The Design of a New Heterocyclic Coupling Agent for Peptide Synthesis.

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This thesis is submitted in part fulfillment of the requirements of the degree of Doctor of Philosophy in the University of Edinburgh. Unless otherwise stated the work described is original and has not been previously submitted, in whole or in part, for any degree at this or any other university.

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Finally I would like to thank all my relatives, friends and colleagues for their much appreciated contributions.

To Mum and Dad,
Stephen Johnathan and Mark.
Thank You.

O Lord, our Lord,
how majestic is Your name in all the
earth!

When I consider Your heavens,
the work of Your fingers,
the moon and the stars,
which You have set in place,
what is man that You are mindful of him

Psalm 8

For from Him and through Him and to
Him are all things.
To Him be the glory for ever!

Romans 11

Abstract

The subject matter of this thesis is concerned with investigations on the synthesis of *N*-hydroxytriazoles and *N*-hydroxyimidazoles and their potential use as auxiliary nucleophiles in peptide synthesis. Synthetic routes to four *N*-hydroxytriazoles; 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole, 1-hydroxy-4-(4-methoxyphenyl)-5-methyl-1*H*-1,2,3-triazole, ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate, ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate and two *N*-hydroxyimidazoles; 2,5-dimethyl-1-hydroxy-4-phenyl-1*H*-imidazole and 1-hydroxy-2-methyl-4-phenyl-1*H*-imidazole were developed.

2,5-Dimethyl-4-phenyl-1*H*-imidazole was tested as a potential mediator in peptide synthesis by the attempted synthesis of the simple test peptide LeuIlePheAlaGly but was found to be unsuitable in this respect. Therefore investigations were concentrated on the *N*-hydroxytriazoles.

The *N*-hydroxytriazoles were generally more soluble in solvents compatible with the peptide synthesiser (eg. DMF and DMF:dioxane) than the *N*-hydroxyimidazoles. All of the *N*-hydroxytriazoles synthesised were tested for use as potential auxiliary nucleophiles in the synthesis of LeuIlePheAlaGly and were found to be capable of mediating peptide bond formation as successfully as HOBt.

Ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate and ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate were also evaluated as coupling agents for the amino acid sequences found in peptides such as Substance P, Luteinizing hormone releasing hormone (LHRH) and Nerve growth factor (NGF) 100-114. Ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate was found to be superior to 1-hydroxybenzotriazole (HOBt) as an auxiliary nucleophile. This superiority can be attributed to a lack of steric hindrance around the *N*-hydroxy group. Moreover the greater solubility and lack of u.v. absorbance of ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate at 302 nm also made it superior to HOBt for use in solid phase peptide synthesis.

Abbreviations.

AA	Amino acid
Boc	*t*-Butyloxycarbonyl
DCM	Dichloromethane
DCCI	*N,N*-Dicyclohexylcarbodiimide
DIC	*N,N*-Diisopropylcarbodiimide
DMAP	4-Dimethylaminopyridine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethyl sulphoxide
EI	Electron impact
ether	Diethyl ether
FAB	Fast atom bombardment
Fmoc	9-Fluorenylmethyoxycarbonyl
HOBt	1-Hydroxy-1,2,3-benzotriazole
Hplc	High pressure liquid chromatography
Il-1-β	Interleukin-1-β
i.r.	Infrared
LHRH	Luteinizing hormone releasing hormone
NGF	Nerve growth factor
n.m.r.	Nuclear magnetic resonance
Pmc	2,2,5,7,8-Pentamethylchroman-6-sulphonyl
Rt	Retention time
SPPS	Solid phase peptide synthesis
Sub P	Substance P
TFA	Trifluoroacetic acid
tlc	Thin layer chromatography
u.v.	Ultraviolet

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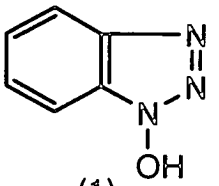

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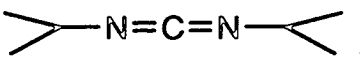

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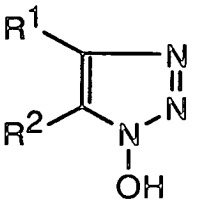

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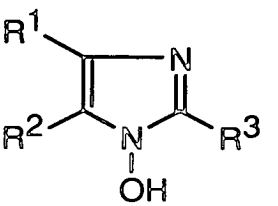

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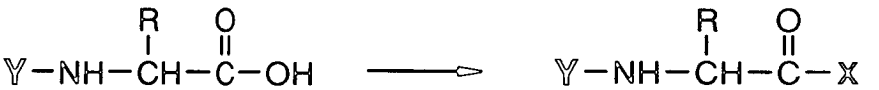
Scheme 1

ACTIVATION



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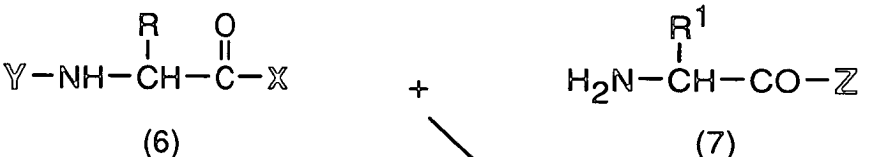
COUPLING



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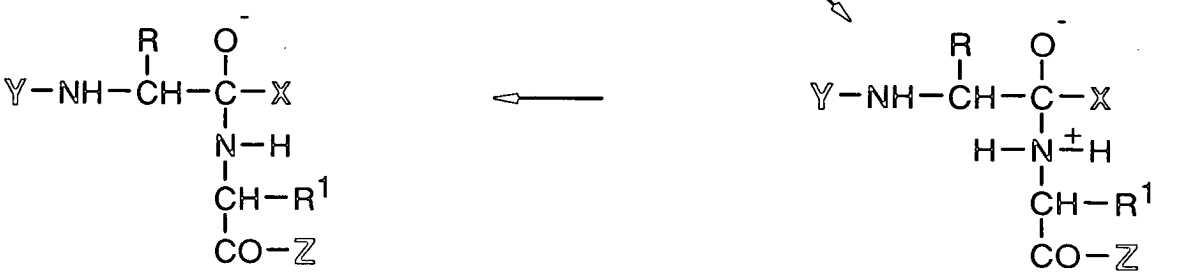
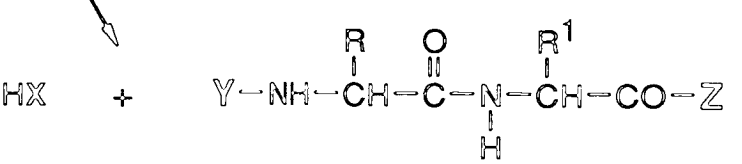




(8)

Scheme 2

1. INTRODUCTION

There are currently various methods for forming a peptide bond between two amino acids in the synthesis of peptide chains. The most widely used method at present [Scheme 1] uses the reagents, 1-hydroxybenzotriazole (HOBt) (1) and diisopropylcarbodiimide (DIC) (2). The present studies are aimed at designing and developing a new *N*-hydroxy heterocyclic coupling agent [Scheme 1] based on *N*-hydroxy-1*H*-1,2,3-triazoles (3) or 1-hydroxy-1*H*-imidazoles (4). The description of the results obtained in these studies is preceded by an introductory survey of:

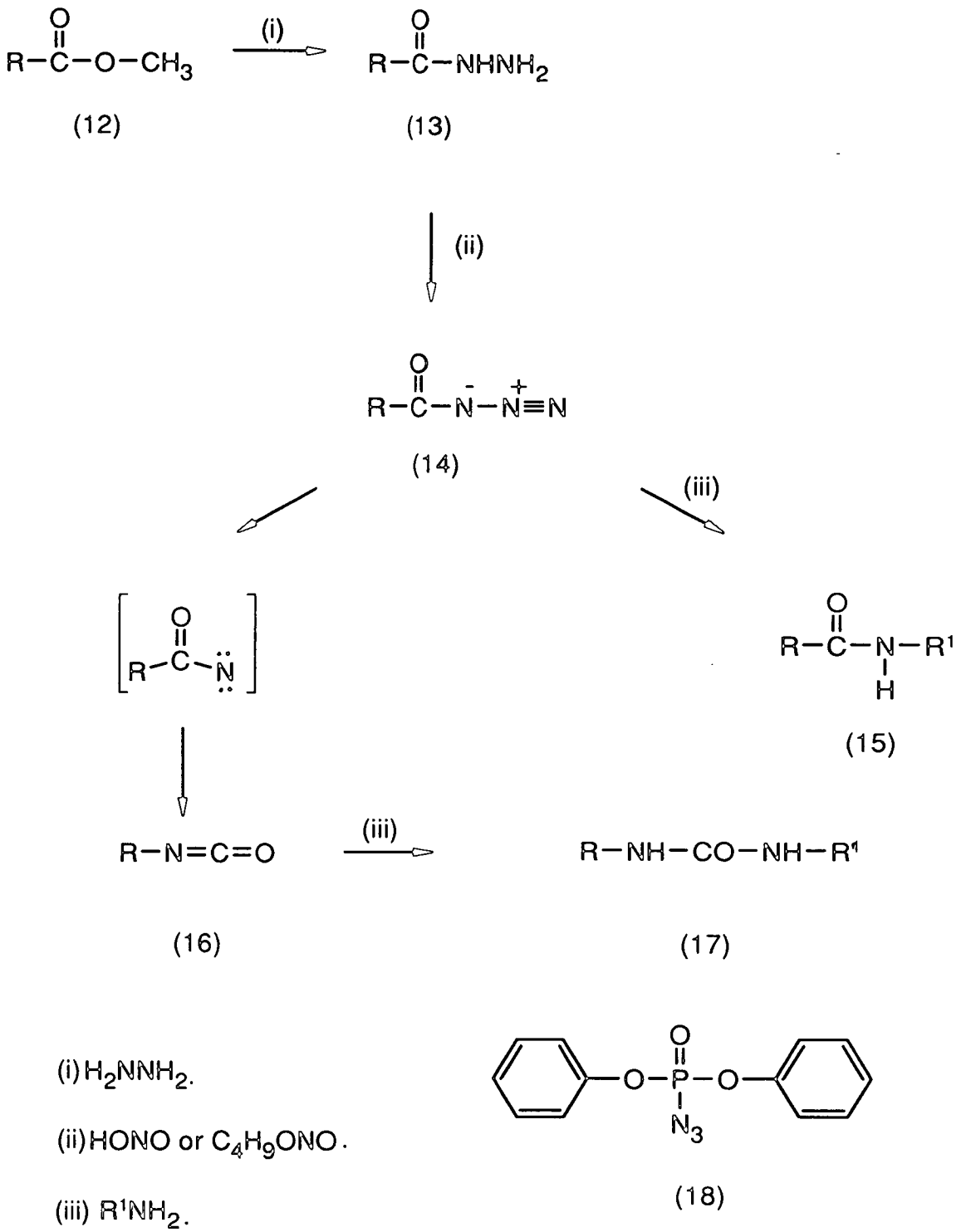
1.1 General Methods for Peptide Coupling.
1.2 Solid Phase Peptide Synthesis.
1.3 Literature Methods for the Synthesis of 1-Hydroxy-1*H*-1,2,3-triazoles and 1-Hydroxy-1*H*-imidazoles.

1.1 General Methods for Peptide Coupling

The chemical requirements for the formation of a peptide bond between two amino acids [Scheme 2] are that the carboxyl group of one (7) and the amino group of the other (6), together with any reactive side chains, are blocked (Y, Z). The free carboxyl group can then undergo nucleophilic attack by the free amino group thus forming only the desired of the four possible peptide bonds (8). This reaction cannot occur spontaneously and must be facilitated by the hydroxyl group of the carboxylic acid (5) being replaced by an electron withdrawing substituent (X), so increasing the electrophilicity of the carbonyl carbon in the activated species (6) and its vulnerability to nucleophilic attack.

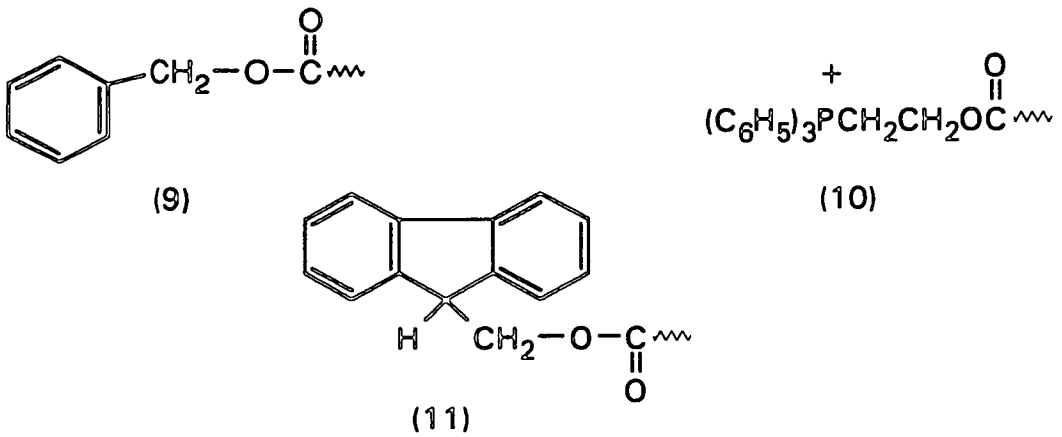
The Acid Chloride Method

It was Fischer[1] who at the turn of the century synthesised the first peptide using the chlorine atom as the electron-withdrawing substituent (X) [Scheme 2]. The acid chloride method however has its problems particularly in regard to unwanted side reactions, racemization and the difficulty of removing protecting groups stable enough to survive the conditions required to convert a carboxylic


(i)
(ii)
(iii)
(iii)
(12)
(13)
(14)
(15)
(16)
(17)
(i) H_2NNH_2.
(ii) HONO or C_4H_9ONO.
(iii) R^1NH_2.
(18)

Scheme 4

acid into an acid chloride. Indeed Fischer[1] had problems in finding a suitable reversible N^{α} protecting group and it was in fact his former student Bergmann

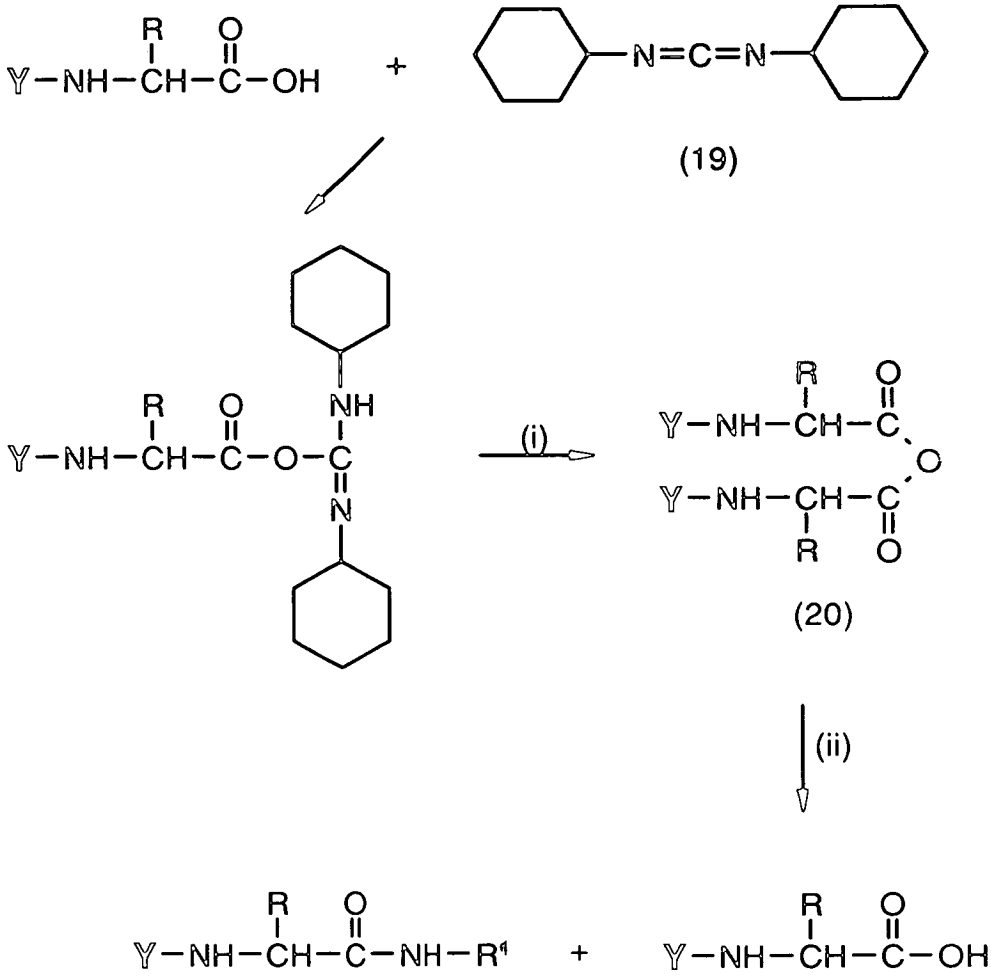


Scheme 3

who, with Zervas[2] introduced [Scheme 3] the benzyloxycarbonyl or Z group (9) as a successful N^{α} protecting group. The acid chloride method was largely neglected due to its problems until, recently, the introduction of new protecting groups led to renewed interest in this mode of coupling. Bechtolsheimer and Kunz[3] used an extremely acid stable protecting group, 2-(triphenylphosphonio)-ethoxycarbonyl (Peoc) (10),[4] which enabled the synthesis of stable acid chlorides of amino and hydroxy acids. Carpino *et al.*[5] have used their base labile 9-fluorenylmethoxycarbonyl (Fmoc) group (11) to protect the N^{α} position and have thereby successfully synthesised several stable Fmoc protected amino acid chlorides.

The Acid Azide Method

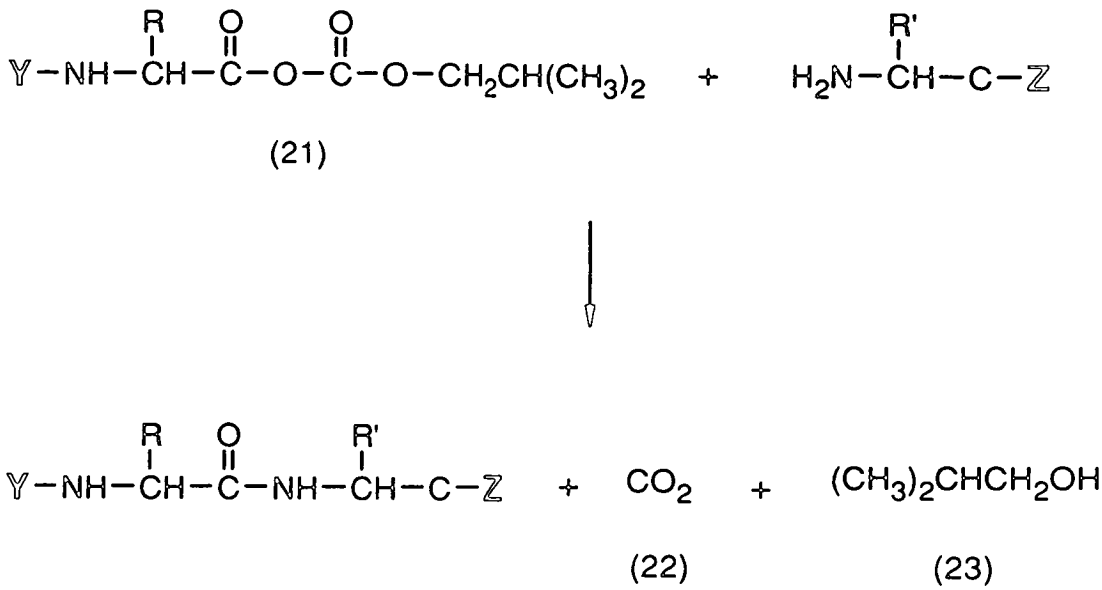
This method was developed by Curtius[6] in 1902 and at that time was the only competitor of the acid chloride method. Conversion [Scheme 4] of the alkyl ester (12) into the corresponding hydrazide (13) and then into the acid azide (14) (using nitrous acid) was the original method of activation of the carboxy terminus for peptide bond formation. However in 1972 Yamada[7] proposed the use of diphenylphosphoryl azide (18), for the direct conversion of carboxylic



(i) $Y-NH-CH(R)-C(=O)-OH$.

(ii) R^1NH_2.

Scheme 5



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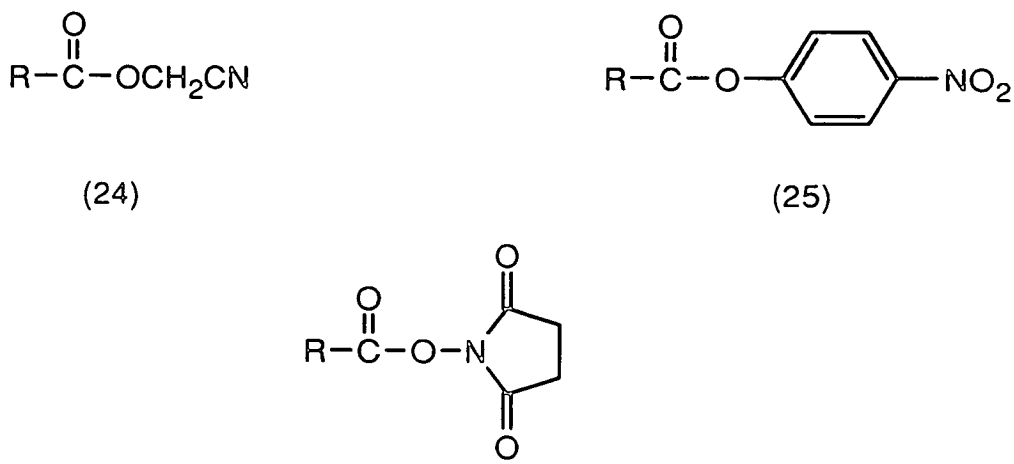
Scheme 6



(24)

(25)

(26)

Scheme 7

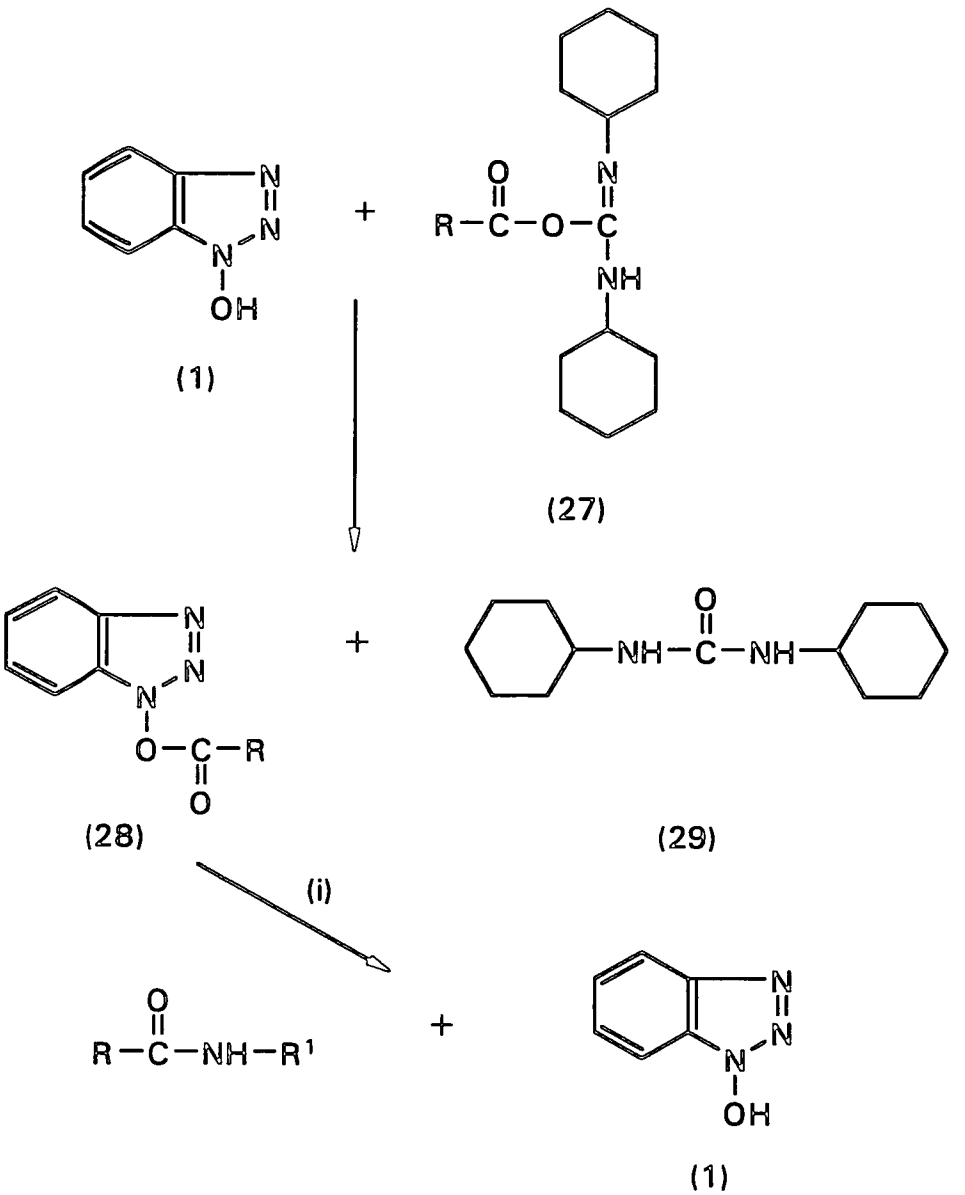
acids to acid azides. This method is popular since it can provide peptides in good yield and with negligible racemization. Unfortunately a major drawback of the acid azide method is the occurrence of a side reaction [Scheme 4] whereby a rearrangement of the azide (14) to the isocyanate (16) then reaction of the latter with the amine component (16) results in formation of the urea derivative (17). Consequently purification becomes very difficult due to the similarity in the properties of the urea (17) and the desired peptide product (15).

The Anhydride Method

The use [Scheme 5] of symmetrical anhydrides (20) in peptide bond formation is reasonably straightforward and efficient. They can be prepared using phosgene or more commonly dicyclohexylcarbodiimide (DCCI) (19) or DIC (2). The main disadvantage of this method is its wastefulness - only one half of the anhydride is incorporated into the peptide. Also, slow and incomplete coupling is encountered when using anhydrides of sterically hindered amino acids to couple with sterically hindered polymer bound peptide. An alternative to this wasteful procedure is the use of mixed anhydrides [Scheme 6] in which it is necessary to direct the nucleophilic attack to the appropriate side of the anhydride. This can be achieved by using electron-withdrawing and/or bulky substituents. Alkylcarbonic acid mixed anhydrides (21) are often used and have the particular advantage of the by-products (22) and (23) being easily removed from the mixture. However, when using this method there is the possibility of racemization, formation of a small amount of the second acylation product or disproportionation to symmetrical anhydrides.

The Active Ester Method

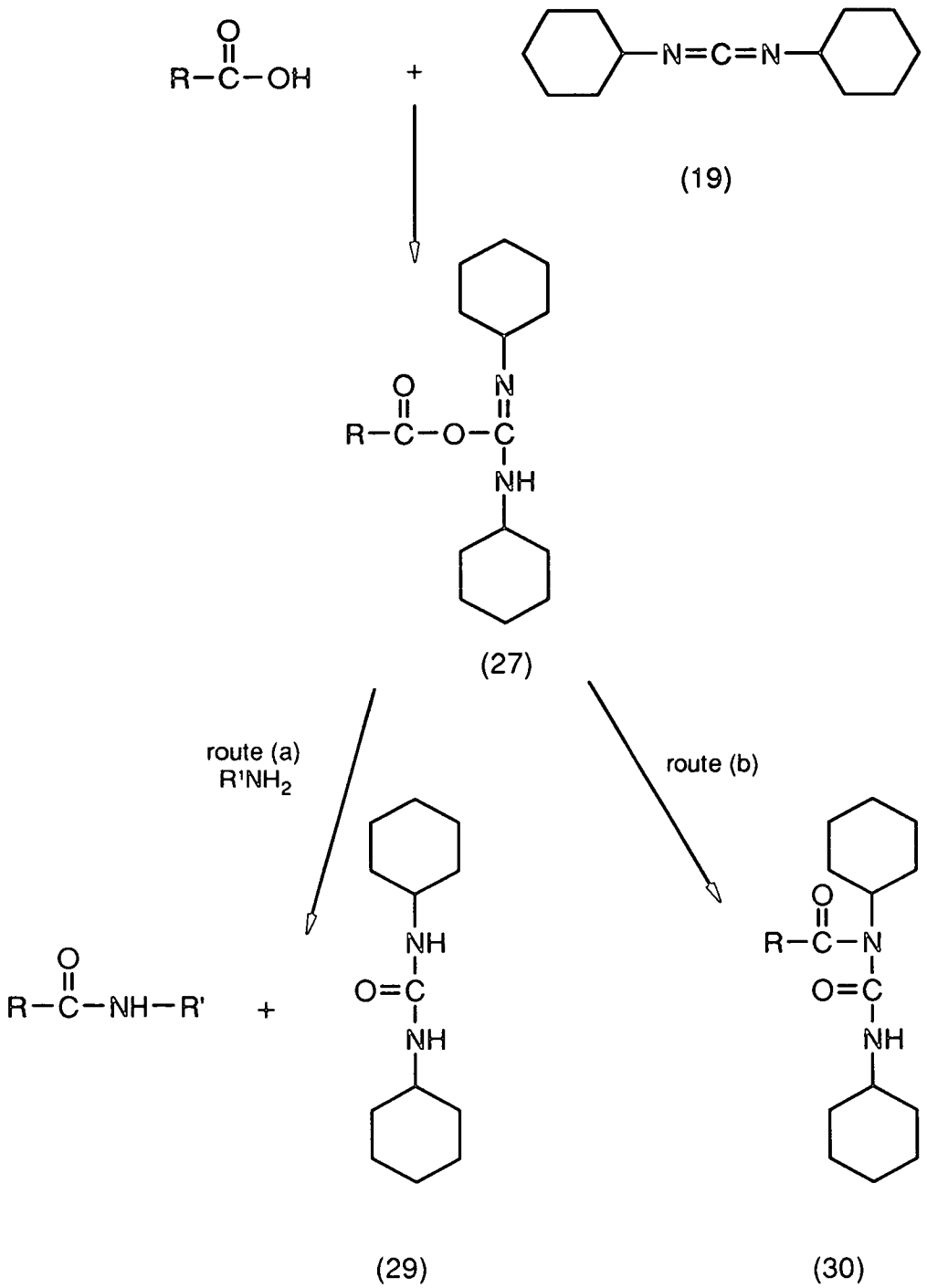
In early peptide chemistry both Fischer[8] and Curtius[9] constructed peptide bonds using amino acid methyl and ethyl esters. However reaction times were slow and it was evident that a more electron-withdrawing substituent on the alcohol component of the ester was necessary to encourage the attack of nucleophiles. The cyanomethyl esters (24) [Scheme 7] were then introduced by Schwyzer *et al.*[10] in 1955, but satisfactory rates of coupling were observed only when reactants were applied in high concentrations. Aryl esters proved more promising and their reactivity could be optimized using electron-withdrawing substituents, as in the case of the *p*-nitrophenyl esters (25) introduced by Bodansky.[11]



(i) R^1NH_2.

