

Ethyl 2-Cyano-2-(hydroxyimino)acetate (Oxyma): An Efficient and Convenient Additive Used with Tetramethylfluoroformamidinium Hexafluorophosphate (TFFH) to Replace 1-Hydroxybenzotriazole (HOBt) and 1-Hydroxy-7-azabenzotriazole (HOAt) during Peptide Synthesis

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The appropriateness of ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) as a substitute for benzotriazole-based additives, for use in the TFFH approach for peptide synthesis, is discussed in terms of its capacity to control racemization, its coupling efficiency in difficult couplings either for stepwise or segment coupling in solution- and solid-phase coupling. In addition, Boc-based solution-phase peptide synthesis and its stability in the presence of growing peptide chains were studied. Oxyma displayed remarkable results in terms of racemization depression together with impressive coupling efficiency in both solution- and solid-phase synthesis. Furthermore, Oxyma suggests a lower risk of explosion than HOBt and HOAt.

For many years acid chlorides were used for activating the carboxyl group of an amino acid for amide bond formation.[1–6] Among peptide coupling reagents, long ago, gained the reputation of being "over activated" and therefore prone to numerous side reactions including loss of configuration.[7] Acid fluorides, on the other hand, are more stable to hydrolysis than acid chlorides and are not subject to the limitation mentioned with regard to *tert*-butyl-based side chain protection. Thus Fmoc-based solid-phase peptide synthesis can be easily carried out via Fmoc amino acid fluorides.[8,9] The conversion of acids into acid fluorides was presented by several methods often involving toxic reagents.[9,10] A remarkable progress was the development of TFFH (tetramethylfluoroformamidinium hexafluorophosphate) (**1**) (Figure 1). TFFH is a non-hygroscopic salt stable to handling under ordinary conditions and acts as an in situ reagent for the preparation of amino acid fluoride during peptide synthesis.[11] TFFH appears to be an ideal coupling reagent for peptide synthesis in solid and solution phase, as well as organic synthesis.[11–16]

For some amino acids, e.g., Fmoc–Aib–OH (Abbreviations are in the last section), it was found that the use of TFFH alone gave results that were less satisfactory than those obtained with isolated amino acid fluorides. The deficiency was traced to inefficient conversion to the acid fluoride, which under the conditions used (2 equivalents of diisopropylethylamine (DIEA)) was accompanied by the corresponding symmetric anhydride and oxazolone.[12,17] On the other hand, it is now shown that if a fluoride additive such as benzyltriphenylphosphonium dihydrogen trifluoride (PTF, **2**) or pyridine–hydrogen fluoride **3**[18] is present during the activation step, the latter two products are avoided and a maximum yield of acid fluoride is obtained. Generation of the amino acid fluoride via TFFH (**1**) is more efficient if PTF is present as shown by model solid-phase syntheses.[18] Presumably this technique can also be used to improve conversion to the isolable acid fluorides.[19]

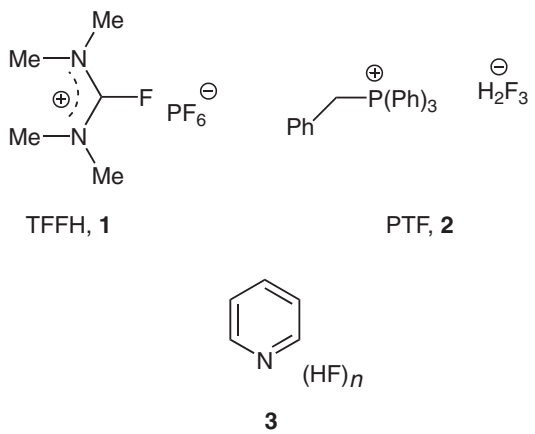

Figure 1. Structures of fluorinating reagents.

