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-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$_2$, 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. Oxyma-B 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 [HBTU (1), HATU (2), HCTU (6)] or as the leaving group.

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

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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. Oxyma-B 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 [HBTU (1), HATU (2), HCTU (6)] or as the leaving group. 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$_2$).

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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. 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. 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.

Results and discussion

Synthesis

Oxyma-B 14 was readily prepared by reaction of 1,3-dimethylbarbituric acid 13 with NaOH, NaN₂, 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) and THF. Furthermore, 14 showed partial solubility in DCM.

Racemization

The undesired loss of configuration at Cα is dramatic in more sensitive building blocks, such as phenylglycine or Cys. In order to check the coupling efficiency and impact of epimerization, stepwise models (1 + 1) (Z-Phg-Pro-NH₂ 15) and (2 + 1) segment (Z-Phe-Val-Pro-NH₂ 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).

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.](image)
than that produced when using HOAt (Table 2).34 The activation of a dipeptide acid is a very interesting phenomenon also occurs when OxymaPure 7 when used as a racemization suppressor and exceeded those achieved with HOAt in 2 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 2 Yield and epimerization during the formation of Z-Phe-Val-Pro-NH2 16 using additives (solution-phase synthesis)4

<table>
<thead>
<tr>
<th>Entry</th>
<th>Coupling reagent</th>
<th>Yield b (%)</th>
<th>LDL/LLL c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DIC/HOBt (1)</td>
<td>94.3</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>DIC/HOAt (2)</td>
<td>91.5</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>DIC/OxymaPure (7)</td>
<td>94.4</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>DIC/Oxyma-B (14)</td>
<td>90.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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-Pro-NH2 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 CH3CN/0.1% TFA in H2O over 15 min, detection at 220 nm and a Phenomex C18 (3 μm, 4.6 × 50 mm) column, tR(LL) = 6.4 min, tR(DL) = 6.8 min.

Table 3 Racemization studies on the solid-phase assembly of H-Gly- AA-Phe-NH2 (where AA = Ser, Cys, Cys(Acm) or His) 15

<table>
<thead>
<tr>
<th>Entry</th>
<th>Coupling model</th>
<th>Coupling reagent</th>
<th>DL/LL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H-Gly-Ser-Phe-NH2 (17)</td>
<td>DIC/HOBt (1)</td>
<td>3.3</td>
</tr>
<tr>
<td>2</td>
<td>H-Gly-Cys-Phe-NH2 (18)</td>
<td>DIC/HOAt (2)</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>H-Gly-Cys(Acm)-Phe-NH2 (19)</td>
<td>DIC/OxymaPure (7)</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>H-Gly-His-Phe-NH2 (20)</td>
<td>DIC/Oxyma-B (14)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

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-NH2, affording tripeptide 16 (Table 2).24 The activation of a dipeptide acid is a very interesting example to test the performance of an additive, since oxazoline 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.
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 HOAt 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 OxymaPure7. 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. 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 BCBK83348 V); HOAt (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 I9300 system (Electrothermal, UK) and are uncorrected. NMR spectra (1H NMR and 13C 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 2010 UFLC-MS using an YMC-Triart C18 (5 μm, 4.6 × 50 mm) column, λ 220 nm and 0.1% DIEA was used. However, further optimization with 0.1% formic acid in H2O; and buffer B: 0.1% formic acid in CH3CN.

Synthesis of 5-(hydroxymino)-1,3-dimethylpyrimidine-2,4,6-(1H,3H,5H)-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. 1H NMR (400 MHz, CDCl3) δ 3.43 [s, 3H, CH3], 3.46 [s, 3H, CH3], 15.94 [br, 1H, OH, D2O exchangeable]. 13C NMR (CD3CN): δ 26.93, 27.82, 135.66, 149.99, 157.45. HRMS (ESI+) m/z calcd for C6H7N3O4: [M − H]+ 260.0117; found [M − H]+ 260.0120.

General method for the racemization experiments

0.125 mmol of an acid (Z-Phg-OH or Z-Phe-Val-OH), 0.125 mmol of H-Pro-NH2, 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 CH3CN–H2O (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$_3$CN/0.1% TFA in H$_2$O 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$_3$CN/0.1% TFA in H$_2$O 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, $t_R$(LLD) = 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

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/Fmoc and Ser(Fmoc) protection strategy. Glycine was introduced in order to achieve better separation of LL and DL isomers than those of 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. The colorless solution was filtered, and the resin was washed with CH$_2$Cl$_2$ (3 × 0.5 mL). The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude peptide was precipitated with cold Et$_2$O (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 40% of 0.1% TFA in CH$_3$CN/0.1% TFA in H$_2$O 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/Fmoc and Cys(Acm) protection strategy. Glycine was introduced in order to achieve better separation of LL and DL isomers than those of 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$_2$O–TIS (95:2.5:2.5) for 1 h at room temperature. The colorless solution was filtered, and the resin was washed with CH$_2$Cl$_2$ (3 × 0.5 mL). The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude peptide was precipitated with cold Et$_2$O (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 40% of 0.1% TFA in CH$_3$CN/0.1% TFA in H$_2$O 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

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/Fmoc and the His(Trt) protection strategy. Glycine was introduced in order to achieve better separation of LL and DL isomers than those of 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$_2$O–TIS (95:2.5:2.5) for 1 h at room temperature. The colorless solution was filtered, and the resin was washed with CH$_2$Cl$_2$ (3 × 0.5 mL). The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude peptide was precipitated with cold Et$_2$O (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 40% of 0.1% TFA in CH$_3$CN/0.1% TFA in H$_2$O 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 $m/z = 396.0$.

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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$_2$**

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$_2$O (9 : 1) at room temperature for 2 h. TFA was removed under nitrogen, and the crude peptide was purified with cold Et$_2$O (3 × 10 mL) and lyophilized. The ratio of the pentapeptide and tetra-peptide was determined by HPLC analysis using a Phenomenex C$_{18}$ (3 μm, 4.6 × 50 mm) column, with a linear gradient of 20% to 40% of 0.1% TFA in CH$_3$CN/0.1% TFA in H$_2$O over 15 min, flow rate = 1.0 mL min$^{-1}$, detection at 220 nm. The τ$_r$ 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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**Notes and references**


25. Although, we have forced the side reaction involving the ethyl ester moiety: mixing of the amino component in the presence of OxymaPure (10 equiv.) for 12 h or microwave heating of the amino component with OxymaPure (10 equiv.) at 80 °C for 10 min in DMF or NMP, we did not appreciate the side reaction at all. For more experimental details, see ref. 18.