Scheme 8

A significant event in this area was the discovery by Konig and Geiger[12] in 1973, of the catalytic effect of 1-hydroxybenzotriazole (HOBt) (1) [Scheme 8]. They found that the rate of aminolysis of activated esters in polar solvents increased upon the addition of *N*-hydroxycompounds having the approximate acidity of acetic acid (pK_a=4.0), [eg. HOBt (1)]. *N*-Hydroxysuccinimide esters (26) [Scheme 7] were


R–C–OH
O
+
N=C=N
(19)
R–C–O–C
O
N
NH
(27)
route (a)
R'NH2
route (b)
R–C–NH–R'
O
+
NH
O=C
NH
R–C–N
O=C
NH
(29)
(30)

Scheme 9

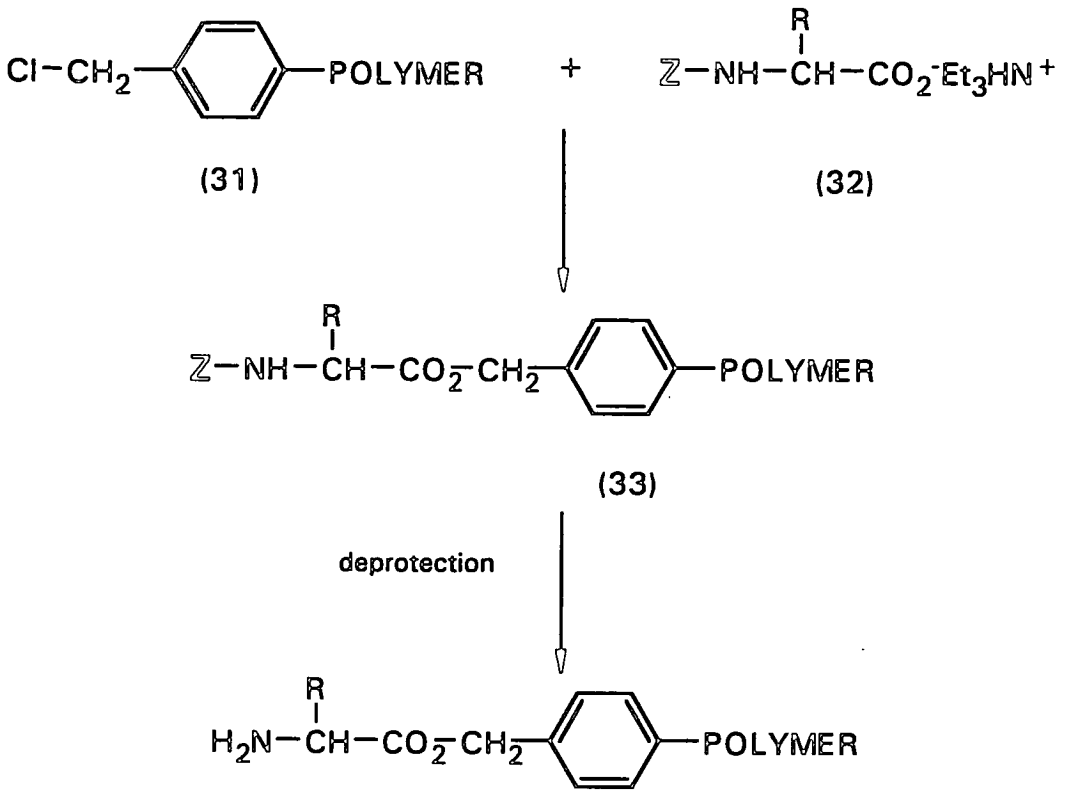
proposed as alternatives to HOBt (1) by Anderson *et al.*[13] Although they are highly reactive there is a problem with the use of these reagents due to competing condensation of the amino component with the carbonyl substituents of the succinimide. Many other activated esters have been employed in peptide coupling.[14]

The Carbodiimide Method

Carbodiimides (chiefly DCC) were introduced by Sheehan and Hess[15] in 1955 as reagents for use in the formation of peptide bonds [Scheme 9, route a]. The mechanism of action of such reagents has been investigated by Khorana *et al.*[16,17] and De Tar *et al.*[18] The reaction proceeds via an *O*-acylurea intermediate (27), powerful activation of which is provided by the presence of the 'N=C' group. The *O*-acylurea (27) can react further by (i) direct attack of the amino component on the *O*-acylurea [Scheme 9 route a] or (ii) formation of a symmetrical anhydride (20) which can then acylate the amine [see Scheme 5].

The by-product of these transformations is dicyclohexylurea (29) which is a solid and therefore easily removed by filtration. However the isourea (27) can spontaneously rearrange [Scheme 9, route b] to the *N*-acylurea (30) which is unreactive towards amines. A superior replacement for DCC is diisopropylcarbodiimide (DIC) (2), which is a liquid, much easier to handle and whose by-products are more soluble then their DCC counterparts - a particular advantage for solid phase peptide synthesis.

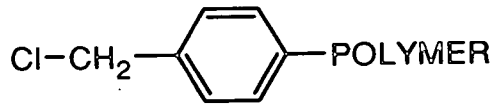
Both racemization and *N*-acylurea formation can be suppressed by addition of auxiliary nucleophiles [Scheme 8]. Thus Wunsch and Drees[19] and Weygand *et al.*[20] used *N*-hydroxysuccinimide, while Konig and Geiger[21] used HOBt and various derivatives. These nucleophilic hydroxy compounds were found to form highly active esters (28) which react rapidly with the amine component. The presence of these compounds in the mixture reduces the concentration of the *O*-acylisourea (27) and therefore the extent of racemization and *O*->*N* acyl migration [Scheme 9] leading to *N*-acylureas (30).



Scheme 10

1.2 Solid Phase Peptide Synthesis

The preparation of peptides containing more than three or four amino acid residues is a very demanding task using conventional methods of organic synthesis. The addition of protecting groups, coupling and deprotection reactions, isolation, crystallization and collection of the products is time consuming and material will inevitably be lost with each operation. However in 1963 Merrifield[22] revolutionized the area of peptide synthesis with an idea for which he later received the Nobel Prize[23] namely the assembly of the peptide chain in a stepwise manner while one end was attached to a solid support. The technique involves the attachment of the first amino acid by a covalent bond to a polymer. The remaining amino acids can then be added in a stepwise fashion until the peptide chain is complete. The finished peptide can then be removed from the solid support. Since the growing peptide is attached to an insoluble support, the reactants and by-products can easily be removed by washing. This procedure lends itself well to automation.



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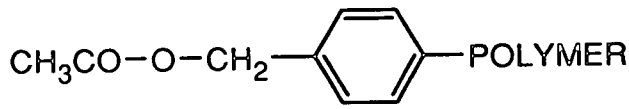

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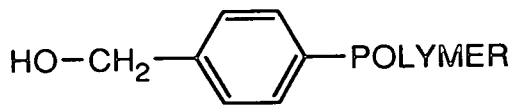


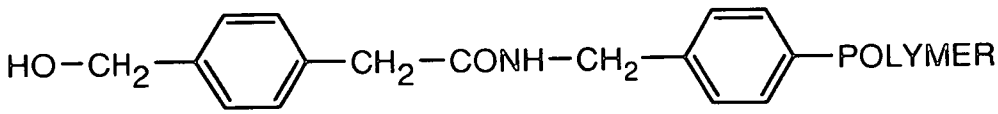
(35)

(i) $CH_3CO_2^-K^+$.

(ii) KOH, EtOH.

(iii) NH_3, MeOH.

Scheme 11



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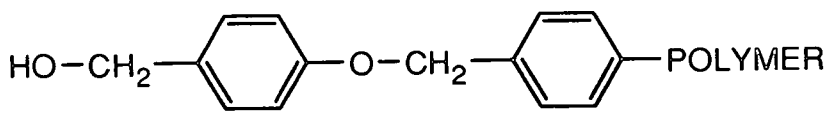


(37)

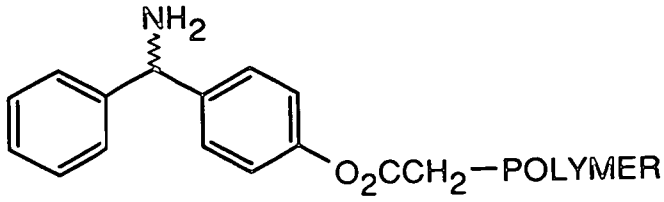
Scheme 12

The insoluble support is a synthetic polymer (resin) bearing reactive groups. Merrifield[22] investigated many potential supports and found [Scheme 10] that a chloromethylated co-polymer (31) of styrene and 1% divinylbenzene was best. This resin was then treated with the triethylammonium salt of the first protected amino acid (32) giving a structure (33) with a substituted benzyl ester linkage. However, problems were encountered in deprotection of the amine. The N^{α} protecting group in use was benzyloxycarbonyl (Z) (9) which can be removed with a solution of HBr in acetic acid. Since this reagent also cleaved the benzyl ester linkage between the first amino acid and the resin, albeit slowly, the cumulative loss of peptide from the resin over repeated cycles was nevertheless a serious problem.

Nitration or bromination[22] of the chloromethylated polymer was introduced as a possible way of overcoming these difficulties. However when the nitrated polymer was used the peptide could not be completely removed from the resin even with prolonged exposure to HBr. In contrast, the brominated polymer was much better, the Z group could be removed with dilute HBr (10% in acetic acid) and the peptide cleaved from the resin in concentrated HBr (30% in acetic acid.) Unfortunately the brominated resin suffered from a reduction in its swelling properties.

The chloromethylated resin (31) may alkylate nucleophilic amino acid side chain substituents, (e.g. the thioether sulphur atom of methionine and the imidazole ring of histidine) during the coupling of the first amino acid to the resin. These problems are avoided[24] if the hydroxymethyl resin (35) [Scheme 11] is used. The chloromethylated resin (31) was heated with a solution of potassium acetate in a high boiling alcohol, to give the corresponding acetate (34) which yields the hydroxymethyl resin (35) upon either saponification with potassium hydroxide or ammonolysis in methanol.

Merrifield[25] introduced [Scheme 12] the hydroxymethyl Pam resin (36) in 1976 which included a "handle" between the resin and peptide so increasing the distance between the polymer and the growing peptide chain thereby alleviating problems of steric hindrance. The increased stability of this link is due to the electron withdrawing para-acetamido group of the benzene ring to which the peptide is attached. The drawback in this case is that vigorous conditions are required to remove the peptide from the resin, for example treatment with anhydrous HF to which large peptides are unstable. The peptide resin link was therefore still unsatisfactory, and a more labile link was sought and found by Wang,[26] the



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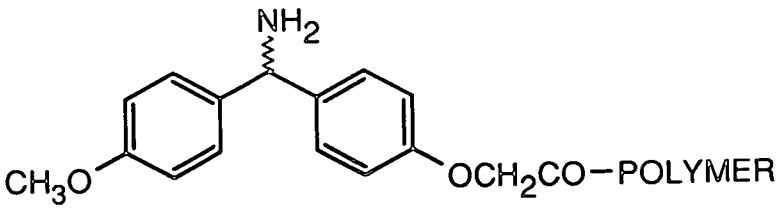

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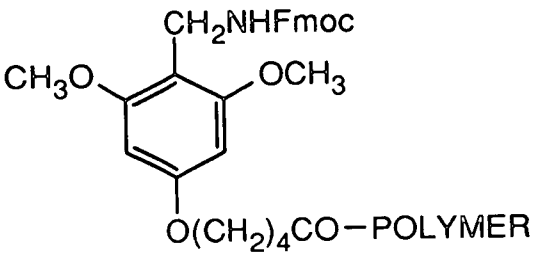

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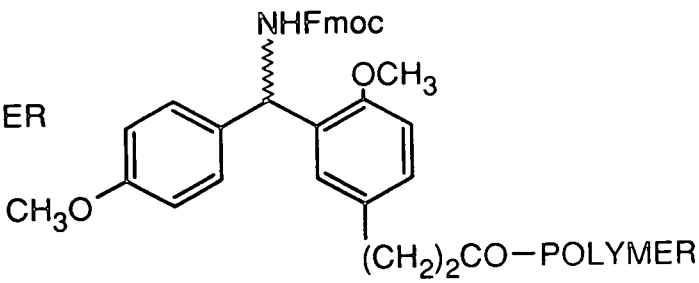

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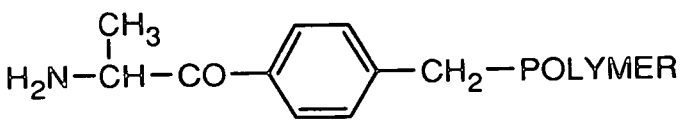

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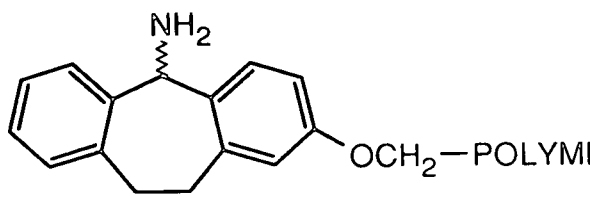

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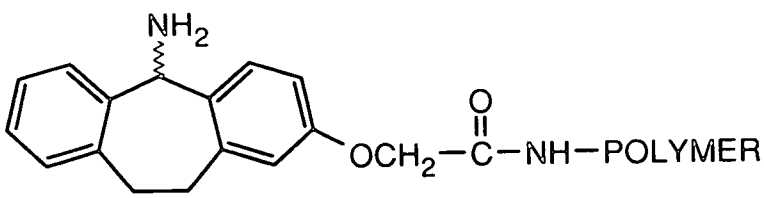


(44)

Scheme 13

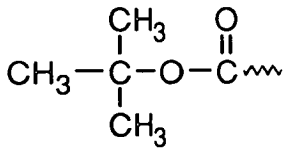
p-alkoxybenzyl alcohol resin (37), the most successful resin of its type to date. The ester linkage between the amino acid and the resin is more acid labile due to electron donation and the peptide can be cleaved easily by TFA. There are many other handles and strategies that have been developed such as fluoride labile linkers by Barany[27] and Ramage,[28] photolabile handles by Rich[29] and Tjoeng[30] and a multi-detachable linker by Tam.[31]

Amide Resins

Many natural peptides such as peptide hormones terminate with a carboxamide function rather than a free carboxyl group at their C termini. In solid phase peptide synthesis (SPPS) ammonolysis of the ester bond anchoring the peptide to the resin affords peptide amides. However this reaction is not without its problems - methanolysis occurs and the methyl esters are gradually converted into the desired amide but this requires a considerable time. A more rational approach would be to functionalize the linker [Scheme 13], so allowing the C-terminal amide to be formed upon cleavage of the peptide-resin link. Such linkers are mostly based on benzylamine or benzhydrylamine derivatives and acidolysis liberates the peptide amide. For example in the case of *p*-acyloxybenzhydrylamine resin (38) (Tam's resin[32]), the peptide amide can be obtained from this resin using HF or TFMSA and hence is compatible with Boc methodology.

A linker was then sought that could be cleaved under mild acid conditions and therefore be more suited to Fmoc methodology. For example, Breipohl[33] used a methoxyl substituted benzhydrylamine system (39) in the synthesis of oxytocin and luteinizing hormone releasing hormone (LHRH). The electron donating methoxyl group aids cleavage of the peptide amide in TFA.

Other examples are the PAL linker (40) developed by Albericio *et al.*[34] and the linker (41) developed by Yajima.[35] An alternative linker is the photolabile α-methylphenacylamido resin (42) developed by Ajayaghosh and Pillai.[36] Work has been carried out recently by McInnes[37] with a view to improving this methodology for the synthesis of peptide amides. The result has been the introduction of a new amide resin (43) which can be cleaved in 1-4% TFA in DCM although in practice a much stronger solution is used in order to simultaneously remove the side chain



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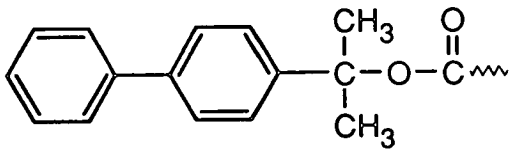

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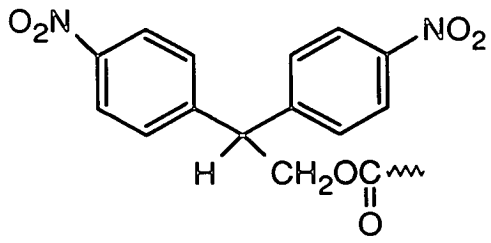
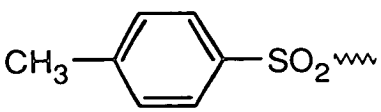


(47)

Scheme 14



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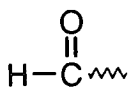

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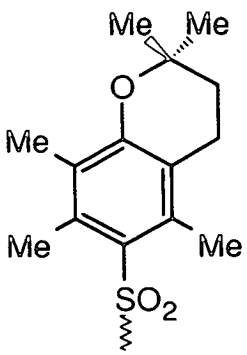


(50)

Scheme 15

protecting groups. This linker can also be attached to the resin through an amide bond (44) as demonstrated by Irving.[38]

N^{α} protection

The most common type of amine protecting group is the ester type [Scheme 14], for example, benzyloxycarbonyl (9) as previously mentioned and *t*-butyloxycarbonyl[39,40] (Boc) (45) introduced by Merrifield.[41] The Boc group (45) is more acid labile and can be removed with 1M HCl in acetic acid or TFA in DCM. The 2-(4-biphenylyl)isopropyloxycarbonyl group (Bpoc) (46) is an even more acid labile group. However its amino acid derivatives are less stable than the corresponding free acids and therefore it is not widely used.

Carpino *et al.*[42] have developed a base-labile N^{α} protecting group namely 9-fluorenylmethoxycarbonyl (Fmoc) (11) which has been applied successfully by Sheppard.[43] This group has gained popularity since it can be removed easily using secondary amines and therefore no harm can be caused to acid labile side chain protecting groups or the acid labile linker. The Fmoc group (11) is also stable to acid, so it may be used in more complex strategies where N-terminal protection of the final product is required.

Ramage[44,45] has recently introduced 2,2-bis(4'-nitrophenyl)ethoxy -carbonyl (Bnpeoc) (47) as another base-labile protecting group which like Fmoc can be removed by base through a β-elimination E1cB mechanism.

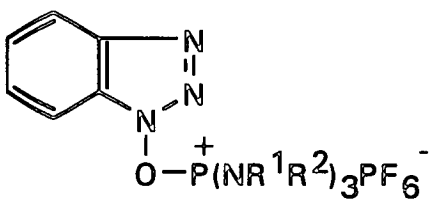
Side chain protecting groups

The N^{α} protecting group strategy used dictates the type of side chain protecting groups that will be employed. There are two commonly used types; benzyl based derivatives and *t*-butyl based derivatives. Boc methodology uses benzyl based derivatives for side-chain protection and depends on graduated acid lability of these groups. The Boc group is removed after each cycle with TFA and the side chain protection is removed using HF. His and Arg can be protected [Scheme 15] using the tosyl group (48) and the indole ring of Trp is protected with the formyl group (49) to prevent alkylation.

The Fmoc based strategy involves an orthogonal approach. A secondary amine is used for removal of the N^{α} protecting group and the side chain protecting groups are acid labile and are usually *t*-butyl based derivatives. The now widely used Arg protecting group is Pmc (50).[46]

Coupling Agents

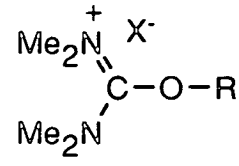
There has been much work done in the area of amino acid coupling and so the last few decades have seen the introduction of many types of coupling reagents. Therefore only a selection of them are covered here, in particular those used for SPPS.

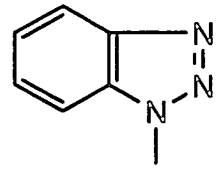
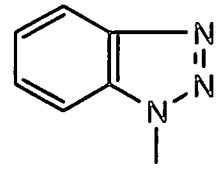
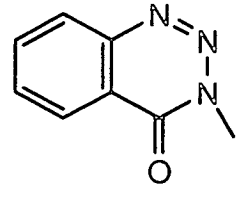
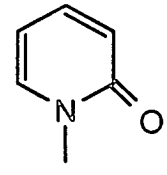
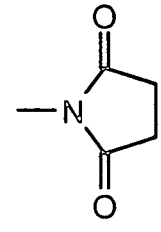
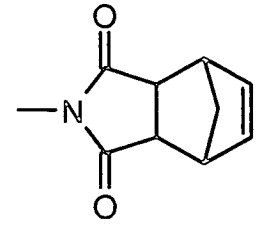


	R^1	R^2
(51) (BOP)	CH_3	CH_3
(52) (PyBOP)	-$(CH_2)_4$-	

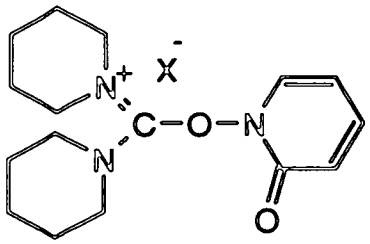
Scheme 16

In 1975, 1,2,3-benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) (51) [Scheme 16] was introduced by Castro[47] as a coupling agent which was for a time considered to be one of the best. In 1988 Hudson[48] recommended the use of BOP (51) with HOBt (1) as an additive as one of the best methods for peptide coupling after doing a comparative study of many different procedures. However a major drawback in the use of BOP is the formation of hexamethylphosphoric triamide (HMPA), a carcinogen. Castro *et al.*[49] then set out to find a safer replacement for BOP. The methyl groups were replaced by ethyl and then morpholino or piperidino groups were substituted for the dimethylamino groups but these compounds were found to be less effectivethan BOP (51). It was the replacement of the dimethylamino groups by pyrrolidino groups as the phosphorus substituents which gave a reagent (PyBOP) (52) capable of yielding coupling rates as good as, if not better than BOP.



	R	X	
(53a)		PF_6^-	HBTU
(53b)		BF_4^-	TBTU
(54)		BF_4^-	TDBTU
(55)		BF_4^-	TPTU
(56)		BF_4^-	TSTU
(57)		BF_4^-	TNTU

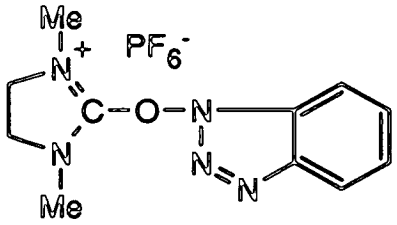
Scheme 17



X

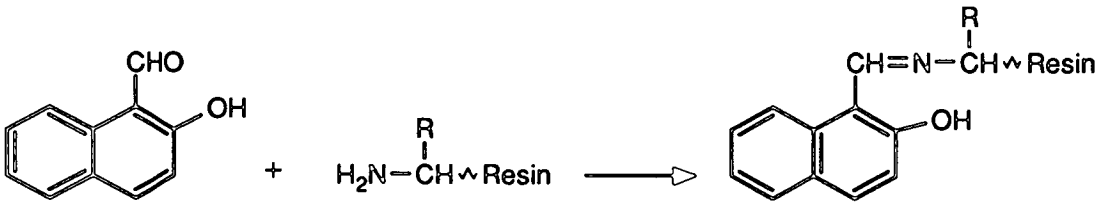
(58) BF_4^-

(59) PF_6^-

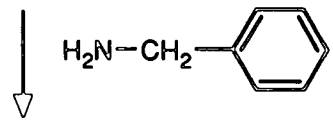


(60)

Scheme 18



(65) (66) (67)



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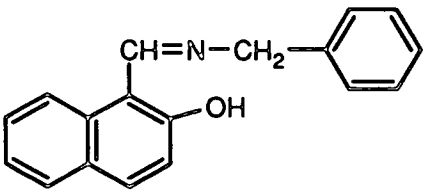


(69)

Scheme 19

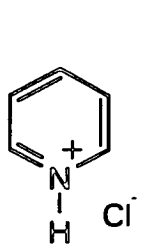
O-1,2,3-benzotriazolyl-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (53a) [Scheme 17] was first synthesised and used in solution phase peptide synthesis by Gross *et al.*[50] then further developed by Knorr *et al.*[51] for solid phase peptide synthesis along with several other new analogues [(53b)-(57)] designed for special purposes. According to Knorr[51] HBTU (53a) and TBTU (53b) are coupling reagents ideally suited to SPPS and they showed that the counterion had no influence on coupling rate or on racemization and that the by-products are soluble in both water and organic solvents.

Further uronium salts have been recently reported [Scheme 18] by Knorr *et al.*[52] for use in SPPS with the Fmoc amino acid strategy, namely 2-(2'-oxo-1(2*H*)-pyridyl)-1,1,3,3-bispentamethyleneuronium tetrafluoroborate (TOPPipU) (58) and its hexafluorophosphate analogue (HOPPipU) (59). These coupling agents are comparable to BOP (51) and TBTU (53b) and have been used successfully in both batch and continuous flow peptide synthesisers. Harmless by-products soluble in both water and organic solvents are generated. The BOI reagent (60) [Scheme 18] introduced by Kiso *et al.*[53] also gives good coupling efficiency.

Monitoring

Reaction rates in peptide synthesis vary considerably as the peptide chain grows due to steric factors and unpredictable conformational effects. These varying rates and the practical time limits cause incomplete reaction and give truncated peptides. A problematic coupling is therefore not always foreseen hence the importance of detecting difficult acylations as they are taking place. Much work has been carried out in this area over the last few decades. In 1964, Merrifield[54] synthesised Bradykinin and checked the extent of acylation by removing a little resin at the end of each coupling for amino acid analysis. Westall *et al.*[55] used propionic acid-hydrochloric acid hydrolysis which although greatly reducing the time usually required for amino acid analysis (overnight hydrolysis in 6N hydrochloric acid) still added an extra two hours to each cycle. Esko *et al.*[56] [Scheme 19] removed a portion of resin (66) and reacted it with an excess of 2-hydroxy-1-naphthaldehyde (65) for twelve hours to form a stable aldimine (67) with the free amino groups on the resin. The chromophore could then be displaced by adding an amine, for example, benzylamine (68) and the amount of soluble aldimine (69) determined spectrophotometrically (420 nm) and hence the amount of

free amine. However it was found that the concentration of reagent was critical and the formation of the Schiff base (67) incomplete.



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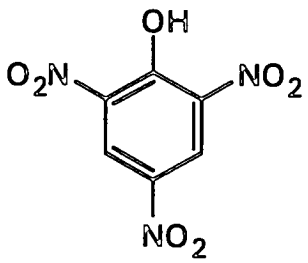
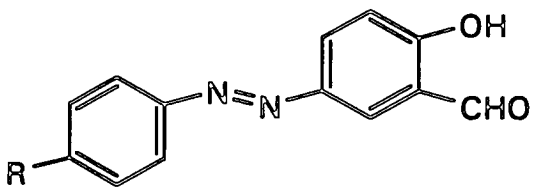
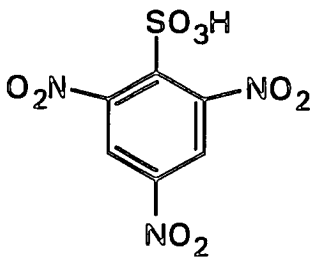


(71)

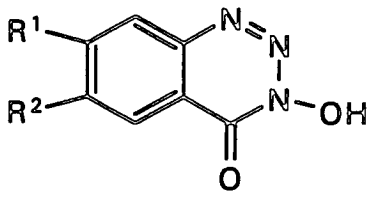


(72) R = H

(73) R = NO_2



(74)



(75) R^1 = H, R^2 = H

(76) R^1 = H, R^2 = NO_2

Scheme 20

There are many methods available for the determination of the number of free amino groups on the resin [Scheme 20]. One such is the Dorman method[57] which measures the amount of chloride bound to the resin after conversion of free amino groups to hydrochlorides using pyridinium chloride (70). Triethylamine is added so displacing the chloride ion from the resin and dilute nitric acid is added to the chilled filtrate and titrated potentiometrically with standard silver nitrate. The amount of silver nitrate required corresponds to the amount of uncoupled resin amine. This method was improved by using pyridinium chloride-36, so allowing the use of radioactivity measurements. In 1972 the use[58] [Scheme 20] of picric acid (71) was developed. The resin is treated with picric acid giving resin supported amine picrates. The resin is washed and treated with diisopropylethylamine which quantitatively releases the picrate into solution where it can be measured spectrophotometrically. However, in 1975, Hancock *et al.*[59] found that as the

peptide chain grew, the polymer support became more polar and the picric acid became increasingly difficult to wash out quantitatively.

