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Oxyma-B, an excellent racemization suppressor for peptide synthesis†

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Peptide-bond formation is a key process in the synthesis of peptide oligomers. Among the many coupling techniques reported, carbodiimides combine strong acylation potency and smooth reaction conditions and are commonly used in the presence of additives. Recently, ethyl 2-cyano-2-(hydroxyimino)acetate (OxymaPure) has emerged as a highly reactive alternative to the classic and explosion-prone benzotriazolic additives, namely 1-hydroxybenzotriazole (HOBt) and 1-hydroxy-7-azabenzotriazole (HOAt). Here we report on a new oxime additive 5-(hydroxyimino)-1,3-dimethylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (Oxyma-B). This new additive showed satisfactory solubility in various solvents (DMF, ACN, and THF). It was also more effective in the control of optical purity during the synthesis of Z-Phg-Pro-NH_2, Z-Phe-Val-Pro-NH_2, H-Gly-Ser-Phe-NH_2, H-Gly-Cys-Phe-NH_2, H-Gly-Cys(Acm)-Phe-NH_2 and H-Gly-His-Phe-NH_2 than related Oxyma- and benzotriazole-based reagents. Oxyma-B also proved to be advantageous compared to the related HONM, because the latter cannot be used with the carbodiimide. Furthermore, Oxyma-B showed satisfactory performance in assembling demanding sequences such as the Aib-enkephalin pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2).

Introduction

The reaction of a carboxylic acid with an amine to render amide derivatives requires activation of the carboxylic acid. This step is commonly carried out through an active ester, which can be previously prepared, isolated, purified, and characterized, or alternatively prepared *in situ* by the use of carbodiimides or onium salts. In all cases, the cornerstone of the process is the leaving group. The most used groups are those derived from phenols and *N*-hydroxy derivatives.[1–7] These include 1-hydroxybenzotriazole (HOBt, **1**),[8] 7-aza-1-hydroxybenzotriazole (HOAt, **2**) (which are reported as the most potent and expensive analogs),[9] and 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt, **3**) (an analog of medium reactivity with a more accessible potency-to-cost ratio).[10] HOBt derivatives are therefore either used in combination with a carbodiimide or another coupling agent or are built into a stand-alone reagent such as an iminium salt[11,12] [HBTU (**4**),[13] HATU (**5**),[14] HCTU (**6**)[15]]. However, the explosive nature of HOBt and its related additives has recently been reported.[16] This observation has led to the reclassification of these compounds under the Class 1 explosive category and has consequently increased transportation difficulties.

Recently, our group reevaluated OxymaPure (ethyl 2-cyano-2-(hydroxyimino)acetate) **7**, which was first reported a few decades ago.[17] OxymaPure **7** displayed remarkable efficiency in inhibiting racemization and showed an impressive coupling efficiency in both automated and manual synthesis. These effects are superior to those shown by HOBt **1** and comparable to those of HOAt **2** (Fig. 1).[18–20]

Later, we devised a third generation of uronium salt, COMU **8**, which carries a morpholonium-based uronium moiety as the proton acceptor and OxymaPure **7** as the leaving group. COMU **8** shows coupling efficiency superior to that of HBTU **4** and comparable to that of HATU **5**.[20–23]

More recently, a new family of uronium salts (HTMU, **10**; HMMU, **11**; and HDmPyMU, **12**) based on isonitroso Meldrum's acid (HONM, **9**) was reported as stand-alone coupling reagents.[24] HONM **9** shows structural similarities to **7**, except for the presence of two carbonyl groups as electron-withdrawing substituents located in the six-membered cyclic structure. This modification should enhance the reactivity of the oxime-based additive as a result of its more powerful

