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Synthesis and Application of a Novel Coupling Reagent, Ethyl 1-Hydroxy-1H -1,2,3-Triazole-4-Carboxylate

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Abstract: An optimal coupling reagent, ethyl 1-hydroxy-1H-1,2,3-triazole-4-carboxylate (HOCt), has been designed and synthesised for application to solid phase peptide synthesis using Fmoc chemistry. It is used in combination with carbodiimide reagents, has very high coupling efficiency, and does not absorb at 302nm, thus allowing real-time monitoring of each coupling cycle. Its applications in the synthesis of endothelin analogues and difficult sequences are also discussed.

Keywords: Triazole; coupling reagent; peptide synthesis.

INTRODUCTION

The principal reaction in the synthesis of a peptide is acylation of an amino group of an amino acid by the carboxyl group of a second amino acid to form an amide bond (coupling). The last few decades have seen the introduction of many types of coupling reagents. Of these, N-hydroxybenzotriazole (HOBt) and its derivatives are of particular importance.

In 1970, HOBt was first introduced into peptide synthesis as an additive to be used with the carbodiimide method by Konig *et al.*[1,2], to suppress racemisation and other side reactions caused by the widely used carbodiimide coupling reagents. Later, phosphonium derivatives of HOBt (BOP reagents) were designed by Castro *et. al.* as efficient coupling reagents for peptide synthesis in solution,[3] and soon became increasingly used as coupling reagents for solid phase peptide synthesis (SPPS).[4,5,6] However, a major drawback with the use of BOP is the formation of hexamethylphosphoric triamide (HMPA), a known carcinogen.[7,8] This has prompted the research for non-toxic coupling reagents. In 1978, Dourtoglou *et al.*[9] reported the synthesis of O-1,2,3-benzotriazolyl-1,2,3-tetramethyluronium hexafluorophosphate (HBTU) as an alternative coupling reagent for peptide synthesis in solution. This reagent was found to be fast in coupling and gave very low levels of

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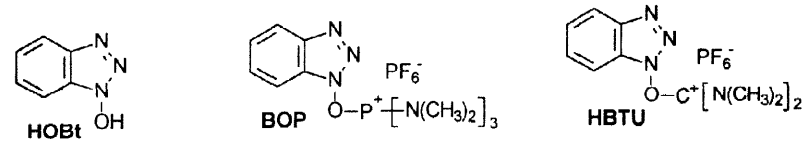
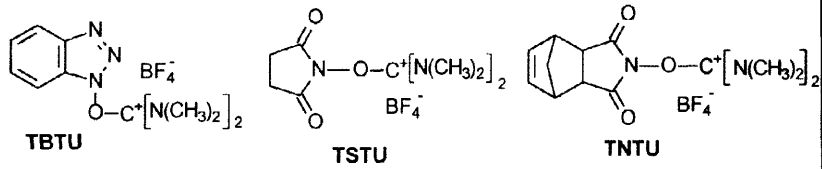
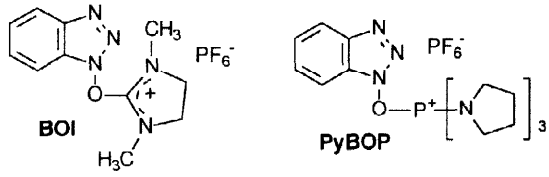
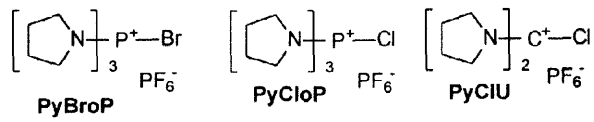
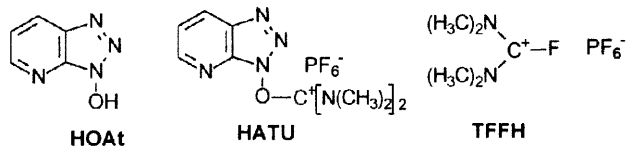
racemisation.[10] Since then, a series of analogues (TBTU, TSTU, TNTU, TSTU) have been developed by Knorr *et al* [11,12] and also other researchers (PyBOP[13] and BOI[14]) and widely used in SPPS as Knorr reagents. The HOBt esters of protected α-amino acids were recognised as the active intermediates involved in the formation of the peptide bond and the high reactivity of the HOBt esters was attributed to anchimeric assistance.[15,16] Further investigation of the original BOP reagents also led to the development of noncarcinogenic, HOBt free coupling reagents, PyBroP,[17] PyCIU and PyCloP.[18] These were found to be more efficient coupling reagents as the coupling intermediate was the more reactive acid chloride.[18]

Another significant development in the field of coupling reagents was the discovery of the 7-azabenzotriazole (HOAt) and the investigation of its coupling efficiency relative to BOP and Knorr reagents.[19,20] The enhanced coupling efficiency and lower level of racemisation reported for the HOAt esters were also attributed to the anchimeric assistance effect.[20] More recently, tetramethylfluoroformamidinium hexafluorophosphate (TFFH) was reported by Carpino *et al.* as a rapid-acting peptide coupling reagent for both solution and solid phase peptide synthesis.[21] The structures of these coupling reagents are listed in Table 1.

Table 1

Structure of Coupling Reagents	References
 HOBt BOP HBTU	1-6
 TBTU TSTU TNTU	9-12
 BOI PyBOP	13,14
 PyBroP PyCloP PyCIU	15,16
 HOAt HATU TFFH	19-21

Stepwise SPPS has been greatly improved by the development of new coupling reagents and methods. However, the efficiency of the coupling reaction varies considerably as the peptide chain grows due to steric factors and conformational effects. Apart from the gradually elongating peptide chain, which obviously accentuates the assembly difficulties, smaller peptides with so called difficult sequences have also been reported to be problematic for synthesis.[22,23] Coupling efficiency is sequence dependent and thus unpredictable, despite the research effort directed at this problem.[24] It was found that amide formation can decrease dramatically at certain amino acids in the synthesis of one peptide, whilst in another peptide the same amino acids can couple very easily. Whenever a serious decrease in coupling occurs during a synthesis, the coupling can normally be improved by a remedial treatment such as extending the coupling time, increasing the ratio of the incoming amino acid, sonication or even a recoupling. It is therefore very important to follow the synthesis using an efficient monitoring system.

The monitoring methods used in the early days of SPPS, i.e. amino acid analysis or Schiff base formation, were time consuming and not compatible with automated synthesis.[25,26] Recent studies have led to the development of more efficient methods of monitoring; however, the majority of these methods require additional reactions to introduce and remove diagnostic reagents, withdrawal of resin samples or construction of new instruments.[27,28,29]

The ideal approach to monitoring a coupling is to examine the solution of the activated amino acid during or after the coupling, without disturbing the synthetic process. Bodansky and Sheehan[30] first introduced this approach to Boc SPPS using *p*-nitrophenyl esters of the N^{α}-protected amino acids. As free *p*-nitrophenol absorbs at 314nm and the active esters absorb at 270nm, examination of the filtrate of a second coupling cycle which absorbs only at 270nm indicates the completion of the previous coupling. However, this method is not applicable to Fmoc chemistry since Fmoc adducts absorb strongly around 300nm. The current monitoring method used for Fmoc SPPS, was developed some time ago in this group.[31] The efficiency of each coupling is assessed by passing the deprotection mixture through a UV spectrophotometer and measuring the UV absorbance at the isosbestic point at 302nm of the Fmoc piperidine adduct and the corresponding fulvene. However, this monitoring method is also not real time monitoring, which means if a coupling step was found to be inefficient by the Fmoc deprotection peak, it would be too late to improve the coupling since all the amino functions of the penultimate amino acid would have been terminated by capping reagents. Obviously, examination of the acylation process before capping and deprotection, i.e. real time monitoring of such a coupling, would be of great advantage. HOBt and its derivatives (TBTU, HBTU, *etc.*) absorb strongly at 302nm, the wavelength at which the Fmoc piperidine adduct is examined; therefore UV monitoring of the coupling mixture is not compatible with Fmoc methodology. Studies were therefore undertaken to find alternative coupling reagents which do not absorb at 302nm and have better coupling efficiency. This led to the development of a new coupling reagent, ethyl 1-hydroxy-1H -1,2,3-triazole-4-carboxylate (HOCt).