The use of an additive to support different coupling reagents is common in the peptide coupling reactions. A common method for minimizing loss of configuration during stepwise coupling in solution phase is to add in the coupling mixture an additive such as 1-hydroxybenzotriazole (HOBt, **4**).[20] Recently, 1-hydroxy-7-azabenzotriazole (HOAt, **5**) has been described as a more favorable coupling additive for both solution-[21] and solid-phase synthesis.[22] The presence of the latter additives in the coupling medium induces the formation of an active ester, which subsequently undergoes aminolysis to afford the desired peptide bond. It was reported that generation of the amino acid fluoride using TFFH is more efficient in the presence of HOAt

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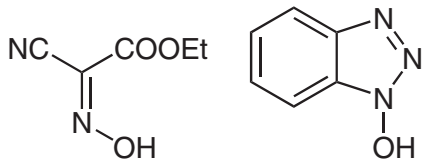

Oxyma (**6**) HOBt (**4**)

Figure 2. Structure of Oxyma (**6**) and HOBt (**4**).

as an additive. The reactivity of the active esters formed is expected to be directly related to the stability of the leaving group anions (OBt^- and OAt^-), and accordingly related to their pK_a values. The pK_a values of the additives HOBt **4** and HOAt **5** are 4.60 and 3.47 respectively.[23]

Recently the explosive properties of HOBt derivatives were reported.[24] This led to their reclassification under a class 1 explosive category and has accordingly increased their transportation difficulties. In a recent paper of our research group, it was reported that ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma, **6**) is an excellent replacement for HOBt and its analogs.[25] Compound **6** (Figure 2) was first reported in 1970 with an acidity similar to that of the HOBt (pK_a value 4.60).[26]

Here the appropriateness of **6** as a substitute for the benzotriazole-based additives is discussed in terms of its capacity to control racemization, its coupling efficiency in difficult couplings either for stepwise or segment coupling in solution- and solid-phase coupling. In addition, Boc-based solution-phase peptide synthesis and its stability in the presence of growing peptide chains were studied.

Results and Discussion

In our search for a class of safe and efficient additives, we came across a family of strongly acidic oximes reported earlier by Itoh.[26] Compound **6** one of the oximes studied, has been tested by our research group as an additive for use in the carbodiimide approach for the formation of peptide bonds. Oxyma displayed a remarkable capacity to inhibit racemization, together with impressive coupling efficiency in both automated and manual synthesis, superior to those of HOBt and HOAt.[25] Calorimetry assays (DSC and ARC) suggested a lower risk of explosion in the case of Oxyma.[25]

Our goal is to test Oxyma as an additive to support the TFFH coupling methodology. Investigations of Oxyma as an additive in the coupling medium, during the generation of the amino acid fluoride using TFFH was carried first by infrared examination. Activation of the protected amino acid Z–Val–OH by means of TFFH in absence of an additive gives the acid fluoride (IR: 1842 cm^{-1}), whereas in case of using Oxyma as an additive, after 5 min activation, only the Oxyma esters (1720 (active ester) and 1710 cm^{-1} (Oxyma ethyl ester)), and a peak at 2251 cm^{-1} corresponding to the CN group were observed.

Further investigation of racemization and the compound's effectiveness of coupling yields were carried out. For the study of racemization, peptide models[27,28] were used for comparison of HOAt, HOBt, and Oxyma. These coupling models are more reliable than those chosen in the previous papers (Figure 3).

With regard to sensitive stepwise coupling (Z–Phg–OH onto H–Pro–NH_2), using 2 min preactivation in the presence of

Z-Phg-Pro-NH_2 (**7**) Z-Phe-Val-Pro-NH_2 (**8**)

Z-Gly-Phe-Pro-NH_2 (**9**) Z-Gly-Phe-Val-OMe (**10**)

Figure 3. Models of peptides.

Table 1. Yield and Racemization during the Formation of Z–Phg–Pro–NH_2 (**7**) in DMF (Solution-Phase Synthesis)[a)]

Entry	Coupling reagent[b)]	Base (equiv)	Yield/%	DL/%
1	TFFH–HOAt	DIEA (2)	78	1.7
2	TFFH–HOBt	DIEA (2)	80	8.2
3	TFFH–Oxyma	DIEA (2)	85	0.54
4	TFFH	DIEA (2)	80	7.4
5	DIC–HOAt[25a]	—	81.4	3.3
6	DIC–HOBt[25a]	—	81.9	9.3
7	DIC–Oxyma[25a]	—	89.9	1.0
8	HOTU[25b]	DIEA (2)	78.9	0.17
9	HATU[25b]	DIEA (2)	78.4	3.1
10	HBTU[25b]	DIEA (2)	80.2	8.2

a) LL- and DL-Forms of the test dipeptide have been described elsewhere.[27] The t_R values of LL and DL were identified by co-injection with authentic and pure samples of LL. HPLC system: Linear gradient of 20 to 80% CH_3CN/H_2O, 0.1% TFA over 30 min, detection at 200 nm Water Symmetry C_{18} 5 μm 4.6 × 150 mm, $t_{R\,LL} = 12.54$ min., $t_{R\,DL} = 13.12$ min. b) A 2 min preactivation time was used.

