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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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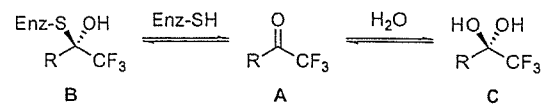
**Abstract**—Trifluoromethyl-β-amino alcohol **11** [(4*S*)-*tert*-butyl 4-amino-6,6,6-trifluoro-5-hydroxyhexanoate] was synthesized in five steps starting from Cbz-L-Glu-OH **5** where the key step involved the introduction of the trifluoromethyl ($CF_3$) group to oxazolidinone **7**, resulting in the 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 $3CL^{pro}$).


## 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**).




Scheme 1. Trifluoromethyl ketone adducts.

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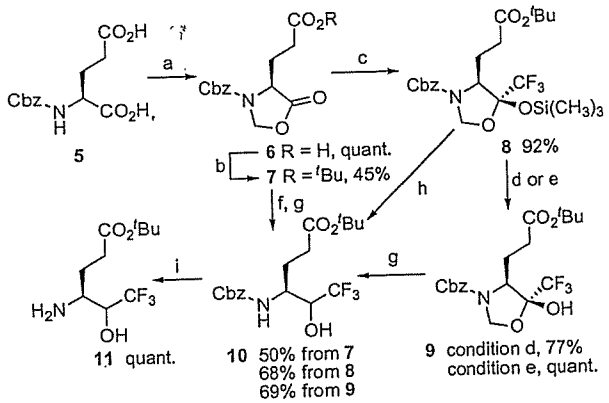
## 2. Results and discussion

### 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.



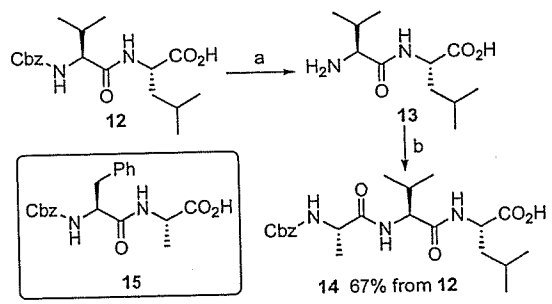

**Scheme 2.** Synthesis of β-amino alcohol **11**. Reagents and conditions: (a) paraformaldehyde, *p*-TsOH·$H_2O$, toluene, reflux, 1.67 h; (b) *t*-BuOH, EDC·HCl, DMAP, $Et_3N$, THF, rt, 16 h; (c) CsF, $CF_3Si(CH_3)_3$, THF, amb. temp, sonication, 2 h; (d) TBAF, THF, 0 °C–rt, 0.5 h; (e) MeOH/water (9:1),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

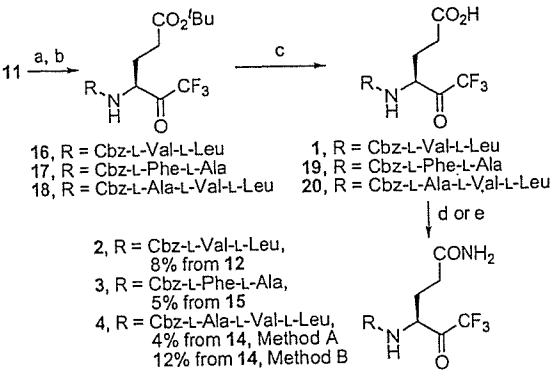
### 2.2. Synthesis of glutamic acid and glutamine peptides with a $CF_3$-ketone unit

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

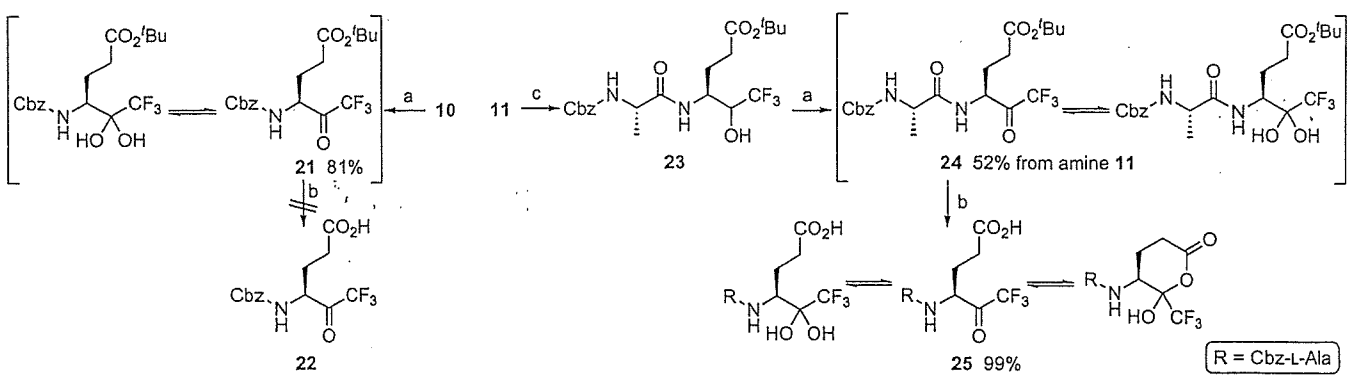
As previously noted, target peptide **1** was predominantly present in the hydrate form in $CDCl_3$. However, as alluded to in the previous section, this might differ depending on the solvent used for the NMR studies. Therefore, we decided to synthesize acid **22**, which is a much simpler molecule than 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 ($^1H$) 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 $\delta$ 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)}; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 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 $^1H$ NMR analysis): $[\alpha]_D^{28}$ +37.8 ($c$ 1.26, $CHCl_3$); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ 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-$d_6$) $\delta$ 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-$d_6$) $\delta$ 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-$d_6$) $\delta$ 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-$d_6$) $\delta$ −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, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ −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, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ 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, $CDCl_3$) $\delta$ −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, $CD_3OD$) $\delta$ 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, $CDCl_3$) $\delta$ 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, $CD_3OD$) $\delta$ 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, $CDCl_3$) $\delta$ −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 $\alpha$ 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.