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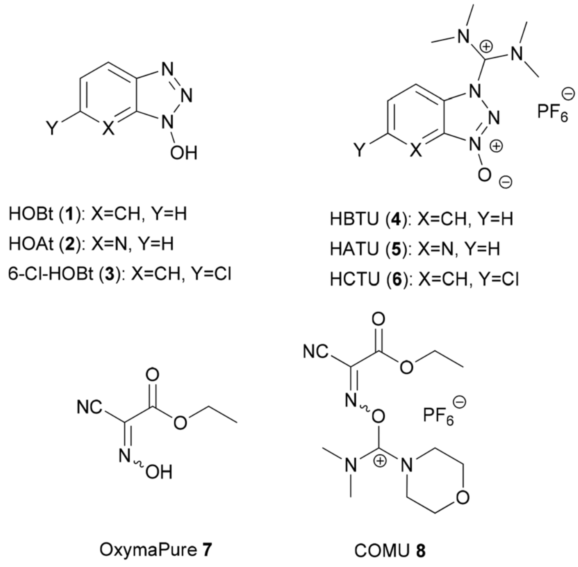
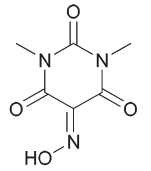


Fig. 1 Structure of benzotriazole additives **1–3** and their coupling reagents **4–5**, OxymaPure **7** and COMU **8**.

electron-withdrawing effect compared with **7**. The cyclic structure of **9** may also be beneficial because the hydroxy function becomes more accessible (Fig. 2).

HONM **9** is highly reactive, even reacting with carbodiimides. Such reactivity translates into a consumption of coupling reagents and a decrease in the yield. However, uronium salts (**10**, **11**, and **12**) show greater reactivity when compared with classical coupling reagents.[24] In parallel, some of our colleagues have reported the inconvenience of a side reaction when removing the ethyl ester moiety from the structure under very specific conditions.[18,25] Here we describe a new additive that has been designed, prepared, and screened for peptide synthesis. Oxyma-B **14**, which has been developed from 1,3-dimethylbarbituric acid **13**, shows a special orientation of the carbonyl moiety, similar to HONM **9**. This orientation can play an assisted basic catalytic role by enhancing the nucleophilicity of the amino function during coupling. In addition, **14** does not hold an ester moiety in its structure and, therefore, there is no risk of a side-reaction.



Oxyma-B **14**

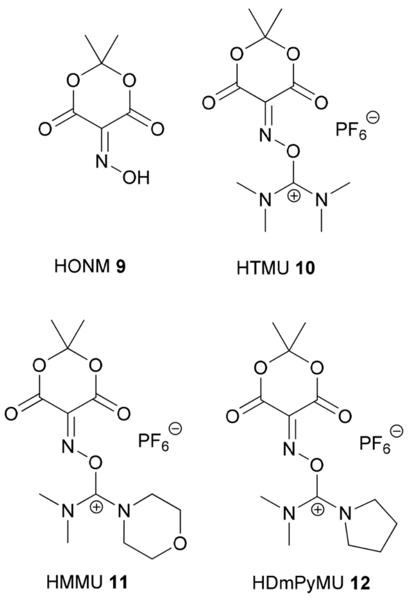


Fig. 2 Structure of HONM **9** and its uronium salts.

Results and discussion

Synthesis

Oxyma-B **14** was readily prepared by reaction of 1,3-dimethylbarbituric acid **13** with NaOH, $NaNO_2$, and AcOH in a mixture of water and methanol for 1 h at 0 °C, followed by acidification by 35% HCl to afford the desired compound as a white solid in 84% yield (Scheme 1).

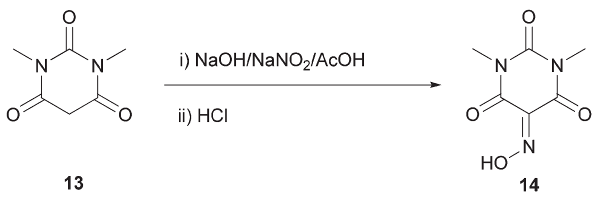
Solubility

Solubility is a key feature in determining the suitability of coupling reagents, especially in the automatic mode. Oxyma-B **14** showed acceptable solubility in DMF (the most widely used solvent in peptide bond formation), ACN (which was reported to be a suitable alternative to DMF in SPPS)[26] and THF. Furthermore, **14** showed partial solubility in DCM.