RESULTS AND DISCUSSIONS

Two factors were considered to be crucial for the formation, and collapse, of the tetrahedral intermediate involved in amide formation (Figure 1). Firstly, steric effects in the union of the amine and activated amino acid must be minimised and, secondly, the activating group must be electronegative and hence a good leaving group. Apart from the nature of the protecting groups in R and R' the only variable that can affect both of these factors resides in the nature of the activating group, Y. Thus in designing new N-hydroxy compounds akin to those discussed previously, the reagent should be more acidic than HOBt, less sterically demanding and, above all, it should not absorb around 302nm, so that the coupling process can be monitored before deprotection of the Fmoc protecting group. A series of N-hydroxy compounds based upon HOBt were synthesised and investigated. The structures of these compounds are listed in Figure 2.

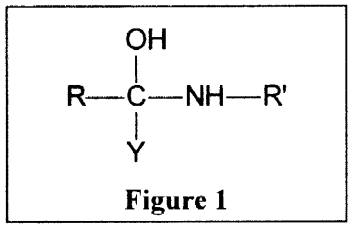
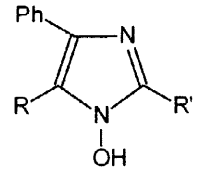
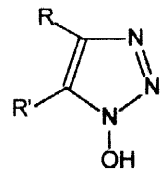


Figure 1



(1) N-hydroxylimidazoles

	R	R'
a.	Me	Me
b.	H	Me



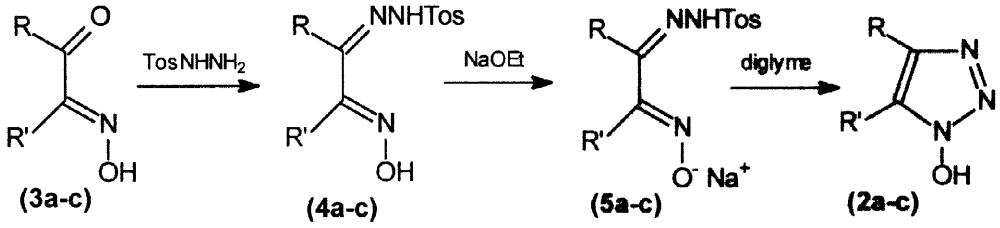
(2) N-hydroxyltriazoles

	R	R'
a.	Ph	Me
b.	p-MeOC$_6$H$_4$	Me
c.	Me	Me
d.	EtO$_2$C	Me
e.	EtO$_2$C	H

Figure 2

The N-hydroxyimidazoles (**1a** & **1b**, Figure 2) were synthesised in order to assess the importance of the triazole ring system and these compounds were found to be very poor auxiliary nucleophiles in amide formation. However, the N-hydroxytriazoles showed some promising activity. It was found that 1-hydroxy-5-methyl-4-phenyl-1H-1,2,3-triazole (**2a**) and 1-hydroxy-4-(4'-methoxy-phenyl)-5-methyl-1H-1,2,3-triazole (**2b**) were as good as HOBt as auxiliary nuleophiles, but less soluble in the solvent system used for SPPS (DMF, dioxane). As would be expected for phenyl- containing compounds, their UV absorbance around 302nm was significant and thus not suitable for real time monitoring. Replacement of the 4-phenyl group with a methyl group led to compound (**2c**), 4,5-dimethyl-1-hydroxy-1H-1,2,3-triazole, however this compound was too

hygroscopic to be isolated as a solid. The general synthetic route used for the synthesis of the three compounds is shown in Scheme 1.



Scheme 1

Since an N-hydroxytriazole with no UV absorbance at 302nm was required, attention was turned to the ester of the triazole carboxylic acid reported by Wolff[32] and Jenkins[33] (Scheme 2). The starting material, ethyl 2-diazo-3-oxobutanoate (7) was obtained in good yield (83%) by the triethylamine catalysed reaction of ethyl 3-oxobutanoate (**6**) with tosyl azide (Scheme 2). The reaction of this diazo-keto-ester (7) with hydroxylamine under aqueous conditions at 80°C afforded the required product, ethyl 1-hydroxy-5-methyl -1H-1,2,3-triazole carboxylate (**2d**) in ~50% yield. The crystal structure is shown in Figure 3 and the crystallographic data will be published independently by S. Parsons.

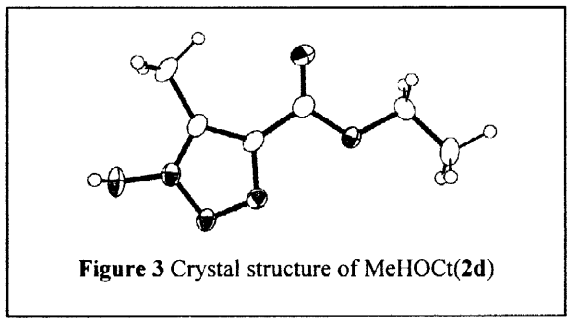
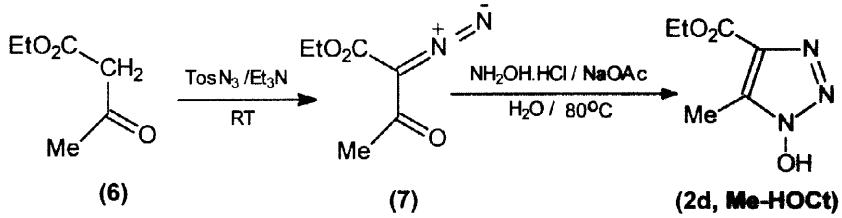
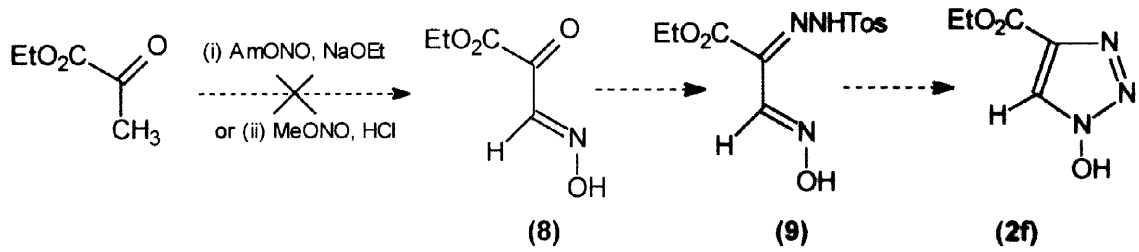


Figure 3 Crystal structure of MeHOCt(**2d**)



Scheme 2

The UV spectrum of this compound was very encouraging since it showed very little absorbance around 302nm. It was very soluble in DMF:dioxane and its pKa was found to be 2.65 (HOBt ~4.0). Further study of this compound as a potential coupling reagent indicated that it was as good as HOBt in the synthesis of the test peptide LeuIlePheAlaGly and also in the synthesis of longer peptides (data not shown). However, it still possessed some degree of steric hindrance near the N-hydroxy group due to the 5-methyl group which might be responsible for the only moderate coupling efficiency. The ideal would be to replace this methyl group with a sterically undemanding H-atom. The corresponding target triazole derivative was the novel auxillary nuleophile, ethyl 1-hydroxy-1H -1,2,3-triazole-4-carboxylate (HOCt, **2e**).[34,35]



(8) (9) (2f)

Scheme 3

At the outset of this work, HOCt was not a known compound and the work presented here describes our investigations leading to the synthesis of this compound. Since it was required as a reagent, the synthetic route had to be short and involve facile isolation and purification of the product. Our initial attempts to obtain HOCt were based on the general procedure used for the synthesis of triazoles (**2a**) and (**2b**) (Scheme 1). In order to proceed by this method, the starting ketoxime (**8**) was required and its synthesis was attempted by the formation of the oxime of ethyl 2-oxopropanoate, firstly by reaction with isoamyl nitrite in the presence of base and secondly by reaction with methyl nitrite in acid. However, neither of these routes were successful in yielding the required ketoxime (**8**). Our attention was then turned to the approach used in the synthesis of ethyl 1-hydroxy-5-methyl-1H-1,2,3-triazole (**2d**, Scheme 2). The application of this approach would require the ethyl 2-diazo-3-oxopropanoate (**13**, Scheme 4) as starting material. The synthesis of this α-diazoaldehyde (**13**) was a known reaction reported by Stojanovic.[36] Ethyl diazoacetate (**10**) reacted with the Vilsmeier reagent (**11**),[37,38,39] followed by hydrolysis of the intermediate (**12**) in aqueous acetic acid to give the expected ethyl 2-diazo-3-oxopropanoate (**13**) as a liquid in a yield of ~50% (route a, Scheme 4). The α-diazoaldehyde (**13**) was then treated with hydroxylamine under the same conditions as shown in Scheme 2, however, only a small amount of solid (<10% yield) was obtained. The analysis data and ^{1}H, ^{13}C NMR spectra were all in agreement with the expected product structure and the pKa was 2.16. The crystal structure of HOCt is shown in Figure 4.