## References and notes

1. Ksiazek, T. G.; Erdman, D.; Goldsmith, C. S.; Zaki, S. R.; Peret, T.; Emery, S.; Tong, S.; Urbani, C.; Comer, J. A.; Lim, W.; Rollin, P. E.; Dowell, S. F.; Ling, A.-E.; Humphrey, C. D.; Shieh, W.-J.; Guarner, J.; Paddock, C. D.; Rota, P.; Fields, B.; DeRisi, J.; Yang, J.-Y.; Cox, N.; Hughes, J. M.; LeDuc, J. W.; Bellini, W. J.; Anderson, L. J.; The SARS Working Group. *N. Engl. J. Med.* **2003**, *348*, 1953–1966.
2. Drosten, C.; Günther, S.; Preiser, W.; van der Werf, S.; Brodt, H.-R.; Becker, S.; Rabenau, H.; Panning, M.; Kolesnikova, L.; Fouchier, R. A. M.; Berger, A.; Burguiére, A.-M.; Cinatl, J.; Eickmann, M.; Escriou, N.; Grywna, K.; Kramme, S.; Manuguerra, J.-C.; Müller, S.; Rickerts, V.; Stürmer, M.; Vieth, S.; Klenk, H.-D.; Osterhaus, A. D. M. E.; Schmitz, H.; Doerr, H. W. *N. Engl. J. Med.* **2003**, *348*, 1967–1976.
3. Rota, P. A.; Oberste, M. S.; Monroe, S. S.; Nix, W. A.; Campagnoli, R.; Icenogle, J. P.; Peñaranda, S.; Bankamp, B.; Maher, K.; Chen, M.; Tong, S.; Tamin, A.; Lowe, L.; Frace, M.; DeRisi, J. L.; Chen, Q.; Wang, D.; Erdman, D. D.; Peret, T. C. T.; Burns, C.; Ksiazek, T. G.; Rollin, P. E.; Sanchez, A.; Liffick, S.; Holloway, B.; Limor, J.; McCaustland, K.; Olsen-Rasmussen, M.; Fouchier, R.; Günther, S.; Osterhaus, A. D. M. E.; Drosten, C.; Pallansch, M. A.; Anderson, L. J.; Bellini, W. J. *Science* **2003**, *300*, 1394–1399.
4. Anand, K.; Ziebuhr, J.; Wadhwani, P.; Mesters, J. R.; Hilgenfeld, R. *Science* **2003**, *300*, 1763–1767.
5. Snijder, E. J.; Bredenbeek, P. J.; Dobbe, J. C.; Thiel, V.; Ziebuhr, J.; Poon, L. L. M.; Guan, Y.; Rozanov, M.; Spaan, W. J.; Gorbalenya, A. E. *J. Mol. Biol.* **2003**, *331*, 991–1004.
6. Bégué, J.-P.; Bonnet-Delpon, D. *Tetrahedron* **1991**, *47*, 3207–3258.
7. Gelb, M. H.; Svaren, J. P.; Abeles, R. H. *Biochemistry* **1985**, *24*, 1813–1817.
8. Patel, D. V.; Rielly-Gauvin, K.; Ryono, D. E.; Free, C. A.; Smith, S. A.; Petrillo, E. W., Jr. *J. Med. Chem.* **1993**, *36*, 2431–2447.
9. Gao, F.; Ou, H.-Y.; Chen, L.-L.; Zheng, W.-X.; Zhang, C.-T. *FEBS Lett.* **2003**, *553*, 451–456.
10. Luesch, H.; Hoffmann, D.; Hevel, J. M.; Becker, J. E.; Golakoti, T.; Moore, R. E. *J. Org. Chem.* **2003**, *68*, 83–91.
11. Walter, M. W.; Adlington, R. M.; Baldwin, J. E.; Chuhan, J.; Schofield, C. J. *Tetrahedron Lett.* **1995**, *36*, 7761–7764.
12. Walter, M. W.; Adlington, R. M.; Baldwin, J. E.; Schofield, C. J. *J. Org. Chem.* **1998**, *63*, 5179–5192.
13. Sato, F.; Omodani, T.; Shiratake, R.; Inoue, Y.; Deguchi, T. PCT Int. Appl. WO 2001044165, 2001; *Chem. Abstr.* **2001**, *135*, 452995.
14. Schmidt, U.; Utz, R.; Lieberknecht, A.; Griesser, H.; Potzolli, B.; Bahr, J.; Wagner, K.; Fischer, P. *Synthesis* **1987**, 236–241.
15. Katritzky, A. R.; Suzuki, K.; Singh, S. K. *Synthesis* **2004**, 2645–2652.
16. Detsi, A.; Micha-Screttas, M.; Igglessi-Markopoulou, O. *J. Chem. Soc., Perkin Trans. 1* **1998**, 2443–2450.
17. The keto and cyclic forms of this compound were not seen in the $^{13}C$ NMR spectrum probably due to the low sensitivity for carbon in NMR.
18. Brimble, M. A.; Trotter, N. S.; Harris, W. R.; Sieg, F. *Bioorg. Med. Chem.* **2005**, *13*, 519–532.
19. We found that $^{19}F$ NMR analysis was the best method to determine the structure of these compounds in solution in cases where the amount of available material for NMR studies was scarce.
20. Zhang, H.-Z.; Zhang, H.; Kemnitzer, W.; Tseng, B.; Cinatl, J., Jr.; Michaelis, M.; Doerr, H. W.; Cai, S. X. *J. Med. Chem.* **2006**, *49*, 1198–1201.
21. Kaldor, S. W.; Hammond, M.; Dressman, B. A.; Labus, J. M.; Chadwell, F. W.; Kline, A. D.; Heinz, B. A. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2021–2026.
22. Kong, J.; Venkatraman, S.; Furness, K.; Nimkar, S.; Shepherd, T. A.; Wang, Q. M.; Aube, J.; Hanzlik, R. P. *J. Med. Chem.* **1998**, *41*, 2579–2587.
23. For details, see Section 4.
24. Lee, B. H.; Miller, M. J. *Tetrahedron Lett.* **1984**, *25*, 927–930.
25. Ducrot, P.; Rabhi, C.; Thal, C. *Tetrahedron* **2000**, *56*, 2683–2692.
26. Oya, M.; Takahashi, T. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 2705–2707.
27. Breddam, K.; Widmer, F.; Johansen, J. T. Eur. Pat. Appl. EP 17485, 1980; *Chem. Abstr.* **1980**, *94*, 137817.
28. (a) Burkhart, J. P.; Koehl, J. R.; Mehdi, S.; Durham, S. L.; Janusz, M. J.; Huber, E. W.; Angelastro, M. R.; Sunder, S.; Metz, W. A.; Shum, P. W.; Chen, T.-M.; Bey, P.; Cregge, J. R.; Peet, N. P. *J. Med. Chem.* **1995**, *38*, 223–233; (b) Doherty, A. M.; Sircar, I.; Kornberg, B. E.; Quin, J., III; Winters, R. T.; Kaltenbronn, J. S.; Taylor, M. D.; Batley, B. L.; Rapundalo, S. R.; Ryan, M. J.; Painchaud, C. A. *J. Med. Chem.* **1992**, *35*, 2–14; (c) Skiles, J. W.; Fuchs, V.; Miao, C.; Sorcek, R.; Grozinger, K. G.; Mauldin, S. C.; Vitous, J.; Mui, P. W.; Jacober, S.; Chow, G.; Matteo, M.; Skoog, M.; Weldon, S. M.; Possanza, G.; Keirns, J.; Letts, G.; Rosenthal, A. S. *J. Med. Chem.* **1992**, *35*, 641–662.
29. Kuo, C. J.; Chi, Y. H.; Hsu, J. T. A.; Liang, P. H. *Biochem. Biophys. Res. Commun.* **2004**, *318*, 862–867.
30. Bacha, U.; Barrila, J.; Velazquez-Campoy, A.; Leavitt, S. A.; Freire, E. *Biochemistry* **2004**, *43*, 4906–4912.
31. Copeland, R. A. *Enzymes: A Practical Introduction to Structure, Mechanism and Data Analysis*, 2nd ed.; Wiley-VCH: New York, NY, 2000.

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# '*O*-Acyl isopeptide method': racemization-free segment condensation in solid phase peptide synthesis

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**Abstract**—We disclosed a novel 'racemization-free segment condensation' based on the '*O*-acyl isopeptide method' in which an N-segment including C-terminal *O*-acyl isopeptide structure with urethane-protected Ser/Thr residue was employed for the segment condensation, suggesting that the use of this method contributes to the effective convergent synthesis of long peptides/proteins.


Total chemical synthesis of peptides/proteins is of great significance to understand biological functions. Toward this purpose, many kinds of convergent synthetic methods have been reported.[1] However, a fundamental drawback of convergent synthesis is that racemization at the C-terminal residue of an N-segment occurs during the condensation reaction with the C-segment. In 'segment condensation',[1g–r] which is one of the important methods in convergent synthesis, a large amount of racemization is generally involved. Particularly, in solid phase segment condensation,[1k–r] the lower reactivity causes a higher extent of racemization as compared with solution phase synthesis. That is because, in contrast to urethane-protected amino acids, peptides easily form chirally labile oxazolones upon C-terminal carboxyl activation, limiting the N-segment to contain either a C-terminal Gly or Pro residue.[1j,o,r]

We have recently disclosed a novel '*O*-acyl isopeptide method'[2] in which a native amide bond at a hydroxy-amino acid residue, for example, Ser, was isomerized to an ester bond, followed by an O–N intramolecular acyl migration reaction (Fig. 1A). The method has been successfully applied to efficiently synthesize difficult sequence-containing peptides such as Alzheimer's disease-related amyloid β peptide (Aβ) 1–42.[2c–g,i] Our studies indicated that isomerization of the peptide backbone at only one position in the whole peptide sequence, that is, formation of a single ester bond, significantly changed the unfavorable secondary structure of the difficult sequence-containing peptide, leading to improved coupling and deprotection efficacy during SPPS. Mutter et al.,[3] Carpino et al.,[4] and Börner and co-workers[5] have also confirmed the efficacy of the '*O*-acyl isopeptide method'. Moreover, very recently, we designed a novel '*O*-acyl isodipeptide unit', that is, Boc-Ser/Thr(Fmoc-Xaa)-OH (Fig. 1B).[2h,i] The use of *O*-acyl isodipeptide units, in which the racemization-inducing esterification reaction on resin could be omitted, allows the '*O*-acyl isopeptide method' to fully automated protocols for the synthesis of peptides/proteins.

Herein, we disclosed a novel 'racemization-free segment condensation' based on the '*O*-acyl isopeptide method' (Fig. 2B). We conceived the idea that the N-segment, which possesses a C-terminal *O*-acyl isopeptide structure, could be coupled to the N-terminal amino group of a C-segment without any undesired racemization because the isopeptide structure includes a urethane-protected Ser/Thr residue. Thus, during the activation of the carboxyl group of the isopeptide, the formation of racemization-inducing oxazolones should be remarkably suppressed.

*Keywords*: *O*-Acyl isodipeptide unit; *O*-Acyl isopeptide method; O–N intramolecular acyl migration; Racemization-free; Segment condensation.
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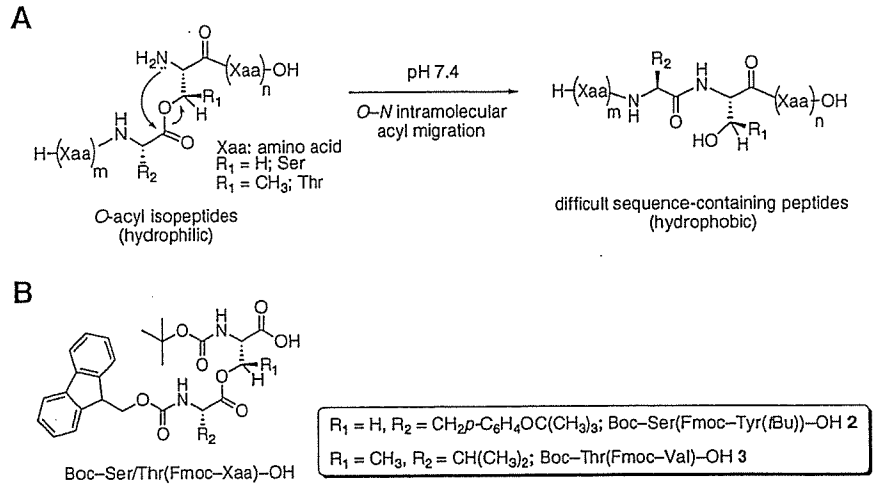



Figure 1. (A) '*O*-Acyl isopeptide method': the synthetic strategy for difficult sequence-containing peptides via the O–N intramolecular acyl migration reaction of *O*-acyl isopeptides; (B) '*O*-acyl isodipeptide units'.

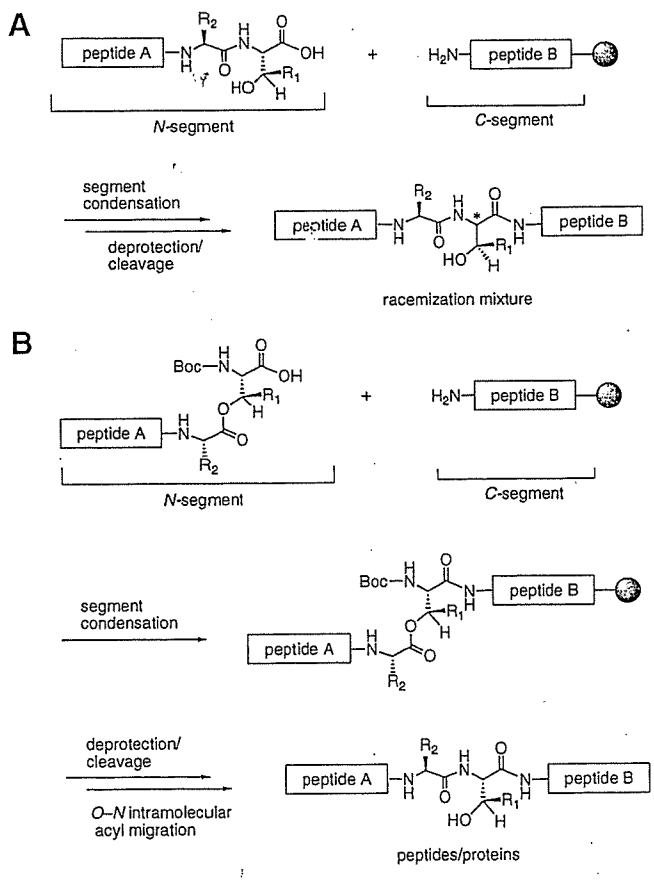



Figure 2. (A) A standard segment condensation; (B) a novel 'racemization-free segment condensation' based on the '*O*-acyl isopeptide method'.