Racemization

The undesired loss of configuration at C_α is dramatic in more sensitive building blocks, such as phenylglycine or Cys. In order to check the coupling efficiency and impact of epimerization,[27,28] stepwise models[12,18,21,24,29–33] (1 + 1) (Z-Phg-Pro-NH_2 **15**) and (2 + 1) segment (Z-Phe-Val-Pro-NH_2 **16**) Oxyma-B-based couplings in solution-phase synthesis were carried out. In the first model of **15**, the α-phenyl moiety in Phg ensured high sensitivity towards epimerization (Table 1).[18]

In this simple model of stepwise coupling of **15**, both OxymaPure **7** and Oxyma-B **14** showed excellent results compared to HOBt **1** and even to HOAt **2** in terms of reducing racemization (entries 3, 4 *vs.* 1, 2).



Scheme 1 Synthesis of Oxyma-B from 1,3-dimethylbarbituric acid.

Table 1 Yield and epimerization during the formation of Z-Phg-Pro-NH_2 **15** using additives (solution-phase synthesis)[a]

Entry	Coupling reagent	Yield[b] (%)	DL/LL[c] (%)
1	DIC/HOBt (**1**)	94.3	11
2	DIC/HOAt (**2**)	91.5	3.9
3	DIC/OxymaPure (**7**)	94.4	0.9
4	DIC/Oxyma-B (**14**)	90.0	1.0

[a] Couplings were performed without preactivation in DMF at room temperature. [b] Conversion yield was calculated by HPLC. Retention times of Z-Phg-OH and Z-Phg-Pro-NH_2 were identified by injection of a pure sample. [c] Retention times for each epimer were identified after co-injection with pure LL and DL samples onto reverse-phase HPLC using a linear gradient of 25 to 50% 0.1% TFA in CH_3CN/0.1% TFA in H_2O over 15 min, detection at 220 nm and a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, t_R(LL) = 6.4 min, t_R(DL) = 6.8 min.

Having evaluated the stepwise system, we tackled the most demanding [2 + 1] coupling for the assembly of dipeptide Z-Phe-Val-OH onto H-Pro-NH_2, affording tripeptide **16** (Table 2).[34] The activation of a dipeptide acid is a very interesting example to test the performance of an additive, since oxazolone formation is promoted as a result of the electron-donating effect of the *N*-aminoacyl substitution.[21,31] The percentages of LDL epimers were considerably higher than in the previous stepwise system (Table 2 and Fig. 3).

In this tripeptide **16** model, Oxyma-B **14** showed better results than OxymaPure **7** and HOAt **2** (entry 4 *vs.* entries 2 and 3), while the worst result was obtained with HOBt **1** (entry 1). These results are important because in our previous work involving OxymaPure **7**, we also detected that it caused less racemization than HOBt **1**, but sometimes slightly more than that produced when using HOAt **2**.

Table 2 Yield and epimerization during the formation of Z-Phe-Val-Pro-NH_2 **16** using additives (solution-phase synthesis)[a]

Entry	Coupling reagent	Yield[b] (%)	LDL/LLL[c] (%)
1	DIC/HOBt (**1**)	96.3	14.8
2	DIC/HOAt (**2**)	97.6	5.9
3	DIC/OxymaPure (**7**)	91.9	7.7
4	DIC/Oxyma-B (**14**)	90.7	5.1

[a] Couplings were performed without preactivation in DMF at room temperature. [b] Conversion yield was calculated by HPLC. Retention times of Z-Phe-Val-OH and Z-Phe-Val-Pro-NH_2 were identified by injection of a pure sample. [c] Retention times for each epimer were identified after co-injection with pure LLL and LDL samples onto reverse-phase HPLC using a linear gradient of 30 to 60% 0.1% TFA in CH_3CN–0.1% TFA in H_2O over 15 min, detection at 220 nm and a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, t_R(LLL) = 5.8 min, t_R(LDL) = 6.9 min.