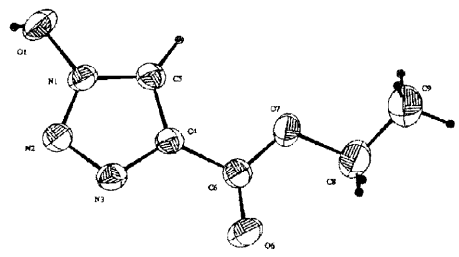
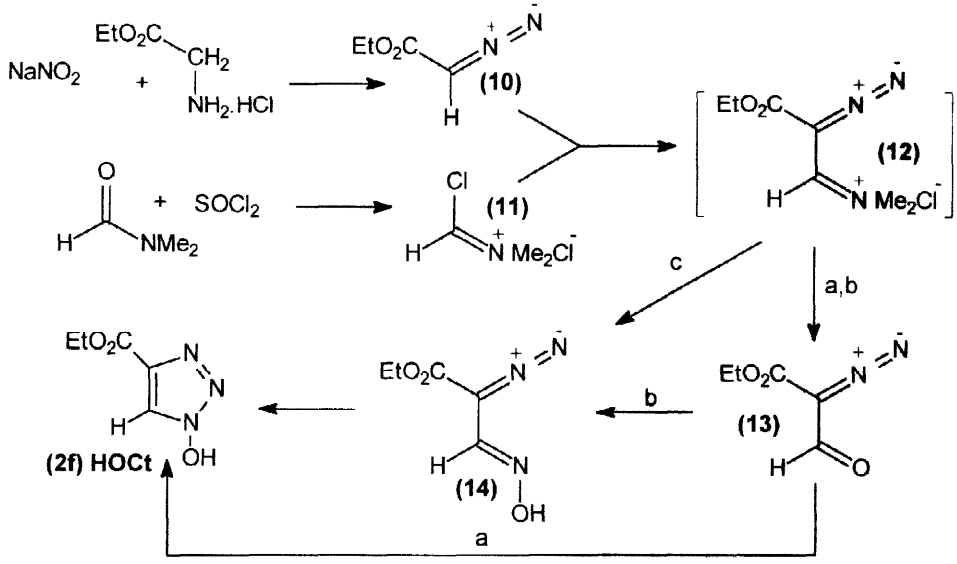


Figure 4 Crystal structure of HOCt (**2e**)

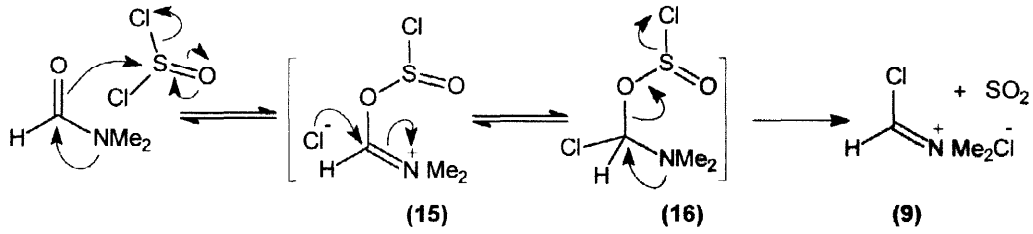
Further study of this reaction was carried out to improve the yield. Since the final product was found to be very soluble in water, the original aqueous conditions were obviously not suitable for the last step. The reaction was thus repeated using anhydrous ethanol as solvent. After work up of the reaction mixture, a bright yellow solid was obtained which, when recrystallised for analysis, gave a colourless solid whose analytical and spectral data were consistent with the data obtained previously for the final product, HOCt (**2e**). Re-investigation of the yellow solid suggested it was the diazo-oxime (**14**), which cyclised under reflux (on

recrystallisation) to give the final product HOCt (route b, Scheme 4). Re-evaluation of the synthetic route also suggested that the intermediate (**12**), which could not be isolated, might undergo reaction with hydroxylamine to form the diazo-oxime (**14**) as was the case with the diazo-aldehyde (**13**). Therefore the reaction between ethyl 2-diazoacetate (**10**) and the Vilsmeier reagent (**11**) was repeated and the intermediate (**12**) was treated with hydroxylamine in anhydrous ethanol to give the expected diazo-oxime (**14**) in ~50% yield (route c, Scheme 4). This was then refluxed in benzene to give the required cyclised product, HOCt.



Scheme 4

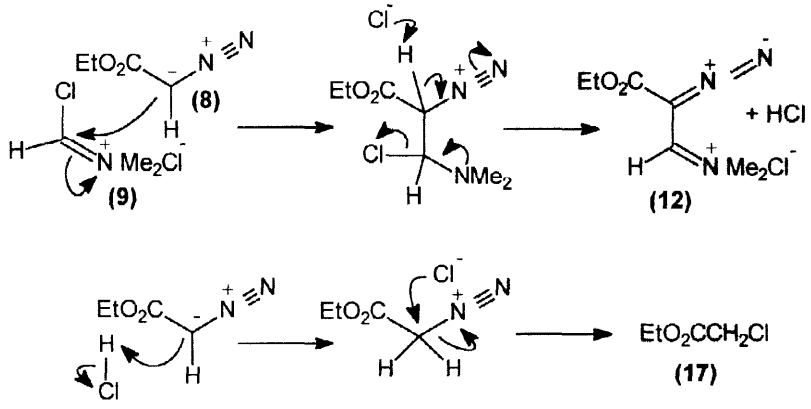
When the synthesis was repeated in order to obtain more material for further study, however, it was found that the overall yield was poor and not reproducible. Moreover, the protocol involved refluxing the diazo oxime (**14**) in benzene to cyclise it, which is hazardous since diazo compounds are potentially explosive, especially at high temperature.



Scheme 5

Further investigation of the whole synthetic route revealed that the poor reproducibility was partially due to the incomplete reaction between DMF and thionyl chloride. This not only reduced the yield of the Vilsmeier reagent, but also made the reagent very unstable due to the presence of the reaction intermediates (**15** and **16**) as indicated by the reaction mechanism (Scheme 5). The removal of the by-product, SO_2 under vacuum

should help to drive the reaction to completion, but one of the starting materials, $SOCl_2$ is also volatile, therefore it must be converted into the reaction intermediate before vacuum could be applied. It was found that this could be done by raising the temperature from 0°C to 40°C and for a longer period of time (2h~3h). The mixture was then evaporated, *in vacuo*, until a white solid was afforded. The temperature of the water bath could also be raised to 40°C. The solid obtained under these conditions was reasonably stable and could be handled in air with care. The reaction between the ethyl 2-diazoacetate (**10**) and the Vilsmeier reagent (**11**) was initially carried out in freshly dried chloroform and was found to be very vigorous, producing both heat and gas. Therefore, the ethyl 2-diazoacetate solution in chloroform was added very slowly while stirring the reaction mixture at 0°C. The reaction was complete soon after the addition of the ethyl 2-diazoacetate (**10**) was finished and the reaction mixture was then evaporated whereupon the product was precipitated from ether as waxy solid. This product was found to be very unstable and should be used immediately. As proposed by Stojanovic,[36] a side reaction was involved in this step thus an extra equivalent of ethyl 2-diazoacetate (**10**) was required to react with the by-product, HCl (Scheme 6). A small amount of the resulting by-product, ethyl chloroacetate could easily be trapped in the product which was a waxy solid, and was very hard to remove due to its relatively high boiling point. This impurity, however, did not affect the succeeding reactions.