To evaluate this hypothesis, we first selected Fmoc-Tyr-Ser-Phe-OH (1) as a model. As a comparative study, 1 was synthesized by the standard segment condensation method.[6] Fmoc-Tyr(*t*Bu)-Ser(*t*Bu)-OH was coupled to H-Phe-O-resin (2-chlorotrityl resin) using the DIPCDI(1,3-diisopropylcarbodiimide, 2.5 equiv)–HOBt(1-hydroxybenzotriazole, 2.5 equiv) method to obtain Fmoc-Tyr(*t*Bu)-Ser(*t*Bu)-Phe-O-resin. After the protected peptide resin was deprotected with TFA, the resulting crude **1** was analyzed by HPLC. As a result, 3.0% of Fmoc-Tyr-D-Ser-Phe-OH was detected in crude **1** (Fig. 3A), which was confirmed by an independent synthesis of the D-Ser derivative. This result indicated that racemization at the activated Ser residue occurred during segment condensation.

On the other hand, in segment condensation based on the '*O*-acyl isopeptide method',[7] *O*-acyl isodipeptide unit, Boc-Ser(Fmoc-Tyr(*t*Bu))-OH[8] (**2**, Fig. 1B) was coupled to H-Phe-O-resin using the DIPCDI(2.5 equiv)–HOBt(2.5 equiv) method to obtain Boc-Ser-(Fmoc-Tyr(*t*Bu))-Phe-O-resin. After deprotection with TFA, the obtained isopeptide H-Ser(Fmoc-Tyr)-Phe-OH·TFA was treated with phosphate buffer (pH 7.4) to induce an O–N intramolecular acyl migration to afford **1**. In HPLC analysis of crude **1**, no detectable racemized compound Fmoc-Tyr-D-Ser-Phe-OH was observed (Fig. 3B), indicating that the *O*-acyl isodipep-

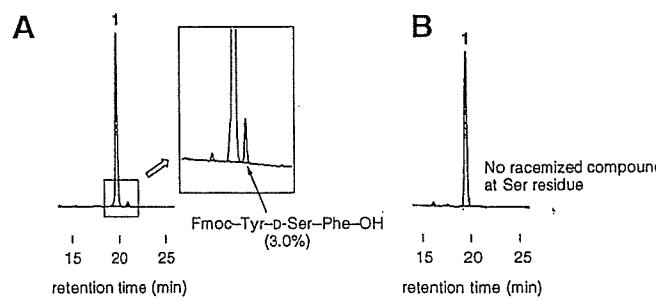



Figure 3. HPLC profiles of crude peptide Fmoc-Tyr-Ser-Phe-OH (1) synthesized using (A) the standard segment condensation and (B) '*O*-acyl isopeptide method'-based segment condensation. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of $CH_3CN$ (35–55% $CH_3CN$, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL $min^{-1}$ (40 °C), detected at 230 nm.

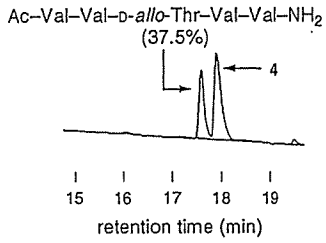



Figure 4. HPLC profile of crude 4 synthesized using a standard segment condensation. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of $CH_3CN$ (0–100% $CH_3CN$, 40 mm) in 0.1% aqueous TFA at a flow rate of 0.9 mL $min^{-1}$ (40 °C), detected at 230 nm.

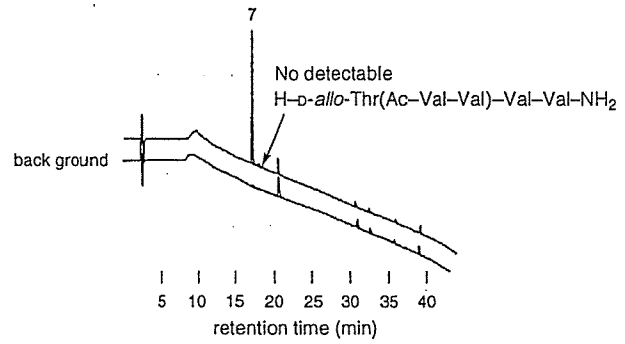
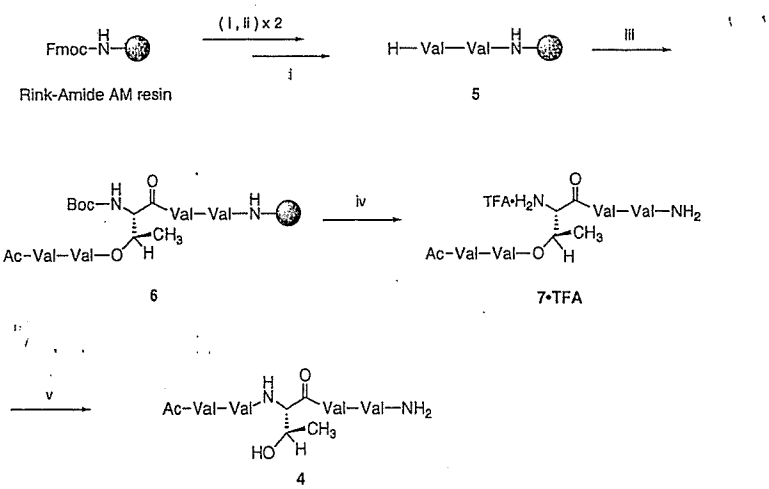



Figure 5. HPLC profile of crude isopeptide 7 (Rt = 17.0 min) synthesized using the '*O*-acyl isopeptide method'-based segment condensation. The retention time of H-D-*allo*-Thr(Ac-Val-Val)-Val-Val-$NH_2$, which was synthesized independently, was 17.8 min. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of $CH_3CN$ (0–100% $CH_3CN$, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL $min^{-1}$ (40 °C), detected at 230 nm.

tide unit could be introduced to the amino group on the resin without any racemization at the activated Ser residue in the isopeptide structure.

To further elucidate the efficacy of this '*O*-acyl isopeptide method'-based segment condensation, pentapeptide Ac-Val-Val-Thr-Val-Val-$NH_2$[2h,i] (4) was adopted. In the condensation of Ac-Val-Val-Thr(*t*Bu)-OH with H-Val-Val-NH-resin (as a standard segment condensation),[9] a large amount of racemization (37.5%) at the activated Thr residue occurred during the DIPCDI–HOBt segment condensation (Fig. 4), which was confirmed by an independent synthesis of Ac-Val-Val-D-*allo*-Thr-Val-Val-$NH_2$. In contrast, in the '*O*-acyl isopeptide method'-based segment condensation (Scheme 1),[10] N-segment Boc-Thr(Ac-Val-Val)-OH,[11] which was synthesized using *O*-acyl isodipeptide unit Boc-Thr(Fmoc-Val)-OH 3 (Fig. 1B),[2h] was coupled to C-segment H-Val-Val-NH-resin (5) to obtain isopeptide resin 6. The DIPCDI(2.5 equiv)–HOBt(2.5 equiv) method in DMF (2 h) was employed for segment condensation, in which N-segment Boc-Thr(Ac-Val-Val)-OH was readily solubilized. The completeness of the coupling was verified by the Keiser test. After TFA treatment, *O*-acyl isopeptide 7·TFA was obtained with an isolated yield of 69%. As shown in Figure 5, HPLC analysis of crude 7 exhibited a high purity of the desired product without any byproduct derived from racemization at Thr, which was confirmed by an independent synthesis of H-D-*allo*-Thr(Ac-Val-Val)-Val-Val-$NH_2$. Moreover, the use of an N-segment with a C-terminal isopeptide did not lead to any additional side reaction. Isopeptide 7 was converted to 4 in phosphate buffered saline at pH 7.4.[2h] These results reveal that a protected *O*-acyl isopeptide with a C-terminal Boc-Thr residue could be introduced to the peptide resin without any racemization at the activated Thr residue, in contrast to the standard method using Ac-Val-Val-Thr(*t*Bu)-OH that involved a significant amount of racemization during condensation to the solid support.

In summary, we herein developed a novel 'racemization-free segment condensation' based on the '*O*-acyl isopeptide method' with the successful synthesis of small peptides. This method allows the use of an N-segment




Scheme 1. Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-Val-OH (2.5 equiv), DIPCDI (1,3-diisopropylcarbodiimide, 2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iii) Boc-Thr(Ac-Val-Val)-OH (2.5 equiv), DIPCDI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iv) TFA–*m*-cresol–thioanisole–$H_2O$ (92.5:2.5:2.5:2.5), 90 min; (v) phosphate buffered saline, pH 7.4, 25 °C.

possessing a C-terminal Ser/Thr residue for segment condensation, without any racemization, as a result of the C-terminal *O*-acyl isopeptide structure with a urethane-protected Ser/Thr residue. Thus, in the synthesis of long peptides/proteins, racemization-free segment condensation becomes possible at not only the C-terminal Gly/Pro but also Ser/Thr residues of the N-segment. Additionally, final deprotected peptides/proteins synthesized using the '*O*-acyl isopeptide method'-based segment condensation are effectively purified by HPLC, because a simple isomerization to an *O*-acyl isopeptide remarkably and temporarily changes the physicochemical properties of the native peptide, and an O–N intramolecular acyl migration triggers the native amide bond formation under physiological conditions.[2] Examples of such studies include membrane peptides/proteins that are difficult to handle in various conditions because of their high self-assembling characters.