Z-Phg-Pro-NH_2 **15**
Z-Phe-Val-Pro-NH_2 **16**
H-Gly-Ser-Phe-NH_2 **17**
H-Gly-Cys-Phe-NH_2 **18**
H-Gly-Cys(Acm)-Phe-NH_2 **19**
H-Gly-His-Phe-NH_2 **20**

Fig. 3 Peptide models used in the racemization test.

Oxyma-B **14** showed the best performance in reducing epimerization, as demonstrated by further epimerization experiments. We selected the tripeptides H-Gly-Ser-Phe-NH_2 **17**,[35] H-Gly-Cys-Phe-NH_2 **18**,[18,36] H-Gly-Cys(Acm)-Phe-NH_2 **19** and H-Gly-His-Phe-NH_2 **20**[37] to study the loss of configuration of the most tricky residues, namely Ser, Cys and His. These amino acids (Ser, Cys, His) gave unusual epimerization during SPPS.[37–41] The tripeptides **17**–**20** were assembled by stepwise solid-phase synthesis with 5 min preactivation and Ser(*t*Bu), Cys(Trt), Cys(Acm) and His(Trt) as protecting groups, respectively.

On the basis of the results shown in Table 3, we conclude that Oxyma-B **14** stands out as one of the most promising additives in peptide synthesis. It showed better results than OxymaPure **7** when used as a racemization suppressor and exceeded those achieved with HOAt **2** in both stepwise and segment coupling in solid- and solution-phase peptide synthesis.

Furthermore, an additional feature of Oxyma-B **14** is that the reaction can be monitored through the change in the color of the solution. The solution is blue at the start of the reaction and after completion becomes a yellowish green. This phenomenon also occurs when OxymaPure **7** is used.

Peptide synthesis

The acylation potency of **14** in hindered couplings was evaluated during SPPS. For this purpose, solid-phase assembly of the Aib-enkephalin pentapeptide **21** was used as a model for SPPS (Table 4).

The design of **21** consisted of introducing the hindered α,α-disubstituted amino acid (Aib) to replace Gly residues (Leu-enkephaline), thereby achieving differences in the coupling performance of each of the additives tested. **21** was stepwise assembled manually on a Fmoc-RinkAmide-AM-PS-resin by means of 1 h coupling times (except Aib–Aib where 1 hour double coupling was applied) with the use of an excess of 3 equiv. of Fmoc-amino acid/additive/carbodiimides.

Table 3 Racemization studies on the solid-phase assembling of H-Gly-AA-Phe-NH_2 (where AA = Ser, Cys, Cys(Acm) or His)

Entry	Coupling model	Coupling reagent	DL/LL (%)
1	H-Gly-Ser-Phe-NH_2 (**17**)	DIC/HOBt (**1**)	3.3
2		DIC/HOAt (**2**)	0.4
3		DIC/OxymaPure (**7**)	0.4
4		DIC/Oxyma-B (**14**)	0.3
5	H-Gly-Cys-Phe-NH_2(**18**)	DIC/HOBt (**1**)	0.5
6		DIC/HOAt (**2**)	0.4
7		DIC/OxymaPure (**7**)	0.3
8		DIC/Oxyma-B (**14**)	0.3
9	H-Gly-Cys(Acm)-Phe-NH_2(**19**)	DIC/HOBt (**1**)	0.4
10		DIC/HOAt (**2**)	0.3
11		DIC/OxymaPure (**7**)	0.3
12		DIC/Oxyma-B (**14**)	0.3
13	H-Gly-His-Phe-NH_2 (**20**)	DIC/HOBt (**1**)	1.1
14		DIC/HOAt (**2**)	1.9
15		DIC/OxymaPure (**7**)	3.0
16		DIC/Oxyma-B (**14**)	1.0