DIEA as base, the performance of Oxyma exceeded that of HOBt and HOAt in terms of yield and optical purity. The yield of the dipeptide model **7** using TFFH/Oxyma was 85% with only 0.54% DL-racemization, while in case of TFFH alone 80% yield was obtained with 7.4% DL-racemization (Table 1, Entries 3 and 4). On the other hand, the use of TFFH/HOAt and TFFH/HOBt as coupling reagents gave 78% and 80% yield with 1.7% and 8.2% DL-racemization respectively. The TFFH/Oxyma combination and the previously reported oxime-based HOTU[25b] gave better conservation of chirality than the lately studied benzotriazole-based coupling reagents (HATU, HBTU, HMDA, and HMDB),[25b,27] DIC in presence of several additives (Table 1, Entries 5–7),[25a,27] and the fluoroamidinuim salts (BTFFH and DFIH).[12b]

In the case of the more racemization-prone well-studied [2 + 1] segment coupling (Z–Phe–Val–OH onto H–Pro–NH_2), 2 min preactivation was used leading to the tripeptide **8**. The segment coupling was performed in the presence of either DIEA or TMP as a base. The yield obtained using TFFH as coupling reagent in the presence of Oxyma in both cases (Table 2, Entries 7 and 8) was very good. Table 2 shows a comparison of the performance of a number of coupling reagents lately used for the segment coupling of the tripeptide **8**.[12b,25,27] In general, it is observed that the degree of racemization in the presence of TMP as base is lower than in case of using DIEA. In addition, the presence of an additive improved the performance of most of the coupling reagents. The use of HOAt as an additive, when using TFFH, BTFFH, DFIH, DIC, HATU, and HAPyU as coupling reagents in the presence of TMP, gave the least degree of racemization, 2.7%, 2.8%, 3.2%, 2.1%, 2.4%, and 1.6% respectively. In the TFFH coupling approach the use of Oxyma as an additive gave comparable optical purity (2.8%) (Table 2,

Table 2. Yield and Racemization during the Formation of Z–Phe–Val–Pro–NH_2 (**8**) (2 + 1) in DMF (Solution-Phase Synthesis)[a)]

Entry	Coupling reagent	Base (equiv)	Yield/%	LDL/%
1	TFFH–HOAt	DIEA (2)	86	15.4
2	TFFH–HOAt	TMP (2)	78	2.7
3	TFFH–HOBt	DIEA (2)	90	28.4
4	TFFH–HOBt	TMP (2)	81	15.3
5	TFFH	DIEA (2)	77	30.9
6	TFFH	TMP (2)	75	23.6
7	TFFH–Oxyma	DIEA (2)	90	22.1
8	TFFH–Oxyma	TMP (2)	86	2.8
9	DIC–HOAt[25a]	—	86.1	2.1
10	DIC–HOBt[25a]	—	78.8	8.9
11	DIC–Oxyma[25a]	—	89.9	3.8
12	HATU[25b]	DIEA (2)	85.8	13.9
13	HATU[25b]	TMP (2)	83.2	5.3
14	HATU–HOAt[25b]	DIEA (2)	75.6	10.9
15	HATU–HOAt[25b]	TMP (2)	72.1	2.4
16	HBTU[25b]	DIEA (2)	89.7	27.4
17	HBTU[25b]	TMP (2)	81.2	14.2
18	HOTU[25b]	DIEA (2)	91.2	23.6
19	HOTU[25b]	TMP (2)	88.7	7.4

a) LLL- and LDL-Forms of the test tripeptide have been described elsewhere[27] and were co-injected with authentic and pure samples. HPLC system: Linear gradient 20 to 80% CH_3CN/H_2O, 0.1% TFA over 30 min, detection at 200 nm Water Symmetry C_{18} 5 μm 4.6 × 150 mm, $t_{R\,LLL} = 6.96$ min, $t_{R\,LDL} = 7.44$ min.