## Acknowledgments

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## References and notes

1. (a) Kiso, Y.; Yajima, H. In *Peptides: Synthesis, Structures, and Applications*; Gutte, B., Ed.; Academic Press: San Diego, 1995; pp 39–91; (b) Bang, D.; Pentelute, B. L.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2006**, *45*, 3985–3988; (c) Lu, Y.-A.; Tam, J. P. *Org. Lett.* **2005**, *7*, 5003–5006; (d) Muralidharan, V.; Muir, T. W. *Nature Methods* **2006**, *3*, 429–438; (e) Ohta, Y.; Itoh, S.; Shigenaga, A.; Shintaku, S.; Fujii, N.; Otaka, A. *Org. Lett.* **2006**, *8*, 467–470; (f) Kawakami, T.; Sumida, M.; Nakamura, K.; Vorherr, T.; Aimoto, S. *Tetrahedron Lett.* **2005**, *46*, 8805–8807; (g) Akaji, K.; Tamai, Y.; Kiso, Y. *Tetrahedron* **1997**, *53*, 567–584; (h) Kawakami, T.; Hasegawa, K.; Teruya, K.; Akaji, K.; Horiuchi, M.; Inagaki, F.; Kurihara, Y.; Uesugi, S.; Aimoto, S. *J. Pept. Sci.* **2001**, *7*, 474–487; (i) Inui, T.; Bódi, J.; Nishio, H.; Nishiuchi, Y.; Kimura, T. *Lett. Pept. Sci.* **2002**, *8*, 319–330; (j) Makino, T.; Matsumoto, M.; Suzuki, Y.; Kitajima, Y.; Yamamoto, K.; Kuramoto, M.; Minamitake, Y.; Kangawa, K.; Yabuta, M. *Biopolymers* **2005**, *79*, 238–247; (k) Benz, H. *Synthesis* **1994**, 337–358; (l) Habermann, J.; Kunz, H. *Tetrahedron Lett.* **1998**, *39*, 4797–4800; (m) Hamuro, Y.; Scialdone, M. A.; DeGrado, W. F. *J. Am. Chem. Soc.* **1999**, *121*, 1636–1644; (n) Barlos, K.; Gatos, D. *Biopolymers* **1999**, *51*, 266–278; (o) Kitagawa, K.; Aida, C.; Fujiwara, H.; Yagami, T.; Futaki, S. *Chem. Pharm. Bull.* **2001**, *49*, 958–963; (p) Nishiuchi, Y.; Nishio, H.; Ishimaru, M.; Kimura, T. In *Peptide Science 2004*, Shimohigashi, Y., Ed.; 2005, pp 131–134.; (q) Dörner, B.; Steinauer, R.; Barthélémy, S.; White, P. D. In *Peptide Science 2004*, Shimohigashi, Y., Ed.; 2005, pp 51–54; (r) Goulas, S.; Gatos, D.; Barlos, K. *J. Pept. Sci.* **2006**, *12*, 116–123.
2. (a) Sohma, Y.; Sasaki, M.; Ziora, Z.; Takahashi, N.; Kimura, T.; Hayashi, Y.; Kiso, Y. *Peptides, Peptide Revolution: Genomics, Proteomics and Therapeutics*; Kluwer Academic: Netherlands, 2003, pp 67–68; (b) Sohma, Y.; Sasaki, M.; Hayashi, Y.; Kimura, T.; Kiso, Y. *Chem. Commun.* **2004**, 124–125; (c) Sohma, Y.; Sasaki, M.; Hayashi, Y.; Kimura, T.; Kiso, Y. *Tetrahedron Lett.* **2004**, *45*, 5965–5968; (d) Sohma, Y.; Hayashi, Y.; Skwarczynski, M.; Hamada, Y.; Sasaki, M.; Kimura, T.; Kiso, Y. *Biopolymers* **2004**, *76*, 344–356; (e) Sohma, Y.; Hayashi, Y.; Kimura, M.; Chiyomori, Y.; Taniguchi, A.; Sasaki, M.; Kimura, T.; Kiso, Y. *J. Pept. Sci.* **2005**, *11*, 441–451; (f) Sohma, Y.; Chiyomori, Y.; Kimura, M.; Fukao, F.; Taniguchi, A.; Hayashi, Y.; Kimura, T.; Kiso, Y. *Bioorg. Med. Chem.* **2005**, *13*, 6167–6174; (g) Taniguchi, A.; Sohma, Y.; Kimura, M.; Okada, T.; Ikeda, K.; Hayashi, Y.; Kimura, T.; Hirota, S.; Matsuzaki, K.; Kiso, Y. *J. Am. Chem. Soc.* **2006**, *128*, 696–697; (h) Sohma, Y.; Taniguchi, A.; Skwarczynski, M.; Yoshiya, T.; Fukao, F.; Kimura, T.; Hayashi, Y.; Kiso, Y. *Tetrahedron Lett.* **2006**, *47*, 3013–3017; (i) Sohma, Y.; Kiso, Y. *ChemBiochem*, published on web on 17-Aug-2006.
3. (a) Mutter, M.; Chandravarkar, A.; Boyat, C.; Lopez, J.; Santos, S. D.; Mandal, B.; Mimna, R.; Murat, K.; Patiny, L.; Saucède, L.; Tuchscherer, G. *Angew. Chem., Int. Ed.* **2004**, *43*, 4172–4178; (b) Santos, S. D.; Chandravarkar, A.; Mandal, B.; Mimna, R.; Murat, K.; Saucède, L.; Tella, P.; Tuchscherer, G.; Mutter, M. *J. Am. Chem. Soc.* **2005**, *127*, 11888–11889.
4. (a) Carpino, L. A.; Krause, E.; Sferdean, C. D.; Schümann, M.; Fabian, H.; Bienert, M.; Beyermann, M. *Tetrahedron Lett.* **2004**, *45*, 7519–7523; (b) Coin, I.; Dölling, R.; Krause, E.; Bienert, M.; Beyermann, M.; Sferdean, C. D.; Carpino, L. A. *J. Org. Chem.* **2006**, *71*, 6171–6177.
5. Hentschel, J.; Krause, E.; Börner, H. G. *J. Am. Chem. Soc.* **2006**, *128*, 7722–7723.
6. Protected peptide Fmoc-Tyr(*t*Bu)-Ser(*t*Bu)-OH (2.5 equiv) was coupled to H-Phe-O-resin (2-chlorotrityl resin, 0.055 mmol) in the presence of DIPCDI (2.5 equiv) and HOBt (2.5 equiv) in DMF for 2 h. The peptide was cleaved from the resin using TFA–thioanisole–*m*-cresol–$H_2O$ (92.5:2.5:2.5:2.5) for 90 min at rt, concentrated in vacuo, washed with $Et_2O$, centrifuged, suspended with water, and lyophilized to give the crude Fmoc-Tyr-Ser-Phe-OH **1**. ESI-MS: calcd for $(M+Na)^+$: 660.2, found: 660.0. The retention time on HPLC (0–100% $CH_3CN$ for 40 min, 230 nm) of synthesized product was identical to that of **1** which was synthesized independently by the standard Fmoc-based SPPS.
7. *O*-Acyl isodipeptide unit, Boc-Ser(Fmoc-Tyr(*t*Bu))-OH[8] **2** (2.5 equiv) was coupled to H-Phe-O-resin (2-chlorotrityl resin, 0.058 mmol) in the presence of DIPCDI (2.5 equiv) and HOBt (2.5 equiv) in DMF for 2 h. The crude H-Ser(Fmoc-Tyr)-Phe-OH was obtained in a same deprotection manner described in Ref. 6. The isopeptide was dissolved in phosphate buffer and stirred for 6 h at rt to give the crude **1**. ESI-MS: calcd for $(M+H)^+$: 638.2, found: 638.0. The retention time on HPLC (0–100% $CH_3CN$ for 40 min, 230 nm) of synthesized product was identical to that of **1** which was synthesized independently by the standard Fmoc-based SPPS.
8. EDC·HCl (623 mg, 3.25 mmol) was added to a stirring solution of Boc-Ser-OBzl[12] (400 mg, 1.35 mmol), Fmoc-Tyr(*t*Bu)-OH (1.5 g, 3.25 mmol), and DMAP (16.6 mg, 0.136 mmol) in dry $CHCl_3$ (40 mL) at 0 °C. The mixture was slowly warmed to rt over 2 h, stirred additionally for

16 h, diluted with AcOEt, and washed successively with water, 1 M HCl, water, a saturated $NaHCO_3$, and brine. The organic layer was dried over $Na_2SO_4$ and the solvent was removed in vacuo. The resulting oil was purified by silica gel column chromatography (AcOEt–hexane 1:3.5) to yield Boc-Ser(Fmoc-Tyr(*t*Bu))-OBzl (889 mg, 1.23 mmol, 91%). After that, Pd/C was added (87 mg) to the stirring solution of the Boc-Ser(Fmoc-Tyr(*t*Bu))-OBzl (856 mg, 1.18 mmol) in AcOEt (36 mL), and the reaction mixture was vigorously stirred under a hydrogen atmosphere for 16 h. The catalyst was filtered off through Celite. The solvent was removed in vacuo and the crude product was filtered via silica gel, at first with AcOEt–hexane 1:2.5 and then the final product was washed out by methanol to give pure **2** (708 mg, 1.09 mmol, 93%). HRMS (FAB): calcd for $C_{36}H_{42}N_2O_9Na$ $(M+Na)^+$: 669.2788, found: 669.2783; HPLC analysis at 230 nm: purity was higher than 95%; NMR ($CD_3OD$, 300 MHz): $\delta$ 7.77 (d, $J=7.4$ Hz, 2H), 7.63–7.60 (m, 2H), 7.38 (t, $J=7.1$ Hz, 2H), 7.32–7.27 (m, 2H), 7.13 (d, $J=8.3$ Hz, 2H), 6.85 (d, $J=8.4$ Hz, 2H), 4.84–4.09 (m, 7H), 3.25–3.15 (m, 1H), 2.91–2.83 (m, 1H), 1.41 (s, 9H), 1.22 (s, 9H).

