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# Racemisation Studies of a Novel Coupling Reagent for Solid Phase Peptide Synthesis

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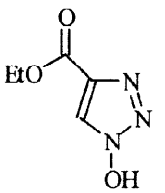
***Abstract:*** A novel coupling reagent ethyl 1-hydroxy-1H-1,2,3-triazole-4-carboxylate has been developed for use in conjunction with diisopropylcarbodiimide for solid phase peptide synthesis. The synthesis and application of this reagent in solid phase peptide synthesis has been reported. Here we report a systematic study into racemisation upon activation and coupling of single amino acids and dipeptides with this reagent. © 1999 Elsevier Science Ltd. All rights reserved.

***Keywords:*** coupling reagent, peptide synthesis, racemisation, epimerisation

## INTRODUCTION

Racemisation still remains the most serious side-reaction during amide bond formation in peptide synthesis and a considerable research effort has been devoted overcoming this problem.[1] Thus, any new coupling reagent designed for solid phase peptide synthesis (SPPS) must be investigated thoroughly using realistic solid phase synthesis conditions. The novel coupling reagent, ethyl 1-hydroxy-1H-1,2,3-triazole-4-carboxylate (HOCt) **(1)** and its use in conjunction with diisopropyl carbodiimide (DIC) in Fmoc solid phase peptide synthesis (SPPS) has been described.[2]

In order to validate the potential of the reagent for use in SPPS an investigation was carried out into whether intermediate Fmoc-amino acid active esters derived from HOCt would be susceptible to racemisation. A series of tripeptides with the general structure Ala-Xaa-Gly were synthesised using HOCt/DIC, where Xaa is derived from a chiral Fmoc-amino acid. Ala-Xaa-Gly was chosen as the model peptide since Gly is not chiral and Ala has a characteristic doublet between 1 and 2 ppm in the $^1$H NMR corresponding to its side-chain methyl group. Thus, if any of the amino acids should racemise with HOCt/DIC a second doublet would then be observed for Ala due to the formation of the D isomer thus providing an easy method of analysis.




**(1)**

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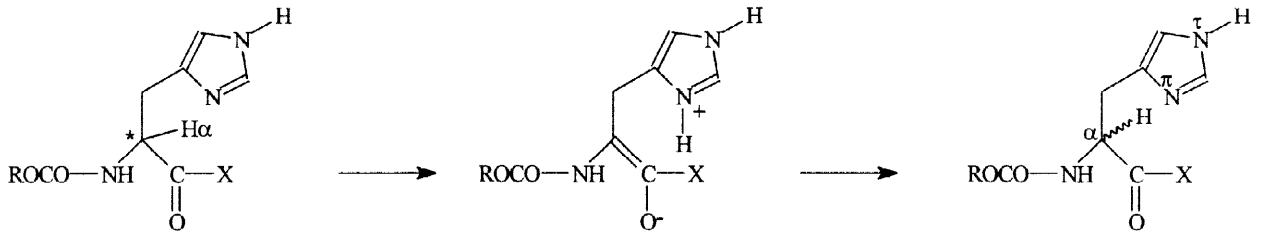
Of all the twenty naturally occurring α-amino acids, $N^{\alpha}$ protected histidine **(2)** when activated is especially prone to racemisation. This is due to the reactivity of the imidazole nucleus.[3] The imidazole ring is a weak base but is strong enough to cause intramolecular proton abstraction to form an intermediate which can protonate to either the D or L amino acid (Figure 1).

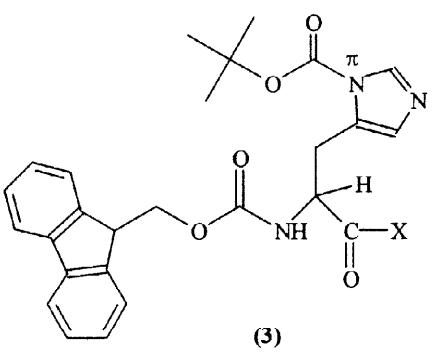



**(2)**

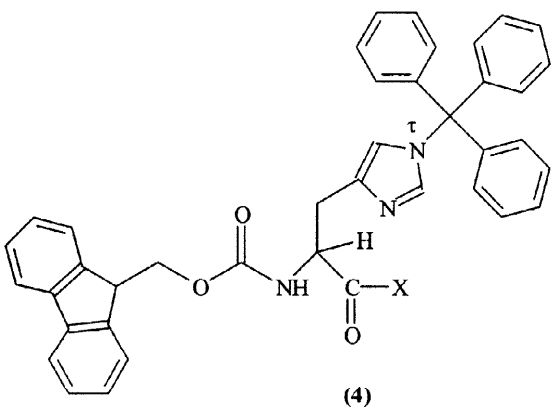
*** = chiral centre**
R = $N^{\alpha}$ protection
X = activating group