5-Phenylazosalicylaldehyde (72) and 5-*p*-nitrophenylazosalicylaldehyde (73) can be used to monitor polymer bound free amino groups.[60] In the presence of free amino groups the resin turns yellow with (72) and red with (73).

Atherton and Sheppard[61] used 2,4,6-trinitrobenzenesulphonic acid (TNBS) (74), which gives a deep red colour when it reacts with free amino groups, as well as ninhydrin tests to monitor the progress of the synthesis of acyl carrier protein (ACP) using N^{α} Fmoc amino acid pentafluorophenyl esters. Konig and Geiger[62] recognized the acylating properties of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HODhbt) (75) but no use in solid phase peptide chemistry was reported until Sheppard *et al.*[63] in 1986 showed that N^{α} Fmoc amino acid esters of HODhbt (75) are easily prepared and stable. They also observed a bright yellow colour during the acylation reaction. This colour was attributed to the ionisation of liberated hydroxy component (HODhbt λ_{max} 440 nm) by resin bound amino groups in the presence of dissolved base. Therefore the intensity of colour gives an indication of the number of residual unreacted amino groups. Using this fact a photometric monitoring system was developed[64] involving monitoring the transmission of light through the resin at 440 nm thus allowing the determination of individual coupling times. Recently[65] derivatives of HODhbt (75) have been synthesised to enable monitoring of the acylation, for example, compound (76) - used as an additive during solid phase peptide synthesis with Fmoc-amino-acid-OPfp esters initially gave a deep red colour to the resin which faded to yellow as the coupling reaction went to completion.

Counterion distribution monitoring[66] continuously monitors the total population of amino groups on the resin using the distribution of a minute quantity of an anionic reporter dye between protonated amine groups and cations in solution. As the acylation proceeds the amount of protonated amine decreases and the anionic dye is displaced into solution whose absorbance therefore increases to a predictable maximum when the reaction is complete.

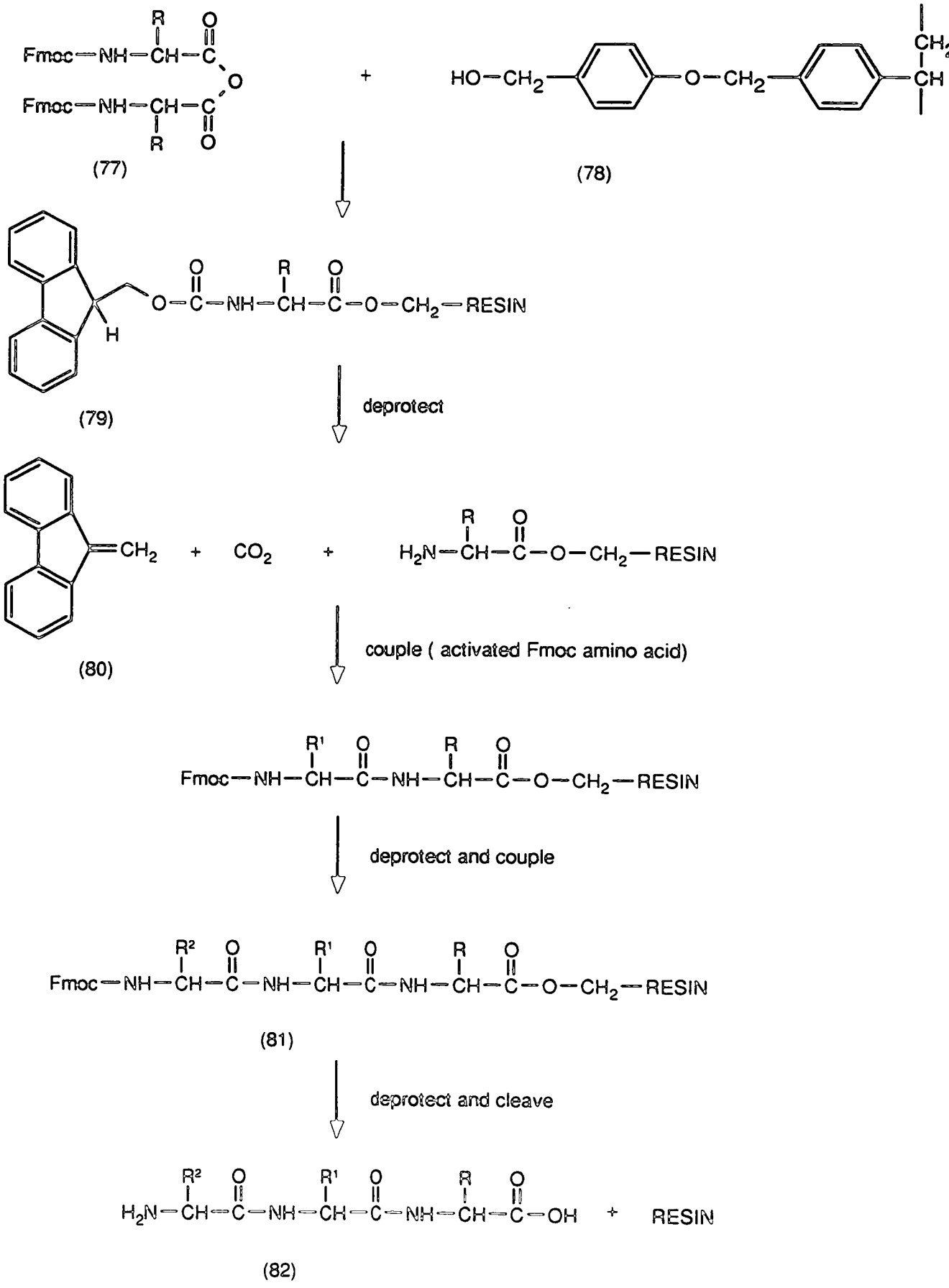
Changes in solvation of the peptide resin at different stages in the synthesis and steric problems cause the accessibility of the N-terminal amine group to be variable. The reliabilty of methods monitoring uncoupled amino groups is therefore

questionable. Also, most of the techniques require additional reactions to introduce and remove a reporter reagent, withdrawal of a resin sample or addition of indicators such as Dhbt esters requiring construction of a photometer and translucent support.

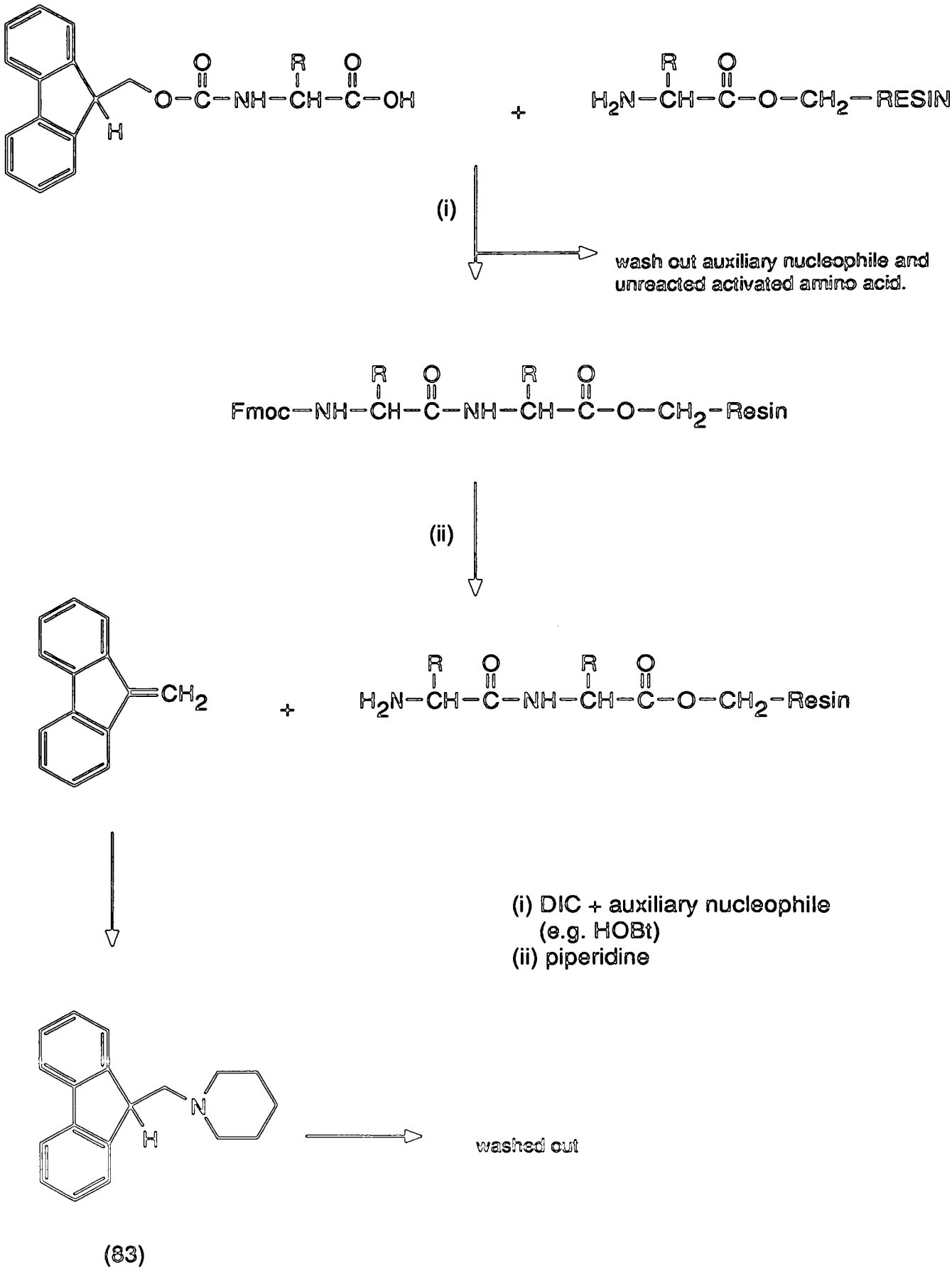
An alternative approach to monitoring is to examine the solution of activated amino acid during or after the coupling. In 1966 Bodansky and Sheehan[67] adopted this approach. Acylation was achieved using *p*-nitrophenyl esters of the protected amino acids, and the filtrate from the reaction was examined by ultraviolet spectroscopy. Absorption at 314 nm was indicative of free *p*-nitrophenol and absorption at 270 nm showed the amount of *p*-nitrophenyl ester present. A second coupling gave a filtrate which had an absorption only at 270 nm, indicating that the first coupling had been complete. Radioactive labelling of the N^{α} protecting group allows acylation to be monitored by the decrease in radioactivity of aliquots of the solution containing the activated amino acid.

In 1986, Dryland and Sheppard[68] carried out the synthesis of a pentadecapeptide under continuous flow conditions using 'Fmoc-polyamide' chemistry. The circulating activated Fmoc amino acid (symmetrical anhydride) and also the deprotection wash are passed through an ultraviolet spectrophotometer (302 nm or 314 nm). During the acylation reaction successive peaks are recorded and the completion of coupling is indicated by the decreasing area under the peaks and final plateauing. However this method is only semi-quantitative, and involves many errors. For example, manipulative losses in anhydride formation and baseline drift of the ultraviolet spectrometer. Nevertheless, this type of monitoring provides reassuring information of the progress of both acylation and deprotection reactions.

McFerran and Walker[69] have recently suggested the use of potentiated conductance as a method for monitoring the progress of both coupling and deprotection reactions. When the peptide bond is formed an acid is released whether using symmetrical anhydrides or active esters. Addition of a sterically hindered base (for example *N*-methylmorpholine or diisopropylethylamine) to the polar solvents used ionizes this acid and the resultant ion pairs give rise to readily detectable conductance signals allowing determination of the amount of the acid and therefore direct estimation of the extent of acylation. During Fmoc deprotection with piperidine, carbon dioxide is evolved which forms a carbamic acid salt with the base so giving rise to conductance signals directly related to the amount of


(77)
(78)
(79)
deprotect
(80)
couple (activated Fmoc amino acid)
deprotect and couple
(81)
deprotect and cleave
(82)

Scheme 21


O-C-NH-CH-C-OH
H
+
H2N-CH-C-O-CH2-RESIN
(i)
wash out auxiliary nucleophile and unreacted activated amino acid.
Fmoc-NH-CH-C-NH-CH-C-O-CH2-Resin
(ii)
CH2
+
H2N-CH-C-NH-CH-C-O-CH2-Resin
(i) DIC + auxiliary nucleophile (e.g. HOBt)
(ii) piperidine
N
H
washed out

(83)

Scheme 22

Fmoc-piperidine adduct. However absorption of carbon dioxide from the atmosphere can cause inaccuracies so solutions must be freshly prepared.

None of the above methods provide accurate quantitative information and there is still much room for improvement and development of an accurate monitoring system for the coupling stage.

Fmoc chemistry is the strategy used in these present studies [Scheme 21]. Syntheses are achieved by SPPS using an ABI430A peptide synthesiser. The first amino acid (the C-terminal residue) is coupled to a *p*-alkoxybenzyl alcohol resin (78) outside the peptide synthesiser *via* a symmetrical anhydride (77). Once on the synthesiser the chain is built up by addition of N^{α} Fmoc protected amino acids coupled *via* a symmetrical anhydride for 20-30 min and a repeated coupling *via* DIC/HOBt activation for 20-30 min. The N^{α} protecting group is removed (79) at each stage by treatment with piperidine giving the dibenzofulvene (80). Once the peptide chain (81) is complete it is cleaved from the resin, side chain protection is removed and the crude peptide (82) is purified to the desired level.

The efficiency of each coupling is assessed [Scheme 22] by passing the deprotection wash [dibenzofulvene-piperidine adduct (83)] through an ultraviolet cell (302 nm). The absorption recorded has a peak area proportional to the amount of adduct present and therefore to the amount of the last amino acid coupled to the growing peptide chain. At this stage if coupling efficiency is seen to be poor, it is one stage too late to do anything about it since all the amine functions have been exposed. However, if the washings of the previous coupling step containing free triazole (or imidazole) and activated amino acid could be monitored by their ultraviolet absorbance then if coupling was poor, the cycle could be interrupted and repeated. It is therefore desirable to find an *N*-hydroxy compound suitable for use as a coupling reagent with an ultraviolet absorbance different from the activated Fmoc-amino-acid. At present, the use of HOBt does not allow efficient monitoring of this type since it absorbs strongly in the same region as the Fmoc group.

Requirements for a good auxiliary nucleophile

The reagent must be:

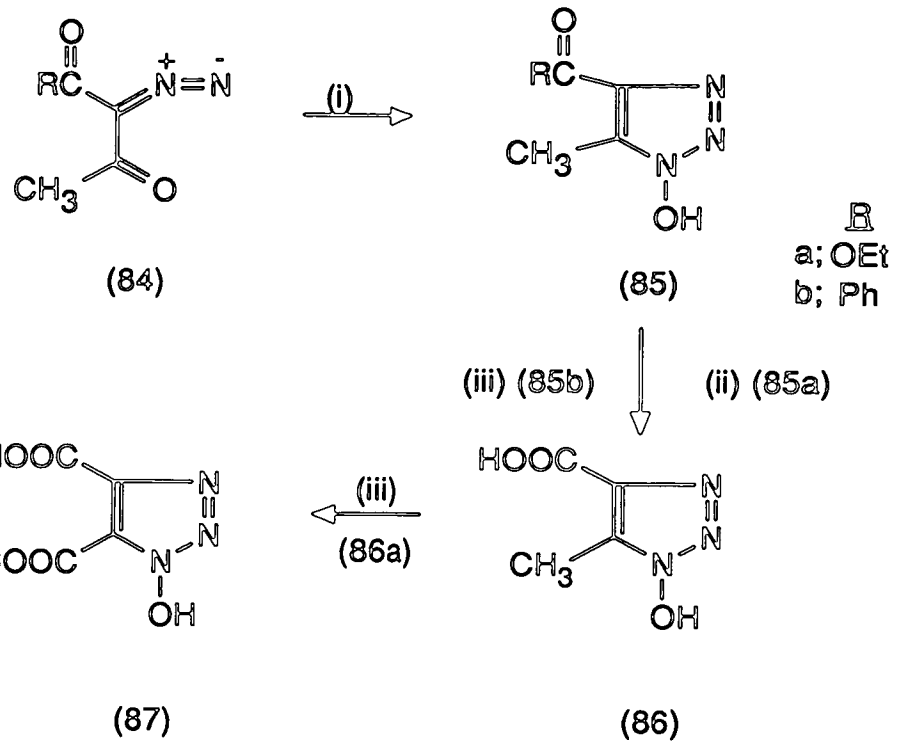
(i) a good activating and leaving group.

(ii) sterically unhindered round the 'N-OH' group.

(iii) readily soluble in solvent systems compatible with the synthesiser (DMF, DCM, dioxane),

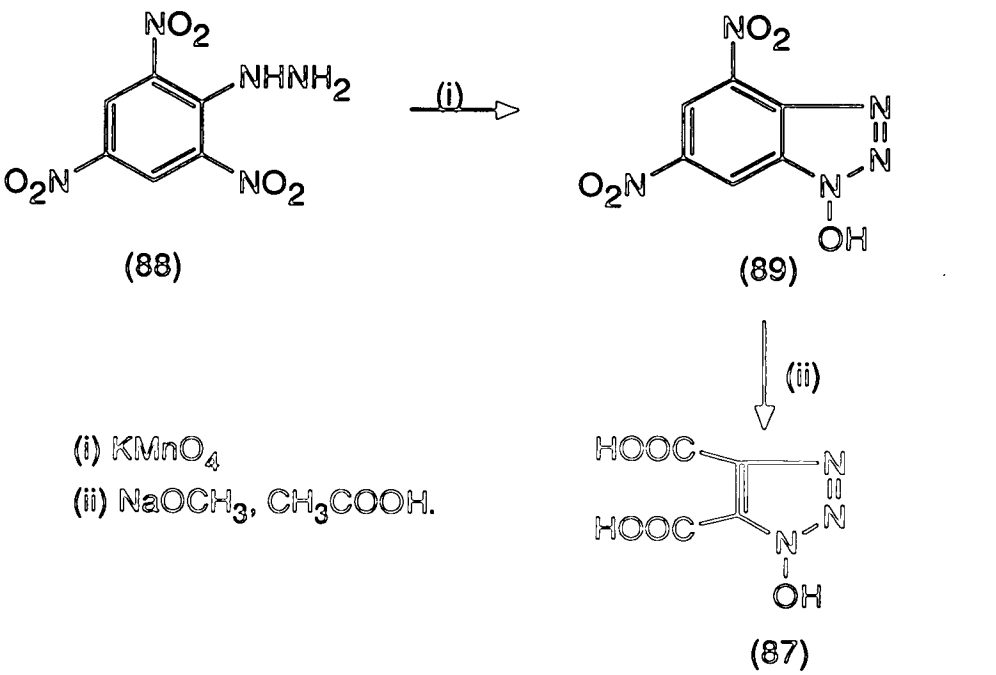
(iv) of a nature which will enable monitoring of the coupling reaction.

HOBt as an auxiliary nucleophile has been used extensively in this research group for many years. However, in these present studies we hoped to synthesise a 1-hydroxy-1*H*-1,2,3-triazole derivative with superior properties to HOBt which would allow the use of a monitoring system as described above and also be improved in its general ability as an auxiliary nucleophile. Investigations leading to an improved auxiliary nucleophile were based on other nitrogen containing heterocycles chiefly, 1-hydroxy-1*H*-1,2,3-triazoles and *N*-hydroxy-1*H*-imidazoles. The substituents on these heterocyclic rings were varied with a view to satisfying the requirements set out above.



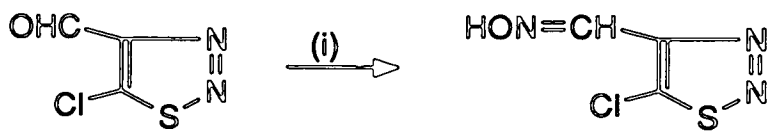
(i) NH_2OH, H_2O, EtOH, reflux.
(ii) saponification with NaOH, H_2O, heat.
(iii) $KMnO_4$, EtOH, heat.

Scheme 23



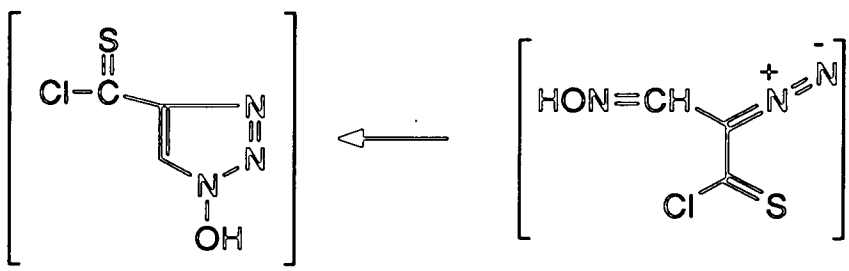
(i) $KMnO_4$
(ii) $NaOCH_3$, CH_3COOH.

Scheme 24



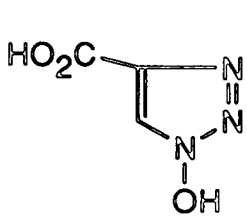
(90)

(91)



(93)

(92)



(94)

(i) NH_2OH, H_2O, MeOH, room temp.

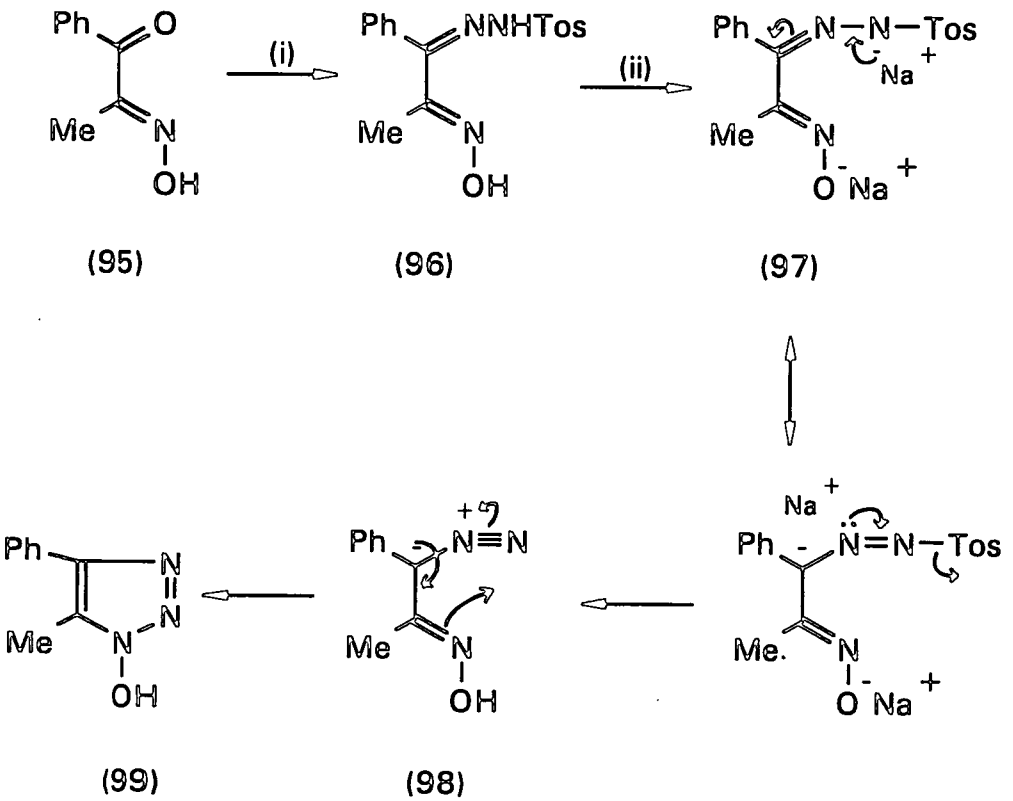
(ii) DMSO, room temp.

(iii) H_2O, room temp.

Scheme 25

1.3 Literature Methods for the Synthesis of 1-Hydroxy-1*H*-1,2,3-triazoles and 1-Hydroxy-1*H*-imidazoles.

1-Hydroxy-1*H*-1,2,3-triazoles.



(95) (96) (97)

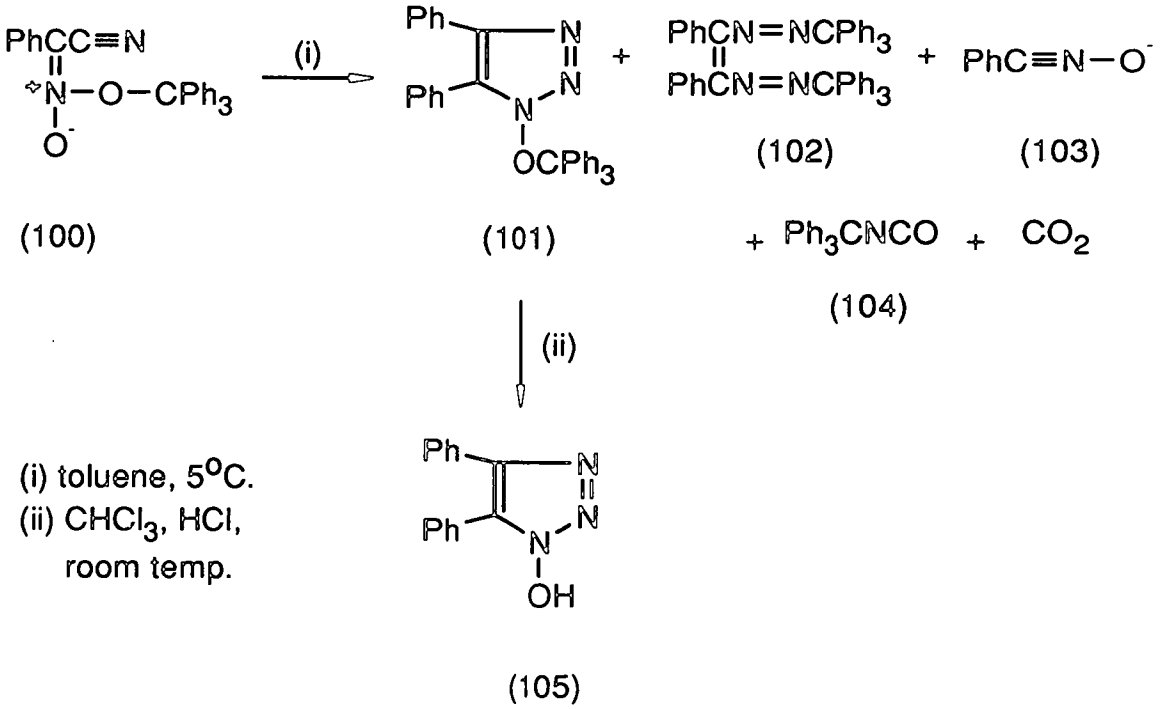
(99) (98)

(i) $TosNHNH_2$, ethanol, reflux.

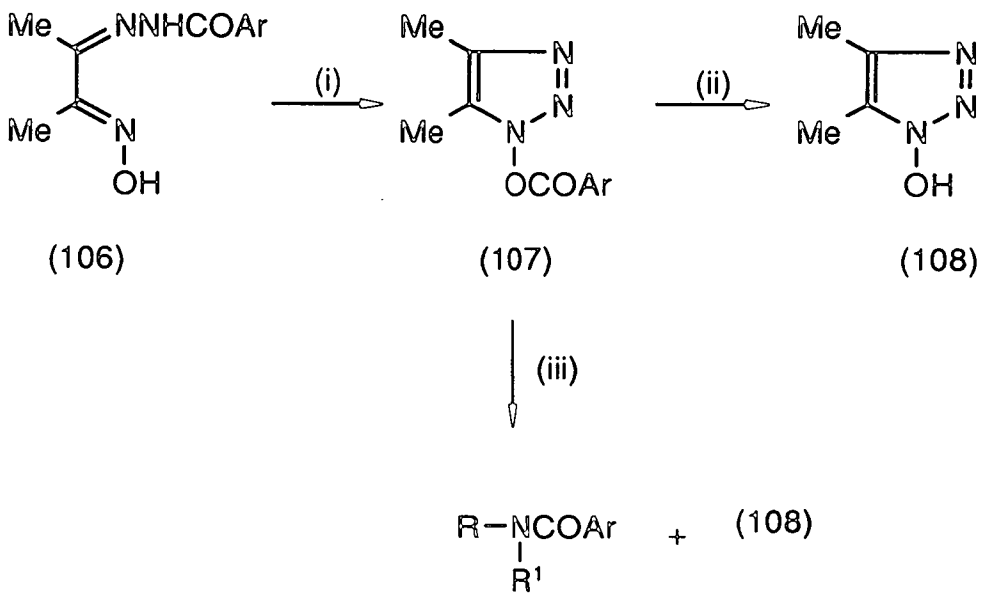
(ii) Na, ethylene glycol, heat.