9. The protected peptide Ac-Val-Val-Thr(*t*Bu)-OH (2.5 equiv) was coupled to H-Val-Val-NH-resin (Rink-amide AM resin, 0.065 mmol) in the presence of DIPCDI (2.5 equiv) and HOBt (2.5 equiv) in DMF for 2 h. Crude **4** was obtained in a similar deprotection manner described in Ref. 6. ESI-MS: calcd for $(M+Na)^+$: 579.4, found: 579.3. The retention time on HPLC (0–100% $CH_3CN$ for 40 min, 230 nm) of the synthesized product was identical to that of **4** which was synthesized previously.[2h]
10. After the preparation of the H-Val-Val-NH-resin (Rink-amide AM resin, 0.009 mmol), Boc-Thr(Ac-Val-Val)-OH[11] (2.5 equiv) was coupled in the presence of DIPCDI (2.5 equiv) and HOBt (2.5 equiv) in DMF for 2 h at rt. The crude *O*-acyl isopeptide **7**·TFA was obtained in a similar deprotection manner described in Ref. 6. Yield: 69% (calculated from the original loading of Rink-amide AM resin). HRMS (FAB): calcd for $C_{26}H_{45}N_6O_7$ $(M+H)^+$: 557.3663, found: 557.3656; HPLC analysis at 230 nm: purity was higher than 95%. The retention time on HPLC (0–100% $CH_3CN$ for 40 min, 230 nm) of the synthesized product was identical to that of **7** which was synthesized previously.[2h]
11. After *O*-acyl isodipeptide unit, Boc-Thr(Fmoc-Val)-OH[2h] **3** was loaded to 2-chlorotrityl resin, subsequent coupling with Fmoc-Val-OH, N-acetylation using $Ac_2O$, 0.1% TFA treatment, and HPLC purification gave pure Boc-Thr(Ac-Val-Val)-OH. ESI-MS: calcd for $(M+Na)^+$: 482.3, found: 482.1.
12. Skwarczynski, M.; Sohma, Y.; Noguchi, M.; Hayashi, Y.; Kimura, T.; Kiso, Y. *J. Org. Chem.* **2006**, *71*, 2542–2545.

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# "Click Peptides"—Chemical Biology-Oriented Synthesis of Alzheimer's Disease-Related Amyloid β Peptide (Aβ) Analogues Based on the "*O*-Acyl Isopeptide Method"

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*A clear understanding of the pathological mechanism of amyloid β peptide (Aβ) 1–42, a currently unexplained process, would be of great significance for the discovery of novel drug targets for Alzheimer's disease (AD) therapy. To date, though, the elucidation of these Aβ1–42 dynamic events has been a difficult issue because of uncontrolled polymerization, which also poses a significant obstacle in establishing experimental systems with which to clarify the pathological function of Aβ1–42. We have recently developed chemical biology-oriented pH- or phototriggered "click peptide" isoform precursors of Aβ1–42, based on the "O-acyl isopeptide method", in which a native amide bond at a hydroxy-amino acid residue, such as Ser, is isomerized to an ester bond, the target peptide subsequently being generated by an O–N intramolecular acyl migration reaction. These click peptide precursors did not exhibit any self-assembling character under physiological conditions, thanks to the presence of the one single ester bond, and were able to undergo migration to give the target Aβ1–42 in a quick and easy, one-way (so-called "click") conversion reaction. The use of click peptides could be a useful strategy to investigate the biological functions of Aβ1–42 in AD through inducible activation of Aβ1–42 self-assembly.*

## 1) Introduction

Amyloid β peptides (Aβs) are the main proteinaceous components of the pathognomonic amyloid plaques found in the brains of Alzheimer's disease (AD) patients.[1] These Aβs have been found to be neurotoxic in vivo and in vitro.[2] Although the predominant forms of Aβ consist of 40- and 42-residue peptides, designated Aβ1–40 and Aβ1–42, respectively, Aβ1–42 is thought to play a more critical role than Aβ1–40 in amyloid formation and pathogenesis of AD.[3] Aβs are produced through the proteolytic processing of amyloid precursor protein (APP) and, according to the "amyloid cascade hypothesis", reduction of Aβ in the brain is considered to be a therapeutic intervention in AD. One of the most attractive classes of drug candidates in the treatment of AD are BACE1 inhibitors, as BACE1 (β-site APP cleaving enzyme, β-secretase) is a membrane-bound aspartic protease that triggers Aβ formation by cleaving at the N terminus of the Aβ domain.[4] In over a decade of inhibitor studies on aspartic proteases,[5] we have reported potent BACE1 inhibitors containing an unnatural amino acid—phenylnorstatine (Pns = (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid)—as a substrate transition-state mimic (Scheme 1).[6] A clear understanding of the toxicological mechanism of Aβ against neuronal cells, currently an unknown process, would also open doors for the discovery of novel drug targets, so "chemical biology"-based research represents one of the most powerful tools for identifying the pathological function of Aβ in AD. In this article we review our recent achievements in the "*O*-acyl isopeptide method" as a novel synthetic method in the field of peptide chemistry and its application to the chemical biology-oriented synthesis of Aβ analogues, leading to the development of "click peptides" (Scheme 2).[7–14]

## 2) The "*O*-Acyl Isopeptide Method"—Efficient Synthesis of Peptides Containing "Difficult Sequences"

The synthesis of peptides containing "difficult sequences" is one of the most problematic areas in peptide chemistry.[15] These peptides are often obtained with low yields and purities in solid-phase peptide synthesis (SPPS), the difficult sequences generally being hydrophobic and promoting aggregation in solvents during synthesis and purification. This aggregation is attributed to intermolecular hydrophobic interaction and hydrogen bond networks between resin-bound peptide chains, resulting in the formation of extended secondary structures such as β-sheets.[16]

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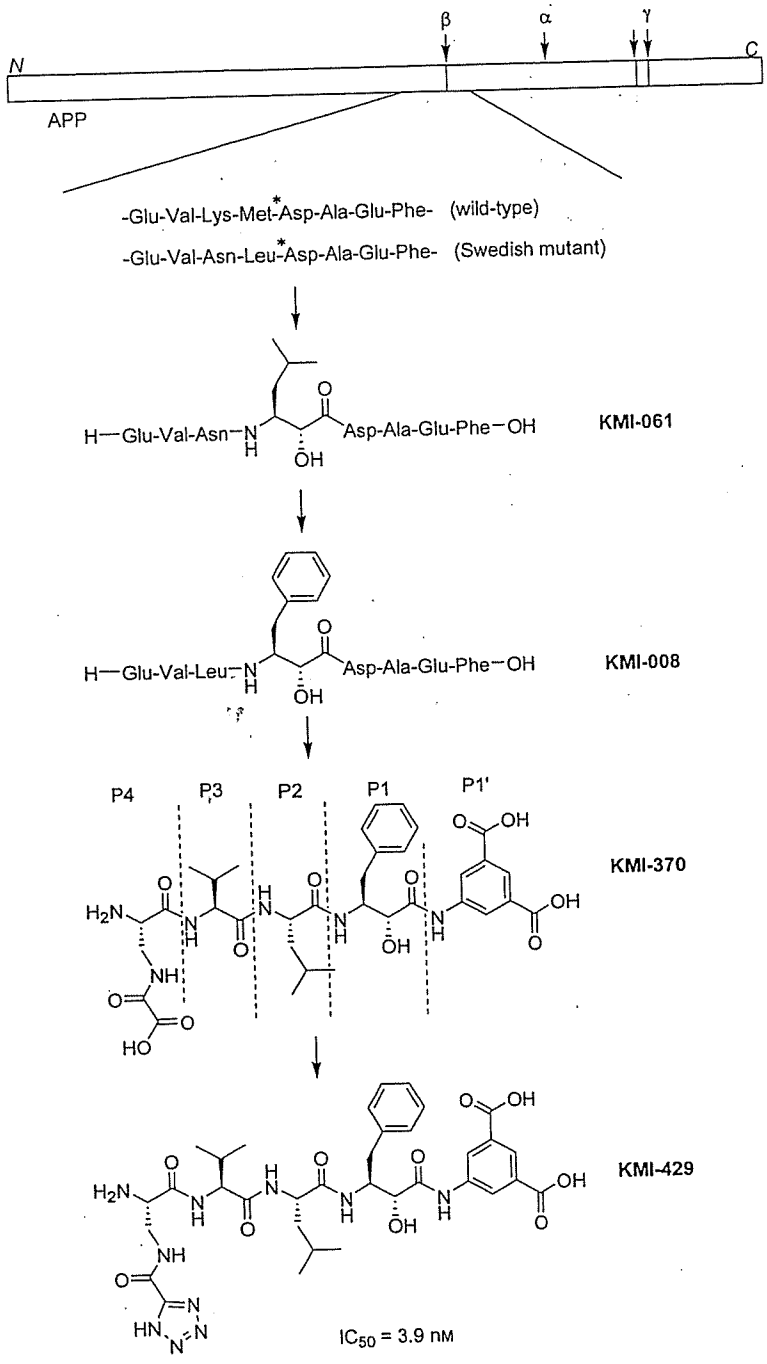


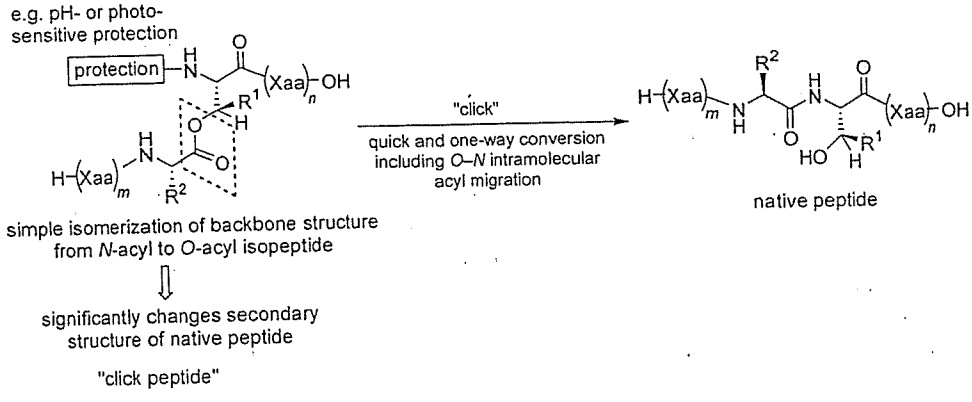

**Scheme 1.** BACE1 inhibitors containing an unnatural amino acid—phenylnorstatine [(2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid]—as a substrate transition state mimic.