Table 3. Yield and Racemization during the Formation of Z–Gly–Phe–Pro–NH_2 (**9**) (2 + 1) in DMF (Solution-Phase Synthesis)[a)]

Entry	Coupling reagent	Base (equiv)	Yield/%	LDL/%
1	TFFH–HOAt	DIEA (2)	90.3	2.1
2	TFFH–HOAt	TMP (2)	85.9	1.1
3	TFFH	TMP (2)	80.1	10.6
4	TFFH–HOBt	DIEA (2)	89.2	6.4
5	TFFH–HOBt	TMP (2)	83.9	4.2
6	TFFH–Oxyma	DIEA (2)	89.9	2.9
7	TFFH–Oxyma	TMP (2)	86.9	1.4

a) LLL- and LDL-Forms of the test tripeptide have been described elsewhere[28] and were co-injected with authentic and pure samples. HPLC system: Linear gradient 20 to 80% CH_3CN/H_2O, 0.1% TFA over 30 min, detection at 200 nm Water Symmetry C_{18} 5 μm 4.6 × 150 mm, $t_{R\,LLL} = 11.81$ min, $t_{R\,LDL} = 12.83$ min.

Entry 8) to that of the HOAt additive (2.7%) (Table 2, Entry 2). In addition, TFFH/Oxyma gave higher optical purity than the oxime-based HOTU[25b] (Table 2, Entry 19) and the DIC/Oxyma[25a] coupling approach (Table 2, Entry 11).

For the rather non-sensitive case of segment coupling of Z–Gly–Phe–OH to H–Pro–NH_2, which leads to tripeptide **9**, Oxyma and HOAt additives again perform better than HOBt in term of optical purity and yield (Table 3).

In the case of formation of the tripeptide Z–Gly–Phe–Val–OMe (**10**) which is a less sensitive case the previous results were confirmed. The coupling reaction was performed in the presence of DIEA or TMP as base showing comparable results of Oxyma and HOAt clearly better than that seen with HOBt (Table 4). In addition, an improvement in the coupling efficiency of TFFH in the presence of an additive was observed.

Table 4. Yield and Racemization during the Formation of Z–Gly–Phe–Val–OMe (**10**) (2 + 1) in DMF (Solution-Phase Synthesis)[a)]

Entry	Coupling reagent	Base (equiv)	Yield/%	LDL/%
1	TFFH–HOAt	DIEA (2)	90	1.7
2	TFFH–HOAt	TMP (2)	87	0.8
3	TFFH–HOBt	DIEA (2)	89	5.7
4	TFFH–HOBt	TMP (2)	85	3.9
5	TFFH	DIEA (2)	88	6.2
6	TFFH	TMP (2)	86	5.6
7	TFFH–Oxyma	DIEA (2)	90	1.8
8	TFFH–Oxyma	TMP (2)	89	1.1

a) LLL- and LDL-Forms of the test tripeptide have been described elsewhere[28] and were co-injected with authentic and pure samples. HPLC system: Linear gradient 20 to 80% CH_3CN/H_2O, 0.1% TFA over 30 min, detection at 200 nm Water Symmetry C_{18} 5 μm 4.6 × 150 mm, $t_{R\,LLL} = 18.06$ min, $t_{R\,LDL} = 18.62$ min).

Further racemization experiments were carried out for the stepwise coupling of the four previously studied model systems, Z–Val–Val–OCH_3 (**11**), Z–Val–Ala–OCH_3 (**12**), Z–Phe–Val–OCH_3 (**13**), and Z–Phe–Ala–OCH_3 (**14**),[28] using TFFH as coupling reagent in the presence and in absence of Oxyma. In the presence of Oxyma as an additive an improvement in yield was observed but the optical purity of the studied models was almost the same (less than 1%) as observed from NMR analysis. The OCH_3 units of the esters **11**, **12**, **13**, and **14** were monitored at 3.72, 3.74, 3.68, and 3.70 ppm respectively. These peaks correspond to the LL-enantiomers. The slightly down shielded peaks at 3.87, 3.88, 3.83, and 3.85 ppm correspond to the DL-enantiomers respectively.