Scheme 26

One of the earliest reports [Scheme 23] of 1-hydroxy-1*H*-1,2,3-triazoles was from Wolff[70] in 1902, who synthesised triazoles (85a) and (85b) from the reaction of the corresponding diazo compounds (84a) and (84b) with hydroxylamine. More recently Jenkins[71] has also synthesised ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (85a) by the reaction of ethyl 2-diazo-3-oxobutanoate (84a) with



Scheme 27



Scheme 28

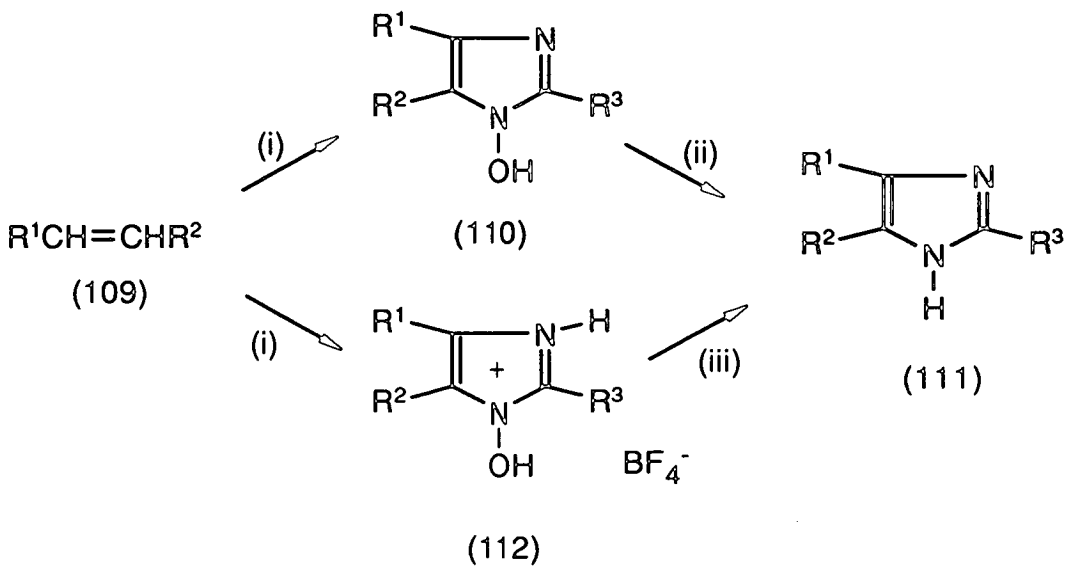
hydroxylamine. Wolff[70] found that saponification of the ethyl carboxylate (85a) with sodium hydroxide gave the corresponding monocarboxylic acid (86), further oxidation of this triazole (86) with potassium permanganate gave the dicarboxylic acid (87). Permanganate oxidation of *N*-hydroxytriazole (85b) again gave the monocarboxylic acid (86).

In 1967, Huisgen and Weberndorfer[72] [Scheme 24] also reported the synthesis of the dicarboxylic acid (87) by the oxidation of the dinitrobenzotriazole (89) with potassium permanganate. The benzotriazole derivative (89) was obtained from the reaction of 2,4,6-trinitrophenylhydrazine (88) with sodium acetate and acetic acid.

L'abbé *et al.*[73] have reported [Scheme 25] the preparation of *N*-hydroxytriazole (94) from a 1,2,3-thiadiazole (90). Treatment of the aldehyde derivative (90) with hydroxylamine gave 5-chloro-4-hydroxyiminomethyl-1,2,3-thiadiazole (91) which gave 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylic acid (94) when stirred in DMSO containing a few drops of water. This took place through the ring opening of the thiadiazole (91) and subsequent cyclisation to give the thiocarboxylic chloride (93) leading to the *N*-hydroxytriazole product (94) on hydrolysis.

In 1967, Bartlett and Stevens[74] reported [Scheme 26] the synthesis of 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (99) by the thermal decomposition of the tosylhydrazone sodium salt (97). The tosylhydrazone (96) was prepared by the reaction of tosylhydrazine with the starting keto-oxime (95) and was then heated in a solution of sodium in ethylene glycol forming its disodium salt (97) which decomposed to give the *N*-hydroxytriazole (99) via the mechanism shown. The intermediate sodium salt (97) was not isolated by Bartlett and Stevens.[74] However, Jenkins[71] further developed this synthesis and was able to isolate the sodium salt (97) prior to its cyclisation to 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (99) by treatment of the tosylhydrazone (96) in dimethoxyethane (DME) with a solution of sodium in ethanol. Jenkins[71] found that the thermal decomposition of the sodium salt (97) in DME gave the *N*-hydroxytriazole (99) in 34% yield, yet, when the salt (97) was refluxed in diglyme the yield was greatly improved to 77%.

Boyer *et al.*[75] obtained 1-hydroxy-4,5-diphenyl-1*H*-1,2,3-triazole (105) [Scheme 27] by the acid hydrolysis of 4,5-diphenyl-1-triphenylmethoxy-1,2,3-triazole (101) which was formed when the nitronate ester (100) fragmented in toluene at 5^{0}C giving compounds (101) to (104) and carbon dioxide. Jenkins[71] was also successful



(i) $NO^+BF_4^-$, R^3CN, room temp.

(ii) $TiCl_3$, MeOH, room temp.

(iii) Red-Al, room temp.

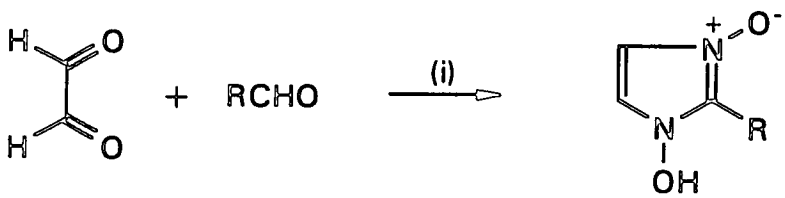
	R^1	R^2	R^3
a;	$PhCH_2$	H	CH_3
b;	Bu^t	H	CH_3
c;	$(CH_3)_2CHCH_2$	H	CH_3
d;	n-C_4H_9	H	CH_3
e;	n-C_5H_{11}	H	CH_3
f;	CH_3	H	CH_3
g;	CH_3	CH_3	CH_3
h;	C_6H_5	H	CH_3
i;		-$(CH_2)_4$-	CH_3
j;	CH_3	H	C_6H_5

Scheme 29

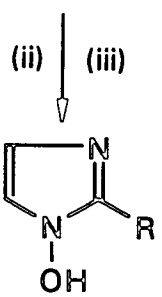
in obtaining this *N*-hydroxytriazole (105) from its corresponding tosylhydrazone by the method [Scheme 26] reported by Bartlett and Stevens.[74]

Alexandrou *et al.*[76] have reported [Scheme 28] the synthesis of 4,5-dimethyl-1-hydroxy-1*H*-1,2,3-triazole (108) from the hydrolysis of 1-aroyloxy-4,5-dimethyl-1,2,3-triazoles (107a) to (107c). These *N*-aroyloxytriazoles (107a) to (107c) were obtained in poor yield from the lead tetra-acetate oxidation of α-hydroxyiminoaroylhydrazones of biacetyl (106a) to (106c). The *N*-hydroxytriazole (108) was also formed when the *N*-aroyloxytriazoles (107a) to (107c) were used as acyl transfer agents.

1-Hydroxy-1*H*-imidazoles



(113) (114) (115)

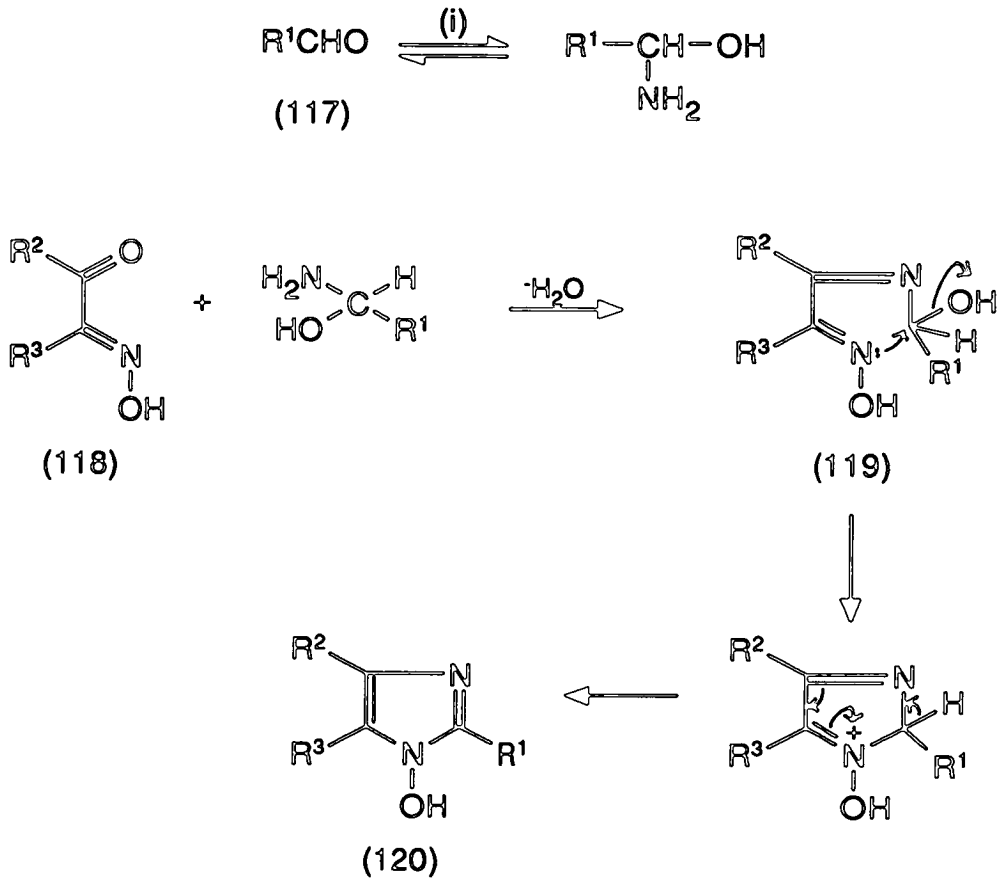


(116)

	R
a;	H
b;	CH_3
c;	CH_2CH_3

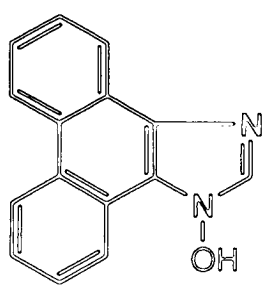
(i) $NH_2OH.HCl$, MeOH, H_2O, <20°C.
(ii) H_2, Pd/C, HCl, H_2O,
(iii) $KHCO_3$, H_2O, room temp.

Scheme 30



(i) aqueous ammonia, room temp.

	R¹	R²	R³
a;	Ph	Ph	Ph
b;	Ph	-$C_{12}H_8$-	
c;	Ph	Me	Me
d;	Me	Me	Me



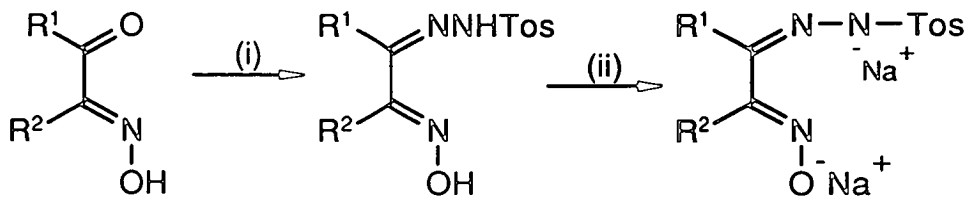
(120b)

Scheme 31

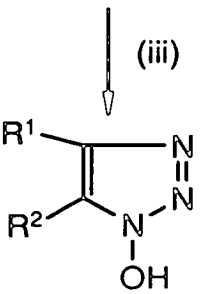
In contrast to 1-hydroxy-1*H*-1,2,3-triazoles more is known about 1-hydroxy-1*H*-imidazoles. In 1984, Lipshutz and Morey[77] reported [Scheme 29] the synthesis of *N*-hydroxyimidazoles (110a) to (110e) by the reaction of the corresponding alkene with nitrosyl fluoroborate in acetonitrile. Their aim was to synthesise imidazoles (111a) to (111e) by the method [Scheme 29, (iii)] reported ten years earlier by Scheinbaum and Dines,[78] that is by the reduction of the *N*-hydroxyimidazolium ions (112f) to (112j) with Red-Al [sodium bis(2-methoxyethoxy)aluminium hydride). The imidazolium ions (112f) to (112j) were prepared as above by the reaction of the corresponding alkene with nitrosyl fluoroborate in nitrile media. However, Lipshutz and Morey[77] found this method of reduction to be irreproducible. They obtained the *N*-hydroxyimidazoles (110a) to (110e) which they were only able to reduce with $TiCl_3$ and not with Red-Al.

Laus *et al.*[79] have reported [Scheme 30] the synthesis of several *N*-hydroxyimidazoles (116a) to (116c) by the reduction of 1-hydroxyimidazole 3-oxides (155a) to (155c), prepared by the reaction of an aqueous solution of glyoxal (113) and the appropriate aldehyde (114) with hydroxylamine.

In 1964, two groups reported [Scheme 31] more straightforward routes to *N*-hydroxyimidazoles. Volkamer and Zimmermann[80] reported the condensation of α-oximinoketones (118a) and (118b) with benzaldehyde (117a) in the presence of ammonia to give the corresponding *N*-hydroxyimidazoles (120a) and (120b). Similarly Allan and Allan[81] also took this approach, condensing butane-2,3-dione monoxime (118c) or (118d) with the appropriate aldehydes in aqueous ammonia to give the *N*-hydroxyimidazoles (120c) (from benzaldehyde) and (120d) (from acetaldehyde). Allan and Allan[81] have proposed that the reaction proceeds via intermediate (119). These latter methods provide a straightforward route to *N*-hydroxyimidazoles using easily available starting materials and therefore, have been applied in the present studies of *N*-hydroxyimidazoles.



(121) (122) (123)



(124)

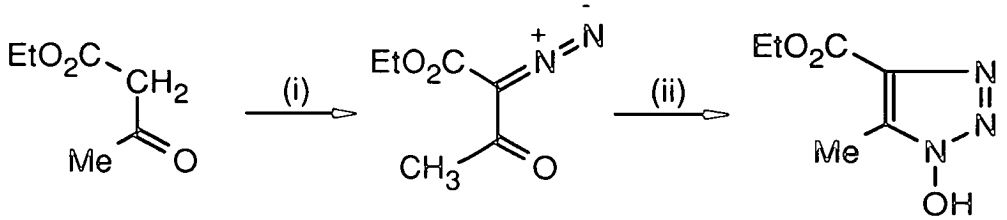
(i) $TosNHNH_2$, EtOH, reflux.

(ii) NaOEt, DME, room temp.

(iii) diglyme, reflux.

	R^1	R^2
a;	Ph	Me
b;	p-$MeOC_6H_4$	Me
c;	Me	Me

Scheme 32



(125) (126) (127)

(i) $TosN_3$, Et_3N, MeCN, room temp.

(ii)$NH_2OH.HCl$, NaOAc, H_2O, 80°C.

Scheme 33

2. DISCUSSION

The following section describes the synthesis or attempted synthesis [Scheme 2] of several *N*-hydroxytriazoles (3) and *N*-hydroxyimidazoles (4) and their further investigation as potential auxiliary nucleophiles in peptide synthesis. The stimulus for these investigations was the need for an auxiliary nucleophile that would improve acylation times, and allow the development of a monitoring system whereby the progress of the coupling could be followed in conjunction with the monitoring of the deprotection, so allowing the detection of inefficient coupling prior to deprotection. The description of the results is presented in 5 sections:

2.1 Investigations of the Synthesis of Aryl and Alkyl 1-Hydroxy-1*H*-1,2,3-triazoles.

2.2 Investigations into the Use of Aryl and Alkyl 1-Hydroxy-1*H*-1,2,3-triazoles as Reagents in Peptide Coupling.

2.3 Investigations of the Synthesis of 1-Hydroxy-1*H*-imidazoles.

2.4 Investigations into the use of 2,5-Dimethyl-1-hydroxy-4-phenyl-1*H*-imidazole (160a) as a Reagent in Peptide Coupling.

2.5 Monitoring and Concluding Remarks.

2.1 Investigations of the Synthesis of Aryl and Alkyl 1-Hydroxy-1*H*-1,2,3-triazoles.

The first task was to determine if *N*-hydroxytriazoles other than HOBt would be satisfactory reagents in the mediation of peptide bond formation. The first *N*-hydroxytriazole that was synthesised [Scheme 32] was 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a), a known compound previously reported by Jenkins[71] and Bartlett and Stevens.[74] The starting material for the synthesis of this *N*-hydroxytriazole (124a) was 1-phenylpropane-1,2-dione-2-oxime (121a) which was obtained in reasonable yield (79%) by nitrosation of propiophenone using

methyl nitrite in the presence of hydrogen chloride. The corresponding tosylhydrazone (122a) was obtained in good yield (89%) from the reaction of the keto-oxime (121a) with tosylhydrazine. The subsequent cyclisation of the tosylhydrazone (122a) was accomplished through the thermal decomposition of its disodium salt (123a) to give the desired *N*-hydroxytriazole in 77% yield, as confirmed by i.r. and ^{1}H n.m.r. spectra. The i.r. spectrum exhibited a broad OH absorption between 3100-2300 cm^{-1}. The ^{1}H n.m.r. spectrum showed a multiplet from the aromatic protons and a singlet at δ2.42 corresponding to the 5-methyl group.

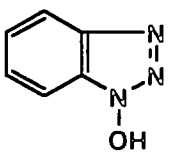
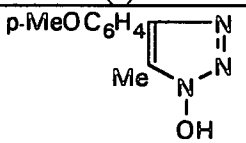
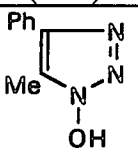
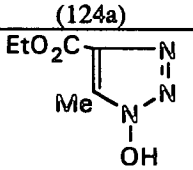
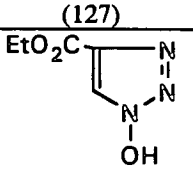
N-Hydroxytriazole	Absorbance (302nm)
 (1)	off scale >2.0
 (124b)	off scale >2.0
 (124a)	1.2
 (127)	0.1
 (132)	0.04

Figure 1. A comparison of the absorbances at 302 nm of equal concentrations of the coupling reagents in DMF.

1-Hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a) was then tested as a potential auxiliary nucleophile [Section 2.2] and found to be as good as HOBt. As would be expected for a compound containing a phenyl group, its u.v. absorbance (Fig. 1) although less than that of HOBt was still significant and the solubility of this triazole (124a) in DMF and DMF:dioxane was found to be no better than that of HOBt. If a methoxy group was added to the phenyl ring in the para position it would not be expected to satisfy our u.v. requirements. However it would be interesting to discover the effect of an electron donating group on the triazoles' ability to mediate peptide coupling. Therefore the synthesis of 1-hydroxy-4-(4'-methoxyphenyl)-5-methyl-1*H*-1,2,3-triazole (124b) was attempted.

The previously unknown starting keto-oxime (121b) [Scheme 32] was synthesised in good yield (62%) by the nitrosation of 4-*p*-methoxypropiophenone with methyl nitrite in the presence of anhydrous hydrogen chloride. The oximino-ketone (121b) analysed correctly and gave mass, i.r. and ^{1}H n.m.r. spectra consistent with its assigned structure. Thus the i.r. spectrum exhibited a broad OH band and a characteristic carbonyl absorption at 1650 cm^{-1}. The ^{1}H n.m.r. spectrum showed two singlets at δ2.0 and 3.82 corresponding to the two methyl and methoxy groups respectively and the aromatic protons were split into a characteristic AA,BB pattern for a *p*-substituted compound. The corresponding triazole (124b) was synthesised as in the case of the *N*-hydroxytriazole (124a) by heating the oximino-ketone (121b) with tosylhydrazine in ethanol under reflux to give the tosylhydrazone (122b) which was cyclised *via* its disodium salt (123b) to the desired *N*-hydroxytriazole (124b) in 73% yield. Again the analytical data and the mass, i.r. and ^{1}H n.m.r. spectra of the tosylhydrazone (122b) and *N*-hydroxytriazole (124b) were in agreement with the expected structures. The ^{1}H n.m.r. spectra for each of these compounds (122b) and (124b) showed the aromatic protons to have the same characteristic splitting pattern as above, confirming a *p*-substituted compound, and also exhibited the required methyl and methoxy peaks. In addition, the spectrum for the tosylhydrazone (122b) also contained peaks corresponding to the protons of the tosyl group and to the NH group. When this *N*-hydroxytriazole (124b) was tested as a potential peptide coupling agent it was found to be as good as HOBt and 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a) but was not quite as soluble in DMF and DMF:dioxane. The u.v. absorbance (Fig. 1) of this *N*-hydroxytriazole (124b) at 302 nm was much stronger than that of *N*-hydroxytriazole (124a). 1-Hydroxy-4-(4-methoxyphenyl)-5-methyl-1*H*-1,2,3-triazole (124b) did not appear to be better in

any way than HOBt or 1-hydroxy-4-phenyl-5-methyl-1*H*-1,2,3-triazole (124a) and since our main aim was the development of a monitoring system for the coupling stage we then turned our attention to obtaining a product with no u.v. absorbance at 302 nm.

The first change to make would be to remove the phenyl group at the 4 position, and so the synthesis of 4,5-dimethyl-1-hydroxy-1*H*-1,2,3-triazole (124c) was attempted. This *N*-hydroxytriazole (124c) had previously been prepared by Jenkins[71] as a gum in 40% yield by the method used above to prepare *N*-hydroxytriazoles (124a) and (124b) and was characterized by the synthesis of its *N*-phenylurethane derivative. Alexandrou *et al.*[76] have reported (p 35) this *N*-hydroxytriazole (124c) although they obtained it in very poor yield. Therefore, synthesis of 4,5-dimethyl-1-hydroxy-1*H*-1,2,3-triazole (124c) was repeated in the hope of obtaining a solid product. The readily available starting material, butane-2,3-dione 2-oxime (121c) was converted to the corresponding tosylhydrazone (122c) (96%) (as confirmed by comparison with an authentic sample - mass and i.r. spectra) when refluxed with tosylhydrazine in ethanol. However, when an attempt was made to cyclise the tosylhydrazone (122c) to the required *N*-hydroxytriazole (124c) by heating its disodium salt (123c) under reflux in diglyme an oil resulted. When the oil was dissolved in water and freeze dried an apparent solid was obtained which quickly returned to a gummy state when exposed to the atmosphere. Attempts to triturate the oil with various solvents were also unsuccessful. Such a hygroscopic compound was impractical for use at this stage since the auxiliary nucleophile was put onto the peptide synthesiser in the form of preweighed cartridges. Therefore, further investigations into this compound were not carried out.

Since the above attempt to obtain an *N*-hydroxytriazole with no u.v. absorbance around 302 nm failed our attention turned [Scheme 33] to the carboxylate triazole (127) reported by Jenkins[71] and Wolff.[70] Our hope was that this *N*-hydroxytriazole (127) would satisfy our u.v. requirements and also be suitable for use with DIC as a peptide coupling agent. The precursor to the required product (127), ethyl 2-diazo-3-oxobutanoate (126) was obtained in good yield (83%) from the triethylamine catalysed reaction of ethyl oxobutanoate (125) with tosyl azide. The reaction of this diazo-keto-ester (126) with hydroxylamine under aqueous conditions at 80°C afforded the required product (127) in a disappointing yield of 43%. The melting point and i.r. spectrum of this *N*-hydroxytriazole (127) were in agreement with the same data of an authentic sample[71] of the compound (127).

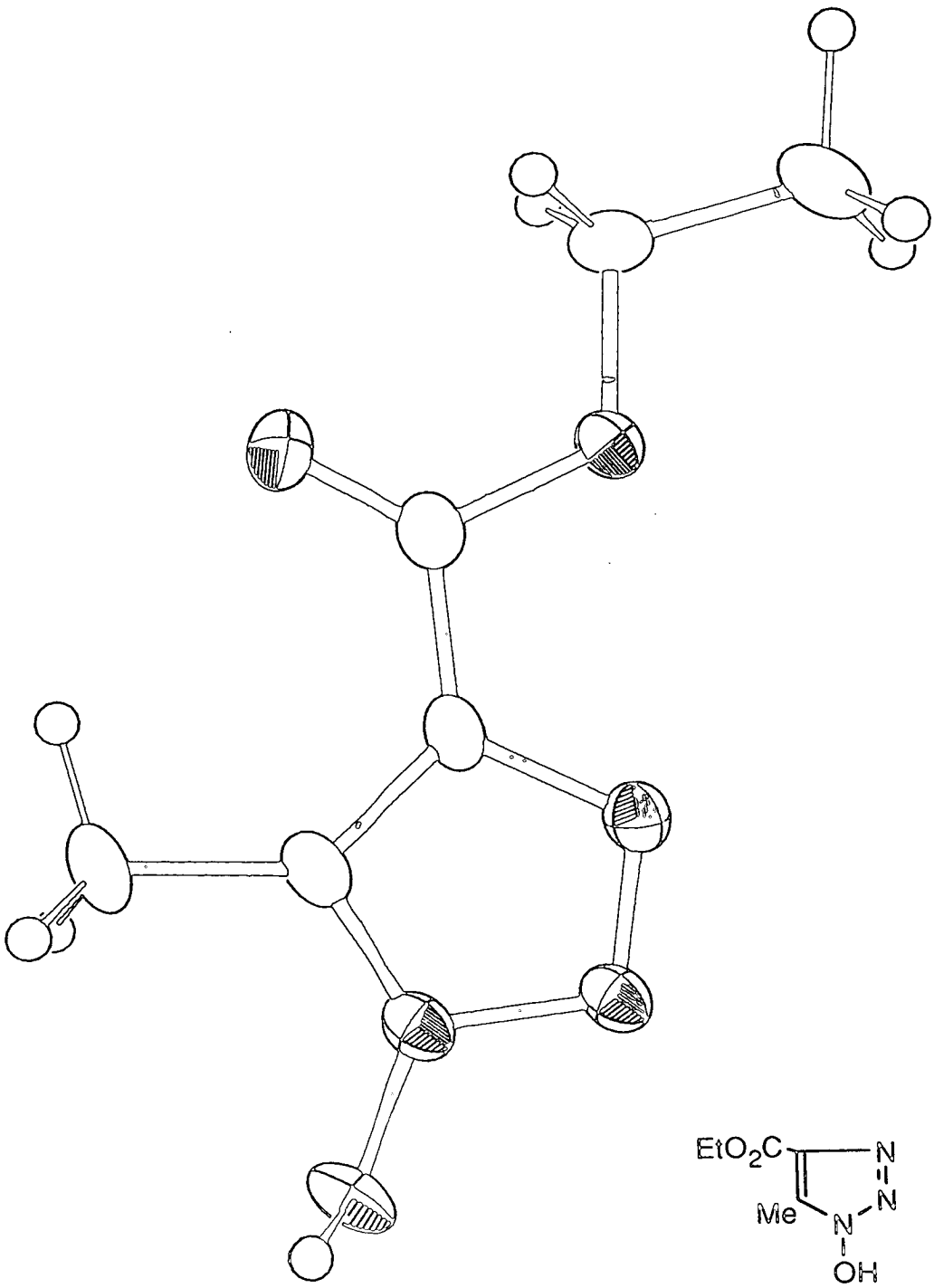


Figure 2(a).