Several years ago, when we tried to synthesize some peptide derivatives including phenylnorstatine for the study of aspartic protease inhibitors, some of the synthesized compounds could not be purified by preparative-scale HPLC, due to their extremely low solubilities in various solvents. These peptide derivatives thus came to be viewed as "difficult sequence"-containing peptides.

For over a decade we have been using a pH-dependent "*O*–*N* intramolecular acyl migration", a well known reaction seen in Ser/Thr-containing peptides,[17] in the design of a novel class of water-soluble prodrugs of HIV-1 protease inhibitors[18] and antitumor taxoid derivatives.[19] These prodrugs, which are *O*-acyl isoforms of parent drugs possessing α-hydroxy-β-amino acids, had higher water solubility because of the presence of a newly formed and ionized amino group. Moreover, migration to afford the *N*-acyl parent drugs could be achieved in short times under physiological conditions with no side reactions.

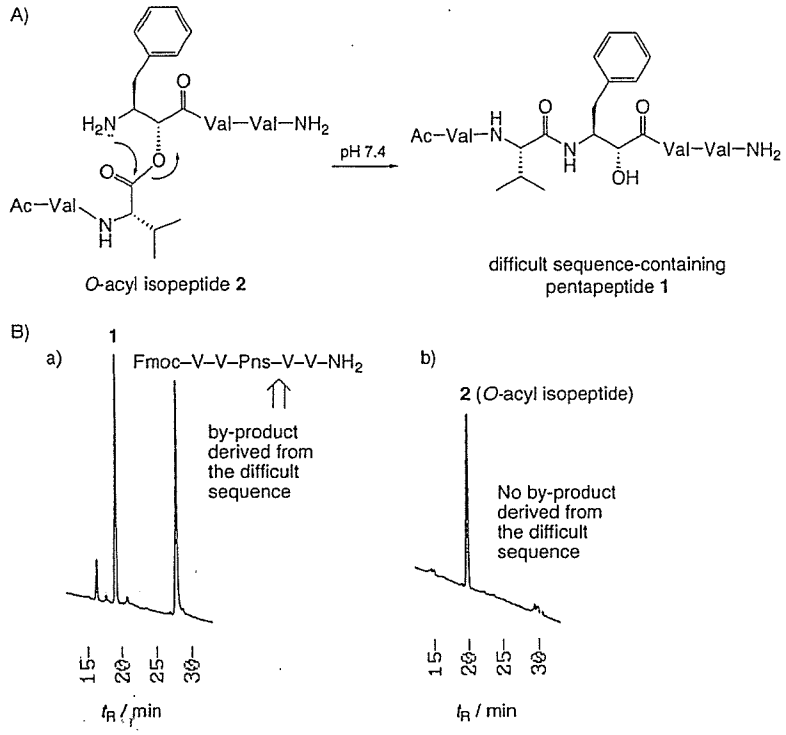
In 2003,[7,8] we conceived the idea in this context that the use of hydrophilic "*O*-acyl isopeptides" derived from phenylnorstatine-containing peptide derivatives should allow the solubility problem in HPLC purification to be overcome, and to evaluate this hypothesis a model of a peptide containing a difficult sequence (Ac-Val-Val-Pns-Val-Val-$NH_2$, **1**, Scheme 3A) was selected. During this research, however, we made the surprising discovery that not only did the "*O*-acyl isopeptide" possess higher solubility in various media, but the coupling and deprotection efficacy during SPPS had also been improved by modifying the nature of the difficult sequence (Scheme 3B). Namely, the isomerization of the peptide backbone from the *N*-acyl to the *O*-acyl isopeptide structure (i.e., the formation of a single ester bond) had significantly changed the unfavorable secondary structure of the difficult sequence-containing peptides. This finding was to result in the development of the "*O*-acyl isopeptide method" as a novel and efficient synthetic method in peptide chemistry (Scheme 4A).

In the case of the "*O*-acyl isopeptide method" based on the synthesis of **2** (Scheme 3A), Boc-Pns-OH was coupled to H-Val-Val-NH-resin, and subsequent acylation with Fmoc-Val-OH at the α-hydroxy

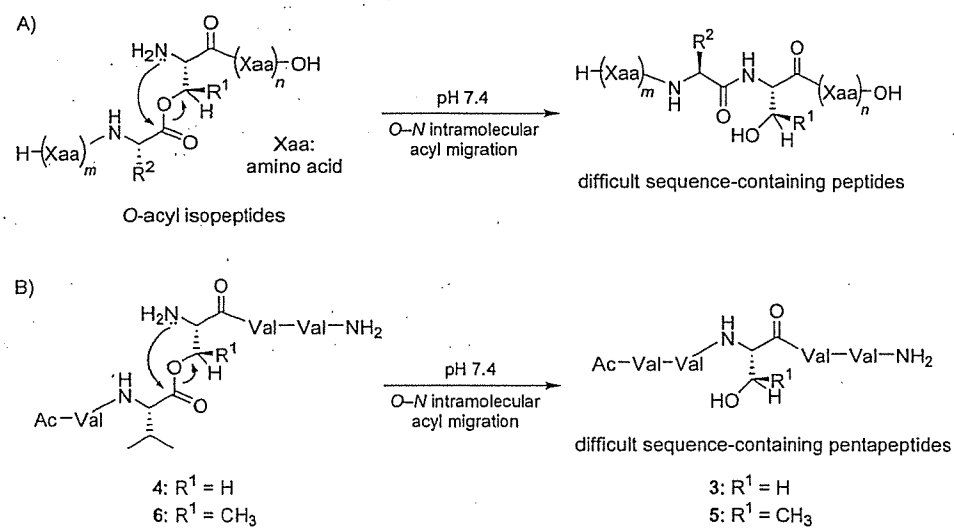



**Scheme 2.** "Click peptides" based on the "*O*-acyl isopeptide method".




**Scheme 3.** A) Synthesis of a model difficult sequence-containing pentapeptide **1** (Ac-Val-Val-Pns-Val-Val-$NH_2$) from its *O*-acyl isopeptide **2** through *O–N* intramolecular acyl migration. B) HPLC profiles after final deprotection: a) peptide **1** synthesized by a standard Fmoc-based SPPS, and b) its *O*-acyl isopeptide **2**.



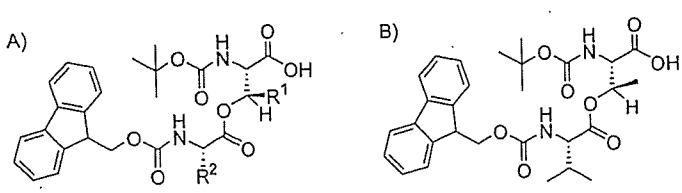

**Scheme 4.** A) "*O*-Acyl isopeptide method": the synthetic strategy for difficult sequence-containing peptides through *O–N* intramolecular acyl migration reactions of *O*-acyl isopeptides. B) Application of the "*O*-acyl isopeptide method" for the synthesis of pentapeptides **3** and **5**.

peptide-resin was efficiently synthesized with no interference from the difficult sequences. We hypothesized that the modification of **1** to the ester structure **2** had changed the secondary structure of the peptide to a form more favorable for Fmoc removal. Additionally, since H-Pns-Val-Val-$NH_2$ was not formed as a by-product, 1) the esterification of the secondary hydroxy group of Pns was successfully completed on the solid support, 2) the ester bond formed was stable to both piperidine and TFA treatments, and 3) no diketopiperazine was formed when the last Fmoc group was removed. Moreover, the solubility of **2**·TFA in polar solvents was significantly higher than that of *N*-acyl peptide **1**, because of the ionized amino group in the isopeptide. Accordingly, a solution of **2**·TFA could easily be subjected to preparative HPLC to provide pure **2** in a reasonable overall yield.[7,8,11] Similar positive results were obtained in an application of the *O*-acyl isopeptide method to the synthesis of Ac-Val-Val-Ser-Val-Val-$NH_2$ (**3**, Scheme 4B).[8,10,11]

Very recently,[14] a novel "*O*-acyl isodipeptide unit" (i.e. Boc-Ser/Thr(Fmoc-Xaa)-OH; Scheme 5A) was designed for efficient synthesis of a difficult sequence-containing peptide by the *O*-acyl isopeptide method. In this method, the racemization-prone esterification reaction could be omitted. In the synthesis of *O*-acyl isopeptide **6**—H-Thr(Ac-Val-Val)-Val-Val-$NH_2$ (Scheme 4B)—a high degree of racemization of the esterified Val residue (21%) had occurred with the DIPCDI/DMAP method (Figure 1A). Such a degree of racemization is significantly higher than that observed in the esterification between Val and Ser in **4** (0.8%),[8,10,11] and is probably due to steric hindrance at the secondary hydroxy group in Thr in relation to Ser. To avoid this problem, we adapted an *O*-acyl isodipeptide unit—Boc-Thr-(Fmoc-Val)-OH **7** (Scheme 5B)—for the synthesis of **5** by the *O*-acyl isopeptide method. *O*-Acyl isodipeptide **7**, which readily

group of Pns was carried out by the DIPCDI/DMAP (DIPCDI = 1,3-diisopropylcarbodiimide) method in $CH_2Cl_2$ to provide an ester. After coupling with another Val residue, *N*-acetylation, and TFA treatment, *O*-acyl isopeptide **2**·TFA was obtained without formation of the Fmoc-containing byproduct (Scheme 3Bb), whereas in the synthesis of **1** by standard Fmoc-based SPPS, an undesired Fmoc-Val-Val-Pns-Val-Val-$NH_2$ was obtained at a similar rate to peptide **1** (Scheme 3Ba). Hence, the protected




$R^1$ = H; Boc–Ser(Fmoc–Xaa)–OH
$R^1$ = $CH_3$; Boc–Thr(Fmoc–Xaa)–OH
"O-acyl isodipeptide unit"

Boc–Thr(Fmoc–Val)–OH, 7

Scheme 5. A) General structure of *O*-acyl isodipeptide unit, and B) *O*-acyl isodipeptide unit **7**.