The performance of Oxyma was further tested in the manual synthesis of Leu–enkephalin (H–Tyr–Gly–Gly–Phe–Leu–NH_2, **15**) in solution phase using TFFH–Oxyma. Boc chemistry was used in the solution phase except for the last amino acid coupling. Fmoc–Tyr(O*t*-Bu)OH was used and the Fmoc group was deblocked by using 30% diethylamine in acetonitrile for 1 h and then the crude peptide was treated with TFA–DCM (1:1) for two hours at room temperature.

For testing the efficiency of TFFH/Oxyma in Boc chemistry, the pentapeptide H–Tyr–Gly–Gly–Phe–Leu–NH_2 was stepwise elongated using Boc–amino acids. Boc–Phe–OH was preactivated using TFFH/Oxyma/DIEA (1:1:2) for 2 min in DMF and then coupled to H–Leu–NH_2 for 30 min to afford the dipeptide Boc–Phe–Leu–NH_2. After deblocking of the Boc protecting group, using TFA–DCM (1:1) for 2 h at room temperature, the dipeptide TFA salt is further coupled to Boc–Gly–OH in the presence of TFFH/Oxyma using the same strategy to give the tripeptide. The crude tripeptide was treated directly with TFA–DCM followed by subsequent coupling with Boc–Gly–OH. The crude tetrapeptide was treated directly with TFA–DCM.

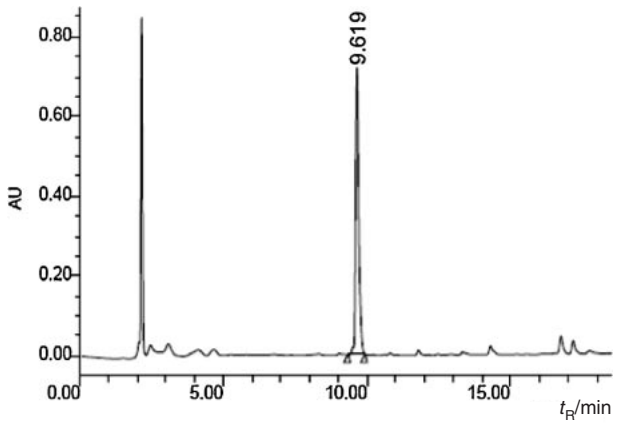

Figure 4. HPLC analysis of the crude sample of TFA·Tyr–Gly–Gly–Phe–Leu–NH_2 (**15**). TFFH–Oxyma is used as coupling reagent. Conditions: HPLC system: Linear gradient 20 to 80% CH_3CN/H_2O, 0.1% TFA over 30 min, detection at 200 nm Water Symmetry C_{18} 5 μm 4.6 × 150 mm. $t_R = 9.619$ min (98.3%).

Table 5. The Percentages of Des-Aib (H–Tyr–Aib–Phe–Leu–NH_2) Obtained during Solid-Phase Assembly of the Pentapeptide (H–Tyr–Aib–Aib–Phe–Leu–NH_2, **16**)[a),b)]

Coupling reagent	Base (equiv)	Pentapeptide/%	Des-Aib/%
TFFH–HOAt	DIEA (2)	95	5
TFFH–HOBt	DIEA (2)	64	36
TFFH	DIEA (2)	95	5
TFFH–Oxyma	DIEA (2)	98	2

a) Tetrapeptide (des-Aib) was confirmed by peak overlap in the presence of an authentic sample. The crude H–Tyr–Aib–Aib–Phe–Leu–NH_2 was analyzed by HPLC [Symmetry Waters C_{18} (4.6 × 150 mm, 4 μm), linear gradient over 30 min of 20 to 80% CH_3CN in H_2O/0.1% TFA, flow rate 1.0 mL min^{-1}, t_R penta = 11.3 min, t_R des-Aib = 11.56 min]. b) HPLC-MS showed the right mass for the pentapeptide at 612.0.