Scheme 6

The reaction intermediate (**12**), which should be used immediately, was added as a solid to a solution of 1.2 eq. of $NH_2OH.HCl$ and 0.6 eq. of Na_2CO_3 in ice-cold H_2O (pH ~7.5). A bright yellow solid was soon precipitated after the addition of the orange coloured diazo intermediate (**12**). The solid was filtered off after 5 min, washed with cold water and dissolved in benzene. A small amount of water was isolated using a separating funnel and the solution dried over anhydrous Na_2SO_4, filtered, then refluxed to give the final product. The yield of the diazo-oxime (**14**) was similar to the ethanol method (50%), but it offers the advantage that no solvent has to be removed. This is very important as evaporating diazo compounds can be hazardous. The other advantage

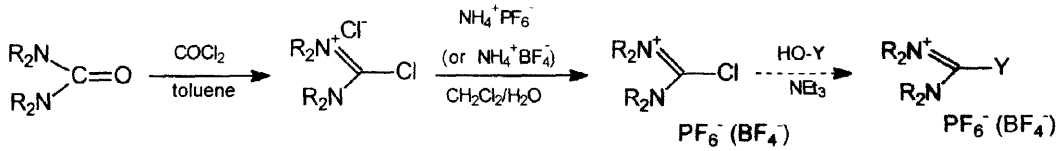
of this protocol was that all water soluble impurities could be removed which simplied the recrystallisation of the final product.

The only drawback remaining at this stage was the final cyclisation which was carried out under reflux in benzene. The problem was solved fortuitously. When an NMR sample of this yellow solid in $CDCl_3$ solution was left at room temperature for two days, the yellow colour disappeared, resulting in a completely colourless solution, which gave exactly the same ^1H NMR as the final product, HOCt. The diazo oxime (**14**) which precipitated from water was thus dissolved in chloroform, after removal of the remaining trace of water by phase separation, the chloroform solution was dried over Na_2SO_4 **(The diazo oxime (14) must not be dried in the solid state due to its instability)**. A drop of acetic acid was added to the $CHCl_3$ solution as catalyst, and the solution was left at room temperature until the yellow colour disappeared after about two days. Light was also found to promote the cyclisation. The HOCt was isolated as a colourless solid which can be recrystallised easily from ethyl acetate and the final product was very stable and can be stored in air for years.

A procedure for the synthesis of HOCt was thus developed as a very convenient protocol which can be carried out easily in two working days plus the final recrystallisation. An average overall yield of 30~35% can be achieved reproducibly. It should be pointed out that while this work was in progress, Bennett *et al.*[40] reported the synthesis of the same triazole by Stojanovic's method although no experimental details were given.

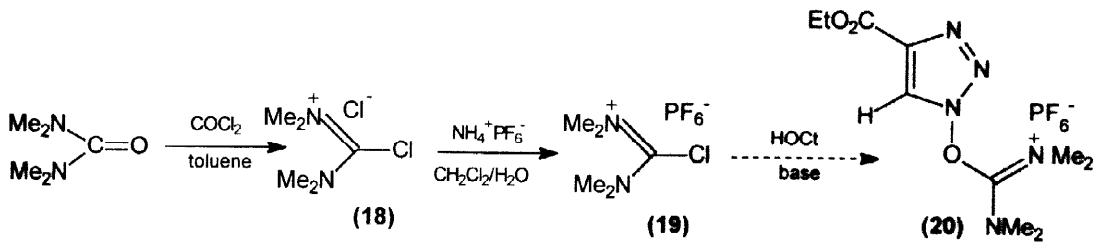
Attempted synthesis of the HOCt Knorr reagents

Knorr reagents was first synthesised by Gross *et al.* [9,10] and further developed by Knorr *et al.* as superior coupling reagents for peptide synthesis.[11-14,17,18] A general synthetic procedure is shown in Scheme 7.[9,10] The product precipitated from the reaction mixture as the uronium salt which was isolated by filtration and purified by recrystallisation.

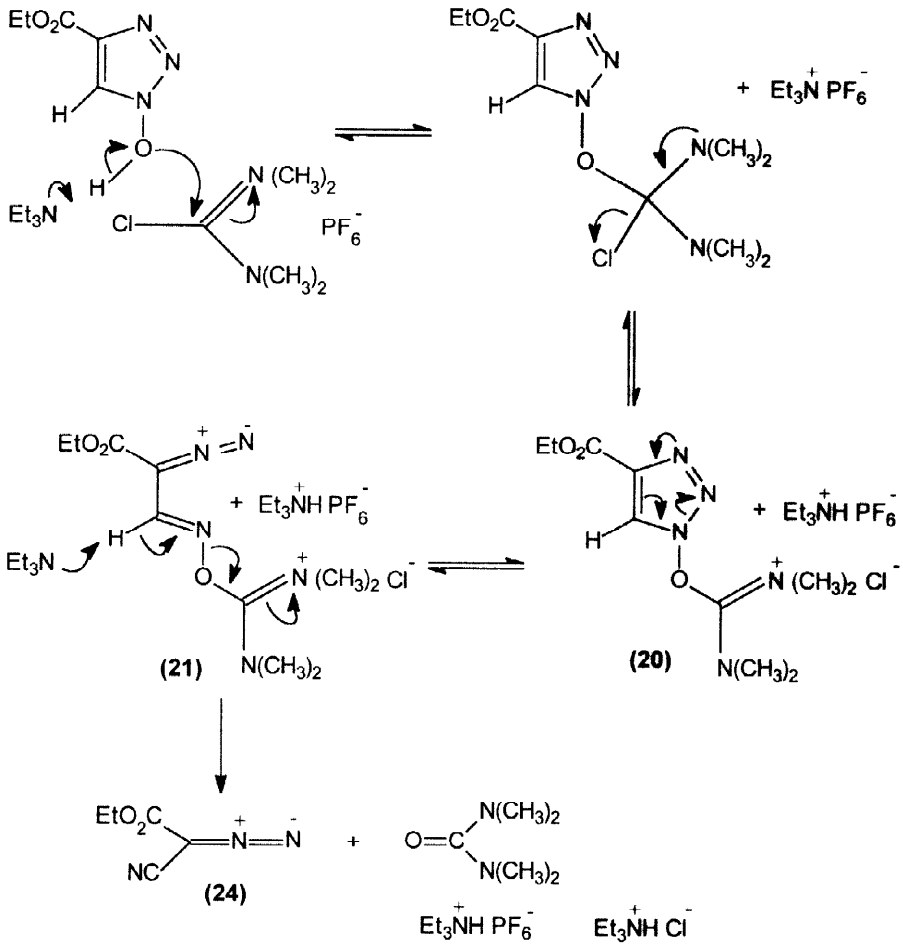


Scheme 7

The same procedure was followed in order to synthesise the corresponding HOCt Knorr reagents (Scheme 8). However, none of the expected product (**20**) precipitated. Instead, a yellow colour appeared in the solution indicating the formation of diazo compound(s). IR spectrum of the reaction mixture showed two peaks at 2229cm^{-1} and 2135cm^{-1} characteristic for a nitrile group and a diazo group respectively. Attempts to triturate the product, however, were largely unsuccessful. The electrospray mass spectrum of the reaction mixture showed a peak of the original starting material 1,1,3,3-tetramethylurea (m/z 116) on the positive ion mode and a