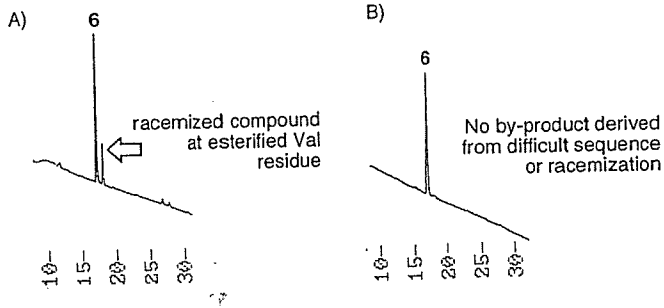



Figure 1. HPLC profiles of crude isopeptide **6** after final deprotection, A) without or B) with the use of *O*-acyl isodipeptide **7**.

dissolved in DMF, was coupled to H-Val-Val-NH-resin by the standard DIPCDI/HOBt (1-hydroxybenzotriazole) method to provide Boc-Thr(Fmoc-Val)-Val-Val-resin. As shown in Figure 1 B, HPLC analysis of crude **6** synthesized from *O*-acyl isodipeptide unit **7** was of high purity, with no byproducts arising either from the difficult sequence or from racemization. This suggests that the use of *O*-acyl isodipeptide units, enabling the racemization-inducing esterification reaction to be omitted, allows the application of the "*O*-acyl isopeptide method" to fully automated protocols for the synthesis of long peptides or proteins.

*O*-Acyl isopeptides (**2**, **4**, and **6**) were stable in a solid state (TFA salt) at 4 °C for at least 2 years. On the other hand, when an isopeptide was dissolved and stirred in phosphate-buffered saline (PBS, pH 7.4) at room temperature, quantitative *O*–*N* intramolecular acyl migration to the corresponding parent peptide was observed with no side reaction. Isopeptides **2** and **6** exhibited faster migration—with half-lives of 1 and 23 min, respectively—than that observed in the Ser-containing **4** (half-life = 2 h). The faster migration in **2** and **6** may be attributed to a unique interlocking effect of the phenyl group in Pns and the β-methyl group in Thr as a result of conformational restrictions, such as a *gem*-effect through the *geminal* methyl substitution.[20] Finally, the *N*-acyl peptides (**1**, **3**, and **5**) were formed as white precipitates from the corresponding isopeptides.

Interestingly, shortly after we had disclosed the "*O*-acyl isopeptide method", Carpino et al. and Mutter et al. also reconfirmed the efficacy of this method.[21] Carpino et al. efficiently synthesized the Jung–Redemann 26-residue peptide by utilizing the "*O*-acyl isopeptide method", whereas this peptide could not be synthesized by standard SPPS.[21b] Mutter et al. confirmed by CD-based (CD = circular dichroism) analyses that the secondary structure of an *O*-acyl isopeptide was significantly different from those of corresponding *N*-acyl native peptides,[21a,c] which agrees with our hypothesis. These reports indicate that the "*O*-acyl isopeptide method" is widely advantageous for synthesizing peptides containing difficult sequences through the disruption of the unfavorable secondary structures of the native peptides.

## 3) A "Click Peptide" by the "*O*-Acyl Isopeptide Method"—Efficient Production of Aβ1–42 from pH-Triggered Aβ1–42 Analogues

The pathological self-assembly of Aβ1–42 in amyloid plaque formation, a currently unexplained process, is very difficult to demonstrate in vitro due to uncontrolled polymerization. For example, synthesized Aβ1–42 already contains variable oligomeric forms,[22] as Aβ1–42 undergoes time- and concentration-dependent aggregation in the aqueous TFA/acetonitrile solution used in HPLC purification.[23] Moreover, the Aβ1–42 monomer easily forms aggregates even in a standard storage solution such as dimethylsulfoxide (DMSO).[24] Uncontrolled self-assembly in an in vitro experiment might cause considerable discrepancy in biological data, so this highly aggregative feature of Aβ1–42 is a significant obstacle to overcome in establishing a reliable in vitro biological experiment system with which to investigate the major causative agents of AD. The highly aggregative properties of Aβ1–42 in various media also result in synthetic difficulties with this peptide:[25] that is, this "difficult sequence"-containing peptide. In particular, in conventional reversed-phase HPLC purification (aqueous TFA/acetonitrile system) of synthesized Aβ1–42 the removal of impurities accumulated during SPPS is very laborious, due to the peptide's low solubility and broad elution profiles under either acidic or neutral conditions.

We considered that an "in situ" system that would deliver an intact Aβ1–42 (**9**) monomer under physiological experimental conditions while suppressing the spontaneous self-assembly of Aβ1–42 under storage conditions would be advantageous for understanding the inherent pathological functions of aggregative Aβ1–42 in AD. For this purpose we used the "*O*-acyl isopeptide method" as the basis for designing a novel Aβ1–42 isopeptide, "26-*O*-acyl isoAβ1–42 (26-AIAβ42, **10**, Figure 2), in which a native $Gly^{25}$–$Ser^{26}$ amide bond in Aβ1–42 had been isomerized to the β-ester bond.[9–12]

After careful selection of the appropriate resin and evaluation of the side reactions in the *O*-acyl isopeptide method,[11] protected 26-AIAβ42-resin was synthesized on 2-chlorotrityl chloride resin with minimal side reactions, and deprotected crude **10** was easily purified by HPLC thanks to its high purity and narrow elution profile with reasonable water solubility. Carpino et al. also reported similar positive results in the synthesis of Aβ1–42.[21b] This suggests that only one insertion of the isopeptide structure into the 42-residue peptide can suppress the unfavorable nature of the peptide's difficult sequence.

The water solubility of **10** (TFA salt) was 15 mg mL$^{-1}$, 100 times higher than that of Aβ1–42 (**9**, 0.14 mg mL$^{-1}$). Interest-

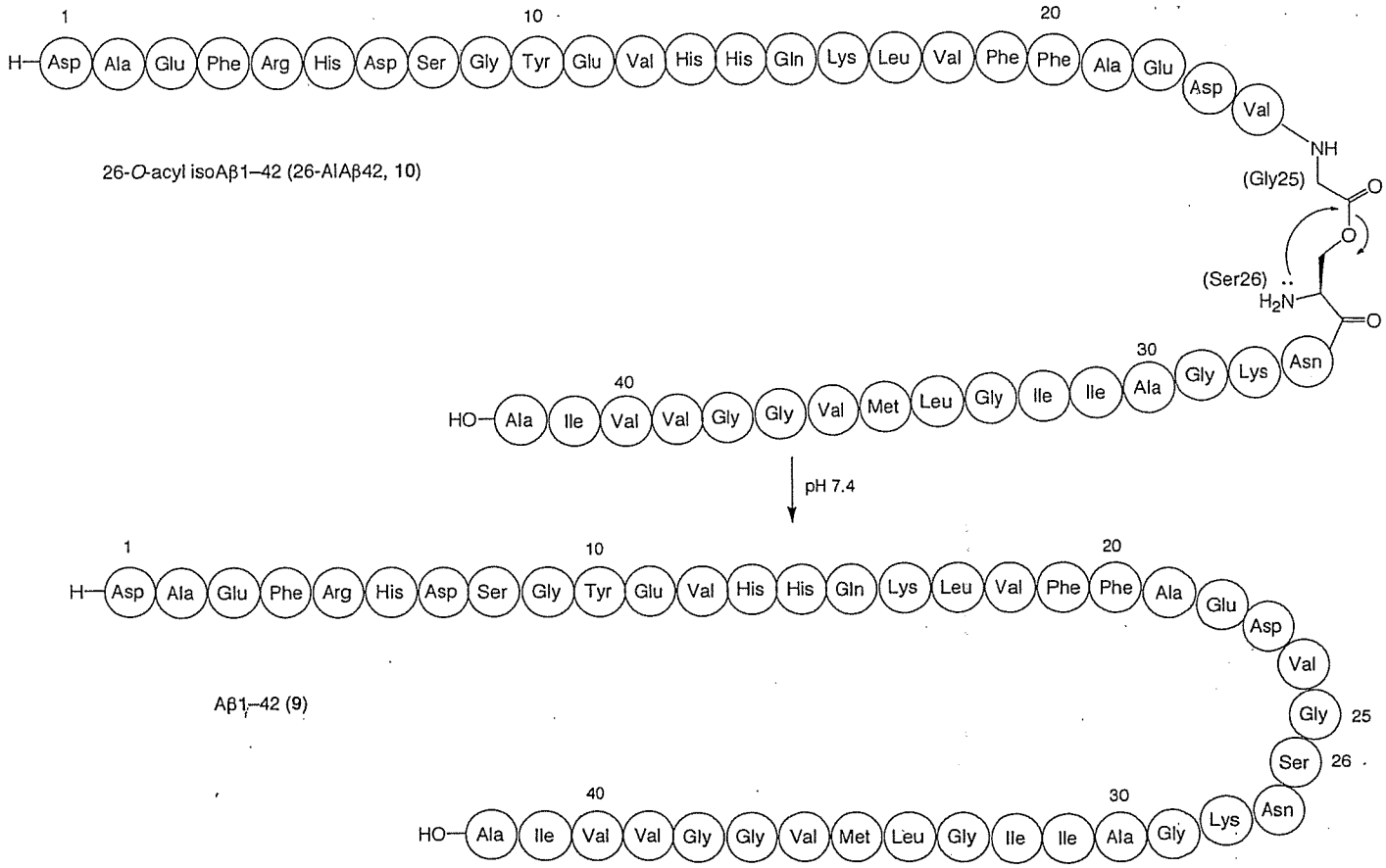



**Figure 2.** A pH-triggered "click peptide" based on the "*O*-acyl isopeptide method": production of Aβ1–42 (**9**) through the pH-dependent *O–N* intramolecular acyl migration of 26-AIAβ42 (**10**).