The TFA salt of the tetrapeptide was used directly without purification to couple with Fmoc–Tyr(OBu)–OH under the same condition used before. Then the Fmoc group was removed by treatment with 30% diethylamine in acetonitrile at room temperature for 1 h. The crude product was then treated with a mixture of TFA–DCM (1:1) at room temperature for 2 h to remove the butyl group. The crude pentapeptide was obtained in yield (68.9%). The purity by HPLC was 98.3 (at $t_R = 9.62$ min) (Figure 4). The HPLC-MS showed the right mass for the pentapeptide at 555.3.

Hindered Amino Acids. In a more demanding example, the Leu–enkaphlin derivative H–Tyr–Aib–Aib–Phe–Leu–NH_2[29] (**16**) was manually assembled on Fmoc-Rink Amide AM-resin using amino acid/activator (3 equiv), DIEA (6 equiv), using 30 min coupling time, except for the case of Aib–Aib, which required 1 h. Percentages of incorporation for the coupling of Fmoc–Aib–OH onto the Aib-containing resin were determined by reverse-phase HPLC analysis, after cleavage of the peptide from the resin by treatment with TFA/H_2O (9:1) for 2 h at room temperature (Table 5). Little improvement was observed with TFFH in the presence of Oxyma as an additive when compared to HOAt/TFFH or TFFH alone. The percentage of the desired pentapeptide was being 98%, where Oxyma gave a better result than HOBt.

Conclusion

Oxyma was used as an additive in the TFFH approach for peptide synthesis. It displayed remarkable results in terms of racemization depression during stepwise and segment coupling. The TFFH/Oxyma combination showed superiority to a number of coupling reagents previously reported. As would be expected, the use of HOAt as an additive with all coupling reagents confirmed its superiority to HOBt in terms of both coupling yields and retention of configuration. Remarkably, Oxyma often gave results comparable or even better than those obtained with HOAt. In addition, Oxyma can be handled with a considerably lower risk than the explosive benzotriazole and its derivatives, which is an extra advantage.

Experimental

General. All peptides were identified by HPLC analysis of the crude sample. Conditions: a Waters Symmetry C_{18} column (4.6 × 150 mm, 5 μm), linear gradient over 30 min of 20 to 80% CH_3CN in H_2O/0.1% TFA, flow rate 1.0 mL min^{-1}. Dipeptides were identified by ^{1}H NMR. NMR spectra were recorded on a JEOL 500 MHz spectrometer at room temperature. Tetramethylsilane (TMS) was used as reference for all NMR spectra with chemical shifts reported as δ units (part per million, ppm) relative to TMS. HPLC-MS electrospray mass spectroscopy was used for identification of peptides.

Racemization Tests with Model Peptides 7–10 in Solution Phase. Test couplings were carried out as previously described for Z–Phg–Pro–NH_2,[27] Z–Phe–Val–Pro–NH_2,[27] Z–Gly–Phe–Pro–NH_2,[28] and Z–Gly–Phe–Val–OMe.[28]

Formation of Z–Phg–Pro–NH_2 (**7**): 0.125 mmol of Z–Phg–OH, 0.125 mmol coupling reagent, and 0.25 mmol of DIEA as base were dissolved in 1 mL DMF at 0 °C. The reaction mixture was preactivated for 2 min. Then 0.125 mmol of H–Pro–NH_2 was added. The reaction mixture was stirred at 0 °C for 1 h and at room temperature overnight. The reaction mixture was diluted with 25 mL of ethyl acetate and extracted with 1 M HCl (2 × 5 mL), 1 M $NaHCO_3$ (2 × 5 mL), and saturated NaCl (2 × 5 mL), dried over anhydrous $MgSO_4$, the solvent was removed, and the crude peptide was directly analyzed by HPLC.

Formation of Z–Phe–Val–Pro–NH_2 (**8**): 0.125 mmol of Z–Phe–Val–OH, 0.125 mmol coupling reagent, and 0.25 mmol of base (DIEA or TMP) were dissolved in 1 mL DMF at 0 °C. The reaction mixture was preactivated for 2 min. Then 0.125 mmol of H–Pro–NH_2 was added. The reaction mixture was stirred at 0 °C for 1 h and at room temperature overnight, followed by the workup described above.