**Figure 1**

Masking the π-nitrogen in the imidazole function would seem to be a worthwhile objective, however most protecting groups for histidine actually block the τ nitrogen. The only commercially available analogue with protection of the π-nitrogen is Fmoc-His(Bum)[4] **(3)**, the manufacture of which is a multi-step synthesis with relatively poor overall yield and consequently it is very expensive. Protecting groups such as trityl (Trt) on the τ-nitrogen **(4)** have been shown to suppress enantiomerisation[5] by reducing the basicity of the imidazole through electronic effects as well as its bulk helping to prevent the base catalysed rearrangement which leads to racemisation. Unfortunately it cannot circumvent the problem completely. Thus rigorous conditions have to be set out for any new coupling reagent when activating Fmoc-His(Trt).




**(3)**




**(4)**

## RESULTS AND DISCUSSION

## RACEMISATION STUDIES UPON ACTIVATION AND COUPLING OF SINGLE AMINO ACIDS TO FORM PEPTIDE Ala-Xaa-Gly

All peptides unless otherwise stated were synthesised manually using 1mmol of each Fmoc-amino acid and coupling *via* HOCt/DIC, using a sonic bath for mixing. The completed peptides were cleaved from their resins using standard TFA cleavage conditions with the appropriate scavenger. The nature of Xaa and side-chain protecting groups, if applicable, are shown below in brackets after the amino acid.

Protected Fmoc-Xaa-OH = Ala, Arg(Pmc), Asn(Mbh, Trt), Asp(O*t*-Bu), Cys(Acm, *t*-Bu, Trt), Gln(Trt), Glu(O*t*-Bu), His(Trt), Ile, Leu, Lys(Boc), Met, Phe, Pro, Ser(*t*-Bu), Thr(*t*-Bu), Trp (indole NH not protected), Tyr(*t*-Bu) and Val.

Each tripeptide was analysed for racemisation using a 360MHz $^1$H NMR and d$^6$DMSO as the solvent. The results are tabulated below in Table 1.

| AMINO ACID = Xaa | RACEMISATION CALCULATED (%) |
|---|---|
| Ala , Arg, Asn, Asp, Cys, Gln, Glu, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val | 0 |
| His | 50 |

**Table 1**

The results clearly show no evidence of racemisation on activation of any of the Fmoc-amino acids with HOCt/DIC with the exception of Fmoc-His(Trt) which racemised. Thus histidine racemisation was investigated further in order to devise a protocol of activation and coupling of this amino acid which would avoid racemisation. The results for the various experiments carried out are summarised in Table 2 below.

| TEMPERATURE (°C) | ACTIVATION TIME (min) | HOCt (mmol) | RACEMISATION CALCULATED (%) |
|---|---|---|---|
| ambient | 15 | 1 | 50.0 |
| ambient | 10 | 1 | 31.6 |
| ambient | 5 | 1 | 11.5 |
| ambient | 1 | 1 | 8 |
| zero | 15 | 1 | 0 |
| ambient | 1 | 2 | 1.6 |
| ambient | 1 | 3 | 0.1 |

**Table 2**

Figure 2 illustrates a comparison between a 360MHz $^1$H NMR obtained from an experiment in which histidine racemised and an experiment in which histidine racemisation was suppressed. Racemisation was measured by measuring the difference in the ratios of the integrals for the two doublets between 1 and 2ppm.