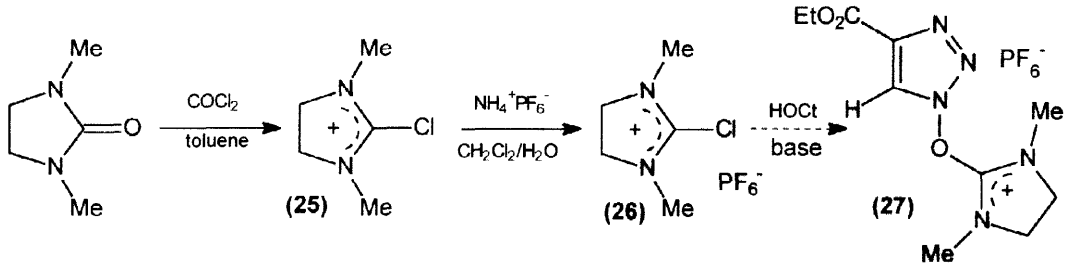
Scheme 8



Scheme 9

peak of PF_6 (m/z 144) on the negative ion mode, but did not show any peak of the expected product (**20**, $C_{10}H_{18}N_5O_3^+PF_6^-$ 256/145). This mixture was then separated by flash chromatography allowing the isolation of one of the products as a yellow oil. The IR spectrum of this oil showed three typical peaks, a nitrile group at 2229cm^{-1}, a diazo group at 2135cm^{-1}, and a carbonyl group at 1716cm^{-1}. The ^{1}H NMR spectrum showed one ethyl group (δ^1H 200MHz, $CDCl_3$, 1.31, t, 3H, J=7.1; 4.30, q, 2H, J=7.1) while the ^{13}C NMR spectrum (200MHz, $CDCl_3$) showed one CH_3 group (13.66), one CH_2 group (62.72) and two quaternary carbon atoms (106.7 and 160.4). The data indicated that the triazole ring had undergone ring-opening and that the yellow oily product was the diazonitrile derivative (**24**, Scheme 9). The expected mass of (**24**)(MH^+ 140) was observed in the electrospray mass spectrum only under very mild conditions (cone 10 Volts, ion energy 0.1 Volts, source temperature 50°C). A proposed mechanism for the formation of this compound is shown in Scheme 9, which is very similar to the mechanism proposed by Dulcere for the conversion of aldoximes to nitriles using the Vilsmeier reagent (**11**).[38]

Since no product was isolated under the above reaction conditions, a weaker base, imidazole was used instead of triethylamine. The resulting by-product, imidazole hydrochloride precipitated and was removed by filtration. The filtrate was evaporated to give an oily, yellow residue and isolation of the product again was unsuccessful. Similar results were obtained when the BOI analogue of HOCt was attempted (**27**, Scheme 10), which suggested that the HOCt Knorr reagents could not be isolated as stable solids.



Scheme 10

The synthesis and study of HOCt active esters

Active esters are coupling reagents which can be useful in SPPS. An attempt was therefore made to synthesise the active esters of HOCt. FmocLeuOH was chosen as the standard for studies of reaction conditions since the side chain is not functionalised but still provides reasonable steric hindrance. DCCI was used as the dehydrating reagent since the by-product DCU was not soluble in DCM and could be removed easily by filtration. The reaction was carried out at various temperatures, monitoring by TLC(DCM/MeOH = 9:1), which indicated the presence of the starting materials even after stirring overnight. It was then found that this was due to the high reactivity of the active ester, which was not stable under TLC conditions. The reaction was thus repeated and the reaction mixture was filtered then the filtrate was evaporated to yield a colourless solid, which

was examined by ^{1}H NMR spectroscopy. A singlet was observed at 8.0ppm which could not be attributed to any of the starting materials. This was found to belong to the H-5 of the active ester. However, ^{1}H NMR data also indicated that there was ~20% unreacted HOCt present in the mixture. Further investigation showed that the active ester is very sensitive to moisture being prone to hydrolysis on prolonged stirring and attempted purification. Therefore the active esters of all of the common amino acids were prepared under dry conditions. The Fmoc amino acid and HOCt were dissolved in dry DCM in exactly 1:1 ratio and the mixture was stirred under nitrogen. After 30 min, the insoluble DCU formed was filtered off and the filtrate evaporated under vacuum. The residue was then dissolved in $CDCl_3$ for ^{1}H NMR spectroscopy. The amount of the free triazole in the mixture should be equal to that of the unreacted amino acid. The percentage of active ester of each amino acid was thus calculated from the ^{1}H NMR data. However, the results indicated that most of the active esters obtained contained a significant amount of unreacted amino acid, sometimes as much as 50%.

In order to confirm the formation of the active ester, an indirect method was designed. FmocLeuOH and HOCt (0.5mmol each) were dissolved in dry DCM to which was added DCCI (0.5mmol). The mixture was stirred at RT under nitrogen for 30 min and the DCU which precipitated was filtered off (under nitrogen) and the filtrate evaporated until dry. The residue was then dissolved in DMF/dioxane (1:1, 4ml) and the mixture was added to a reaction vessel, which contained 0.25mmol H_2N-Ala-Wang resin. The mixture was vortexed on the peptide synthesiser for 30 min. and the coupling yield was determined by measuring the Fmoc deprotection and amino acid analysis. The deprotection of Leu was 85% of that of Ala, according to the Fmoc deprotection peaks, but amino acid analysis showed Leu/Ala (1:1) indicating 100% coupling. As no other activating reagent was added, the active intermediate could only have been the active ester which suggested that the HOCt worked through its active ester just as HOBt. However, since the active esters were too hygroscopic to handle they were considered unsuitable as coupling reagents.

Considering these results HOCt alone was investigated as coupling reagent in association with DIC.

Racemisation studies of HOCt

Racemisation remains a major consideration in peptide synthesis. Technically, it is very important to detect a small amount of racemisation as this can be crucial to the biological activity of the peptide synthesised. As racemisation can happen in the process of activation, as well as in the coupling, the method should take both processes into account. High field proton NMR spectroscopy provides a very sensitive way to distinguish protons with subtle differences in chemical environment. It was thus decided to study the racemisation by NMR spectroscopy. A trial peptide Ala-His-Gly was made using DIC as the coupling reagent to give the maximum amount of racemisation. As expected, two doublets showed up in the ^{1}H NMR spectrum, each corresponding to the methyl group of D or L-Ala, which were in c.a 1:1 ratio and 0.15ppm apart from each other. Careful integration of the two doublets would then allow calculation of the percentage racemisation. A series of trimers

with the general structure of Ala-X-Gly were designed. This sequence was chosen as Gly was not chiral and Ala was the amino acid least prone to racemisation. If the amino acid at the middle position racemised, the percentage racemisation could be calculated from ^{1}H NMR data.

No racemisation was observed for eighteen N$^{\alpha}$-protected native amino acids (neglecting Gly). The exception was His, which is especially prone to racemisation due to the imidazole side chain group. The first trimer studied was thus Ala-His-Gly. When FmocHis(Trt)OH in which the τ-nitrogen of His is protected, was used in the synthesis of this trimer, 20% D-His was observed in the product under normal coupling conditions using HOCt compared with ~10% while using HOBt. However, the racemisation level was reduced to a negligible level when excess (3eq.) of HOCt was used in the coupling mixture, or if the coupling is carried out at 0°C in a sonic bath.

The introduction of a protecting group on the π-nitrogen of His has proven to be advantageous in overcoming the racemisation problem.[41,42] However, the synthesis of the π-nitrogen protected histidine is very difficult and the only commercially available analogue, FmocHis(Bum), can only be prepared in very poor yield and of not very satisfactory purity.[43] When the π–nitrogen protected His(Bum) was used in the synthesis of the trimer, no racemisation was detected from the ^{1}H NMR, using either HOBt or HOCt as coupling reagent. However, as the reagent itself was not pure, other unpredictable impurities were found in the final product.

Monitoring of the coupling

One of the objective of this study was to develop a new coupling reagent which did not absorb at 302nm, so that the coupling process could be monitored before deprotection of the Fmoc protecting group. This would present the opportunity for improvement when a poor coupling occurred. The reaction mixture was passed through a UV detector connected to the peptide synthesiser at the end of each coupling cycle, so that the unreacted Fmoc amino acid or its activated isomer could be examined. To reduce the error caused by uneven delivery during different stages of the synthesis, an external standard, fluorene, was introduced. The amount of unreacted Fmoc amino acid can thus be deduced from the peak area. A comparison is made between this real-time monitoring and the previously developed deprotection monitoring method, and a typical profile is shown in Figure 5.