ingly, since isomerization of the peptide chain to the *O*-acyl isopeptide structure seems to increase the solubility of the insoluble original peptide with 42 residues drastically, this suggests that *O*-acyl isopeptides totally break the secondary structures responsible for the insolubility of the original peptide. In addition, HPLC analysis of **10** revealed a sharp peak even in a slow gradient system, while **9** was eluted as a broad peak under the same elution condition, as reported.[25] Recent solution-state NMR studies of Aβ1–40 and Aβ1–42 have indicated that the Ser26 residue produces turn- or bendlike structures that bring two β-sheets into contact and so cause hydrogen bonding interactions between peptide chains, which is associated with β-aggregation.[3d] As we have demonstrated that the use of *O*-acyl isopeptides allows the unfavorable natures of pentapeptides containing difficult sequences to be suppressed, permitting the synthesis of **10**. Thus, this method might be a powerful strategy for increasing the solubilities of even larger peptides.

As shown in Figure 3, purified **10** could be quantitatively converted into Aβ1–42 (**9**) by *O–N* intramolecular acyl migration in PBS (pH 7.4) with no side reactions such as hydrolysis of the ester bond. In PBS (pH 7.4) at 37 °C, this migration was very rapid, with a half-life of approximately 1 min, and the migration was complete after 30 min. This fast migration may be attributed to the presence of the less sterically hindered Gly25

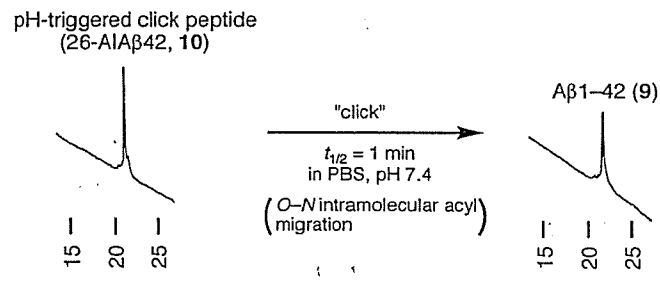



**Figure 3.** pH-triggered "click": HPLC profiles of the conversion of pH-triggered click peptide (26-AIAβ42, **10**) into the corresponding Aβ1–42 (**9**) in PBS (pH 7.4) at 37 °C.

residue. On the other hand, the TFA salt of **10** was stable at 4 °C either in the solid state or in DMSO solution. Moreover, slower migration was observed at pH 4.9, with a half-life of 3 h, with no migration at pH 3.5 after incubation for 3 h. This rapid migration under physiological conditions enables the production of an intact monomer Aβ1–42 in situ for investigation of the inherent biological function of Aβ1–42 in AD.

Not only is wildtype Aβ1–42 observed in AD, but missense mutations inside the Aβ-coding region in the APP gene are also well known. These mutations, known as Flemish- (A21G),[26] Arctic- (E22G),[27] Dutch- (E22Q),[28] Italian- (E22 K),[29] and Iowa-type (D23N)[30] are found at positions 21–23 in Aβ.

Recently, a Japanese-Tottori-type (D7N) mutation has also been reported.[31] Recent studies have discussed several differences between Aβ mutants in amyloid formation, metabolism, and elimination that are related to the progression of AD-like diseases,[32] so more detailed studies comparing these features between Aβ mutants should afford crucial information for understanding the mechanism of the diseases. With the aid of this background information, novel water-soluble isopeptides of each Aβ1–42 mutant, "26-AIAβ42 mutant", were also successfully synthesized,[12] suggesting that the *O*-acyl isopeptide method is a universal strategy for increasing the water solubility of poorly soluble peptides. Moreover, these isopeptides were converted into their corresponding intact Aβ1–42 mutants with no significant differences in half-life values under physiological conditions.

We named these *O*-acyl isopeptides pH-triggered "click peptides", since the isopeptides had the capability for "quick and one-way conversion" to the parent Aβ1–42 through pH-dependent *O–N* intramolecular acyl migration (Figures 2 and 3). These pH-triggered click peptides should provide a novel tool for biological evaluation in AD research, with the click peptides being storable in a solubilized form before use and rapidly producing intact Aβ1–42 in situ during biological experiments.

## 4) A "Click Peptide" Based on the "*O*-Acyl Isopeptide Method"—Efficient Phototriggered Production of Aβ1–42 from an Aβ1–42 Analogue

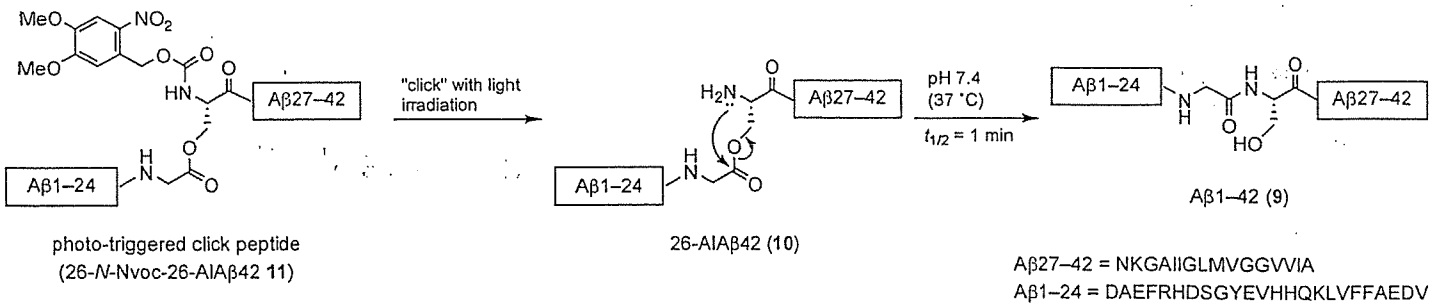
A clear understanding of the currently unexplained processes of pathological folding, self-assembly, and aggregation of Aβ1–42 would be of great significance in AD research. However, elucidation of these Aβ1–42 dynamic events is also a difficult issue due to uncontrolled polymerization.[22–24]

"Caged" compounds—synthetic molecules with their biological activities masked by covalently attached photocleavable protecting groups—are generally considered to be advantageous for the study of the dynamic processes of peptides or proteins, because, upon photoactivation, only a short duration of time is required to control the spatiotemporal dynamics of the native compounds.[33,34] However, the attachment of small photocleavable groups would be unlikely to be able to mask the spontaneous self-assembly potency of aggregative peptides, since this sort of potency is generally extremely strong and attributable to large sections of the peptide structure. To overcome this issue, Imperiali et al. introduced an additional cationic fibrilinhibitory unit, covalently attached through a photocleavable linker to an aggregative peptide derived from prion protein.[35] This analogue suppressed the self-assembling nature of the original aggregative peptide, although the fibrils formed from the original peptide released by photolysis were insufficiently dense because of a side effect involving the co-released fibril-inhibitory unit.

In a different approach to the development of a phototriggered Aβ1–42 analogue with effective inactivation of the self-assembling nature, a strategy based on an *O*-acyl isopeptide protected by a photocleavable group was planned. The *O*-acyl isopeptide was expected to be nonaggregative and to be able to convert into the inherent aggregative peptide by photo-irradiation "click" without the presence of any additional fibril-inhibitory unit. Consequently, we designed and synthesized a phototriggered "click peptide" of Aβ1–42 (**9**)—26-*N*-Nvoc-26-AIAβ42 (**11**)[13]—in which a photocleavable 6-nitroveratryloxycarbonyl (Nvoc) group[36] had been introduced at the α-amino group of Ser26 in **10**, to establish a novel biological evaluation system in which the activation of the self-assembly process could be readily controlled (Scheme 6). Mutter et al. have also presented a similar concept, in the form of a pH- or enzyme-triggered "switch-peptide" to control self-assembly of Aβ-derived peptides.[21c] These systems could be crucial in current AD-related research.

In size-exclusion chromatography, a peak corresponding to an oligomer (~octamer) of **9** ($t_R$ = 15 min) increased with incubation time at the expense of the monomer peak ($t_R$ = 27 min), while, in the click peptide **11**, the monomeric form was clearly retained even after 24 h incubation (Figure 4A). Similarly, Th-T fluorescence intensity, which corresponds to the extent of fibril formation,[37] increased with time in **9**, while fluorescence intensity in **11** remained unchanged during 24 h incubation (Figure 4B). These results clearly indicate that click peptide **11** is nonaggregative and that isomerization of the peptide backbone at only one position in the whole peptide sequence (i.e., the formation of a branched ester structure in **11**) had significantly changed the secondary structure of **9**, resulting in the complete masking of the aggregative nature of **9**.

Under nonphotolytic conditions, click peptide **11** demonstrated only slight hydrolysis (< 2%) at the ester bond between Gly25 and Ser26 after 6 h incubation in PBS (pH 7.4) at




**Scheme 6.** Phototriggered click peptide (26-*N*-Nvoc-26-AIAβ42, **11**): the production of Aβ1–42 (**9**) by phototriggered click, followed by the *O–N* intramolecular acyl migration reaction of 26-AIAβ42 (**10**).