Formation of Z–Gly–Phe–Pro–NH_2 (**9**) (2 + 1) and Z–Gly–Phe–Val–OMe (**10**) (2 + 1) were performed by the same procedure mentioned previously for the segment coupling.

Crude products were analyzed by reverse-phase HPLC (with a waters Symmetry C_{18}, 5 μm, 4.6 × 150 mm column), linear gradient over 30 min of 20 to 80% CH_3CN in H_2O/0.1% TFA, flow rate 1.0 mL min^{-1}, detection at 220 nm. In the Z–Phg–Pro–NH_2 model, the t_R values of the LL- and DL-epimers were 12.54 and 13.12 min, respectively, whereas in the Z–Phe–Val–Pro–

NH_2 case, the t_R values of the LLL- and LDL-epimers were 6.96 and 7.44 min, respectively. In the Z–Gly–Phe–Pro–NH_2 model, the t_R values of the LLL- and LDL-epimers were 11.81 and 12.83 min, respectively, whereas in the Z–Gly–Phe–Val–OMe case, the t_R values of the LLL- and LDL-epimers were 18.06 and 18.62 min, respectively.

Racemization Tests with Model Dipeptides 11–14 in Solution Phase. Test couplings were carried out as previously described for Z–Val–Val–OCH_3 (**11**), Z–Val–Ala–OCH_3 (**12**), Z–Phe–Val–OCH_3 (**13**), and Z–Phe–Ala–OCH_3 (**14**).[28]

To a solution of 0.1 mmol of Z–Val–OH or Z–Phe–OH and 0.2 mmol of DIEA in 2 mL of DMF was added 0.1 mmol of the appropriate coupling reagent at 0 °C. The reaction mixture was preactivated for 2 min, followed by the addition of a solution of 0.1 mmol of Val–OMe·HCl or Ala–OMe·HCl and 0.1 mmol of DIEA in 1 mL of DMF. The reaction mixture was stirred at 0 °C for 1 h and at room temperature overnight. After dilution with 25 mL of ethyl acetate, the organic phase was washed with 1 M HCl (3 × 10 mL), saturated $NaHCO_3$ (3 × 10 mL) and saturated NaCl (3 × 10 mL) and dried over $MgSO_4$ anhydrous. After removal of the solvent with a rotary evaporator, the residue was recrystallized from CH_2Cl_2/hexane to give the dipeptide.

The extent of racemization during the preparation of the model systems was monitored by proton NMR analysis. The OCH_3 units of esters **11**, **12**, **13**, and **14** were monitored at 3.73, 3.74, 3.68, and 3.70 ppm respectively. These correspond to the LL-enantiomers. The slightly downshielded peaks at 3.87, 3.88, 3.83, and 3.85 ppm correspond to the DL-enantiomers respectively.

Solution-Phase Synthesis of Leu–Enkephalin (H–Tyr–Gly–Gly–Phe–Leu–NH_2, 15) Using TFFH–Oxyma. The pentapeptide was manually elongated. TFFH/Oxyma (0.05 mmol each) was added to a solution of H–Leu–NH_2 (0.05 mmol), DIEA (0.1 mmol), Boc–Phe–OH (0.05 mmol) in DMF (2 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and at rt for another 1 h, then ethyl acetate was added (40 mL). The organic layer was washed with saturated Na_2CO_3 (2 × 10 mL), 10% citric acid (2 × 10 mL), saturated NaCl (2 × 10), dried ($MgSO_4$), filtered and the solvent was removed under vacuum. The crude dipeptide was treated with TFA–DCM (1:1, 10 mL) at room temperature for 2 h, then the solvent and TFA were removed under vacuum and then washed with ether. The crude TFA salt was used for the next step for the coupling with Boc–Gly–OH using the same previous coupling techniques and then workup as usual. The crude tripeptide was treated directly with TFA–DCM followed by subsequent coupling with Boc–Gly–OH as mentioned before. The TFA salt of the tetrapeptide was used directly without purification to couple with Fmoc–Tyr(OBu)–OH under the same condition used before. Then the Fmoc group was removed by treatment with 30% diethylamine in acetonitrile (10 mL) at room temperature for 1 h. The solvent was removed under vacuum and then the crude product was treated with 10 mL of a mixture of TFA–DCM (1:1) at room temperature for 2 h. The solvent and TFA were removed under vacuum and then the crude pentapeptide was washed with ether to give slight brown solid in yield (68.9%). The purity by HPLC using linear gradient over 30 min of 20 to 80% CH_3CN in H_2O/0.1% TFA, was 98.3% (at t_R 9.62 min). The HPLC-MS showed the right mass for the pentapeptide at 555.3.