It was found that by decreasing the activation time from 15 minutes to 1 minute the racemisation of Fmoc-His(Trt) was reduced to a level which was comparable to 1-hydroxybenzotriazole (HOBt), (8%)[2]; however, this was still unacceptable. A low temperature experiment (0°C) showed no evidence of racemisation from the 360MHz $^1$H NMR obtained. However, low temperatures are difficult to achieve using the automated peptide synthesisers generally available and therefore it was proposed that, by using an excess of HOCt (standard coupling uses equimolar), racemisation of histidine could be suppressed by protonation of the imidazole ring due to the acidity ($pK_a$ = 2.1) of HOCt. It was found that 2 equivalents of HOCt reduced racemisation to 1.6% and this could be improved further by introducing another equivalent of HOCt (3 equivalents in total) decreasing racemisation to 0.1%. This excess of HOCt did not appear to hinder the coupling efficiency which remained quantitative according to the Fmoc deprotection profile obtained from the monitoring system.[6]

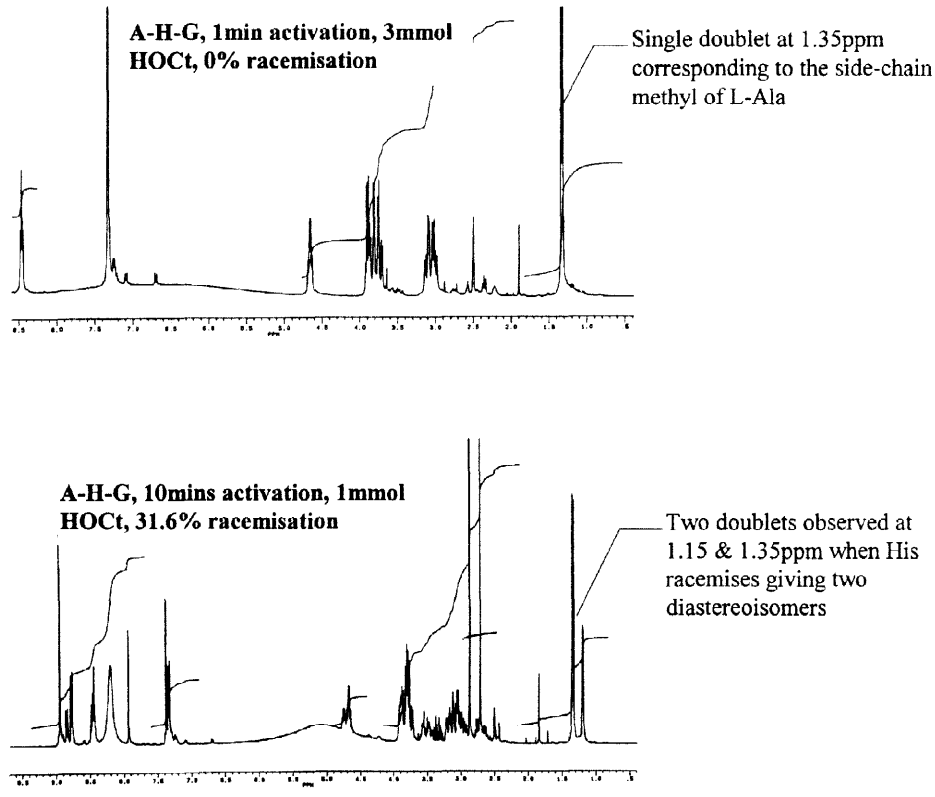



**Figure 2**

## EPIMERISATION STUDIES UPON ACTIVATION AND COUPLING OF DIPEPTIDES TO Gly-RESIN TO FORM PEPTIDE Ala-Xaa-Gly

The scope and utility of HOCt in peptide fragment coupling is currently under investigation for the synthesis of proteins by the convergent strategy.[7] In order to investigate this method thoroughly several experiments were carried out to elucidate whether peptide fragments, activated with HOCt/DIC, would be prone to epimerisation. Since there are many possible selections of the sites for union of peptide fragments for protein synthesis the racemisation study concentrated on the most likely choices of participating amino acids. In order to do this the

study had to be kept simple; thus, several dipeptides were chosen and, after activation with HOCt/DIC, these were coupled to Wang resin previously loaded with Fmoc-Gly. The dipeptides were protected as their Z derivatives as neither Fmoc nor Boc dipeptides were commercially available. All dipeptides were coupled manually using 1min activation time and a sonic bath for mixing. Each coupling reaction was allowed to proceed for 2h at RT before the tripeptides were isolated by cleavage with 90% TFA/$H_2O$ followed by complete Z removal with HBr/AcOH. The two methods of analysis which were used were 600MHz NMR followed by the Izumiya[8] test which is an accurate procedure for the determination of the degree of epimerisation through separation of diastereoisomers by an amino acid analyser. Thus the corresponding tripeptides with the d-amino acid in the Xaa position were also synthesised so that the retention time of any epimerised product could be evaluated on the amino acid analyser. All experiments were run consecutively on the same day to avoid ambiguity and the results are summarised in Table 3. The results from 600MHz NMR clearly showed no evidence of epimerisation for any of the tripeptides as only one doublet was observed for the side-chain methyl of Ala. These results were confirmed by the IZUMIYA test which showed a single peak eluting from the column for all tripeptides each having a different retention time to their corresponding epimerised product.