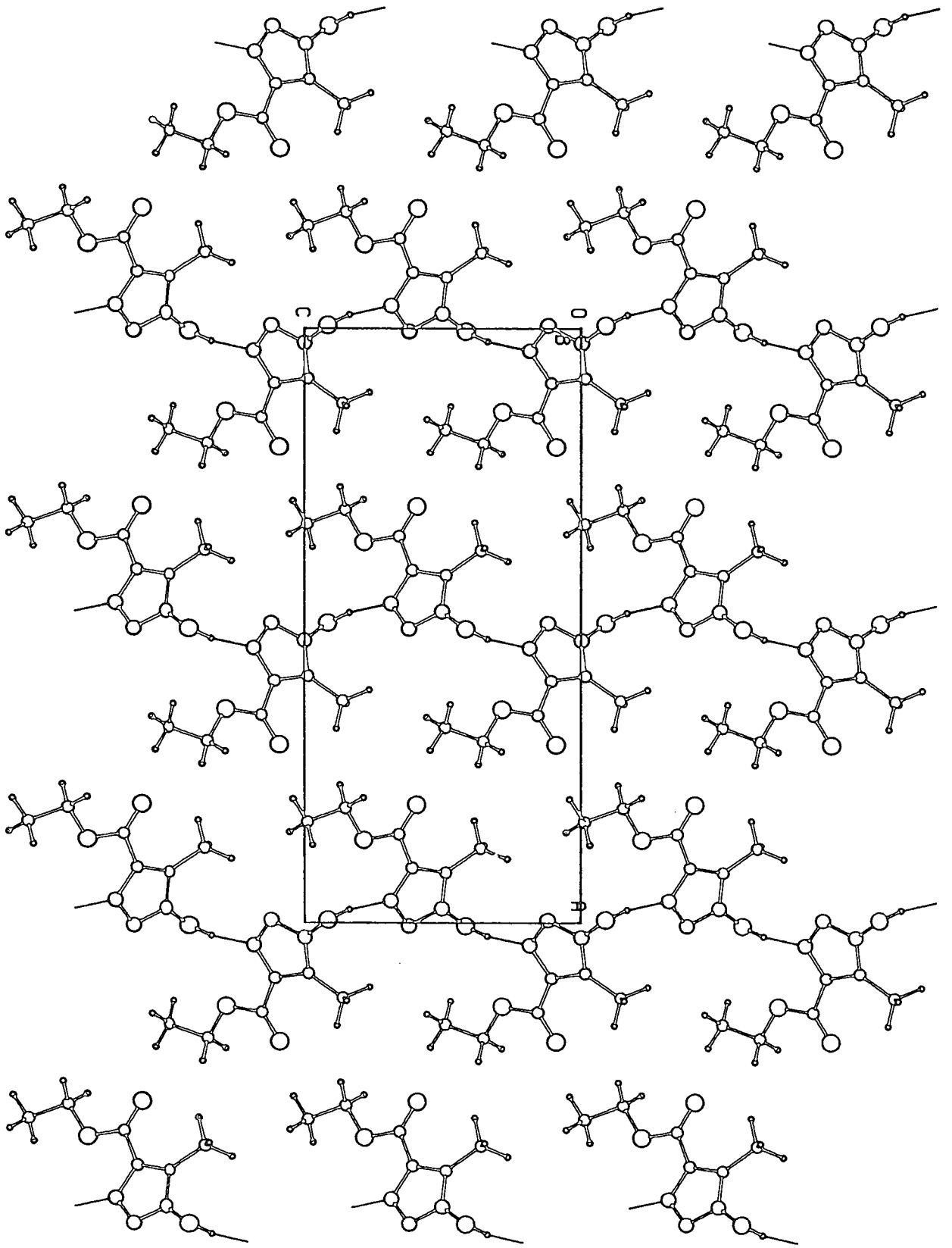


Figure 2(b).

N(1) - O(1)	1.354(4)	C(4) - C(6)	1.455(5)
N(1) - N(2)	1.340(4)	C(5) -C(5M)	1.498(6)
N(1) - C(5)	1.344(5)	C(6) - O(6)	1.203(5)
O(1) - H(1)	0.96(5)	C(6) - O(7)	1.339(5)
N(2) - N(3)	1.316(5)	O(7) - C(8)	1.455(5)
N(3) - C(4)	1.358(5)	C(8) - C(9)	1.491(6)
C(4) - C(5)	1.386(5)		

O(1) - N(1) - N(2)	119.5(3)	N(1) - C(5) - C(4)	102.7(3)
O(1) - N(1) - C(5)	126.3(3)	N(1) - C(5) -C(5M)	122.9(3)
N(2) - N(1) - C(5)	113.9(3)	C(4) - C(5) -C(5M)	134.4(4)
N(1) - O(1) - H(1)	106.5(31)	C(4) - C(6) - O(6)	124.9(4)
N(1) - N(2) - N(3)	104.9(3)	C(4) - C(6) - O(7)	110.7(3)
N(2) - N(3) - C(4)	110.3(3)	O(6) - C(6) - O(7)	124.4(4)
N(3) - C(4) - C(5)	108.2(3)	C(6) - O(7) - C(8)	117.6(3)
N(3) - C(4) - C(6)	123.1(3)	O(7) - C(8) - C(9)	107.1(3)
C(5) - C(4) - C(6)	128.7(4)		

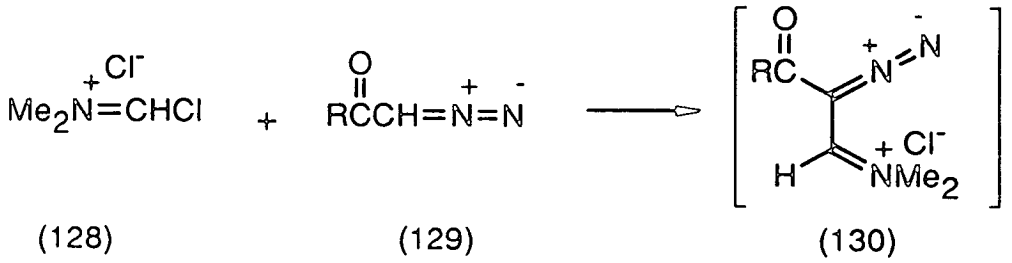
N(2) - N(1) - O(1) - H(1)	101.7(33)	N(3) - C(4) - C(5) - N(1)	-0.1(4)
C(5) - N(1) - O(1) - H(1)	-84.1(33)	N(3) - C(4) - C(5) -C(5M)	179.2(4)
O(1) - N(1) - N(2) - N(3)	174.9(3)	C(6) - C(4) - C(5) - N(1)	176.9(4)
C(5) - N(1) - N(2) - N(3)	-0.1(4)	C(6) - C(4) - C(5) -C(5M)	-3.8(8)
O(1) - N(1) - C(5) - C(4)	-174.4(3)	N(3) - C(4) - C(6) - O(6)	179.0(4)
O(1) - N(1) - C(5) -C(5M)	6.2(5)	N(3) - C(4) - C(6) - O(7)	0.5(5)
N(2) - N(1) - C(5) - C(4)	0.1(4)	C(5) - C(4) - C(6) - O(6)	2.4(7)
N(2) - N(1) - C(5) -C(5M)	-179.3(3)	C(5) - C(4) - C(6) - O(7)	-176.1(4)
N(1) - N(2) - N(3) - C(4)	0.1(4)	C(4) - C(6) - O(7) - C(8)	175.7(3)
N(2) - N(3) - C(4) - C(5)	0.1(4)	O(6) - C(6) - O(7) - C(8)	-2.8(6)
N(2) - N(3) - C(4) - C(6)	-177.2(4)	C(6) - O(7) - C(8) - C(9)	178.8(3)

Figure 2(c). Bond lengths (A), angles (degrees) and torsion angles (degrees) with standard deviations.

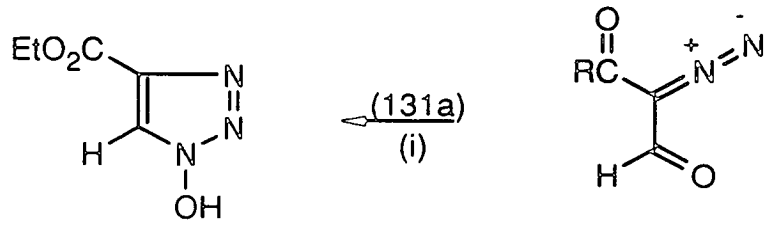
N-Hydroxytriazole (127) formed very good crystals and so an X-ray structure was obtained (Fig. 2) to show the orientation of the functional groups particularly round the N-OH group. The X-ray showed the molecules to be arranged (Fig. 2b) very neatly with intermolecular hydrogen bonding. The u.v. spectrum (Fig. 1) of ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) was very encouraging since it showed very little absorbance around 302 nm. The triazole (127) was also readily soluble in DMF and DMF:dioxane (50:50) and its pK_a was found to be 2.65. It was therefore evaluated as a potential coupling agent in the synthesis of the test peptide LeuIlePheAlaGly and found to be as good as HOBt [Section 2.2]. In summary, ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) (i) was found to be a good activating and leaving group; (ii) was readily soluble in solvent systems compatible with the peptide synthesiser such as DMF and DMF:dioxane (50:50); and (iii) exhibited a u.v. absorbance compatible with the proposed u.v. monitoring of the coupling reaction. Therefore, since three of the four requirements set out earlier (p 28) were satisfied our studies of this *N*-hydroxytriazole (127) were continued.

The peptide coupling ability of the triazole (127) was further tested in the synthesis of longer peptides (Section 2.2) and further investigations were carried out to improve the disappointing yield (43%) of the required product (127). The diazo-keto-ester (126) was reacted with hydroxylamine under anhydrous ethanolic conditions. However the starting diazo compound (126) was obtained rather than the *N*-hydroxytriazole (127). Starting material (126) was also obtained when the reaction was carried out in anhydrous dimethoxyethane (DME) or anhydrous *n*-butanol. Due to the lack of success obtained when anhydrous conditions were applied to the synthesis of triazole (127) further attempts were made to improve the yield under aqueous conditions. Jenkins[71] treated the diazo-keto-ester (126) with hydroxylamine in one portion for 8 hours; however, when this reaction was monitored by u.v. spectrometry the reaction was seen to be complete in 4 hours. Therefore the reaction time was altered to a total of 5 hours and the hydroxylamine was added in two portions. Unfortunately the highest yield of the product (127) obtained was under these latter conditions and was only 53%.

Although ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) was successful in meeting several of our requirements it still possessed a considerable degree of undesirable steric bulk around the *N*-hydroxy group due to the methyl group at position 5 of the triazole ring. Our aim was therefore to obtain an



(128) (129) (130)



(132) (131)

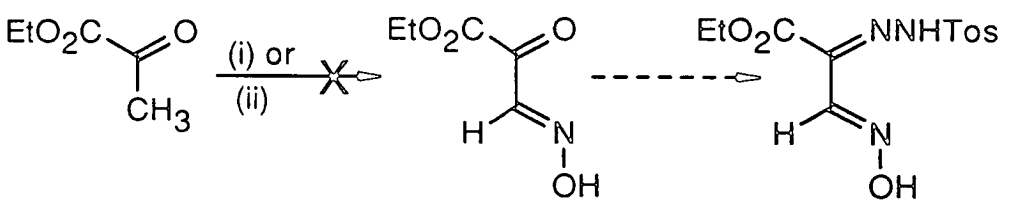
(i) hydroxylamine

R

a; OEt

b; Ph

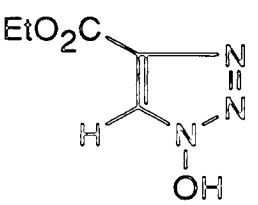
Scheme 34



(133) (134) (135)

(i) AmONO, NaOEt, EtOH, room temp.

(ii) MeONO, HCl, ether, room temp.

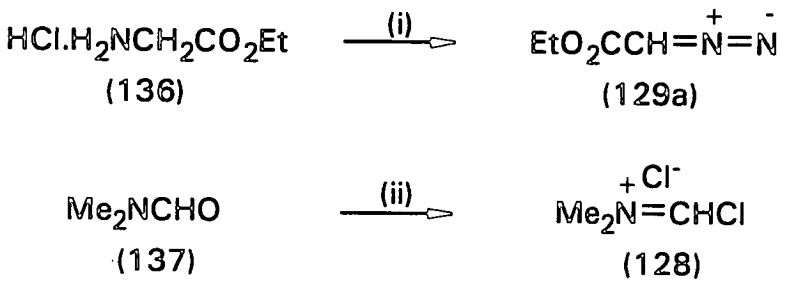


(132)

Scheme 35

N-hydroxytriazole unsubstituted at this 5 position, hence investigations were commenced into the synthesis [Scheme 34] of ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132).

At the outset of this work *N*-hydroxytriazole (132) was not a known compound and the work presented here describes our investigations leading to the synthesis of triazole (132). This has since been reported by Brooks *et al.*[82] who prepared it from the reaction of the diazoaldehyde (131) reported by Stojanovic[83] with hydroxylamine, although no experimental details were given. Our initial attempts to obtain the triazole (132) were based on the general procedure [Scheme 32] used for the synthesis of triazoles (124a) and (124b) whereby a ketoxime (121) was converted to the corresponding tosylhydrazone (122) which was then cyclised to the triazole (124). In order to proceed by this method [Scheme 35], the starting ketoxime (134) was required and its synthesis was attempted by the oximation of ethyl 2-oxopropanoate (133), 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 (134).



(i) $NaNO_2$, H_2SO_4, DCM, H_2O, chill.

(ii) $SOCl_2$, room temp.

Scheme 36

Our attention then turned to the approach used [Scheme 32] in the synthesis of ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) which was obtained by the reaction of the corresponding diazo-keto-ester (126) with hydroxylamine. The application of this approach [Scheme 34] to ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-

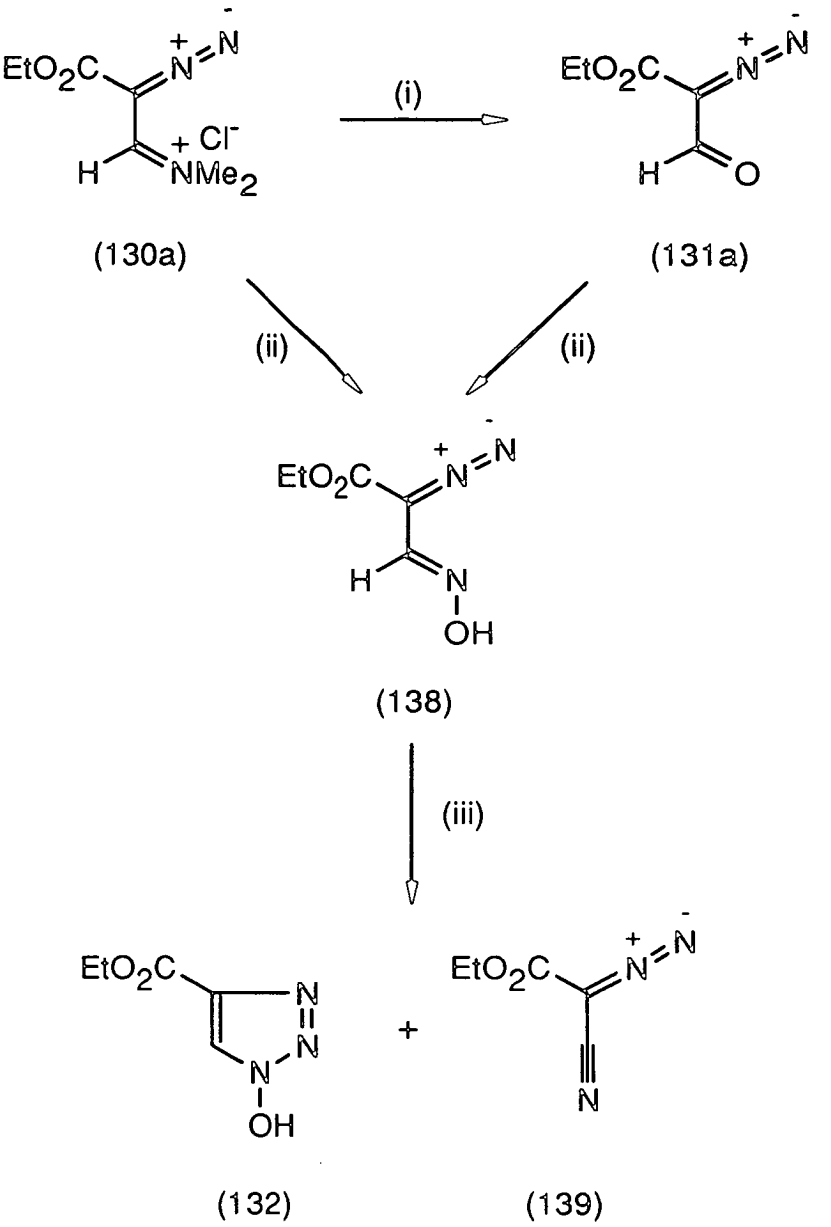
carboxylate (132) would require the α-diazoaldehyde, ethyl 2-diazo-3-oxopropanoate (131a).

In 1967, Stojanovic[83] reported the synthesis of two previously unknown α-diazoaldehydes (131a) and (131b), the former being the diazo compound needed for the synthesis of the required *N*-hydroxytriazole (132). Stojanovic[83] obtained ethyl 2-diazo-3-oxopropanoate (131a) from the reaction of ethyl diazoacetate (129a) with dimethylchloromethyleneammonium chloride (128).

These starting materials were easily prepared [Scheme 36] by literature methods. Ethyl diazoacetate[84] (129a) was synthesised in 97% yield by the reaction of ethyl 2-aminoethanoate hydrochloride (136) with sodium nitrite in acid and the hygroscopic dimethylchloromethyleneammonium chloride[85] (128) was synthesised from DMF (137) and thionyl chloride under argon. Due to the difficulty in handling the DMF-chloride it was used immediately without purification.

The α-diazoaldehyde (131a) (50% yield) was obtained by the hydrolysis in aqueous acetic acid of the proposed[83] non-isolable intermediate (130a) formed from the reaction of ethyl diazoethanoate (129a) with the DMF-chloride (128) and identified by i.r. and u.v. spectroscopy. In an effort to improve this yield the hydrolysis was carried out in a two-phase mixture of ether and an aqueous buffer (sodium acetate and acetic acid, pH7). However the yield was not improved. The diazoaldehyde (131a) was then treated with hydroxylamine under aqueous conditions at 80^0C for 7 hours. However, only a very small amount of solid (9%) was obtained. The formulation of this solid as the expected *N*-hydroxytriazole (132) followed from its mass spectrum and combustion analysis data which were consistent with the molecular formula $C_5H_7N_3O_3$. Thus its i.r. spectrum contained a broad band at 3200-2000 cm^{-1} due to the OH and a distinctive carbonyl band at 1730 cm^{-1} and its ^1H n.m.r. spectrum showed proton resonances characteristic of an ethyl group at δ4.3 and 1.29 in addition to a signal due to the proton at the 5-position of the triazole ring.

Since the yield of the required *N*-hydroxytriazole (132) was very poor (9%) under aqueous conditions, the synthesis was repeated using anhydrous ethanol rather than water as the reaction solvent. After work up of the reaction mixture a bright yellow solid (47%) was obtained which when recrystallized for analysis gave a colourless solid whose analytical and spectral data were consistent with the data obtained



(i) aqueous acetic acid, room temp.

(ii)$NH_2OH.HCl$, Na_2CO_3, EtOH, room temp.

(iii) benzene, reflux.

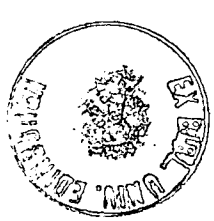
Scheme 37

previously for the *N*-hydroxytriazole (132). However, the i.r. spectral data of the yellow solid obtained prior to recrystallisation exhibited a strong absorbance at 2100 cm^{-1} indicating the presence of a diazo group. Therefore, it became apparent [Scheme 37] that the yellow solid was actually the diazo-oxime (138) which had obviously cyclised during recrystallisation to give the corresponding ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132). The synthesis of the diazo-oxime (138) was repeated giving the product in 40% yield. The progress of the reaction was monitored by u.v. spectroscopy indicating the oximation of the diazoaldehyde (131a) to be complete within 20 mins hence, the reaction time was reduced from 17 h to 1 h.

Formation of the required *N*-hydroxytriazole (132) was then easily accomplished by the thermally assisted cyclisation of the diazo-oxime (138). Various solvents were used for this step such as ethyl acetate (35%), DME (45%) and benzene (88%), the latter giving the greater yield of product (132). The progress of the reaction could be followed easily as the reaction mixture changed from bright yellow to pale yellow in 45 min - 1 hour. As the benzene cooled colourless crystals formed whose spectral data was consistent with the expected structure of the *N*-hydroxytriazole (132).

This compound (132) was then tested as a potential peptide coupling agent in the synthesis of the test peptide LeuIlePheAlaGly and was found to be as good as HOBt. Up to this point ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) had met all the requirements that had been set at the beginning of this work: it (i) was a good activating and leaving group; (ii) was very soluble in DMF and DMF:dioxane (50:50); (iii) exhibited a negligible u.v. absorbance (Fig. 1) around 302 nm; and (iv) possessed no steric bulk around the *N*-hydroxy group. Therefore the triazole (132) was examined more extensively in the synthesis of various other peptides (Section 2.2) and further attempts were made to improve its synthesis.

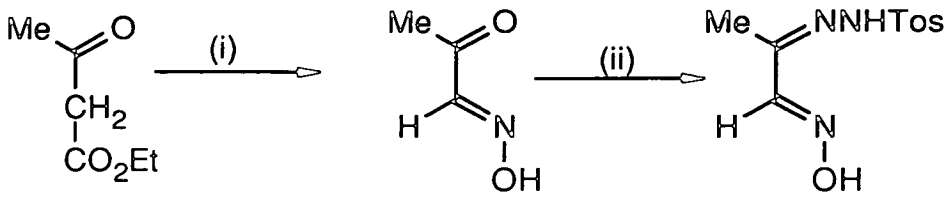
Stojanovic[83] proposed [Scheme 37] the non-isolable intermediate formed during the preparation of the ethyl 2-diazo-3-oxopropanoate (131a) to be ethyl 2-diazo-3-dimethyliminium propanoate chloride (130a). It was thought that this intermediate (130a) could perhaps undergo reaction with hydroxylamine to form the diazo-oxime (138) just as the diazoaldehyde (131a) does and if the proposed intermediate (130a) could be isolated and used in this way then time and material could be saved. Therefore, the reaction of the DMF-chloride (128) with ethyl 2-diazoethanoate



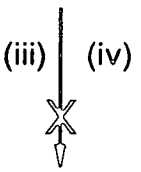
(129) was repeated but the residue (130a) which was previously treated with aqueous acetic acid to give the diazo-aldehyde (131a) was dried *in vacuo* and kept under nitrogen or argon. Since it appeared to be sensitive to moisture it was not possible to purify and characterise this intermediate and purification was also found to be unnecessary. The diazo-oxime (138) was successfully obtained from the treatment of ethyl 2-diazo-3-dimethyliminium propanoate chloride (130a) with hydroxylamine in 41% yield. The product (138) is sparingly soluble in water and the yield was found to be greater if during the work up of the reaction mixture cold water was used. Various alterations were made to this procedure in an attempt to improve the yield of the diazo-oxime (138). Anhydrous acetonitrile instead of anhydrous ethanol gave 29% yield. An increase in reaction time and changing the order of addition of reactants also did not improve the yield (15%). A brown intractable solid was obtained when the hydroxylamine hydrochloride was treated with sodium ethoxide instead of sodium carbonate and again no product was obtained when the hydroxylamine was generated in DME rather than ethanol.

The order of events during the work up was shown to influence the quantity and quality of the product (138) obtained. After the reaction of the diazoiminium compound (130a) with hydroxylamine the solvent was evaporated; leaving a residue. It was found that in order to obtain a clean product (not oily) the residue had to be washed with water before being dissolved in DCM; which gave the product (138) on evaporation. If the residue was washed with a two phase mixture of water and DCM an oil was obtained from the organic portion. Despite many attempts, the yield of this reaction could not be significantly improved.

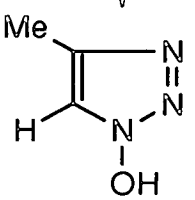
More recently Jiang[86] has found [Scheme 37] that the pale yellow colour which remains at the end of the cyclisation of diazo-oxime (138) to triazole (132) is due to the presence of the diazo-cyano compound (139), whose i.r. spectrum showed cyano and diazo absorption at 2230 cm^{-1} and 2137 cm^{-1} while its ^{1}H n.m.r. spectrum showed proton resonances characteristic of an ethyl substituent at δ4.1 and 1.1 and its ^{13}C n.m.r. spectrum further confirmed the proposed structure (139) by exhibiting absorbances corresponding to carbonyl diazo and cyano groups and also to a methylene and methyl group. It would appear that heating the diazo-oxime (138) may cause it to decompose slightly. A sample of the diazo-oxime (138) in deuterated chloroform (a yellow solution) was prepared for ^{1}H n.m.r.; the following day the solution was colourless and was found to be *N*-hydroxytriazole (132) as evidenced by ^{1}H n.m.r. Deuterated chloroform contains a trace of acid and



(140) (141) (142)

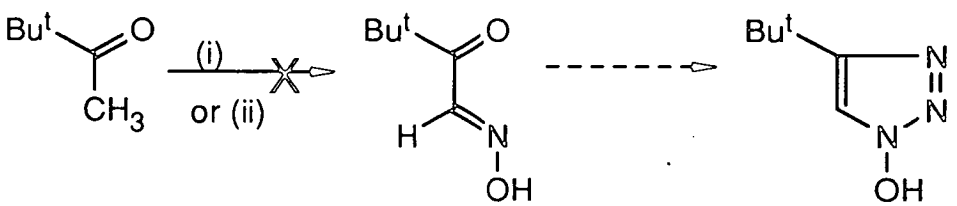


(i) $NaNO_2$, H_2SO_4, H_2O, 0^oC.
(ii) $TosNHNH_2$, EtOH, reflux.
(iii) NaOEt, DME, room temp.
(iv) diglyme, reflux.



(143)

Scheme 38



(144) (145) (146)

(i) AmONO, NaOEt, EtOH, room temp.

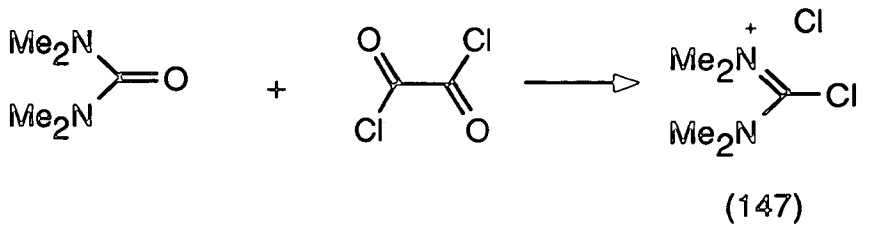
(ii) MeONO, HCl, ether, room temp.

Scheme 39

it was thought that this may have catalysed the cyclisation. Therefore a solution of the diazo-oxime (138) in chloroform was prepared and a few drops of acetic acid were added and the mixture was left in the cold room (8^0C) for two days, after which time the previously yellow solution was colourless and gave an almost quantitative yield of required product (132). It is therefore preferable that the cyclisation of the diazo-oxime (138) is carried out in slightly acidic conditions rather than subjecting it to high temperatures.

Although we had been successful in obtaining an *N*-hydroxytriazole (132) as good as HOBt with no steric bulk around the *N*-hydroxy group and a negligible u.v. absorbance around 302 nm investigations were still continued into the synthesis of *N*-hydroxytriazoles with no substitution at position 5 of the triazole ring, chiefly, 1-hydroxy-4-methyl-1*H*-1,2,3-triazole (143) and 4-*t*-butyl-1-hydroxy-1*H*-1,2,3-triazole (146). The synthetic route [Scheme 32] that was used to obtain *N*-hydroxytriazoles(124a) and (124b) whereby the corresponding keto-oxime (121) was prepared and taken through to the triazole (124) *via* its tosylhydrazone (122) was again applied [Schemes 38 and 39] in the attempted synthesis of *N*-hydroxytriazoles (143 and 146). The synthesis [Scheme 38] of the keto-oxime (141) was readily accomplished in a reasonable yield (66%) by the acid catalysed reaction of ethyl 3-oxobutanoate (140) with sodium nitrite. The corresponding tosylhydrazone (142) was obtained (76%) under the usual conditions and its structure was confirmed by its spectral data. Thus its i.r. spectrum contained bands at 3500 and 3200 cm^{-1} due to the NH component and a broad absorption at 3500-2500 cm^{-1} due to the OH group and its ^{1}H n.m.r. showed proton resonances at δ2.38 and 1.95 due to the two methyl groups and at δ7.3 corresponding to the oximinol proton in addition to signals due to the protons of the phenyl group. However the attempted base catalysed cyclisation of the tosylhydrazone (142) was unsuccessful. This reaction gave only a brown intractable oil which yielded no identifiable material and so the synthesis was not further investigated.

Undeterred, the synthesis [Scheme 39] of *N*-hydroxytriazole (146) was also attempted beginning with the synthesis of the keto-oxime (145). Two methods for the synthesis of this compound (145) from 3,3-dimethylbutan-2-one (144) were attempted. Oximation of the ketone (118) using isoamyl nitrite in the presence of sodium ethoxide gave no identifiable material and likewise oximation with methyl nitrite in the presence of anhydrous hydrogen chloride gave only an intractable oil, with no evidence for the formation of the required oximino-ketone (145). Therefore

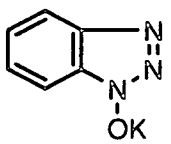


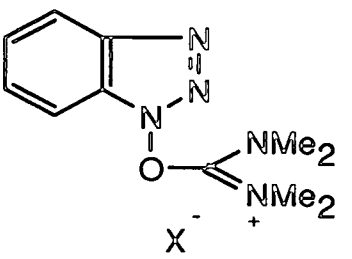
(147)

X

(53a) PF_6^-

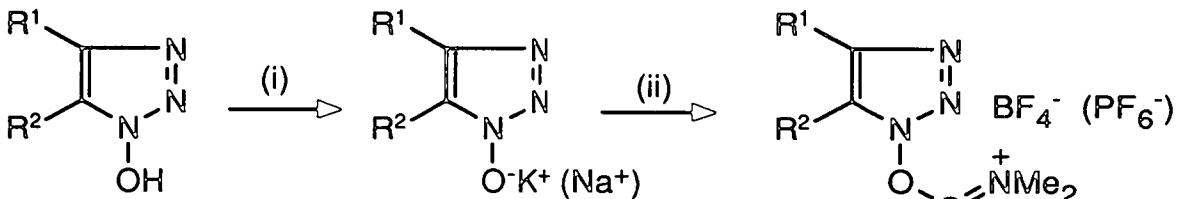
(53b) BF_4^-

(i) KX,  , EtOH, room temp.



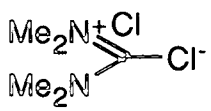
(53)

Scheme 40



(148) (149) (150)

(i) KOH (or NaOH), EtOH, room temp.

