 (m, 2H), 4.90 (br s, 0.3H), 4.38–4.06 (m, 2H), 2.42–2.11 (m, 3H), 1.98–1.84 (m, 1H), 1.44 (s, 4H), 1.42 (4) (s, 4H), 1.42 (1) (s, 1H), 1.39–1.34 (m, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.6 (q, J_{C-F}=34.7 Hz), 189.5 (q, J_{C-F}=34.7 Hz), 175.0, 174.8, 173.6, 173.2, 172.8, 172.3, 172.1, 156.3, 156.2, 156.0, 136.0, 135.9, 135.8, 128.5, 128.3, 128.2 (0), 128.1 (6), 128.0 (7), 128.0 (3), 128.0 (1), 123.1 (q, J_{C-F}=288.8 Hz), 115.5 (q, J_{C-F}=292.6 Hz), 94.3 (q, J_{C-F}=30.9 Hz), 94.2 (q, J_{C-F}=30.5 Hz), 81.7, 81.6, 81.3, 81.2, 67.3, 67.1, 53.9, 53.8, 53.7, 50.9, 50.7, 50.1, 31.8, 30.9 (2), 30.9 (0), 29.7, 27.9, 24.8, 23.4, 23.2, 18.4, 18.1; ^{19}F NMR (376 MHz, $CDCl_3$) δ −76.5 (keto), −82.0 (hydrate), −82.1 (hydrate) (one signal obscured or overlapping); MS (ESI−) m/z 459 (M−H, 38%), 351 (100).

4.1.23. 4-[*N*-(Benzyloxycarbonyl)-L-alanyl]amino-6,6,6-trifluoro-5-oxohexanoic acid 25. TFA (0.10 mL, 1.30 mmol) was added dropwise to a solution of ester **24** (40.0 mg, 0.087 mmol) in CH_2Cl_2 (5 mL) at room temperature. The reaction mixture was then stirred at room temperature for 24 h before being concentrated under reduced pressure to give the title compound **25** (34.9 mg, crude yield 99%) as a yellow oil and ca. 90% pure (as judged by HPLC analysis) and as a ca. 6:2:1 mixture of cyclic, keto, and hydrate forms (as judged by ^{19}F NMR analysis) and the cyclic form existed as a ca. 1:1 mixture of rotamers (as judged by ^{19}F NMR analysis): $[\alpha]_D^{24}$ +10.5 (c 0.77, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD) δ 7.38–7.25 (m, 5H), 5.08 (s, 2H), 4.18 (q, J=7.3 Hz, 1H), 3.04–2.86 (m, 1H), 2.58 (t, J=6.5 Hz, 1H), 1.38 (d, J=7.3 Hz, 3H), 1.46–1.23 (m, 2H) (signal for one proton was obscured by the signal for methanol); ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.5, 176.9, 155.9, 136.0, 128.5, 128.2, 128.1, 122.4 (q, J_{C-F}=283.1 Hz), 75.0 (q, J_{C-F}=31.7 Hz), 67.1, 49.5, 34.0, 29.7, 27.7, 18.3; ^{13}C NMR (101 MHz, CD_3OD) δ 176.5, 176.0, 158.4, 138.2, 129.4, 129.0, 128.8, 75.8 (q, J_{C-F}=30.1 Hz), 67.5, 50.8, 35.3, 28.2, 17.9 (signal due to CF_3 group carbon could not be discerned and one signal was obscured or overlapping); ^{19}F NMR (376 MHz, $CDCl_3$) δ −74.6 (2) (cyclic), −74.6 (4) (cyclic), −76.2 (keto), −82.2 (1) (hydrate), −82.2 (3) (hydrate), −82.3 (0) (hydrate), −82.3 (2) (hydrate). (The appearance of four signals for the hydrate form of this compound in ^{19}F NMR is probably due to partial racemization over time at the α position of this compound. The extent of racemization at the time ^{19}F NMR was measured was less than 5%.[28]) MS (ESI−) m/z 403 (M−H, 46%), 222 (69), 199 (100).

4.2. Enzyme inhibitory assay

The inhibitory assay was performed using a commercially available fluorogenic substrate Dabcyl-KTSAVLQSGFRKME-Edans (Genesis Biotech, Taiwan) corresponding to the N-terminal autocleavage site of SARS 3CL^{pro}.[29] The change in fluorescence intensity was monitored in a Cary Eclipse fluorescence spectrophotometer (Varian) with 355 and 538 nm for excitation and emission wavelengths, respectively. Kinetic measurements were performed at 25 °C in buffer containing 10 mM sodium phosphate (pH 7.4), 10 mM sodium chloride, 1 mM EDTA, and 1 mM TCEP. The inhibition constant, K_i, was determined by measuring the apparent kinetic parameters at a constant substrate concentration with varying inhibitor concentrations (0–1 mM). The protease (final concentration of 1 mM) was incubated with inhibitor for 10 min at room temperature and the reaction was initiated by adding the substrate (a volume corresponding to a final concentration of 5 mM in the reaction mixture). The dependence of activity on the inhibitor concentration was analyzed in a manner similar to what was reported earlier.[30] Briefly, the kinetic parameters were determined by global nonlinear regression analysis to the equation.

$$v_I/v_0 = V_{max}[S]/\{[S] + K_m(1 + [I]/K_i)\}$$

where v_I and v_0 are the rate of substrate cleavage in the presence and absence of inhibitor, respectively. V_{max} is the

maximal rate, [*S*] is the substrate concentration, [*I*] is the inhibitor concentration, and K_m is the Michaelis constant.[31]

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Supplementary data

NMR spectra for all new compounds **1**, **2**, **3**, **4**, **8**, **9**, **10**, **11**, **21**, **24**, and **25**, and HPLC chromatograms of compounds **1**, **2**, **3**, and **4**. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2006.06.052.

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