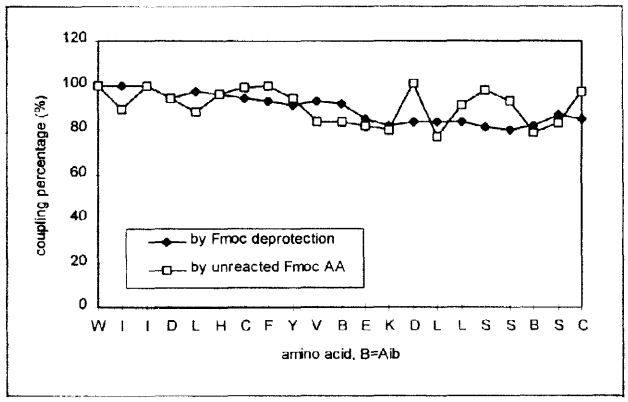


Figure 5 Coupling efficiency of an Et-1 analogue

The two methods correspond with each other reasonably well, but errors in measurement do occur. This is because the solubility of some Fmoc amino acids is not good enough, and therefore they cannot be transferred to the reaction vessel completely. Even for the more soluble ones, reagent delivery can also vary slightly. This is reasonable since the synthesiser was not specifically designed for this monitoring process. The slight variation will not seriously affect the coupling process, but it does affect the accuracy of the coupling efficiency deduced from the data. More accurate monitoring, therefore, will rely on improvements in the solubility of the amino acids (i.e. better solvent, elevated temperature etc.), and also the delivery system of the synthesiser.

Synthesis of endothelin analogues and difficult peptides using HOCt

Endothelin (ET) is a highly potent vasoconstrictor which was discovered in 1988[44]. The biological activity and SAR studies of ET-1 analogues will be published elsewhere. However, the synthesis of some endothelin analogues, have been found to be difficult, especially when bulky amino acids such as Aib are included in the sequence.[45] A comparative study was thus carried out and some typical coupling results monitored by the Fmoc deprotection method are shown in Figures 6 and 7.

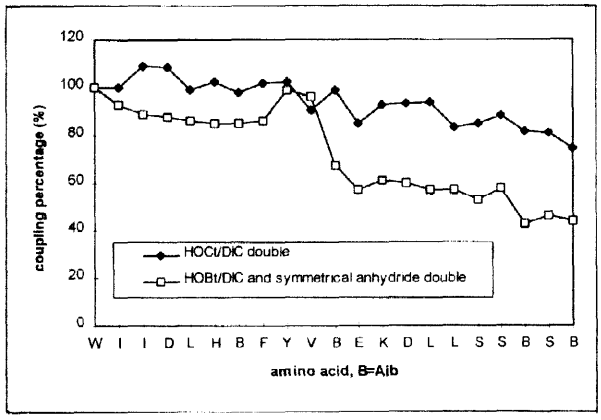


Figure 6 Coupling efficiency of ET-1 analogue using HOCt or HOBt/symmetrical anhydride method

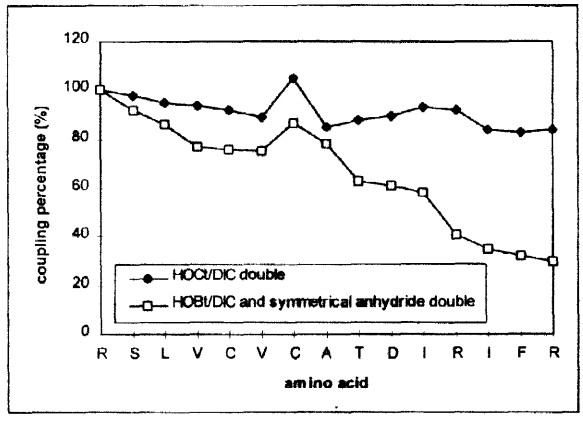


Figure 7 Coupling efficiency of NGF fragment using HOCt or HOBt/symmetrical anhydride method

In Figure 6, two coupling methods, HOCt/DIC double coupling and the combination of HOBt/DIC and symmetrical anhydride couplings, are compared for the synthesis of ET-1 analogues, which involves the bulky amino acid, Aib, at four positions. The overall coupling yield with HOCt is nearly 30% higher. Another typical example was the synthesis of an NGF fragment.[46] This small peptide caused many problems in its assembly, and a low yield was obtained in the first few syntheses before HOCt became available. When HOCt was used as coupling reagent, the overall coupling percentage was improved from 30% to 80% (Figure 7), and the

peptide was isolated in very good purity without any difficulty.[46] HOCt has thus been adopted in this laboratory as the standard coupling reagent and has been used for the chemical synthesis of many peptides and proteins.

MATERIAL AND METHODS

All chemicals were purchased from Aldrich or Sigma except the Fmoc amino acids, which were purchased either from Bachem or Novabiochem. The peptides were synthesised on an adapted ABI 430A peptide synthesiser at 0.25mmol scale as was reported previously,[47] except that HOCt was used instead of HOBt.

Synthesis of imidazoles and triazoles

Hazard: diazo compounds are potentially explosive and therefore must be handled with care. The diazoesters are toxic and prone to cause development of specific sensitivity. A well-ventilated fume hood should be used for the entire procedure.

2,5-Dimethyl-1-hydroxy-4-phenyl-1H-imidazole (1a) A solution of 1-phenylpropane-1,2-dione 2-oxime (8.2g, 0.05mol) in ethanol (150ml) was treated at RT with acetaldehyde (4.4g, 0.1mol) then concentrated ammonia solution (10.0ml, 0.2mol) and the resulting mixture was stirred at RT overnight. The mixture was evaporated to give an oil which was dissolved in H_2O (20ml). A solid precipitated almost immediately from the aqueous solution and was collected, washed with ether and dried in vacuo to give the N-hydroxyimidazole derivative as a colourless solid. (17.5g, 80%), m.p. 168-170°C(from acetonitrile-ethanol), δ^1H NMR(200MHz, DMSO-d_6): 7.36-7.60(5H, m, Ar), 3.77(1H, br, s, OH), 2.27 (3H, s, CH_3), 2.25 (3H, s, CH_3). Found: C,69.9; H,6.3; N,14.8%, 188 (m/z, EIms); $C_{11}H_{12}N_2O$ calculated: C,70.3; H,6.4; N,14.9%, 188 (M^+).

1-Hydroxy-2-methyl-4-phenyl-1H-imidazole (1b) 1-Phenylethane-1,2-dione 2-oxime was prepared (40%) by the sodium ethoxide catalysed reaction of acetophenone with isoamyl nitrite as described by Claisen and Manasse,[48] and had m.p. 123-125°C(lit.[48] 126-128°C). A solution of this compound (1.5g, 0.01mol) in ethanol (40ml) was treated with acetaldehyde (0.9g, 0.02mol) and concentrated ammonia solution (2.0ml, 0.04mol) and the mixture was stirred at RT overnight. The mixture was evaporated to give an oil which was treated with H_2O (16.0ml) and the resulting solution acidified by the addition of concentrated HCl, then extracted with ether to give the starting ketoxime (0.3g, 20%), identified by comparison with an authentic sample. Precipitated solid from the acidic aqueous portion was collected and combined with further material obtained by adjusting the pH to 6.0 with ammonia to give product (1b) (0.5g, 26%). m.p. 176-178 °C, ^{1}H NMR(200MHz, DMSO-d_6): 7.20-7.75 (5H, m, Ar), 2.27 (3H, s, CH_3). Found: C,69.1; H,5.8; N,15.9%; 174(m/z, EIms); $C_{10}H_{10}N_2O$ calculated: C,69.0; H,5.8; N,16.1%; 174 (M^+).

1-Phenylpropane-1,2-dione-2-oxime (3a) 1-Phenylpropane-1,2-dione 2-oxime was prepared (yield 79%) by the reaction of propiophenone with methyl nitrite in the presence of anhydrous HCl as described by Hartung and Crossley,[49] and was isolated as a colourless solid, m.p. 115-116°C (lit.[49] 112-113°C).

1-Phenylpropane-1,2-dione-2-oxime 1-toluene-4-sulphonylhydrazone (4a) The title compound was prepared (yield 89%) by reaction of 1-phenylpropane-1,2-dione 2-oxime with tosylhydrazine as described by Bartlett[50] and Jenkins[32], and had m.p. 181-183 (lit.[32] 177-179°C).