(ii)  KBF_4 (or KPF_6),

(147)

	R^1	R^2
a;	Ph	Me
b;	EtO_2C	Me
c;	EtO_2C	H

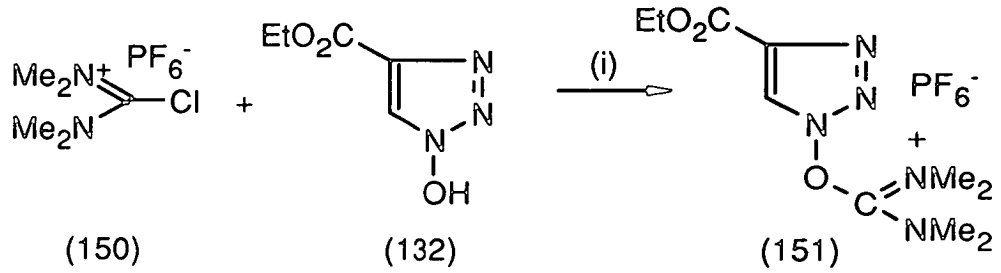
Scheme 41

investigations into the synthesis of 4-*t*-butyl-1-hydroxy-1*H*-1,2,3-triazole (146) were discontinued.

The *N*-hydroxytriazoles that were successfully prepared and subsequently tested as auxiliary nucleophiles for peptide synthesis were 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a), 1-hydroxy-4-(4-methoxyphenyl)-5-methyl-1*H*-1,2,3-triazole (124b), ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) and ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132). All were found to be satisfactory auxiliary nucleophiles for peptide synthesis and a comparison of these triazoles with each other and HOBt is summarized in Section 2.2.

As explained in the introduction (p 21) Knorr[51] has reported [Scheme 40] the synthesis of uronium salts of HOBt, that is HBTU (53a) and TBTU (53b). These compounds (53a) and (53b) are solids which are used as coupling reagents in peptide synthesis. They are commercially available and avoid the need for separately added HOBt. Attempts were made [Scheme 41] to prepare the Knorr derivatives of the *N*-hydroxytriazoles (124a), (127) and (132) with the aim of obtaining coupling reagents superior to HBTU and TBTU. In each case the potassium or sodium salt (149) of the corresponding *N*-hydroxytriazole was prepared and treated with tetramethyluronium chloride (147) formed from the reaction of tetramethylurea with oxalyl chloride.

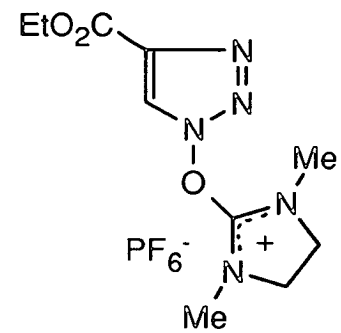
In the case of 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a) its potassium salt (149a) was prepared and then added to the hygroscopic chloroformamidinium chloride with potassium tetrafluoroborate or potassium hexafluorophosphate. In both these reactions, one using the fluoroborate and the other the hexafluorophosphate, an oily product was obtained rather than a well defined solid as might have been expected considering the same method when used to prepare the Knorr reagent gives the product as a solid in reasonable yield. Attempts to triturate the oily products were largely unsuccessful, yielding only a little of a white solid (1%) from the reaction which used tetrafluoroborate as the counterion. This product gave accurate mass and combustion analysis data consistent with the molecular formula $C_{14}H_{20}N_5O^+BF_4^-$ and its ^1H n.m.r. spectrum was in total agreement with the assigned structure (150a) exhibiting proton resonances at δ3.14 and 2.84 due to the methyl groups of the uronium portion of the compound and the δ2.25 due to the methyl group of the *N*-hydroxytriazole in addition to signals due to the protons of a phenyl group. Further attempts at this difficult synthesis failed to give greater



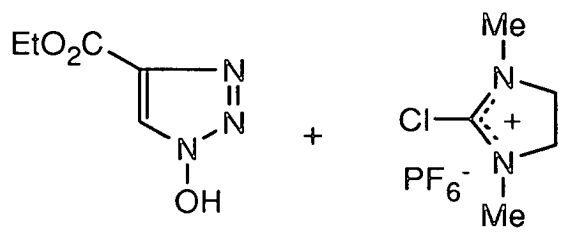
(150) (132) (151)

(i) Et_3N, DCM, room temp.

Scheme 42

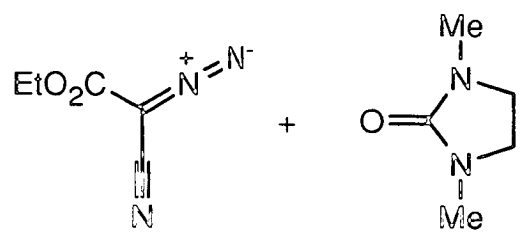


(152)



(132) (153)

(i)



(139) (154)

(i) Et_3N, DCM, O^oC.

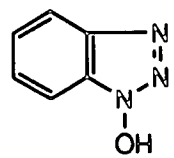
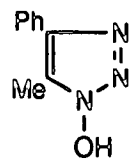
Scheme 43

quantities of the desired product (150a) and it was decided therefore to use the triazole alone as an auxiliary nucleophile for peptide synthesis.

The potassium salt of ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (149b) was prepared in 50% yield and added to the tetramethyluronium chloride with potassium tetrafluoroborate or potassium hexafluorophosphate. However all attempts to obtain the Knorr derivative of this triazole (150b) were unsuccessful yielding only intractable oils. Again, this triazole was used alone with DIC.

When ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) was obtained attempts were again made to obtain the corresponding Knorr derivative (150c). However, obtaining the potassium or sodium salts of this triazole proved unsuccessful. Jiang[86] has since attempted to synthesise the Knorr derivative (151) [Scheme 42] avoiding the use of the sodium or potassium salt of the triazole (132) by using the tetramethyluronium hexafluorophosphate salt (150) but was also unsuccessful. The reason for these difficulties can perhaps be seen in the attempted synthesis [Scheme 43] of compound (152). The *N*-hydroxytriazole (132) and 2-chloro-1,3-dimethylimidazolidinium hexafluorophosphate (153) were added together and triethylamine was added slowly to the mixture. An oil was obtained and from this two products (139) and (154) were isolated by column chromatography. Products (139) and (154) were identified according to their spectral data. ^{1}H n.m.r. of product (154) exhibited proton resonances at δ3.35 and 2.8; the former corresponds to two methylene groups and the latter to two methyl groups. The spectral data for compound (139) was in agreement with that obtained previously [Scheme 37]. It would appear that the *N*-hydroxytriazole (132) is unstable and its triazole ring opens.

Considering these results the triazole alone was investigated [Section 2.2] as a coupling reagent and was found to be very successful in meeting all of our requirements. Also, unlike HOBt this triazole is stable at room temperature, is not sensitive to light and does not need to have water associated with it for storage purposes.

Resin	Auxiliary	Amino Acid (mmol)	Acylation time (min)	Yield (from deprotection) (%)	Crude peptide obtained from peptide resin.	Amino Acid Analysis
0.5mmol ($0.46mmolg^{-1}$)	 (1)	1.0	45	91	0.06g from 0.23g	Gly,1.0;Ala,1.01; Phe,0.95; Ile,0.89; Leu,0.95.
0.5mmol ($0.59mmolg^{-1}$)		"	30	83	0.084g from 0.34g	Gly,1.0; Ala,0.96; Phe,0.94; Ile,0.86; Leu,0.92.
0.5mmol ($0.59mmolg^{-1}$)		"	15	88	0.083g from 0.43g	Gly,1.0; Ala,0.95; Phe,0.92; Ile,0.85; Leu,0.91.
0.5mmol ($0.46mmolg^{-1}$)	 (124a)	1.0	45	96	0.03g from 0.175g	Gly,1.0; Ala,1.03; Phe,0.98; Ile,0.88; Leu,0.82.
0.5mmol ($0.59mmolg^{-1}$)		"	30	88	0.08g from 0.32g	Gly,1.0; Ala,0.91; Phe,0.84; Ile,0.77; Leu,0.82.
0.5mmol ($0.59mmolg^{-1}$)		"	15	80	0.08g from 0.33g	Gly,1.0; Ala,0.94; Phe,0.92; Ile,0.83; Leu,0.89.

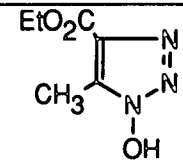
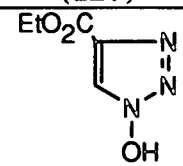
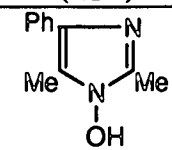
Resin	Auxiliary	Amino Acid (mmol)	Acylation time (min)	Yield (from deprotection) (%)	Crude peptide obtained from peptide resin.	Amino Acid Analysis
0.5mmol ($0.53mmolg^{-1}$)	 (127)	1.0	45	100	0.07g from 0.3g	Gly,1.0; Ala,0.98; Phe,0.97; Ile,0.91; Leu,0.94.
0.25mmol ($0.8mmolg^{-1}$)	 (132)	0.5	30	94	0.05g from 0.15g	Gly,1.0; Ala,1.02; Phe,0.91; Ile,0.89; Leu,0.99.
0.5mmol	 (160a)	1.0	45	0	/	2% of AlaGly on the resin.

Figure 3. Synthesis of LeuIlePheAlaGly using different coupling agents and coupling times.

2.2 Investigations into the Use of Aryl and Alkyl 1-Hydroxy-1*H*-1,2,3-triazoles as Reagents in Peptide Coupling.

1-Hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a)

The first test (Fig. 3) for our new auxiliary was the pentapeptide LeuIlePheAlaGly, a straightforward synthesis which would indicate immediately if the *N*-hydroxytriazole (124a) was capable of suitably activating an amino acid for coupling and worthy of further investigation. The synthesis of this pentapeptide was known to proceed smoothly and in excellent yield when using DIC/HOBt activation. Therefore as a comparison the synthesis was repeated using 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a) instead of HOBt, with a coupling time of 45 min.

The deprotection traces showed that each amino acid coupled in close to 100% yield therefore it can be concluded that this *N*-hydroxytriazole (124a) is very capable of activating amino acids for peptide synthesis. The pentapeptide was completed and examined (Fig. 3) by amino acid analysis and hplc after cleavage from the resin. The synthesis was repeated for both HOBt and triazole (124a) using 30 and 15 min coupling times. The results for these studies can be seen in Figure 3. Originally the deprotection samples taken were dependent on the gas pressure in the synthesiser; this of course was variable, at times causing unusual deprotection results. However, the sample is now taken using a loop measurement system which is consistent between samples.

The hplc traces for LeuIlePheAlaGly synthesised using HOBt and those for the same peptide synthesised using the *N*-hydroxytriazole (124a) were very similar to each other and each showed a good degree of purity. The deprotection traces and amino acid analysis results did not show any significant differences when HOBt or triazole (124a) was used. LeuIlePheAlaGly was obtained in good yield in each case, even when coupling for only 15 min. 1-Hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a) is as effective an activating agent for the synthesis of this pentapeptide as HOBt is.

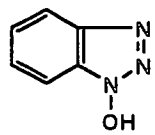
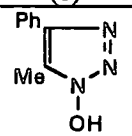
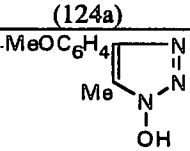
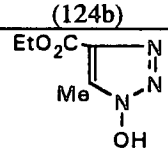
Resin	Auxiliary	Weight of Peptide Resin	Yield by Deprotection	Yield by Amino Acid Analysis
0.54mmol g^{-1}	 (1)	0.64g	81%	100%
0.6mmol g^{-1}	 (124a)	0.65g	83%	100%
0.54mmol g^{-1}	 (124b)	0.64g	74%	100%
0.54mmol g^{-1}	 (127)	0.63g	72%	100%

Figure 4. Synthesis of AlaGlyResin using one equivalent of activated amino acid to GlyResin.

A further study (Fig. 4) was carried out whereby Ala was coupled on to GlyResin using DIC/1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a) and also using DIC/HOBt. Equimolar quantities of amino acid, triazole (124a) and GlyResin were used in a double couple cycle rather than the usual two fold excess of activated amino acid to GlyResin. Again the *N*-hydroxytriazole (124a) was as good as HOBt in achieving the synthesis of AlaGlyResin.

Although 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a) proved to be successful in mediating peptide coupling in these cases, it did not satisfy our requirements concerning the steric hindrance round the *N*-hydroxy group or its ultraviolet absorbance. Figure 1 (p 40) shows the absorbance at 302 nm to be strong

although not as strong as HOBt; nevertheless this is not acceptable for the proposed monitoring.

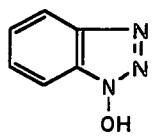
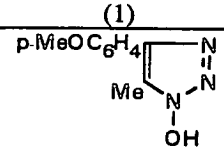
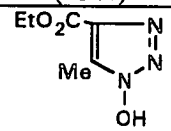
Auxiliary	Weight of PeptideResin	Yield by Deprotection	Yield by Amino Acid Analysis
 (1)	0.74 g	87%	72%
 (124b)	0.7 g	/ (m.f.)	79%
 (127)	0.7 g	88%	77%

Figure 5. Synthesis of PheAlaGlyResin using two equivalents of activated amino acid to GlyResin and a single couple cycle. (m.f. = mechanical failure).

Figures 3-5. The results shown here serve to confirm the ability of the *N*-hydroxytriazoles shown to mediate peptide coupling. Synthesis of these simple peptides is straightforward and all the triazoles tested gave similar results for deprotection, weight and amino acid analysis. Therefore, selection of a superior replacement for HOBt can only be made by considering other criteria and by presenting the better candidates with more difficult sequences to synthesise.

1-Hydroxy-4-(4'-methoxyphenyl)-5-methyl-1*H*-1,2,3-triazole (124b)

This triazole (124b) was also found to be capable of assisting in peptide bond formation. AlaGlyResin (Fig. 4) was prepared using equimolar quantities of amino acid, triazole (124b) and GlyResin employing a double couple cycle. The deprotection peaks and weight of the product (121) indicated that this triazole (124b) was as good as the previous triazole (124a) and HOBt.

PheAlaGlyResin was also synthesised (Fig. 5) using equimolar quantities of activated amino acid and GlyResin but this time using a single couple cycle. In this case the deprotection trace and amino acid analysis results show a slight improvement on those obtained in the corresponding synthesis using DIC/HOBt. However, this triazole (124b) was not a satisfactory replacement for HOBt either. It had a strong ultraviolet absorbance around 302 nm (Fig. 1, p 40), also steric effects and solubility in solvents suitable for use in the peptide synthesiser were not improved, compared with HOBt and triazole (124a).

Both of these triazoles (124a) and (124b) although capable of assisting peptide bond formation were not satisfactory replacements for HOBt and therefore were not further investigated. Our attention turned to the *N*-hydroxytriazole carboxylates (127) and (132) which were in turn examined as potential replacements for HOBt.

Ethyl 1-Hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127)

DIC/ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) activation was used to synthesise the test peptide LeuIlePheAlaGly (Fig. 3), employing a twofold excess of activated amino acid with respect to the Gly-resin and a double couple cycle. The deprotection traces indicated an excellent yield (99%) of the pentapeptide. The peptide was then cleaved from the resin and examined. Amino acid analysis and hplc results were in agreement with the deprotection traces, indicating a good yield and high degree of purity.

Two further studies were carried out to validate the coupling efficiency of the triazole (127) when using only one equivalent of activated amino acid. Firstly employing a double cycle in the synthesis of AlaGlyResin (Fig. 4) and secondly employing a single couple cycle in the synthesis of PheAlaGlyResin (Fig. 5). The deprotection traces and amino acid results show no significant difference between the coupling ability of HOBt and the *N*-hydroxytriazole (127) in use here.

The solubility of this *N*-hydroxytriazole (127) in DMF and DMF:dioxane (50:50) is slightly better than HOBt and triazoles (124a) and (124b) and its ultraviolet absorbance at 302 nm is insignificant compared with HOBt (Fig. 1), therefore, it may be possible to develop a monitoring system for the acylation stage. Further

syntheses were then attempted of peptides having sequences which presented the *N*-hydroxytriazole (127) with a more difficult task in order to validate whether the 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) is a feasible replacement for HOBt or not.

(a) Peptide Il-1-β pre127-138.

H-ArgAspSerGlnGlnLysSerLeuValMetSerGly-OH (155)

The first large peptide attempted was Il-1-β pre127-138 which at the time was being studied in this research group. An interleukin is a peptide produced by leukocytes and active during the immune response, affecting the growth and function of cells. Il-1-β pre127-138 is a more arduous synthesis than LeuIlePheAlaGly and this peptide was required by Maclean[87] for further study. Maclean[87] synthesised Il-1-β pre102-138 using symmetrical anhydride and DIC/HOBt couplings, in low yield and found the couplings between amino acids 127-138 to be particularly poor. This peptide (155) was then synthesised using ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) as the coupling agent. The activated amino acids were added in two equivalents with respect to the Gly-functionalised resin, using a double couple cycle (2 x 30 min). Gln was used without side chain protection and the resulting peptide (127) was obtained in 71% yield (by weight). The deprotection traces indicated a 76% yield up to Asp, however the coupling of Arg to Asp caused the yield to drop to 56%. Amino acid analysis was carried out on the peptide resin emphasizing again the poor synthesis. The peptide was cleaved from the resin in the appropriate TFA/scavenger mixture and hplc on the crude peptide also confirmed the poor synthesis by showing many peaks.

(b) Luteinizing Hormone Releasing Hormone (LHRH)

H-PyrHisTrpSerTyrGlyLeuArgProGly-NH_2 (156)

Luteinizing hormone releasing hormone is released from the hypothalamus and it controls the release of luteinizing hormone and growth hormone releasing hormone which mediates the secretion of growth hormone. LHRH is a peptide amide, that is, it has an amide function at the C-terminal, consisting of 10 amino acids. LHRH was

obtained in 43% yield by Breipohl[33] when using HOBt activated amino acids and its synthesis was therefore attempted using ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) as the coupling agent.

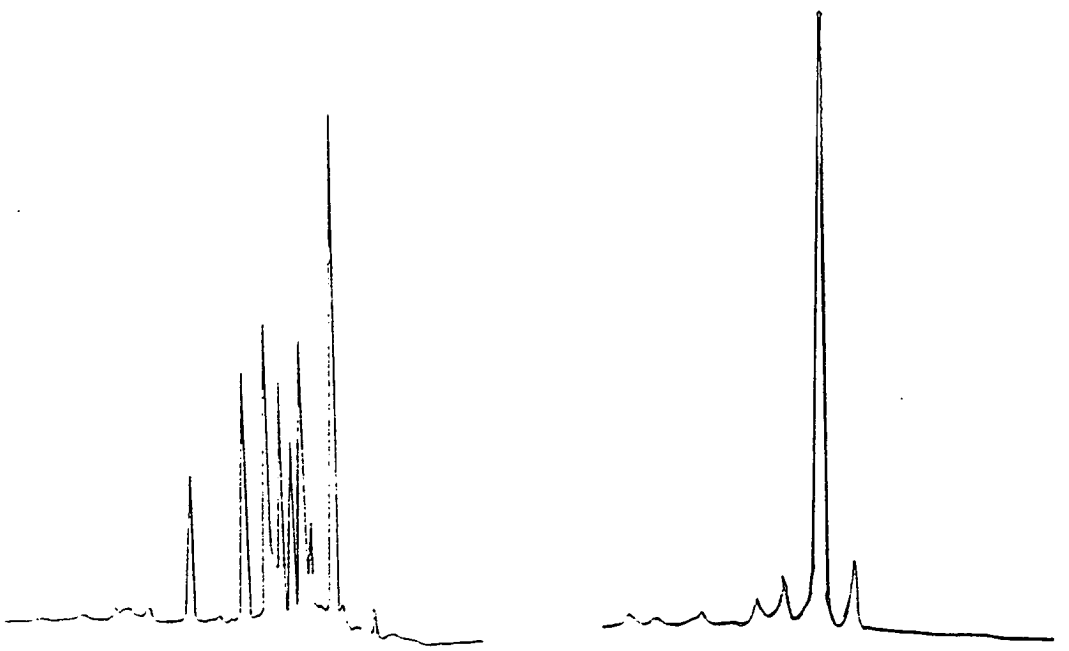
The amide resin (44) used was prepared by Irving[38] and the amino acids were activated using DIC/ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) and added in two equivalents with respect to the amide resin (44). A double couple cycle was employed throughout.

The completed peptide (137) was obtained in only 32% yield (by weight of peptide resin). The monitoring of the deprotection wash was unsuccessful due to the low concentration of the deprotection solution after the addition of Tyr. Amino acid analysis confirmed the low yields indicated above therefore the synthesis of LHRH was repeated using a treble couple cycle throughout except for Pro. The weight of the completed peptide resin indicated an 85% yield although the deprotection monitoring indicated 60% yield. The peptide was cleaved from the resin giving a correct mass result of 1181.7 (M^+) [expected 1182.3 (M)] also confirmed by amino acid analysis.

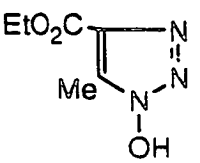
(c) Substance P.

H-ArgProLysProGlnGlnPhePheGlyLeuMet-NH_2 (157)

Sub P is a neuropeptide found in the brain; it is also a peptide amide consisting of eleven amino acids and is involved with the transmission of pain. The amide resin (44) used was prepared by Irving[38] and Gln was used without side chain protection. Synthesis of this peptide[37] using HOBt indicated the problematic couplings to be after the first Gln, up to this point, the couplings of Gly to Phe_2 proceed smoothly and in good yield. Therefore amino acids Met, Leu and Phe (x2) were added using DIC/HOBt activation, then, amino acids Gln_1 to Arg were added using DIC/1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) activation, in two equivalents with respect to the starting resin functionality. Monitoring of the synthesis failed due to a blocked sinter in the synthesiser. However, manual deprotection of Fmoc-peptide-resin indicated a 36% yield (calculated from the ultraviolet absorbance at 302 nm of the deprotection solution). The weight of peptide-resin indicated a 41% yield. These results compare favourably with the



(a) Crude Substance P synthesised using



(b) Crude Substance P synthesised using

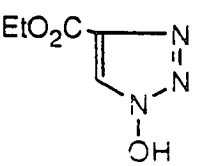


Figure 7. A comparison (by hplc) of the crude products obtained when Substance P was synthesised using *N*-hydroxytriazoles (127) and (132).

deprotection monitoring for the same synthesis using HOBt/symmetrical anhydride which indicated an 18% yield. The peptide (157) was cleaved from the resin and identified by its mass spectrum [1346.7 (M^+-1): expected 1347.74 (M^+)], amino acid analysis and hplc.

Peak	Assignment
1346.7	molecular ion
1232.6	-Arg + Ac.
1135.7	-Arg -Pro + Ac.
1007.2	-Arg -Pro -Lys +Ac.
612.9	-Arg -Pro -Lys -Pro -Gln -Gln.

Figure 6. Mass spectral data for crude Substance P synthesised using *N*-hydroxytriazole (127) with no Gln protection.

Although, the yield for this synthesis (36%) was better than that obtained when HOBt was used, it was still very poor and a lot of deletion peptides were obtained as can be seen in the mass spectrum (Fig. 6) and hplc (Fig. 7).

Peak	Assignment
1347.4	molecular ion
1233.1	-Arg +Ac.
1135.9	-Arg -Pro +Ac.
1007.4	-Arg -Pro -Lys +Ac.
910.4	-Arg -Pro -Lys -Pro +Ac.
782.2	-Arg -Pro -Lys -Pro -Gln +Ac.
654.8	-Arg -Pro -Lys -Pro -Gln -Gln +Ac.

Figure 8. Mass spectral data for crude Substance P synthesised using an excess of the *N*-hydroxytriazole (127) with no Gln protection.

The synthesis was repeated and an excess of the triazole (127) was added; this however, did not improve the yield (23% by deprotection monitoring, 41% by weight). The mass spectrum (Fig. 8) of the crude peptide again indicated various deletion peptides. The crude peptide was purified by preparative hplc giving a pure single peak product.

The synthesis was repeated, coupling all the amino acids (except Gly) with DIC/ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127), using an excess of the triazole (127). Gln was used with trityl

Peak	Assignment
1347.0	molecular ion
1232.6	-Arg + Ac.

Figure 9. Mass spectral data for crude Substance P synthesised using *N*-hydroxytriazole (127) with Gln (trityl) protection.

protection. The yield (87% according to deprotection monitoring); (55% by weight) was improved and the mass spectrum (Fig. 9) of the crude peptide indicated a great decrease in the number of deletion peptides present. The peptide was purified by preparative hplc to give a pure single peak product (138). The same synthesis carried out with HOBt as the auxiliary nucleophile indicated an 81% yield by deprotection.

It appears that this triazole (127) meets our requirements in some areas such as: (i) its capability of activating amino acids for coupling; (ii) increased solubility in DMF and DMF:dioxane (50:50) compared with HOBt and triazoles (124a) and (124b); and (iii) reduced u.v. absorbance at 302 nm. However it is still unsatisfactory as a replacement for HOBt, because it is not superior when presented with more difficult sequences and does not meet the requirement for less steric hindrance around the *N*-hydroxy group. This requirement was met in the synthesis of ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132), which was then investigated as a potential mediator for peptide bond formation.

Ethyl 1-Hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132)

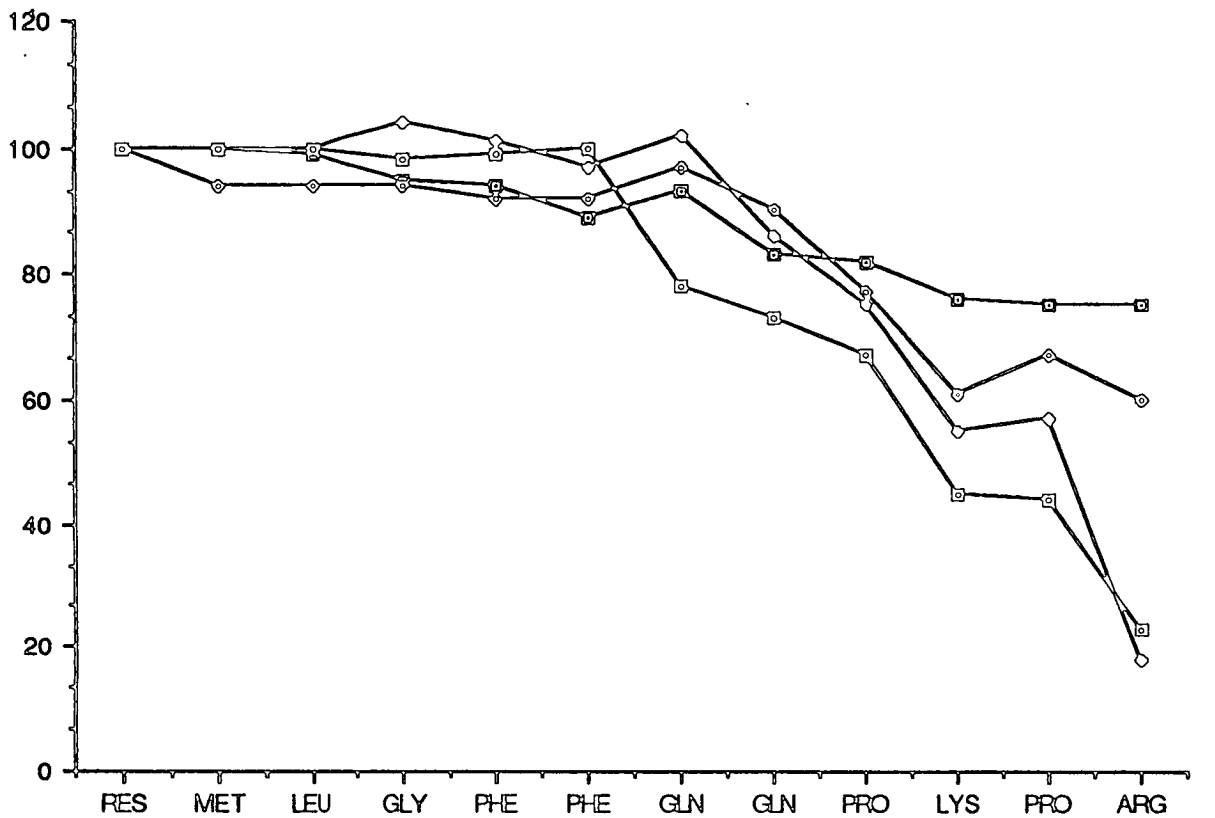
Once again the first peptide synthesised was the test peptide LeuIlePheAlaGly. The activated amino acids were added in two equivalents with respect to the Gly-resin using a double couple cycle. The synthesis went to completion in excellent yield, as confirmed by amino acid analysis and hplc (Fig. 3). The next step was then to give the *N*-hydroxytriazole a more difficult test of its ability. Therefore, as before the synthesis of Substance P was attempted.

(a) Substance P.

H-ArgProLysProGlnGlnPhePheGlyLeuMet-NH_2 (157)

The synthesis of this peptide amide (157) was again achieved using the amide resin (44) prepared by Irving.[38] Gln was used without side chain protection and the amino acids were added in two equivalents with respect to the resin. Since the initial couplings of up to the second Phe were known to proceed in good yield and also because there was insufficient triazole (132) available at this time DIC/HOBt activation was used for amino acids Leu and Phe (x 2), the remaining amino acids (except Gly) were added using DIC/ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) activation. The peptide was completed in 60% yield (by deprotection monitoring; 57% by weight). This yield is excellent considering the same synthesis with HOBt and using unprotected Gln gave <20% yield and when using ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) a 36% yield was obtained. The peptide (157) was cleaved (Fig. 7) from the resin and examined by mass spectrometry and amino acid analysis, then further purified by preparative hplc giving pure Sub P exhibiting a single peak on hplc and a clean mass spectrum.