Table 4 Percentage of tetrapeptide des-Aib (H-Tyr-Aib-Phe-Leu-NH_2) during solid-phase assembly of pentapeptide **21** (H-Tyr-Aib-Aib-Phe-Leu-NH_2)[a]

Entry	Coupling reagent	Preactivation time	Penta (%)	des-Aib[b] (%)
1	DIC/HOBt (**1**)	3 min	8.4	83.1
2	DIC/HOAt (**2**)	3 min	37.5	60.2
3	DIC/OxymaPure (**7**)	3 min	42.8	50.4
4	DIC/Oxyma-B(**14**)	No preactivation	19.4	72.6
5	DIC/Oxyma-B(**14**)	3 min	26.4	61.1
6	DIC/Oxyma-B(**14**)	3 min[c]	10.5	79.7
7	DIC/Oxyma-B(**14**)	7 min	9.4	82.8

[a] One-hour coupling times were generally applied, except for Aib–Aib (one-hour double coupling). [b] Deletion tetrapeptide (des-Aib) was identified by peak overlap in HPLC with an authentic sample obtained in the solid phase. The crude H-Tyr-Aib-Aib-Phe-Leu-NH_2 was analyzed by reverse-phase HPLC using linear gradient of 30 to 60% 0.1% TFA in CH_3CN–0.1% TFA in H_2O over 15 min, detection at 220 nm and a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, t_R = 6.68 (pentapeptide), 6.78 (des-Aib) min. [c] 0.1% DIEA was used.

Our first idea was to apply a range of preactivation times with the use of **14** and DIC. A 3 min preactivation gave the best result (entry 5 *vs.* entries 4 and 7). In order to enhance the coupling potency of **14**, 0.1 equiv. of DIEA was used. However, it showed less reactivity than expected (entry 5 *vs.* 6). In comparison with other additives, **14** showed a better performance than HOBt **1** (entry 5 *vs.* 1) and a worse performance than HOAt **2** and OxymaPure **7** (entry 5 *vs.* entries 2 and 3, respectively).

Conclusions

Our results indicate that Oxyma-B **14** is a superior racemization suppressor than OxymaPure**7**. Moreover, **14** performs better than HOAt **2**, which to date was considered to be the most outstanding in that regard. Also, we envisaged that an additional effect, namely the presence of the carbonyl groups oriented in the same direction as the *N*-OH group in this molecule, would increase the reactivity of Oxyma-B **14**. Hence, these groups enhanced basic catalysis; thereby improving the nucleophilicity of the amine function during the coupling, and occurs when HOAt **2** and HONM **9** are used.[24] Furthermore, Oxyma-B does not contain any ethyl ester, which can lead to side reactions.

The preparation and study of the applications of the corresponding uronium salt are underway and will be published elsewhere.