Synthesis of H–Tyr–Aib–Aib–Phe–Leu–NH_2 (16) in Solid Phase. 100 mg of Fmoc–RA–PS (0.7 mmol g^{-1}) were deblocked by 10 mL of 20% piperidine in DMF for 10 min, washed with DMF (2 × 10 mL), DCM (2 × 10 mL), and then DMF (2 × 10 mL). Fmoc–Leu–OH (3 equiv), TFFH/additive (3 equiv), and DIEA (6 equiv) were mixed in 0.5 mL DMF and activated for 1–2 min and then added to the resin and stirred slowly for 1 min and let to couple for 30 min (1 h double coupling only for the Aib–Aib), (ninhydrin test was negative). The resin was washed with DMF, and then deblocked by 20% piperidine in DMF for 7 min. The resin was washed with DMF, DCM, and DMF, then coupled with the next amino acid. Coupling and deblocking steps were repeated to provide the penta peptide. The percentage of des-Aib (4-mer) (Tyr–Aib–Phe–Leu–NH_2) during solid-phase assembly of the pentapeptide (Tyr–Aib–Aib–Phe–Leu–NH_2) was confirmed by peak overlap in the presence of an authentic sample. The crude H–Tyr–Aib–Aib–Phe–Leu–NH_2 was analyzed by HPLC [Symmetry Waters C_{18} (4.6 × 150 mm, 4 μm), linear gradient over 30 min of 20 to 80% CH_3CN in H_2O/0.1% TFA, flow rate 1.0 mL min^{-1}, t_R penta = 11.3 min, t_R des-Aib = 11.56 min]. The HPLC-MS analysis showed the right mass for the pentapeptide at 612.0.

Abbreviations

Aib: α-aminoisobutyric acid, Boc: *t*-butyloxycarbonyl, BTFFH: bis(tetramethylene)fluoroformamidinium hexafluorophosphate, DFIH: 1,3-dimethyl-2-fluoro-4,5-dihydro-1*H*-imidazolium hexafluorophosphate, DCM: dichloromethane, DIC: diisopropylcarbodiimide, DIEA: diisopropylethylamine, DMF: dimethylformamide, HOBt: 1-hydroxybenzotriazole, HOAt: 7-aza-1-hydroxybenzotriazole, Oxyma: ethyl 2-cyano-2-(hydroxyimino)acetate, HAPyU: 1-(1-pyrrolidinyl-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene)-*N*-methylmethanaminium hexafluorophosphate *N*-oxide, HATU: *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene)-*N*-methylmethanaminium hexafluorophosphate *N*-oxide, HBTU: *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide, HDTU: *O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,2,3,3-tetramethyluronium hexafluorophosphate, HMDA: 1-[(dimethylimino)(morpholino)-methyl]-3*H*-[1,2,3]triazolo[4,5-*b*]pyridine-1-ium-3-olate hexafluorophosphate, HMDB: 1-[(dimethylimino)(morpholino)-methyl]-3*H*-benzo[1,2,3]triazolo-1-ium-3-olate hexafluorophosphate, HOTU: *O*-[cyano(ethoxycarbonyl)methylidene)-amino]-1,1,3,3-tetramethyluronium hexafluorophosphate, TFFH: tetramethylfluoroformamidinium hexafluorophosphate, TFA: trifluoroacetic acid, TMP: 2,4,6-trimethylpyridine (collidine), PTF: benzyltriphenylphosphonium dihydrogen trifluoride, Z: benzyloxycarbonyl. Amino acids and peptides are abbreviated and designated following the rules of the IUPAC-IUB Commission of Biochemical Nomenclature (*J. Biol. Chem*. **1972**, *247*, 977).

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Supporting Information

HPLC analysis of **7** (Table 1, Entry 3), HPLC analysis of **8** (Table 2, Entry 8). This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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