The synthesis of Sub P was repeated as above using an excess of the *N*-hydroxytriazole (132). The results obtained under these conditions were even more impressive. The overall yield according to the deprotection monitoring was 75% and 82% by weight of the peptide resin. The peptide was cleaved from the resin and identified by mass spectrometry [1348.2 (MH^+); expected 1347.74 (M^+)], amino acid analysis and hplc. All results confirmed that the synthesis had proceeded in good yield. Considering the excellent results



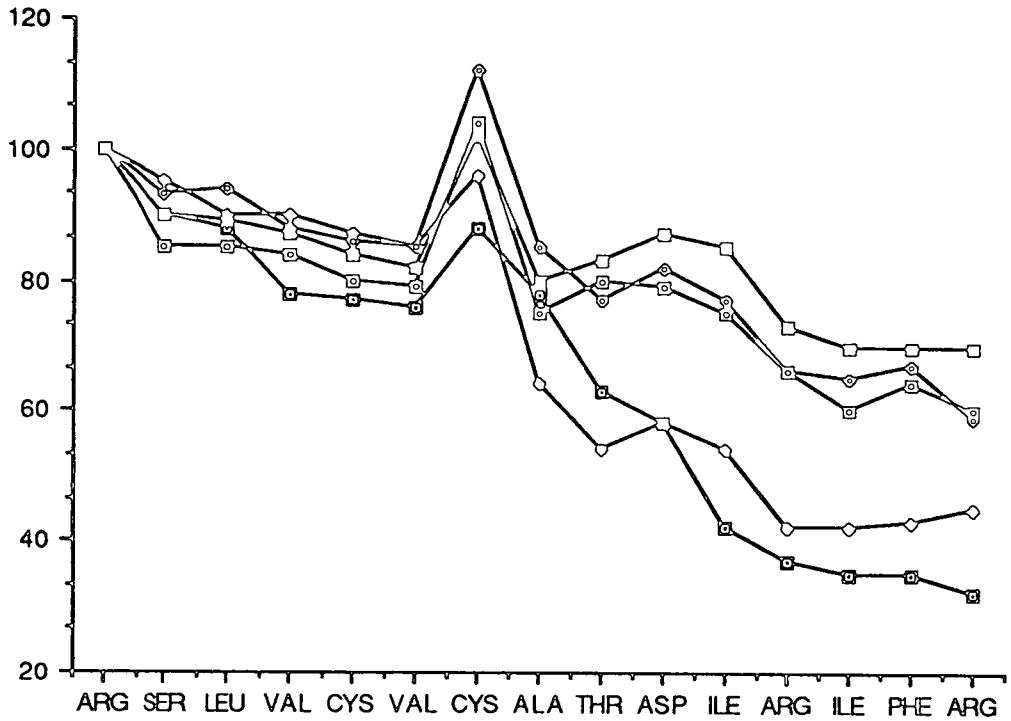
—◇— HOBt (0.5 mmol).

—⊡— *N*-hydroxytriazole (127) (0.75 mmol).

—◈— *N*-hydroxytriazole (132) (0.5 mmol).

—⊡— *N*-hydroxytriazole (132) (1.0 mmol).

Figure 10.Synthesis of Substance P without Gln protection.
In each case a standard double cycle was used adding two equivalents (0.5 mmol) of amino acid with respect to the resin.



—□— HOBt (0.5 mmol), d.c., 30 min.

—◇— *N*-hydroxytriazole (132), (1.0 mmol), s.c., 30 min.

—◎— *N*-hydroxytriazole (132), (0.5 mmol), d.c., 30 min.

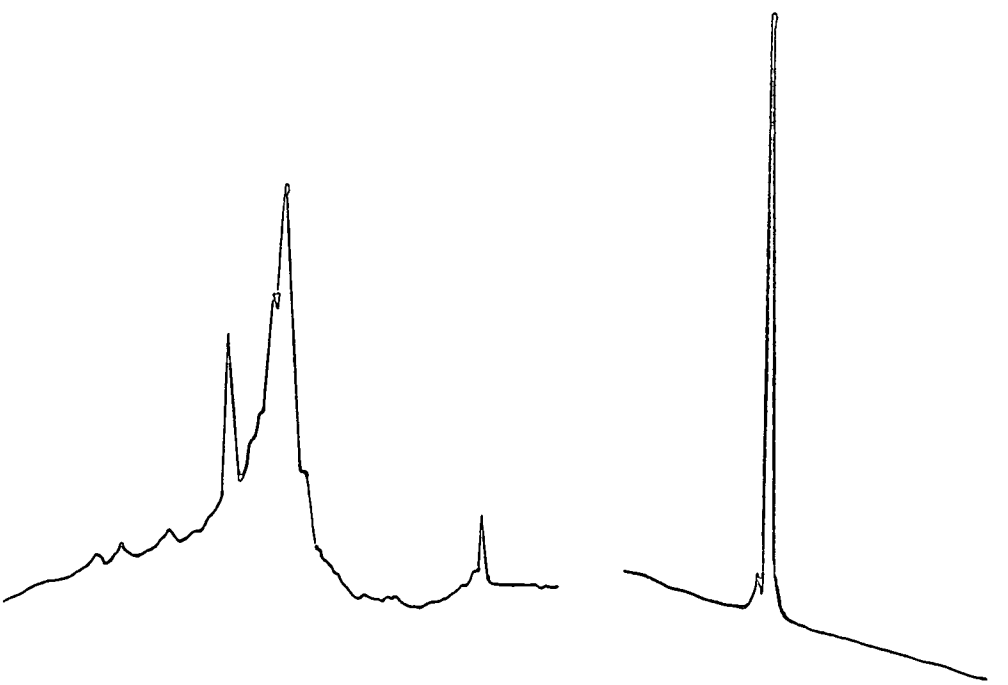
—▣— *N*-hydroxytriazole (132), (1.0 mmol), d.c., 15 min.

—▣— *N*-hydroxytriazole (132), (0.5 mmol), d.c., 15 min with addition of 2,6-lutidine.

Figure 11.Synthesis of NGF 100-114.

In each case the amino acids (0.5 mmol) were added in two equivalents with respect to the resin.

[d.c.= double couple cycle, s.c.= single couple cycle].



(a) (b)

Figure 12.(a) Analytical hplc of crude protected NGF 100-114
(b) Analytical hplc of purified unprotected NGF 100-114.

(Fig. 10) obtained in this case ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) was applied as an auxiliary nucleophile to another synthesis (NGF 100-114) which was known to be problematic, in order to ascertain the reproducibility of the successful couplings obtained for Sub P.

The graphs (Fig. 10 and 11) show the percentage yield of each coupling according to the deprotection of Fmoc from the appropriate amino acid. (100% yield is the integral value of the peak recorded by the uv cell corresponding to the deprotection of the first amino acid).

(b) Nerve Growth Factor (NGF) 100-114.

H-ArgPheIleArgIleAspThrAlaCysValCysValLeuSerArg-OH (158)

The changes initiated by NGF act to promote the survival and maintenance of the cholinergic neurons in the central nervous system. Synthesis of this sequence using symmetrical anhydride, then DIC/HOBt by Kelly[88] gave a poor yield of product (33%). This sequence was required for further investigation and biological testing by Kelly,[88] therefore it was important that the yield should be improved. The yield (Fig. 11) was found to drop between Cys108 and Arg100. Therefore, amino acids 109-113 were coupled using symmetrical anhydride for the first coupling and HOBt for the second coupling with extended coupling times. DIC/ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) was then used to couple the remaining amino acids. Using a single couple cycle (30 min) the activated amino acids were added in two equivalents with respect to the Arg-Resin and an excess of the triazole (132) was added. The yield (44%) was slightly improved (Fig. 11) compared to that obtained when using HOBt (33%). The graph shows the results from the deprotection monitoring. The Fmoc group on the last Arg was not removed, but was left on the completed peptide-resin which was given to Kelly[88] for further study. The final yield (44%) was calculated by deprotecting a small sample of Fmoc-peptide-resin manually and examining the ultraviolet absorbance at 302 nm of the deprotection solution.

The synthesis was repeated, again using an excess of the triazole (132), employing a double couple cycle (2 x 15 min) this time. Manual deprotection of Fmoc-Arg-peptide-resin indicated a 63% yield, two times better than the original synthesis

using HOBt. The same yield (63%) was obtained when no excess of triazole (132) was used and the double couple cycles were 30 minutes each. This indicated the importance of using a double couple cycle rather than single couple.

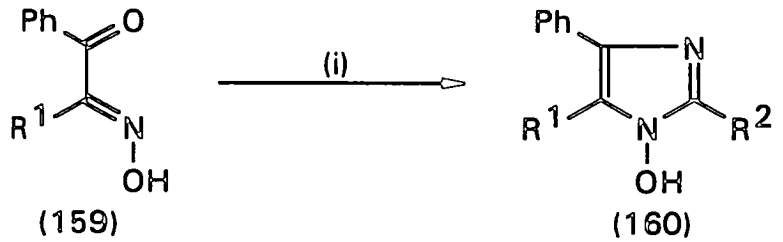
In 1973, when Konig and Geiger[12] discovered the catalytic effect of HOBt, they found that the rate of aminolysis of activated esters in polar solvents increased upon the addition of *N*-hydroxy compounds having the approximate acidity of acetic acid (pK_a=4.0). However, measurement of the pK_a of ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) showed it to be more acidic than acetic acid; it had a pK_a of 2.16. Hence, the acidity of this triazole (132) may be an important factor and cause an unnecessary drop in yield. If so, the free triazole (132) liberated after coupling takes place could be neutralised by addition of 2,6-lutidine, therefore, this approach was attempted.

Synthesis of NGF 100-114 (158) was repeated as before, using a double couple cycle (2 x 15 min), two equivalents of activated amino acid with respect to the Arg-resin and 2,6-lutidine was also added. The peptide was completed in 70% yield which is the best yield obtained for this fragment to date. The peptide was cleaved from the resin to yield the crude cysteine protected peptide (Fig. 12). The *t*-butyl groups were removed using tributylphosphine to give the unprotected reduced peptide, which was oxidised and purified to give the correct product (Fig. 12), m/z FAB 1751.94115 (expected 1751.94122).

Although the yield for this synthesis was 70%, the highest obtained for this sequence, the use of 2,6-lutidine with this *N*-hydroxytriazole has not been continued. This is because 2,6-lutidine absorbs at 302 nm and also, a more recent synthesis of NGF 100-114 using DIC/ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) activation throughout has given a 70% yield. Therefore, the difference that the base makes in this case is not significant, indicating that the acidity of the triazole does not cause problems.

The *N*-hydroxytriazole (132) used here is the most successful replacement for HOBt obtained to date. It not only satisfied all the requirements which had been set at the beginning of this work but it is also superior to HOBt in that it is stable at room temperature, is not sensitive to light and does not need to have water associated with it.

2.3 Investigations of the Synthesis of 1-Hydroxy-1*H*-imidazoles.



(159) (160)

(i) R^2CHO, NH_3, room temp.

	R^1	R^2
a;	Me	Me
b;	Me	H
c;	H	Me

Scheme 44

Our aim was to investigate not only *N*-hydroxytriazoles as potential auxiliary nucleophiles but also *N*-hydroxyimidazoles in order to ascertain the ability of the imidazole ring to mediate in the activation of amino acids. Therefore, the synthesis of several *N*-hydroxyimidazoles was attempted using the route [Scheme 44] developed by Jenkins[71] whereby the appropriate keto-oxime was treated with an aldehyde in ammonia.

The syntheses of three *N*-hydroxyimidazoles in particular were attempted. The first one being the known compound 2,5-dimethyl-1-hydroxy-4-phenyl-1*H*-imidazole (160a). 1-Phenylpropane-1,2-dione 2-oxime (159a) was treated with acetaldehyde in ammonia, initially in equimolar quantities but this gave a very poor yield (11%) of product (160a). However when the amount of acetaldehyde was increased twofold and ammonia fourfold the yield of product (160a) was much improved (80%). The product gave elemental and mass data consistent with the molecular formula and its ^1H n.m.r. spectrum was in complete agreement with the expected structure. The ^1H n.m.r. spectrum exhibited proton resonances corresponding to the two methyl groups and a multiplet from the remaining aromatic protons.

Unfortunately this *N*-hydroxyimidazole had a significant u.v. absorbance around 302 nm and its solubility in solvents suitable for use in the peptide synthesiser was poor. Nevertheless, its ability as a reagent for peptide coupling (Section 2.4) was

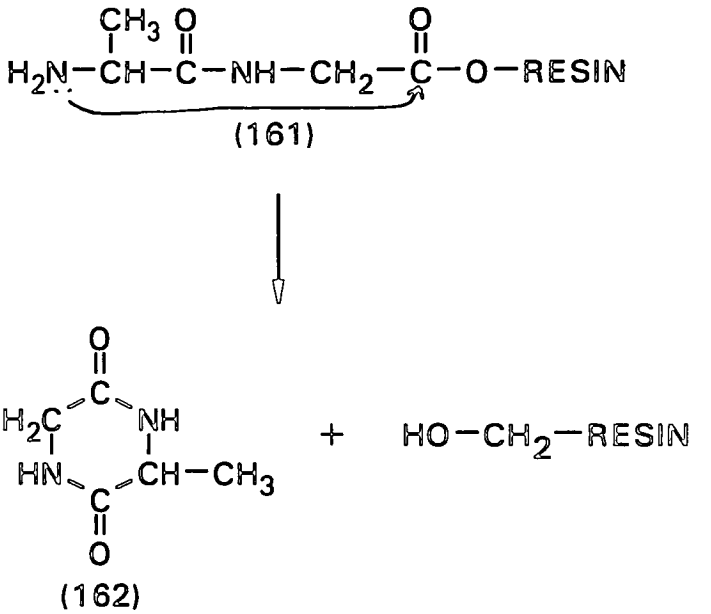
assessed in the attempted synthesis of the test peptide LeuIlePheAlaGly with disappointing results. It appears that the imidazole structure is unsuitable for the activation of amino acids.

Meanwhile the synthesis of 1-hydroxy-5-methyl-4-phenyl-1*H*-imidazole (160b) was attempted by the reaction of 1-phenylpropane-1,2-dione 2-oxime (159a) with aqueous formaldehyde and concentrated ammonia. However, the starting keto-oxime (159a) was obtained in 44% yield and no further identifiable material was isolated.

The synthesis was repeated using formaldehyde sodium bisulfite addition compound in place of aqueous formaldehyde. But again these conditions yielded the starting materials; the bisulfite addition compound was recovered in 78% yield and the keto-oxime (159a) was recovered in 88% yield.

The synthesis of 1-hydroxy-2-methyl-4-phenyl-1*H*-imidazole (160c) was also attempted, the starting material being 1-phenylethane-1,2-dione 2-oxime (159c). This keto-oxime (159c) was prepared (40%) by nitrosation of acetophenone using isoamyl nitrite in the presence of sodium ethoxide and subsequently treated with acetaldehyde (four fold excess) and ammonia (two fold excess). The starting keto-oxime (159c) was returned in 20% yield and a solid (26%) was also obtained which was shown from a satisfactory elemental analysis and mass spectrum to be the required product (160c). Spectral data for this solid further confirmed these results.

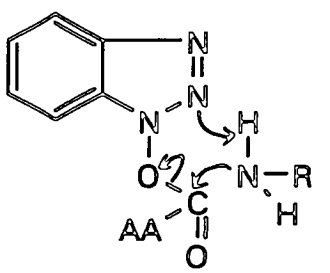
This imidazole was not tested as an auxiliary nucleophile for peptide coupling since the properties of *N*-hydroxyimidazole (160c) were not very different from imidazole (160a) which had failed to mediate peptide bond formation. The only change in imidazole (160c) was the removal of the methyl group at position 5 of the imidazole ring. It would have been more informative had we been able to test *N*-hydroxyimidazoles without the methyl group at position 2 or without any substitution at positions 2 and 5, perhaps an interesting project for future work.



(161)

(162)

Scheme 45



(163)

Scheme 46

2.4 Investigations into the use of 2,5-Dimethyl-1-hydroxy-4-phenyl-1*H*-imidazole (160a) as a Reagent for PeptideCoupling.

The test (Fig. 3, p 62) for this potential coupling agent (160a) was as before, LeuIlePheAlaGly. Gly functionalized resin was used and the amino acids were activated using DIC/2,5-dimethyl-1-hydroxy-4-phenyl-1*H*-imidazole (160a) (two equivalents with respect to the resin). The deprotection peak relating to the coupling of Ala to Gly indicated a 68% yield but there was no deprotection peak for the coupling of Phe to AlaGly. The synthesis was aborted and the resin examined by amino acid analysis, which indicated only 2% of the dipeptide AlaGly on the resin. This would indicate that AlaGly had become detached from the resin, probably due to diketopiperazine formation [Scheme 45] which is more likely to occur in Gly containing peptides since lack of steric hindrance allows the cyclisation to occur.

Further investigations leading to a new auxiliary nucleophile were directed towards the *N*-hydroxytriazoles since the *N*-hydroxyimidazole framework was unsuccessful in this case. The success of the triazoles rather than the imidazoles may be explained [Scheme 46] by looking at the six membered intramolecular general base catalysis proposed by Konig and Geiger.[21] If this is actually the mechanism of action of HOBt then it can be seen why imidazoles are unsuccessful.

2.5 Monitoring

One of the main aims running throughout these investigations was that of obtaining an *N*-hydroxytriazole without any u.v. absorbance around 302 nm. This was to enable the development of a monitoring system for the coupling stage. As explained [Scheme 22] in the introduction the syntheses of peptides are monitored at the deprotection stage. The deprotection wash is passed through an ultraviolet cell (302 nm) and a peak is recorded whose area is proportional to the amount of amino acid coupled in the previous acylation. However, at this stage if coupling efficiency is seen to be poor, it is one stage too late to do anything about it since all the amine functions have been exposed. Therefore, the aim was to develop a system whereby the coupling reaction could be monitored in a similar way to the deprotection stage by removing samples of the coupling solution to pass them through an ultraviolet cell (302 nm). The samples should contain free triazole and activated amino acid; as the coupling proceeds the amount of activated amino acid will decrease, hence, the Fmoc absorption should decrease. Providing the absorbance of the triazole does not interfere with the Fmoc absorption, then, the recorded peak for each sample of coupling solution will be smaller than the previous one.

We have been successful in setting up a coupling monitoring system on the peptide synthesiser which was modified and developed throughout the syntheses in the text with the aim of establishing a method whereby it would be possible to allow a software feedback system to interrupt the synthesis if the coupling was not satisfactory prior to the next capping/deprotection. Initially the main difficulties encountered were technical problems, for example, the first sample of the activated amino acid was taken before it was added to the resin, subsequent samples were taken from the reaction vessel during coupling. This caused an abnormal drop between the first and second peaks recorded due to the dilution factor involved in adding the activated amino acid to the swollen resin. This problem was corrected and the first sample was taken immediately after the activated amino acid was added to the resin. However, it was found that after three samples there was insufficient solvent present to allow efficient vortexing of the resin. Therefore the sample size and dilution had to be altered. It was also important that transfer lines for the sample going to the u.v. cell were free of any traces of amino acid. Each alteration to the monitoring system required changes in the timing of other cycles such as the activation cycle.

As syntheses were attempted many more problems relating to the peptide synthesiser were discovered and as these were tackled more realistic results were approached although not in full agreement with the deprotection monitoring. The results obtained for coupling monitoring latterly (in the synthesis of NGF and Sub P) were still unreliable and appeared to be sensitive to slight variations in the weighing of amino acids, temperature, dilution and gas pressure.

Further work has been carried out by Shaw[89] and the synthesiser is now in a temperature controlled environment and the coupling agent is delivered in solution from a stock bottle of the triazole and DIC in DMF instead of being put on the machine as a solid like the amino acids. More realistic results are being obtained and for each coupling to be monitored a standard is recorded (0.25 mmol of fluorene) and only one sample at the end of the coupling is taken. It is hoped soon to have total agreement between deprotection and coupling results.

Concluding Remarks.

The syntheses of many potential auxiliary nucleophiles were investigated. Four *N*-hydroxytriazoles (124a), (124b), (127) and (132) and one *N*-hydroxyimidazole (161a) were obtained and tested as coupling agents in the synthesis of small peptides. Unfortunately, the *N*-hydroxyimidazole (161a) was found to be quite unsuccessful as a coupling agent and therefore we concentrated on investigating *N*-hydroxytriazoles. However further investigations into *N*-hydroxyimidazoles and their apparent inability to act as efficient auxiliary nucleophiles could be an interesting project for future consideration.

1-Hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a), 1-hydroxy-4-(4-methoxyphenyl)-5-methyl-1*H*-1,2,3-triazole (124b), ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) and ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) were all as good as HOBt in achieving peptide bond formation but when other qualities were considered one triazole (132) in particular stood out from the rest. Our aim had been to find a superior replacement for HOBt and the requirements set out were that the reagent must be:

(i) a good activating and leaving group.
(ii) readily soluble in solvent systems compatible with the synthesiser (DMF, dioxane).
(iii) of a nature which will enable monitoring of the coupling reaction (ie.having no u.v. absorbance around 302 nm).
(iv) sterically unhindered round the 'N-OH' group.

N-Hydroxytriazoles (124a) and (124b) were unsuitable because of their large u.v. absorbance at 302 nm and the steric bulk around the *N*-hydroxy group. Also the triazole (124b) was not easily soluble in DMF and DMF:dioxane (50:50). *N*-Hydroxytriazoles (127) and (132) looked more promising with negligible u.v. absorbances at 302 nm. Both of these triazoles were investigated more thoroughly by using them in the synthesis of larger peptides such as Substance P and NGF 100-114. It became clear that ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) was superior to ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) and 1-hydroxybenzotriazole. This triazole (132) met all our requirements, achieved the syntheses of NGF 100-114 and Substance P in better yield than HOBt, and was

found to be stable at room temperature, stable to light and did not need to have water associated with it.

This work has now been continued by Jiang[86] and is progressing well. Ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) is now used throughout this research group and has been used to synthesise many more peptides in good yield, such as other NGF fragments, Ubiquitin 69-76 and many different fragments from endothelin-1 and big endothelin. Where the same peptides have been made with HOBt the yield of peptide obtained when *N*-hydroxytriazole has been used is much better than the yields obtained with HOBt. Stability studies have been carried out and ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) has been found to be very stable.

3. EXPERIMENTAL.

3.1 General Experimental Details.

Infra-red spectra were recorded from nujol suspensions or thin films using a Perkin Elmer 781 or Bio-Rad FT87 spectrophotometer.

^1H n.m.r. spectra were recorded on a Bruker WP80 (80MHz) or a Bruker WP200 (20MHz) in the solvent stated using tetramethylsilane (TMS) as the internal standard (δ=0.00).

Ultraviolet spectra were recorded on a Varian Cary 210 spectrophotometer in the solvent indicated.

Routine mass spectra were measured on a Kratos MS9. High and low resolution fast atom bombardment (FAB) spectra were measured on a Kratos MS50TC machine using thioglycerol as matrix.

Melting points were determined in open capillaries on a Buchi 510 melting point apparatus or on a Koffler hot-stage.

Elemental analyses were carried out on a Carlo-Erba Elemental Analyser, Model 1106.

Thin layer chromatography (tlc) was carried out using plastic sheets precoated with silica gel 60GF-254 (Merck 5735) in the solvent system indicated. Detection of compounds was *via* ultraviolet absorption at 254 nm.

Crude solid products isolated by filtration from reaction mixtures were dried *in vacuo* at room temperature unless otherwise stated. All organic extracts were dried over anhydrous magnesium sulphate prior to rotary evaporation under reduced pressure.

Solvents were of technical grade unless otherwise stated. When anhydrous solvents were used they were dried using the reagents given in parentheses: benzene (sodium

wire), diethylene glycol dimethyl ether (calcium hydride), dimethoxyethane (calcium hydride), dichloromethane (calcium hydride), chloroform (calcium chloride), xylene (sodium wire), acetonitrile (calcium hydride), ethanol (iodine, magnesium), diethyl ether (sodium wire), according to procedures set out in Perrin.[90]

All amino acid derivatives were purchased from Novabiochem except FmocArgPmc (Raylo) and were of the L-configuration. All peptides unless otherwise stated were synthesised on an Applied Biosystems 430A automated peptide synthesiser. The synthesiser was fitted with a monitoring system consisting of either a Hewlett Packard HP 3396A integrator or an Applied Biosystems 759A absorbance detector and a Spectra Physics Chrom Jet integrator. The dimethylformamide (DMF) and 1,4-dioxane used were of peptide synthesis grade, supplied by Rathburn Chemicals, Scotland.

Amino acid analysis was performed on an LKB 4151 amino acid analyser subsequent to Carius tube hydrolysis with 6N hydrochloric acid (acid hydrolysis) or with a solution of toluene-4-sulphonic acid and tryptamine in water (tryptophan method) at 110°C for 18 to 36 hours (as indicated).

High performance liquid chromatrography (hplc) was carried out using an Applied Biosystems 151A separation system. Analytical columns were Applied Biosystems Aquapore RP300 C_{18} reversed phase silica (300A° pore size, 7 μm spherical silica, 4.6 mm internal diameter) or Vydac Protein and Peptide C_{18} reverse phase column (218 TP254). Columns were eluted with a gradient of acetonitrile with 0.1% TFA (solvent A) as specified in the text. The acetonitrile was ultraviolet grade (Rathburn Chemicals) as was the TFA (Applied Biosystems). Preparative hplc was carried out on the same equipment using ABI aquapore PREP 10 columns (300A° pore size, 200 μm spherical silica, 10 μm internal diameter) unless otherwise noted.

The base labile 9-fluorenylmethoxycarbonyl (Fmoc) group was used for N^{α}-amino protection. This was complemented by the use of acid-labile side chain protection and an acid labile peptide-resin linkage *p*-alkoxybenzyl alcohol resin (Wang linker[26]) or amide resin (44). [38]

The following Fmoc amino acids used were unprotected: Leucine, Isoleucine, Alanine, Phenylalanine, Glycine, Valine, Proline, Methionine, Tryptophan.

The following amino acids used the indicated side chain protection: Arginine (pentamethylchromansulphonyl - Pmc), Cystine ($SSBu^t$), Lysine (Boc), Aspartic acid (OBu^t), Tyrosine (Bu^t), Serine (Bu^t) and Threonine (Bu^t). The side chain protecting groups used for glutamine and histidine will be specified for each peptide.

Most residues were double coupled unless otherwise stated using the method of activation specified in the text. Glycine was always single coupled as a symmetrical anhydride. The syntheses were monitored by passing the deprotection solution (0.2 ml) (diluted with DMF) through a u.v. detector (314 or 302 nm). The progress of the coupling reactions was also monitored by removal of a sample of the reaction mixture (0.2 ml diluted with DMF) to be passed through the u.v. detector (314 or 302 nm) at 1, 16 and 31 minute intervals. This coupling monitoring system was modified and developed with the aim of establishing a method whereby it would be possible to allow a software feedback system to interrupt the synthesis if the coupling was not satisfactory prior to the next capping/deprotection.

Deprotection, activation, coupling and capping were achieved by programmed cycles as summarized below.

1 Capping - the unreacted amine sites were capped using acetic anhydride in DMF (0.5 M, 2.0 ml) and pyridine in DMF (0.5 M, 2.0 ml) for 2 min, drained, washed, then capped again for 4 min.

2 Wash - the resin was washed with DMF: dioxane (50:50) (x5).

3 Deprotection - the resin was deprotected (3 min) with 20% piperidine in DMF then washed with DMF (x4). Deprotection with 20% piperidine in DMF was repeated (1 min) and the resin was again washed with DMF (x4).

4 Wash - the resin was washed with DMF: dioxane (50:50) (x5).

5 Coupling - the amino acid was preactivated for 20 min prior to addition to the resin using DIC/HOBt or DIC/triazole. The resin was coupled (for a time period specified in the text) with the activated amino acid.

6 Wash - the resin was washed with DMF:dioxane (50:50) (x6). The coupling cycle was usually repeated and following washing the next cycle commenced.

Cleavage Of Peptide-Resin

Peptide-resin (0.1 g-1.0 g) was treated with 95% aqueous TFA (10.0 ml) containing an appropriate scavenger mixture (specified in the text) and stirred at room temperature for 2 to 4 h. The resin was removed by filtration, chloroform was added to the filtrate and concentrated *in vacuo*. The resulting residue was treated with ether, so precipitating the peptide as an off-white solid which was collected, washed with ether and dried.

Coupling the C-Terminal Amino Acid onto the *p*-Alkoxybenzyl Alcohol Resin

A solution of the Fmoc amino acid (14.4 mmol) in DMF (20.0 ml) was treated with diisopropylcarbodiimide (7.2 mmol) and sonicated for 15 min. The resulting solution was added to *p*-alkoxybenzyl alcohol resin (2.4 mmol) previously swollen in DMF (15.0 ml) in the presence of DMAP (0.5mmol). The mixture was sonicated at room temperature for 2.5 h. The resin was then filtered, washed well with DMF, DCM and then ether, dried and the substitution was determined by an ultraviolet deprotection study.

3.2 Experimental.

1-Phenylpropane-1,2-dione-2-oxime (121a).