Experimental

Materials

The solvents used were of HPLC reagent grade. Chemicals and amino acid derivatives were purchased from Sigma-Aldrich, Fluka, Gl Biochem (Shanghai) Ltd, Iris Biotech GmbH, or Merck Millipore. The following coupling reagents were used: DIC (Fluka, lot number BCBK8348 V); HOBt (Gl Biochem (Shanghai) Ltd, Lot number GLS110604-00602); HOAt (Gl Biochem (Shanghai) Ltd, lot number GLS121115-00601); and OxymaPure (Luxembourg Biotech., Batch number 1301117008). Melting points were determined with a Barnstead IA9300 system (Electrothermal, UK) and are uncorrected. NMR spectra (^{1}H NMR and ^{13}C NMR) were recorded on a Bruker AVANCE III 400 MHz spectrometer. Chemical shift values are expressed in ppm downfield from TMS as an internal standard. IR was recorded using a Bruker-ALPHA spectrophotometer. Follow-up of the reactions and checks of the purity of the compounds was performed by TLC on silica gel-protected aluminium sheets (Type 60 GF254, Merck Millipore, Bedford, MA, USA), and the spots were detected by exposure to an UV-lamp at λ 254 nm for a few seconds. Analytical HPLC was performed on an Agilent 1100 system, and the Chemstation software was used for data processing. LC-MS was performed on Shimadiz 2020 UFLC-MS using an YMC-Triart C_{18} (5 μm, 4.6 × 150 mm) column, and data processing was carried out by the LabSolution software. Buffer A: 0.1% formic acid in H_2O; and buffer B: 0.1% formic acid in CH_3CN. High-resolution mass spectrometric data were obtained using a Bruker micrOTOF-Q II instrument operating at room temperature and a sample concentration of approximately 1 ppm.

Synthesis of 5-(hydroxyimino)-1,3-dimethylpyrimidine-2,4,6 (1*H*,3*H*,5*H*)-trione (Oxyma-B 14)

15.6 g (0.1 mol) of 1,3-dimethylbarbituric acid **14** was dissolved in 60 mL of water containing 6.0 g (0.15 mol) NaOH. Next, 10 mL of methanol was added to the transparent solution obtained, then a solution of 7.6 g (0.11 mol) of sodium nitrite in 30 mL of water was poured into the mixture, which was then stirred for 2 to 3 min. The mixture was cooled to 10 °C, and then 18 g (0.3 mol) of acetic acid was added dropwise. The mixture was kept at 25 °C for 1 h. Next, 25 mL of 30% hydrochloric acid was added to the mixture and stirred for 10 min. The precipitate was filtered, and washed first with 50% methanol and then with water. The product was recrystallized from aqueous methanol 2 : 1 to obtain Oxyma-B **14** as a white crystal in 84.0% yield (15.56 g); m.p. 140–142 °C. IR (ATR): 3200–2300 (br, OH), 1667 (s, CO, amide) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.43 (s, 3H, CH_3), 3.46 (s, 3H, CH_3), 15.94 (br, 1H, OH, D_2O exchangeable). ^{13}C NMR (CD_3CN): δ 26.93, 27.82, 135.66, 149.99, 157.45. HRMS (ESI+) *m*/*z* calcd for $C_6H_7N_3O_4$: $[M - H]^-$ 184.0358; found $[M - H]^-$ 184.0369.

General method for the racemization experiments[18,33,34,42–44]

0.125 mmol of an acid (Z-Phg-OH or Z-Phe-Val-OH), 0.125 mmol of H-Pro-NH_2, and 0.125 mmol of the corresponding additive were dissolved in 2 mL of DMF, and the solution was cooled in an ice bath and treated with 0.125 mmol of DIC. The mixture was stirred at 0 °C for 1 h and at room temperature overnight. An aliquot (10 μL) of the solution was then taken and diluted to 1 mL with a mixture of CH_3CN–H_2O (1 : 2). Next, 5 μL of this solution was injected into a reverse-phase HPLC apparatus.

Z-Phg-Pro-NH$_2$

A linear gradient of 25–50% 0.1% TFA in CH_3CN/0.1% TFA in H_2O over 15 min was applied, with a flow rate of 1.0 mL min^{-1} and detection at 220 nm using a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, t_R(LL) = 6.4 min, t_R(DL) = 6.8 min, t_R(Z-Phg-OH) = 9.1 min.

Z-Phe-Val-Pro-NH$_2$

A linear gradient of 30–60% 0.1% TFA in CH_3CN/0.1% TFA in H_2O over 15 min was applied, with a flow rate of 1.0 mL min^{-1} and detection at 220 nm using a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, t_R(LLL) = 5.8 min, t_R(LDL) = 6.9 min, t_R(Z-Phe-Val OH) = 8.1 min.