| 600MHz NMR & IZUMIYA TEST DATA OBTAINED FROM COUPLING Ala-Xaa to Gly-RESIN | | | |
|---|---|---|---|
| **PEPTIDE** | **EPIMERISATION (%) CALCULATED FROM 600MHz NMR** | **Rt (min) FROM IZUMIYA** | **COMMENTS ON RESULTS** |
| A-A-G | 0 | 29.567 | no evidence of A-dA-G, Rt = 35.133 |
| A-V-G | 0 | 31.633 | no evidence of A-dV-G, Rt = 38.400 |
| A-P-G | 0 | 32.567 | no evidence of A-dP-G, Rt = 38.500 |
| A-I-G | 0 | 37.700 | no evidence of A-dI-G, Rt = 43.533 |

**Table 3**

## CONCLUSIONS

We have thoroughly investigated racemisation which can occur during the amino acid activation/coupling processes involving stepwise urethane protected amino acid addition and protected dipeptide activation/coupling. In these cases chiral integrity can be lost by direct α-proton abstraction or *via* oxazolone formation. It has been shown that for HOCt/DIC activation this is negligible for all the amino acids investigated with the exception of histidine. This particular amino is prone to racemisation with any coupling reagent but we have found a method which avoids racemisation completely. Epimerisation occurring during peptide fragment coupling *via* the HOCt active ester was also investigated and was found to be negligible. Thus, in this and the paper previously published in Tetrahedron[2] we have shown that HOCt, when used in conjunction with DIC, is a valuable coupling reagent for use in stepwise SPPS and the fragment coupling strategy since it is superior to reagents such as HOBt and is racemisation free.

## MATERIALS AND METHODS

Wang resin, Fmoc amino acids and Z-dipeptides were purchased from either Novabiochem or Bachem. Acetonitrile, piperidine, DMF and 1,4-dioxane were purchased from Rathburn Chemicals, acetic anhydride and EDT were from Fluka, HBr/acetic acid, DMAP, DIC and thioanisole were purchased from Aldrich, TFA and DIEA were from Perkin Elmer and phenol from Sigma. Those peptides assembled by automation were synthesised on an adapted ABI 430A peptide synthesiser on a 0.25mmol scale.

## SYNTHESIS OF Ala-Xaa-Gly-OH AND Ala-dXaa-Gly

For synthesis the following protected amino acids and dipeptides were used.

Protected Fmoc-Xaa-OH, where Xaa = Ala, Arg(Pmc), Asn(Mbh, Trt), Asp(O*t*-Bu), Cys(Acm, *t*-Bu, Trt), Gln (side-chain COOH not protected), Gln(Trt), Glu(O*t*-Bu), His(Trt), Ile, Leu, Lys(Boc), Met, Phe, Pro, Ser(*t*-Bu), Thr(*t*-Bu), Trp (indole NH not protected), Tyr(*t*-Bu) and Val

Protected Fmoc-dXaa-OH, where Xaa = Ala, His, Ile, Pro, Val

Z-Ala-Xaa-OH where Xaa = Ala, Ile, Pro and Val

***Resin Loading***

Fmoc-Gly-OH (19.1g, 64.2mmol) was dissolved in DMF and DIC (5ml, 32.1mmol) was added. The resulting solution was allowed to stand for 15 minutes before adding it to Wang resin (10g, 1.07mmol/g, pre-swollen in DMF) and a catalytic amount of DMAP. The resulting mixture was allowed to sonicate for 4h before the resin was isolated by filtration through a sinter. The resin was washed with copious DMF, 1,4-dioxane, DCM and ether.