1-Hydroxy-5-methyl-4-phenyl-1H-1,2,3-triazole (2a)[32] A suspension of 1-phenylpropane-1,2-dione-2-oxime 1-toluene-4-sulphonylhydrazone (12.6g, 0.036mol) in anhydrous 1,2-dimethoxyethane (180ml) was stirred and treated with a solution of sodium (2.5g, 0.11mol) in anhydrous ethanol (360ml). The mixture was stirred at RT for 30 min, then evaporated under vacuum. The solid obtained was broken up, anhydrous diglyme (360ml) was added and the mixture was stirred and heated under reflux for 15min. then cooled. Any lumps of solid were broken up and stirring and heating under reflux were continued for a further 30min. The mixture was evaporated under vacuum and the residue was dissolved in H_2O (140ml) and the solution chilled and acidified to pH1 by addition of 2M HCl. The precipitated solid was collected and combined with further material which separated from the aqueous layer on standing to give the product (2a) (4.9g, 77%) which was purified by recrystallisation from aqueous methanol, m.p. 164-166°C (lit.[32] 168-169°C), δ^1H NMR (200MHz, $CDCl_3$): 2.42(3H, s, CH_3), 7.34-7.88 (5H, m, Ar).

1-(4-Methoxyphenyl)propane-1,2-dione 2-oxime (3b) A solution of 1-(4-methoxyphenyl)-propan-1-one (26.3ml, 0.15mol) in anhydrous ether (100ml) was stirred and treated simultaneously at RT with slow streams of anhydrous HCl and methyl nitrite [generated by dropping a cold 6M sulphuric acid solution into a stirred mixture of sodium nitrite (12.4g), methanol (8.0ml) and water (8.0ml)]. After 40min the flow of methyl nitrite was stopped and the passage of HCl continued for a further 10min. The mixture was then left stirring at RT for 24h and the resulting mixture was extracted with 10% w/v aqueous NaOH solution (10 x 20ml). The combined aqueous solution was poured into a stirred mixture of concentrated HCl (35.0ml) and ice (40g) whereupon precipitated solid was collected, washed with water and dried to give the oxime product(**3b**). (18.0g, 62%) as a cream powder, m.p. 135-138°C, ν_{max} 3500-2500 br (OH) and 1650 (C=O) cm^{-1}; δ^1H NMR(200MHz, DMSO-d_6): 2.00(3H, s, CH_3), 3.82(3H, s, OCH_3), 6.94(2H, d, J=9Hz, ArH), 7.90(2H, d, J=9Hz, ArH). Found: C,61.7; H,5.5; N,7.1%; 193(m/z, EIms); $C_{10}H_{11}NO_3$ calculated: C,62.2; H,5.7; N,7.3%; 193 (M^+).

1-(4-Methoxyphenyl)propane-1,2-dione 2-oxime 1-toluene-4-sulphonylhydrazone (4b) A solution of 1-(4-methoxyphenyl)propane-1,2-dione 2-oxime (3b) (7.7g, 0.04mol) in ethanol (100ml) was added to a solution of tosylhydrazine (7.4g, 0.04mol) in ethanol (50ml) and the resulting mixture was stirred and heated under reflux for 2h. The mixture was filtered to remove inorganic material. The filtrate was cooled and the precipitated solid was collected to afforded the tosylhydrazone derivative (4b) (9.9g, 69%) which formed colourless crystals. m.p. 169-171°C (from toluene-ethanol), ν_{max} 3400-2500 br (OH) and 3200 (NH) cm^{-1}; δ^1H NMR(200MHz, DMSO-

d_6): 1.93(3H, s, CH_3), 2.39 (3H, s, CH_3), 3.78(3H, s, OCH_3), 7.01-7.81(8H, m, ArH), 10.25(1H, s, OH), 11.51(1H, s, NH). Found: C,56.9; H,5.4; N,11.5%; 361(m/z, EIms); $C_{17}H_{19}N_3O_4S$ calculated: C,56.5; H,5.3; N,11.6%; 361(M^+).

1-Hydroxy-4-(4-methoxyphenyl)-5-methyl-1H-1,2,3-triazole (2b) A suspension of 1-(4-methoxyphenyl) propane-1,2-dione 2-oxime 1-toluene-4-sulphonylhydrazone (**4b**) (12.6g, 0.035mol) in anhydrous 1,2-dimethoxyethane (90.0ml) was treated with a solution of sodium (2.5g, 0.11mol) in anhydrous ethanol (350ml) and the resulting mixture was stirred at RT for 30min, then evaporated under high vacuum. The solid obtained was broken up, anhydrous diglyme (350ml) was added and the mixture was stirred and heated under reflux for 1.5h. The mixture was evaporated under high vacuum and the residue was dissolved in water (150ml) with warming, chilled and acidified to pH1 by the addition of concentrated HCl. The precipitated solid was collected and dried to give the title compound as cream microcrystals(5.2g, 75%). m.p. 174-176°C(from ethanol-light petroleum), ν_{max} 3100-2000 br (OH) cm^{-1}; δ^1H NMR(200MHz, DMSO-d_6): 2.33(3H, s, CH_3), 3.70(3H, s, CH_3O), 7.00(2H, d, J=9Hz, ArH), 7.60(2H, d, J=9Hz, ArH), Found: C,58.1; H,5.4; N,20.3%; 205 (m/Z, EIms). $C_{10}H_{11}N_3O_2$ calculated: C,58.5; H,5.4; N,20.5%, 205(M^+).

Butane-2,3-dione 2-oxime 3-tosylhydroazone (3c) The title compound was prepared by the reaction of butane-2,3-dione 2-oxime with toluene-4-sulphonylhydrazine in 96% yield as described by Jenkins and had m.p. 168-170°C (from toluene-ethanol) (lit.[32] 178-179°C).

Attempted synthesis of 4,5-dimethy-1-hydroxyl-1H-1,2,3-triazole (2c) The same procedure was followed as described in the synthesis of 1-hydroxy 4-(4-methoxyphenyl)-5-methyl-1H-1,2,3-triazole (**2b**), but it failed when used to make the expected 4,5-dimethyl-1-hydroxy-1H-1,2,3-triazole (**2c**).

Synthesis of HOCt

Ethyl 2-diazo-3-oxobutanoate (7)[32,33] A solution of ethyl acetoacetate (22.1g, 0.17mol) in anhydrous acetonitrile (280ml) was treated with a single portion of triethylamine (17.2g, 0.17mol). The mixture was stirred and treated at RT with a single portion of toluene-4-sulphonyl azide (33.5g, 0.17mol). The mixture was stirred at RT with exclusion of atmospheric moisture for 1.5h and then evaporated under high vacuum. The residue was treated with ether (200ml) and 30% KOH (200ml). The two layers were separated and the ether layer was washed with two further portions of 30% KOH, then with H_2O (3 x 40ml) and evaporated to give the known product as a yellow liquid (22.0g, 83%); ν_{max} 2120 (N≡N) and 1700 (C=O) cm^{-1}.

Ethyl 1-hydroxy-5-methyl-1H-1,2,3-triazole-4-carboxylate(2d)[32,33] A solution of NaOAc (24.9g, 0.3mol) in H_2O (50ml) was treated with hydroxylamine hydrochloride (20.9g, 0.3mol) until neutral and then added to a solution of ethyl 2-diazo-3-oxobutanoate (15.6g, 0.1ml) in ethanol (50ml). The mixture was then stirred and heated at 80°C for 2h. A solution of NaOAc (16.6g, 0.2mol) in H_2O (40ml) was treated with hydroxylamine hydrochloride (14.0g, 0.7mol) until neutral and then added to the above mixture, then stirred and heated at 80°C

for a further 3h. The mixture was evaporated to give an off-white solid which was dissolved in H_2O (150ml) with warming, then chilled and acidified to pH 1 by addition of concentrated HCl. The precipitated solid was collected, dried to give colourless crystals (9.0g, 53%), m.p.133-136°C (from ethyl acetate), (lit.[32] 145-147°C) (from ethyl acetate); ν_{max} 3000-2000 br (OH) and 1720 (C=O) cm^{-1}; δ^1H NMR(200MHz, DMSO-d_6): 1.24(3H, t, J=7Hz, CH_3), 2.34(3H, s, CH_3), 4.29(2H, q, J=7Hz, CH_2).