Study of serine racemization during assembly of H-Gly-Ser-Phe-NH$_2$ on the solid phase[35,37]

Experiments consisted of the study of the stepwise coupling of Ser and Gly residues onto previously formed H-Phe-Rink-Amide-AM-PS-resin (0.6 mmol g^{-1}, 100 mg), with the use of the Fmoc/*t*Bu and Ser(^{t}Bu) protection strategy. Glycine was introduced in order to achieve better separation of LL and DL isomers than des-Gly dipeptides. Coupling times of 1 h were used after 5 min preactivation of a solution of Fmoc-amino acids (3 equiv.), the corresponding additive (3 equiv.), and DIC (3 equiv.) in DMF (0.5 mL) at room temperature. Fmoc was removed with 20% piperidine in DMF for 7 min. The peptide chain was released from the resin by treatment with TFA–H_2O–TIS (95 : 2.5 : 2.5) for 1 h at room temperature. The colorless solution was filtered, and the resin was washed with CH_2Cl_2 (3 × 0.5 mL). The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude peptide was precipitated with cold Et_2O (3 × 5 mL) and then lyophilized. It was then analyzed by reversed-phase HPLC, with the use of a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, linear gradient 0 to 30% of 0.1% TFA in CH_3CN/0.1% TFA in H_2O over 15 min, with detection at 220 nm. The t_R values of the LL and DL epimers were 5.55 min and 6.06 min, respectively. LC–MS showed the expected mass for the tripeptide at *m*/*z* = 309.0.

Study of cysteine racemization during assembly of H-Gly-Cys-Phe-NH$_2$ on the solid phase[18,36–38,45]

Experiments consisted of the study of the stepwise coupling of Cys and Gly residues onto previously formed H-Phe-RinkAmide-AM-PS-resin (0.6 mmol g^{-1}, 100 mg), with the use of the Fmoc/*t*Bu and the Cys(Trt) protection strategy. Glycine was introduced in order to achieve better separation of LL and DL isomers than des-Gly dipeptides. Coupling times of 1 h were used after 5 min preactivation of a solution of Fmoc-amino acids (3 equiv.), the corresponding additive (3 equiv.), and DIC (3 equiv.) in DMF (0.5 mL) at room temperature. Fmoc was removed with 20% piperidine in DMF for 7 min. The peptide chain was released from the resin by treatment with TFA–H_2O–TIS (95 : 2.5 : 2.5) for 1 h at room temperature. The colorless solution was filtered, and the resin was washed with CH_2Cl_2 (3 × 0.5 mL). The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude peptide was precipitated with cold Et_2O (3 × 5 mL) and lyophilized. It was then analyzed by reversed-phase HPLC, with the use of a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, linear gradient 0 to 40% of 0.1% TFA in CH_3CN/0.1% TFA in H_2O over 15 min, with detection at 220 nm. The t_R values of the LL and DL epimers were 6.79 and 7.95 min, respectively. LC–MS showed the expected mass for the tripeptide at *m*/*z* = 325.0.

Study of cysteine racemization during assembly of H-Gly-Cys(Acm)-Phe-NH$_2$ on the solid phase

Experiments consisted of the study of the stepwise coupling of Cys and Gly residues onto previously formed H-Phe-Rink-Amide-AM-PS-resin (0.6 mmol g^{-1}, 100 mg), with the use of the Fmoc/*t*Bu and Cys(Acm) protection strategy. Glycine was introduced in order to achieve better separation of LL and DL isomers than of des-Gly dipeptides. Coupling times of 1 h were used after 5 min preactivation of a solution of Fmoc-amino acids (3 equiv., excess), the corresponding additive (3 equiv.), and DIC (3 equiv.) in DMF (0.5 mL) at room temperature. Fmoc was removed with 20% piperidine in DMF for 7 min. The peptide chain was released from the resin by treatment with TFA–H_2O–TIS (95 : 2.5 : 2.5) for 1 h at room temperature. The colorless solution was filtered, and the resin was washed with CH_2Cl_2 (3 × 0.5 mL). The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude peptide was precipitated with cold Et_2O (3 × 5 mL) and lyophilized. It was then analyzed by reversed-phase HPLC, with the use of a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, linear gradient 0 to 40% of 0.1% TFA in CH_3CN/0.1% TFA in H_2O over 15 min, with detection at 220 nm. The t_R values of the LL and DL epimers were 6.70 and 7.54 min, respectively. LC–MS showed the expected mass for the tripeptide at *m*/*z* = 396.0.