***Synthesis of Ala-Xaa-Gly from coupling single amino acid components to Gly-resin***

All peptides unless otherwise stated were synthesised manually on a 0.25mmol scale using 1mmol of each Fmoc-amino acid and coupling *via* HOCt/DIC with a sonic bath for mixing. Each protected Fmoc amino acid was allowed to activate for 15 minutes before adding it to the resin and each coupling step was allowed to proceed for 2h. After each complete coupling a capping step (acetic anhydride) and an Fmoc deprotection step (20% piperidine in DMF/1,4-dioxane(1:1)) were carried out. The completed peptides were cleaved from their resins using standard TFA cleavage conditions along with EDT (0.5ml) when Xaa had side-chain protection. The TFA mixture was evaporated *in vacuo* to an oily residue which was taken into $CH_3CN/H_2O$ and lyophilised. The resulting tri-peptides were not purified as it was important to analyse the crude mixture in order to detect any formation of the diastereoisomer.

The peptides were immediately submitted for NMR analysis which was performed on a 360MHz $^1H$ NMR spectrometer using $d^6$DMSO as the solvent. The importance of this was to determine the shift of the side-chain

methyl of alanine; thus, only this has been interpreted from the NMR data. The $d^6$DMSO shift for all spectra was set at 2.5ppm.

| PEPTIDE Ala-Xaa-Gly where X = | CHEMICAL SHIFT OF Ala $CH_3$ (ppm) & J VALUE (Hz) |
|---|---|
| Arg, Asn, Asp, Cys, Gln, Glu, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val | 1.35, J = 7.2 |
| Ala | 1.35, J = 7.2; 1.25, J = 7.2 |
| His | 1.35, J = 7.2; 1.15, J = 7.2 |

***Racemisation study on Ala-His-Gly***

The peptides were synthesised by automation with the exception of the peptide synthesised at 0°C. The activation time and amount of HOCt used for histidine coupling were varied as indicated in Table 2.

***Synthesis of Ala-dXaa-Gly***

The peptides were synthesised by automation and cleaved from their resins using 90% TFA/$H_2O$. The peptides were reduced *in vacuo* and lyophilised from $CH_3CN/H_2O$. The peptides were immediately analysed using 600MHz nmr ($d^6$DMSO) and the Izumiya test (pH 2.2 tri sodium citrate buffer) for racemisation.[6]

| PEPTIDE | CHEMICAL SHIFT OF Ala $CH_3$ AND J VALUE (Hz) | IZUMIYA TEST Rt (min) |
|---|---|---|
| A-dA-G | 1.35, J = 7.2; 1.25 J = 7.2 | 31.633 |
| A-dV-G | 1.35, J = 7.2 | 37.700 |
| A-dP-G | 1.35, J = 7.2 | 38.400 |
| A-dI-G | 1.35, J = 7.2 | 43.533 |
| A-dH-G | 1.15, J = 7.2 | - |

***Synthesis of Ala-Xaa-Gly from coupling Z-Ala-Xaa-OH to Gly-resin***

The peptides were synthesised manually as above using 1mmol of each dipeptide coupling *via* HOCt/DIC with only 1 minute for activation of the dipeptide component. The peptides were cleaved first of all with 90% TFA/$H_2O$ which also partially cleaved the Z protecting group. In order to complete the Z deprotection the peptides were stirred in pentamethylbenzene (0.5ml), thioanisole (0.6ml) and TFA (10ml) then HBr/acetic acid (5M, 0.4ml) was added. The resulting solution was allowed to stir for 60 minutes. The peptides were reduced *in vacuo* and lyophilised from $CH_3CN/H_2O$. The peptides were analysed immediately by 600MHz NMR ($d^6$DMSO) and the Izumiya test.[6] For the Izumiya test the samples were loaded onto an 8 micron polystyrene sulphonated ion exchange resin in tri sodium citrate buffer (pH 2.2) then eluted with tri sodium citrate buffer (pH 3.2) for 6.45 mins at 52°C followed by tri sodium citrate buffer (pH 4.25) for 40 mins at 6°C.

| PEPTIDE | CHEMICAL SHIFT OF Ala $CH_3$ AND J VALUE | IZUMIYA TEST Rt (min) |
|---|---|---|
| A-A-G | 1.35 J = 7.2; 1.25 J = 7.2 | 29.567 |
| A-V-G | 1.35 J = 7.2 | 32.567 |
| A-P-G | 1.35 J = 7.2 | 35.133 |
| A-I-G | 1.35, J = 7.2 | 38.500 |

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