Ethyl 2-diazoethanoate(10) The title compound was prepared by reaction of ethyl glycinate hydrochloride with sodium nitrite in the presence of dilute H_2SO_4 as described by Searle,[51] giving the desired product as a yellow liquid (yield 95~100%), ν_{max} 2110 (N≡N) and 1700 (C=O) cm^{-1}, δ^1H NMR(60MHz, $CDCl_3$): 1.3 (3H, t, CH_3, J=7.11Hz), 4.3 (2H, q, CH_2, J=7.13Hz), 4.8 (1H, s, CH), which was used without further purification.

Dimethylformamidinium chloride, the Vilsmeier reagent (11) N,N-dimethylformamide (DMF) (52.8g, 0.72 mol) was stirred at room temperature under nitrogen. Freshly distilled thionyl chloride (53ml 0.72 mol) was added dropwise with stirring. When all the thionyl chloride had been added, the mixture was warmed up to 40°C *via* a water bath and stirred for 2h until a very sticky mixture was afforded. The mixture was then evaporated *in vacuo* (*via* oil pump) for 2h (water bath up to 40°C), until a colourless hygroscopic solid was afforded, which was used immediately without further purification. Assumed yield 100%.

Ethyl 2-diazo-3-oxopropanoate(13)[36] The title compound was prepared by the reaction of ethyl 2-diazoethanoate with the Vilsmeier reagent(**11**) and hydrolysis of the resulting diazo intermediate as described by Stojanovic.[36] The product was obtained as a yellow liquid (50%), ν_{max} 2150 (N≡N), 1770 (C=O) cm^{-1}, λmax (ethanol) 220 and 250nm ($\log_\varepsilon$ 4.02 and 3.76) (lit.[36] λmax (ethanol) 217 and 249nm ($\log_\varepsilon$4.24 and 4.08).

Ethyl 2-diazo-3-dimethyliminium propanoate chloride (12) A solution of the Vilsmeier reagent(**11**) in chloroform (0.01mol) was stirred and treated over 15min at 0°C with ethyl 2-diazoethanoate (2.3g, 0.02mol). Gas and heat were evolved and the resulting yellow solution was stirred at RT for 30min. The mixture was then evaporated at RT and the residue precipitated with ether to give a pale yellow hygroscopic solid (1.65g, 80%) which was used immediately without further purification.

***Ethyl 1-hydroxy-1H-1,2,3-triazole-4-carboxylate* (2e)**

(a) $NH_2OH.HCl$ (52.5g, 0.76mol) was suspended in commercial ethanol (300ml) and cooled by ice-salt bath. Sodium carbonate (40g 0.38mol) was added to the suspension followed by the addition of ethyl 2-diazo-3-dimethyliminiumpropanoate chloride(130g, 0.63mol) as a solid. The resulting yellow mixture was stirred mechanically for 1h at 0°C. The insoluble inorganic compounds were filtered off and washed with ethanol (x3). The ethanol solutions were combined and concentrated *in vacuo* **(Caution! The solvent should never be evaporated to dryness!)** to give a yellow residue which was diluted with ice cold water to give a bright yellow solid. It was filtered off and washed with a little cold water and redissolved in chloroform. The small amount of water was separated and the chloroform solution dried over anhydrous Na_2SO_4. The solvent was then removed

to give the ethyl 2-diazo-3-oximinopropanoate as a bright yellow solid (61.4g, 62%). m.p. 77-79°C; ν_{max} 2099cm^{-1} (N≡N) and 1711cm^{-1} (C=O); λ_{max} (DCM) 274nm ($\log_\varepsilon$ 3.87); δ^1H NMR (200MHz, $CDCl_3$): 1.30 (3H, t, CH_3, J=7.2Hz), 4.30 (2H, q, CH_2, J=7.2), 7.01 (1H, s, CH), >10 (1H, broad, OH).

Ethyl 2-diazo-3-oximinopropanoate (2.9g, 0.018mol) was dissolved in freshly dried benzene (25ml) and the solution was stirred under reflux for 1.5h. The mixture was cooled and the precipitated solid collected to give the expected product (2.5g, 88%). m.p. 110-112°C; ν_{max} 3200-2000 br (OH) and 1730 (C=O) cm^{-1}; λ_{max} (DCM) 260nm ($\log_\varepsilon$ 3.63); δ^1H NMR(200MHz, $CDCl_3$): 1.34 (3H, t, CH_3, J=7.14), 4.38 (2H, q, CH_2, J=7.16), 8.06 (1H, s, CH), ~12.5 (1H, s, broad, OH); δ^{13}C NMR(200MHz, $CDCl_3$): 13.9 (CH_3) , 61.7 (CH_2) ,121.7 (CH), 136.5 (C), 159.2 (C); Found: C,38.2; H,4.6; N,26.7%; 157 (m/z, EIms), $C_5H_7N_3O_3$ calculated: C,38.2; H,4.5; N,26.8%, 157(M^+).

(b) $NH_2OH.HCl$(44g, 0.63mol) was dissolved in a minimum of water and cooled via ice salt bath. Sodium carbonate (33.6g 0.32mol) was added and stirred until all had dissolved. Ethyl 2-diazo-3-dimethyliminiumpropanoate chloride(130g, 0.63mol) was added as solid and the yellow product soon precipitated. The precipitate was filtered off after 5min, washed with a little ice cold water and dissolved in chloroform. A small amount of water was separated and the chloroform layer dried with anhydrous Na_2SO_4. The drying reagent was then filtered off and the solution left at RT until the cyclisation was completed. (Yellow colour fades but confirm by ^{1}H NMR). The chloroform solution was evaporated *in vacuo* to give a colourless solid which was recrystallised from ethyl acetate to give 45.5g (46%) product. All analytical and spectral data were consistent with the data obtained in the previous protocol.

HOCt active esters

FmocLeuOCt, reaction condition studies

Three reaction mixtures were prepared as follows: FmocLeuOH (177mg, 0.5mmol) and HOCt (78.5mg, 0.5mmol) were dissolved in 5ml dry DCM. To the clear solution was added DCCI (103mg, 0.5mmol). These were stirred at -20° C, 0°C or room temperature. The mixtures were then filtered and the filtrates evaporated to dryness. The residues were checked by ^{1}H NMR. All showed a mixture of free acid and the active ester.

Synthesis of active esters of the commonly used Fmoc amino acids

Fmoc-amino acid (0.5mmol) and HOCt (0.5mmol) were dissolved in anhydrous DCM under nitrogen and stirred at 0°C. For some Fmoc amino acids, such as FmocTrpOH, which were not very soluble in DCM, DMF was added to the solution until dissolution was complete. DCCI (0.5mmol) in 2ml DCM was then added, and the mixture stirred at 0°C for another 30min. The colourless precipitate was filtered off and the filtrate evaporated. To the residue some anhydrous ether was added and the evaporation was repeated. The colourless solid obtained was dried *in vacuo* over P_2O_5. ^{1}H NMR spectra were collected.

Synthesis of the peptides

All peptides mentioned were synthesised on an adapted ABI 430A peptide synthesiser, and cleaved and purified as described in literature[44] except that HOCt/DIC was used instead of HOBt/DIC as coupling reagent wherever appropriate.

The tripeptides synthesised for racemisation studies were cleaved with no other scavengers except H_2O. After removal of the resin, the mixture was evaporated and then freeze dried without any further purification. ^1H NMR was then carried out on a Bruker WH-360 (360MHz) spectrometer.

Monitoring of the peptide coupling

Exactly 0.5 mmol of Fmoc amino acid was weighed into each cartridge. The solvent delivery system was checked carefully and fluorene in DMF was used as an external standard (0.25mmol). After each coupling step, the unreacted Fmoc amino acid, or its activated derivative in the coupling mixture, was passed through a UV spectrometer, followed by the standard solution in an amount corresponding to 0.50mmol Fmoc amino acid in DMF. The amount of the unreacted Fmoc amino acid was thus calculated as follows:

Coupling percentage (%) = (0.5-washing peak/standard peak) x 100/0.25

If more than one coupling is carried out for each residue, the coupling percentages are additive.

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