Study of histidine racemization during assembly of H-Gly-His-Phe-NH$_2$ on the solid phase[37]

Experiments consisted of the study of the stepwise coupling of Ser and Gly residues onto previously formed H-Phe-RinkAmide-AM-PS-resin (0.6 mmol g^{-1}, 100 mg), with the use of the Fmoc/*t*Bu and the His(Trt) protection strategy. Glycine was introduced in order to achieve better separation of LL and DL isomers than des-Gly dipeptides. Coupling times of 1 h were used after 5 min preactivation of a solution of Fmoc-amino acids (3 equiv., excess), the corresponding additive (3 equiv.), and DIC (3 equiv.) in DMF (0.5 mL) at room temperature. Fmoc was removed with 20% piperidine in DMF for 7 min. The peptide chain was released from the resin by treatment with TFA–H_2O–TIS (95 : 2.5 : 2.5) for 1 h at room temperature. The colorless solution was filtered, and the resin was washed with CH_2Cl_2 (0.5 mL × 3). The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude peptide was precipitated with cold Et_2O (3 × 5 mL) and lyophilized. It was then analyzed by reversed-phase HPLC, with the use of a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, linear gradient 5 to 20% of 0.1% TFA in CH_3CN/0.1% TFA in H_2O over 15 min, with detection at 220 nm. The t_R values of the LL and DL epimers were 4.01 min and 4.65 min, respectively. LC–MS showed the expected mass for the tripeptide at $m/z = 359.0$.

Solid-phase synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH_2[12,18,21–24,29–33]

The synthesis was carried out in a plastic syringe attached to a vacuum manifold to achieve the rapid removal of reagents and solvent. The Fmoc-RinkAmide-AM-PS resin (0.6 mmol g^{-1}, 100 mg), was washed with DMF, DCM, and DMF (2 × 10 mL each) and then treated with 20% piperidine in DMF (10 mL) for 10 min. The resin was then washed with DMF, DCM, and DMF (2 × 10 mL each) and acylated with a solution of Fmoc-Leu-OH (3 equiv.), the corresponding additive (3 equiv.) and DIC (3 equiv.) in DMF (0.5 mL, previously preactivated). After peptide coupling, the resin was washed with DMF and then de-blocked by treatment with 20% piperidine in DMF for 7 min. The resin was washed with DMF, DCM, and DMF (2 × 10 mL each). Then, coupling with the next amino acid, as explained previously, and de-blocking were repeated to obtain the pentapeptide. The peptide was cleaved from the resin with TFA–H_2O (9 : 1) at room temperature for 2 h. TFA was removed under nitrogen, and the crude peptide was purified with cold Et_2O (3 × 10 mL) and lyophilized. The ratio of the penta- and tetra-peptide was determined by HPLC analysis using a Phenomex C_{18} (3 μm, 4.6 × 50 mm) column, with a linear gradient of 20 to 40% of 0.1% TFA in CH_3CN/0.1% TFA in H_2O over 15 min, flow rate = 1.0 mL min^{-1}, detection at 220 nm. The t_R values for pentapeptide and des-Aib were 6.68 min and 6.78 min, respectively. LC–MS showed the expected mass for the pentapeptide at $m/z = 611.0$, and also for des-Aib at $m/z = 526$.

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