1-Phenylpropane-1,2-dione-2-oxime (121a) was prepared (yield 79%) by the reaction of propiophenone with methyl nitrite in the presence of anhydrous hydrogen chloride as described by Hartung and Crossley,[91] and was obtained as a colourless solid, m.p. 115-116°C (lit.,[91] 112-113°C).

1-Phenylpropane-1,2-dione-2-oxime 1-toluene-4-sulphonylhydrazone (122a).

The title compound (122a) was prepared (yield 89%) by the reaction of 1-phenylpropane-1,2-dione 2-oxime (121a) with tosylhydrazine as described by Bartlett and Stevens[74] and Jenkins,[71] and had m.p. 181-183°C (lit.,[71] 177-179°C).

1-Hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a).

A suspension of 1-phenylpropane-1,2-dione 2-oxime 1-toluene-4-sulphonyl hydrazone (122a) (12.6 g, 0.036 mol) in anhydrous 1,2-dimethoxyethane (180 ml) was stirred and treated with a solution of sodium (2.5 g, 0.11 mol) in anhydrous ethanol (360 ml). The mixture was stirred at room temperature for 30 min, then rotary evaporated under high vacuum. The solid obtained was broken up, anhydrous diglyme (360 ml) was added and the mixture was stirred and heated under reflux for 15 min, then cooled. Any lumps of solid were broken up and stirring and heating under reflux were continued for a further 30 min.

The mixture was rotary evaporated under high vacuum and the residue was dissolved in water (140 ml), and the solution chilled and acidified to pH1 by addition of 2M aqueous hydrochloric acid. The precipitated solid was collected and

combined with further material which separated from the aqueous mother liquor on standing to give the *N*-hydroxytriazole product (124a) (4.9 g; 77%) which was purified by recrystallization from aqueous methanol, m.p. 164-166°C (lit.,[71] 168-169°C), ν_{max} 3100-2300 br (OH) and 1650 br (C=O) cm^{-1}, δ_H ($CDCl_3$) 7.88-7.34 (5H, m, Ar) and 2.42 (3H, s, CH_3).

1-(4-Methoxyphenyl)propane-1,2-dione 2-oxime (121b).

A solution of 4-methoxypropiophenone (26.3 ml, 0.15 mol) in anhydrous ether (100 ml) was stirred and treated simultaneously at room temperature with slow streams of anhydrous hydrogen chloride and methyl nitrite [generated by dropping a cold aqueous sulphuric acid solution (7.0 ml concentrated sulphuric acid in 13.0 ml water) into a stirred mixture of sodium nitrite (12.4 g), methanol (8.0 ml) and water (8.0 ml)]. After 40 min the flow of methyl nitrite was interrupted and the passage of hydrogen chloride continued for a further 10 min. The mixture was then left stirring at room temperature for 24 h.

The resulting mixture was extracted ten times with 10% w/v aqueous sodium hydroxide solution (10 x 20.0 ml) and the combined aqueous sodium hydroxide extracts were poured into a stirred mixture of concentrated hydrochloric acid (35.0 ml) and ice (40 g). The precipitated solid was collected, washed with water and dried to give the oxime product (121b) (18.0 g; 62%) as a cream powder, m.p.135-138°C (from ethanol-water), ν_{max} 3500-2500 br (OH) and 1650 (C=O) cm^{-1}; δ_H [$(CD_3)_2SO$] 7.90 (2H, d, J9Hz, ArH), 6.94 (2H, d, J9Hz, ArH), 3.82 (3H, s, OCH_3) and 2.0 (3H, s, CH_3).

Found: C,61.7; H,5.5; N,7.1%; m/z (EIms), 193 (M^+).

$C_{10}H_{11}NO_3$ requires: C,62.2; H,5.7; N,7.3%; M, 193.

1-(4-Methoxyphenyl)propane-1,2-dione 2-oxime 1-toluene-4-sulphonylhydrazone (122b).

A solution of 1-(4-methoxyphenyl)propane-1,2-dione 2-oxime (121b) (7.7 g, 0.04 mol) in ethanol (100 ml) was added to a solution of tosylhydrazine (7.4 g, 0.04 mol) in ethanol (50.0 ml) and the resulting mixture was stirred and heated under reflux for 2 h.

The mixture was filtered to remove inorganic material. The filtrate was cooled and the precipitated solid was collected to afford the tosylhydrazone derivative (122b) (9.9 g; 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_H [$(CD_3)_2SO$] 11.51(1H, s, NH), 10.25(1H, s, OH), 7.81-7.01(8H, m, ArH), 3.78(3H, s, OCH_3), 2.39(3H, s, CH_3) and 1.93 (3H, s, CH_3).

Found: C,56.9; H,5.4; N,11.5%; m/z (EIms), 361 (M^+)

$C_{17}H_{19}N_3O_4S$ requires: C,56.5; H,5.3; N,11.6%; M,361.

1-Hydroxy 4-(4-methoxyphenyl)-5-methyl-1*H*-1,2,3-triazole (124b)

A suspension of 1-(4-methoxyphenyl)propane-1,2,-dione 2-oxime 1-toluene-4-sulphonylhydrazone (122b) (12.6 g, 0.035 mol) in anhydrous 1,2-dimethoxyethane (90.0 ml) was treated with a solution of sodium (2.5 g, 0.11 mol) in anhydrous ethanol (350 ml) and the resulting mixture was stirred at room temperature for 30 min, then rotary evaporated under high vacuum. The solid obtained was broken up, anhydrous diglyme (350 ml) was added and the mixture was stirred and heated under reflux for 1.5 h.

The mixture was rotary evaporated under high vacuum and the residue was dissolved in water (150 ml) with warming, chilled and acidified to pH1 by the addition of concentrated hydrochloric acid. The precipitated solid was collected to give the *N*-hydroxytriazole derivative (124b) (5.2 g; 73%) which formed cream

microcrystals m.p.174-176°C [from ethanol-light petroleum (b.p. 40-60°C), ν_{max} 3100-2000 br (OH) cm^{-1}; $\delta_H[(CD_3)_2SO]$ 7.60 (2H, d, J9Hz, ArH), 7.00 (2H, d, J9Hz, ArH), 3.70 (3H, s, CH_3O) and 2.33 (3H, s, CH_3).

Found: C,58.1; H,5.4; N,20.3%; m/z (EIms) 205 (M^+)

$C_{10}H_{11}N_3O_2$ requires: C,58.5; H,5.4;N,20.5%; M,205.

Butane-2,3-dione 2-oxime 3-tosylhydrazone (122c)

The title compound (122c) was prepared (yield 96%) by the reaction of butane-2,3-dione 2-oxime (121c) with toluene-4-sulphonylhydrazine as described by Jenkins,[71] and had m.p.168-170°C [lit.,[71] 178-179°C (from toluene-ethanol)].

The attempted base-catalysed cyclisation of butane-2,3-dione 2-oxime 2-tosylhydrazone (122c)

A solution of butane-2,3-dione 2-oxime 2-tosylhydrazone (122c) (5.4 g, 0.02 mol) in anhydrous 1,2-dimethoxyethane (100 ml) was stirred at room temperature and treated with a solution of sodium (1.4 g, 0.06 mol) in anhydrous ethanol (200 ml). The mixture was stirred at room temperature with exclusion of atmospheric moisture for 30 min then rotary evaporated under high vacuum. The solid obtained was broken up, anhydrous toluene (40.0 ml) was added and the mixture rotary evaporated under high vacuum. Again, the solid obtained was broken up, anhydrous diglyme (200 ml) was added and the mixture was stirred and heated under reflux for 15 min, then cooled. Any lumps of solid present were broken up and stirring and heating under reflux were continued for a further 30 min.

The mixture was hot-filtered and the resulting solid was dissolved in water (40.0 ml) and acidified to pH1 by addition of 2M aqueous hydrochloric acid then adjusted to pH6 by addition of solid sodium acetate and rotary evaporated. The residue was

extracted with hot ethyl acetate to give an oil which was extracted with hot light petroleum. On evaporation the extracts gave only an intractable gum.

Toluene-4-sulphonyl azide

A solution of toluene-4-sulphonyl chloride (34.4 g, 0.018 mol) in ethanol (400 ml) was stirred and treated dropwise at room temperature with a solution of sodium azide (14.0 g, 0.215 mol) in water (40.0 ml). The mixture was stirred at room temperature for 1 h and then poured into water (1500 ml).

The precipated oil was extracted with dichloromethane (4 x 200 ml), which on evaporation (<35°C) gave a clear yellow liquid (33.2 g; 94%), ν_{max} 2130 (N≡N) cm^{-1} and was identified by comparison with an identical sample.[71]

Ethyl 2-diazo-3-oxobutanoate (126)

A solution of ethyl acetoacetate (125) (22.1 g, 0.17 mol) in anhydrous acetonitrile (280 ml) was treated with a single portion of triethylamine (17.2 g, 0.17 mol). The mixture was stirred and treated at room temperature with a single portion of toluene-4-sulphonyl azide (33.5 g; 0.17 mol). The mixture was stirred at room temperature with exclusion of atmospheric moisture for 1.5 h.

The mixture was rotary evaporated (<35°C) under high vacuum. The residue was treated with ether (190 ml) and 30% w/v aqueous potassium hydroxide (190 ml). The two layers were separated and the ether layer was washed with two further portions of 30% potassium hydroxide (95.0 ml), then with water (3 x 37.0 ml) and rotary evaporated (<35°C) to give the product as a yellow liquid (22.0 g; 83%); ν_{max} 2120 (N≡N) and 1700 (C=O) cm^{-1}.

Ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127)

(a) A solution of solid sodium acetate (24.9 g, 0.3 mol) in water was treated with hydroxylamine hydrochloride (20.9 g, 0.3 mol) until neutral and then added to a solution of ethyl 2-diazo-3-oxobutanoate (126) (15.6 g, 0.1 mol) in ethanol (50.0 ml). The mixture was then stirred and heated at 80°C for 2 h.

A solution of sodium acetate (16.6 g, 0.2 mol) in water (40.0 ml) was treated with hydroxylamine hydrochloride (14.0 g, 0.2 mol) until neutral and added to the aforementioned mixture, then stirred and heated at 80°C for a further 3 h.

The mixture was rotary evaporated to give an off-white solid which was dissolved in water (150 ml) with warming, then chilled and acidified to pH1 by addition of concentrated hydrochloric acid. The precipitated solid was collected, dried and combined with a further crop of solid obtained by extraction of the acidic aqueous filtrate with chloroform and trituration of the residue with ether to give the 1-hydroxytriazole derivative (127) which formed colourless crystals (9.0 g; 53%), m.p.133-136°C, (lit.,[71] 145-147°C) (from ethyl acetate), ν_{max} 3000-2000 br (OH) and 1720 (C=O) cm^{-1}; δ_H [$(CD_3)_2SO$] 4.29(2H, q, J7Hz, CH_2), 2.34(3H, s, CH_3) and 1.24 (3H, t, J7Hz, CH_3).

(b) A solution of hydroxylamine hydrochloride (37.8 g, 0.54 mol) in water (100 ml) was treated with solid sodium acetate (45.0 g, 0.54 mol) until neutral. This mixture was then added to a solution of ethyl 2-diazo-3-oxobutanoate (28.0 g, 0.18 mol) in ethanol (90.0 ml) and the resulting mixture was stirred and heated at 80°C (oil bath) for 8 h.

The mixture was evaporated to give a moist solid which was dissolved in water with warming, then chilled and acidified to pH1 by addition of concentrated hydrochloric acid. The precipitated solid (20.0 g) was collected and combined with a further crop of solid (0.7 g) obtained by extraction of the aqueous filtrate with chloroform and trituration of the residue with ethyl acetate and ether. The solid (20.7 g; 67%) obtained formed colourless crystals (13.0 g; 43%) m.p. 144-147°C (from ethyl acetate) and was identified by comparison (m.p., i.r. and ^1H n.m.r. spectra) with the sample obtained in (a) before.

(c) A solution of ethyl 2-diazo-3-oxobutanoate (126) (1.6 g, 0.01 mol) in anhydrous ethanol (10.0 ml) was stirred and treated in one portion with a suspension of hydroxylamine hydrochloride (3.5 g, 0.05 mol) in anhydrous ethanol (25.0 ml). Solid anhydrous sodium carbonate (2.7 g, 0.025 mol) was added and the mixture was stirred at room temperature for 17 h.

The mixture was rotary evaporated under high vacuum ($<35^{o}C$) and the residue was treated with water (10.0 ml) and collected to give a high melting solid. An oil precipitated in the aqueous mother liquor and was extracted with ether to give a yellow liquid (0.8 g; 50%) identified as starting material, by comparison (i.r. spectrum) with an authentic sample.

(d) A suspension of hydroxylamine hydrochloride (3.5 g, 0.05 mol) in anhydrous *n*-butanol (10.0 ml) was treated with anhydrous sodium carbonate (2.7 g, 0.025 mol) at $0^{o}C$ (ice bath) and the mixture was stirred for 15 min then filtered to remove inorganic material. A solution of ethyl 2-diazo 3-oxobutanoate (126) (1.6 g, 0.01 mol) in anhydrous *n*-butanol (5.0 ml) was added to the above hydroxylamine filtrate. The mixture was stirred at room temperature for 1 h, then heated at $95^{o}C$ (oil bath) for 4 h.

The resulting yellow solution was rotary evaporated under high vacuum ($<35^{o}C$) to give a yellow liquid (1.2 g; 75%) identified as ethyl 2-diazo 3-oxobutanoate (126) by comparison of i.r. spectra.

(e) Repetition of the reaction described in (d) but using anhydrous 1,2-dimethyoxyethane as solvent and heating under reflux for the second hour of reaction gave the starting material (88%), identified by comparison (i.r. spectrum) with the sample obtained in (d) before.

The attempted reaction of ethyl 2-oxopropanoate (133) with isoamyl nitrite in the presence of sodium ethoxide.

A solution of sodium (2.3 g, 0.1 mol) in anhydrous ethanol (100 ml) was treated in one portion with isoamyl nitrite (11.7 g, 0.1 mol) and the mixture was stirred and treated dropwise at room temperature with exclusion of atmospheric moisture with ethyl 2-oxopropanoate (11.6 g, 0.1 mol). The mixture was then stirred at room temperature with exclusion of atmospheric moisture for 3 days during which time the initially pale yellow colour of the mixture turned orange then brown.
Workup of the resulting mixture gave no identifiable material.

The attempted reaction of ethyl 2-oxopropanoate (133) with methyl nitrite in the presence of anhydrous hydrogen chloride.

A solution of ethyl 2-oxopropanoate (17.4 g, 0.15 mol) in anhydrous ether (150 ml) was stirred and treated simultaneously at room temperature with slow streams of anhydrous hydrogen chloride and methyl nitrite [generated by dropping a cold aqueous sulphuric acid solution (7.0 ml concentrated sulphuric acid in 13.0 ml water) into a stirred mixture of sodium nitrite (12.4 g), methanol (8.0 ml) and water (8.0 ml)]. After 40 min the flow of methyl nitrite was interrupted and the passage of hydrogen chloride continued for a further 10 min. The mixture was then left stirring at room temperature for 24 h. During the passage of the two gases the initially yellow solution turned to brown. However after the flow of the gases was stopped, the solution returned to its initial yellow colour.

The mixture was extracted ten times with 10% w/v aqueous sodium hydroxide solution (10 x 5.0 ml) and the sodium hydroxide extracts were poured slowly, with stirring onto a mixture of concentrated hydrochloric acid (9.0 ml) and ice (10.0 g). The resulting solution was extracted with dichloromethane to give a yellow liquid (14.9 g) whose t.l.c in chloroform-ethyl acetate (1:1) showed it to be a multicomponent mixture which was not further investigated.

Ethyl 2-diazoethanoate (129a).

Ethyl 2-diazoethanoate (129a) was prepared (yield 97%) by the reaction of ethyl 2-aminoethanoate hydrochloride (136) with sodium nitrite in the presence of sulphuric acid as described by Searle,[84] giving the desired product as a yellow liquid, ν_{max} 2110 (N≡N) and 1700 (C=O) cm^{-1}, which was used without further purification.

Dimethylformamidinium chloride (128).

Dimethylformamidinium chloride (128) was prepared by the reaction of dimethylformamide with thionyl chloride as described by Bosshard *et al.*[92] and was obtained in quantitative yield as a very hygroscopic solid which was therefore used immediately without further purification.

Ethyl 2-diazo-3-oxopropanoate (131a).

(a) Ethyl 2-diazo-3-oxopropoanoate (131a) was prepared by the reaction of ethyl 2-diazoethanoate (129a) with dimethylformamidinium chloride (128) and hydrolysis of the resulting diazo intermediate (130a) as described by Stojanovic and Arnold.[83] The product was obtained as a yellow liquid (50%), ν_{max} 2150 (N=N) and 1740-1650br (C=O) cm^{-1} [lit.,[83] ν_{max} 2145 (N≡N), 1770 (C=O) cm^{-1}], λ_{max} (ethanol) 220 and 250 nm ($\log_{\varepsilon}$ 4.02 and 3.76) [lit.,[83] (ethanol) : λ_{max} 217 and 249 nm ($\log_{\varepsilon}$ 4.24 and 4.08).

(b) A 2M solution of dimethylformamidinium chloride in anhydrous chloroform (25.0 ml, 0.05 mol) was stirred and treated over 15 min at -20°C (dry ice-acetone bath) with exclusion of atmospheric moisture with ethyl 2-diazoethanoate (11.4 g, 0.1 mol) and the mixture was stirred at room temperature with exclusion of atmospheric moisture for 1 h.

The mixture was then rotary evaporated at $<35^{o}C$ and the hygroscopic residue was kept under argon as much as possible. The residue was washed several times with anhydrous ether and then treated with a solution of sodium acetate (1.0 g) in water (10.0 ml) buffered to pH7 with a few drops of acetic acid. Ether (15.0 ml) was added and the resulting two phase mixture was stirred at room temperature for 3 h. The ether extracts were washed three times with 10% w/v aqueous potassium hydrogen carbonate solution (3 x 15.0 ml) and rotary evaporated ($<35^{o}C$) leaving the diazo compound (131a) as an orange liquid (2.9 g; 41%) identical (i.r. and u.v. spectra) with the sample obtained in (a) before.

Ethyl 2-diazo-3-dimethyliminium propanoate chloride (130a).

A 2M solution of dimethylformamidinium chloride (128) in anhydrous chloroform (5.0 ml, 0.01 mol) was stirred and treated over 15 min at $-5^{o}C$ to $+10^{o}C$ (ice-salt bath) with ethyl 2-diazoethanoate (129a) (2.3 g; 0.02 mol). Gas and heat were evolved and the resulting yellow solution was stirred at room temperature for 1 h.

The mixture was then rotary evaporated at $<35^{o}C$ and the residue washed with anhydrous ether and dried *in vacuo* to give a pale yellow hygroscopic solid (1.6 g; 78%) which was therefore used immediately without further purification.

Ethyl 2-diazo-3-oximinopropanoate (138).

(a) A suspension of hydroxylamine hydrochloride (31.5 g, 0.45 mol) in anhydrous ethanol (180 ml) was cooled to $0^{o}C$ (ice-salt bath) and treated with solid sodium carbonate (28.6 g, 0.27 mol) followed by a solution of ethyl 2-diazo-3-dimethyliminium propanoate chloride (130a) (18.3 g, 0.09 mol) in anhydrous ethanol (45.0 ml). The resulting mixture was stirred and allowed to come to room temperature over 30 min, then stirred for a further 30 min at room temperature.

The mixture was filtered and the ethanolic filtrate was rotary evaporated under high vacuum at <35°C to give a residue which was treated with water, collected and dissolved in dichloromethane. Rotary evaporation at <35°C of the dried dichloromethane extract yielded the diazo oxime (138), a bright yellow solid (5.8 g; 41%), m.p.77-79°C; ν_{max} 2099 (N≡N) and 1711 (C=O) cm^{-1}; λ_{max} 228 and 274 nm ($\log_{\varepsilon}$ 4.06 and 3.87).

(b) Repetition of the reaction described in (a) but using freshly distilled anhydrous acetonitrile as solvent gave the product as a yellow solid (yield 29%), m.p.89-90°C, identified by comparison (m.p. and i.r. spectrum) with the sample obtained in (a) before.

(c) Repetition of the reaction described in (a) but with extension of the reaction time to 17 h gave the product as a yellow solid (yield 37%), m.p.94-95°C, identified by comparison (m.p. and i.r. spectrum) with the sample obtained in (a) before.

(d) A solution of ethyl 2-diazo-3-dimethyliminium propanoate chloride (11.0 g, 0.2 mol) in anhydrous ethanol (100 ml) was stirred and treated in one portion at room temperature with a suspension of hydroxylamine hydrochloride (70.0 g, 1.0 mol) in anhydrous ethanol (400 ml). Solid anhydrous sodium carbonate (64.0 g, 0.6 mol) was added and the mixture was stirred at room temperature with exclusion of atmospheric moisture for 17 hours. The mixture was filtered to remove inorganic material and the filtrate was rotary evaporated under high vacuum (<35°C). The residue obtained was triturated with water, collected and dissolved in dichloromethane. Rotary evaporation (<35°C) of the dichloromethane portion after drying gave the product as a yellow solid (yield 15%), m.p.92-93°C, identified by comparison (m.p. and i.r. spectrum) with the sample obtained in (a) before.

(e) Repetition of the reaction described in (d) but the residue previously obtained when the reaction filtrate was evaporated under high vacuum (<35°C) was treated with dichloromethane leaving a gummy residue. The dichloromethane portion gave an oil which was triturated with water and extracted with dichloromethane to give a

yellow solid (yield 28%), m.p.80-82°C, identified by comparison (m.p. and i.r. spectrum) with the sample obtained in (a) before.

(f) A suspension of hydroxylamine hydrochloride (3.5 g, 0.05 mol) in anhydrous ethanol (40.0 ml) was treated with a solution of sodium (1.2 g, 0.05 mol) in anhydrous ethanol (22.0 ml) and the mixture was stirred for 5 min then filtered to remove sodium chloride. The resulting solution of hydroxylamine was then added in one portion at room temperature with stirring to a solution of ethyl 2-diazo-3-dimethyliminium propanoate chloride (130a) (2.1 g, 0.01 mol) in anhydrous ethanol (5.0 ml). The mixture was then stirred at room temperature with exclusion of atmospheric moisture for 17 h. The mixture was rotary evaporated, giving a pale brown intractable solid (1.7 g) which yielded no identifiable material.

(g) A suspension of hydroxylamine hydrochloride (3.5 g, 0.05 mol) in anhydrous dimethoxyethane (10.0 ml) was treated with anhydrous sodium carbonate (2.7 g, 0.025 mol) at 0°C (ice bath) and the mixture was stirred for 15 min, then filtered to remove inorganic material. The filtered hydroxylamine solution so obtained was added to a suspension of ethyl 2-diazo-3-dimethyliminium propanoate chloride (2.1 g, 0.01 mol) in anhydrous dimethoxyethane (10.0 ml) and the resulting mixture was stirred with exclusion of atmospheric moisture at room temperature for 1 h and then at 85°C for 1 h.

The supernatant liquor was decanted from an intractable oil and rotary evaporated under high vacuum at <35°C leaving an intractable oil from which no identifiable material could be obtained.

(h) A solution of ethyl 2-diazo-3-oxopropanoate (131a) (0.7 g, 0.005 mol) in anhydrous ethanol (7.5 ml) was stirred and treated in one portion with a warm solution of hydroxylamine hydrochloride (1.7 g, 0.025 mol) in anhydrous ethanol (30.0 ml). Anhydrous sodium carbonate (1.9 g, 0.025 mol) was added and the mixture was stirred at room temperature with exclusion of atmospheric moisture for 17 h.

The mixture was filtered to remove inorganic material and the filtrate was rotary evaporated at $<35^{o}C$ to leave a residue which was triturated with a little water giving the product as a yellow solid (0.31 g; 47%), m.p.87-89°C, identified by comparison (m.p., i.r. and u.v. spectrum) with the sample obtained in (a) before.

Ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132).

(a) A solution of ethyl 2-diazo-3-oximinopropanoate (138) (2.9 g, 0.018 mol) in freshly dried purified benzene (25.0 ml) was stirred and heated under reflux with exclusion of atmospheric moisture for 1.5 h.

The mixture was cooled and the precipitated solid was collected to give the *N*-hydroxytriazole derivative (2.5 g; 88%) which formed colourless crystals, m.p.106-108°C [from ethyl acetate-light petroleum (b.p.40-60°C)], ν_{max} 3200-2000 br (OH) and 1730 (C=O) cm^{-1}; λ_{max} 212 and 260 nm ($\log\varepsilon$ 4.25 and 3.63); δ_H [$(CD_3)_2SO$] 8.70 (1H, s, CH), 4.30 (2H, q, J7Hz, CH_2) and 1.29 (3H, t, J7Hz, CH_3).

Found: C, 38.2; H, 4.6; N, 26.7%; m/z (EIms), 157 (M^+).

$C_5H_7N_3O_3$ requires: C, 38.2; H, 4.5; N, 26.8%; M, 157.

Evaporation of the benzene mother liquor gave only an intractable oil (0.3 g) whose t.l.c in ethyl acetate-light petroleum (1:1) showed it to be a multicomponent mixture across a small R_f which was not further investigated.

(b) A solution of ethyl 2-diazo-3-oximinopropanoate (138) (0.16 g, 0.001 mol) in anhydrous 1,2-dimethoxyethane (5.0 ml) was stirred and heated under reflux with exclusion of atmospheric moisture for 1 h.

The mixture was rotary evaporated under high vacuum and the residue was triturated with ether to give the 1-hydroxytriazole (132) as a colourless solid

(0.07 g; 45%), m.p.93-97°C, identified by comparison (m.p. and i.r. spectrum) with the sample obtained in (a) before.
Evaporation of the ethereal filtrate gave only an intractable oil which therefore was not investigated further.

(c) A solution of ethyl 2-diazo-3-oximinopropanoate (138) (0.16 g, 0.001mol) in ethyl acetate (5.0 ml) was stirred and heated under reflux with exclusion of atmospheric moisture for 45 min.

The mixture was hot-filtered to remove some insoluble solid and the filtrate was concentrated to give the 1-hydroxytriazole (132) as a colourless solid (0.006 g; 35%), m.p.97-102°C, identified by comparison (m.p. and i.r. spectrum) with the sample obtained in (a) before.
Rotary evaporation of the ethyl acetate mother liquor gave only an intractable oily residue which yielded no identifiable material.

(d) A solution of hydroxylamine hydrochloride (2.1 g, 0.03 mol) in water (10.0 ml) was stirred and treated with solid sodium acetate (2.5 g, 0.03 mol) until neutral. To the resulting solution was added a solution of ethyl 2-diazo-3-oxopropanoate (131a) (1.4 g, 0.01 mol) in ethanol (5.0 ml) and the mixture was then stirred and heated at 80°C for 7 h.

The mixture was rotary evaporated under high vacuum to give a moist solid which was dissolved with warming in water (5.0 ml) and the solution chilled and acidified to pH1 by addition of concentrated hydrochloric acid. The precipitated solid was collected to give the 1-hydroxytriazole (132) (0.15 g; 9%) which formed colourless crystals, m.p.131-134°C (from ethyl acetate, ethanol and methanol), identified by comparison (m.p., i.r., m.s. and ^{1}H n.m.r. spectra) with the sample obtained in (a) before.
Further extraction of the acidic aqueous mother liquor yielded no further product.

Propane-1,2-dione 1-oxime (141).

Propane-1,2-dione 1-oxime (141) was prepared (yield 66%) by the reaction of ethyl 3-oxobutanoate with sodium nitrite in the presence of sulphuric acid as described by Metzger[93] and had m.p. 60-63°C (lit.,[93] 69°C).

Propane-1,2-dione 1-oxime 2-tosylhydrazone (142).

A solution of propane-1,2-dione 1-oxime (141) (1.7 g, 0.02 mol) in ethanol (10.0 ml) was added to a solution of toluene-4-sulphonylhydrazine (3.7 g, 0.02 mol) in ethanol (30.0 ml) and the mixture was stirred and heated under reflux for 2 h.

The mixture was cooled and the precipitated solid was collected and combined with a further crop of solid obtained by rotary evaporation of the ethanolic mother liquor and trituration of the residue with ethanol and dichloromethane to give the tosylhydrazone product (142) (3.0 g; 76%) which formed colourless microcrystals, m.p. 163-165°C (from ethanol-light petroleum), ν_{max} 3500 and 3200 (NH) and 3500-2500 br (OH) cm^{-1}, $\delta_H[(CD_3)_2SO]$ 11.58(1H, s, NH), 10.7(1H, s, OH), 7.80-7.41(4H, m, ArH), 7.30(1H, s, CH), 2.38(3H, s, CH_3) and 1.95(3H, s, CH_3).

Found: C,47.0; H,5.3; N,16.4%; m/z (EIms) no parent ion observed.

$C_{10}H_{13}N_3O_3S$ requires: C,47.1; H,5.1; N,16.5%; M,255.

The attempted base-catalysed cyclisation of propane-1,2-dione 1-oxime 2-tosylhydrazone (142)

A solution of propane-1,2-dione 1-oxime 2-tosylhydrazone (142) (12.7 g, 0.05 mol) in anhydrous 1,2-dimethoxyethane (100 ml) was treated with a solution of sodium (3.5 g, 0.5 mol) in anhydrous ethanol (500 ml). The mixture was stirred at room temperature with exclusion of atmospheric moisture for 30 min, then rotary evaporated under high vacuum. The solid obtained was broken up, anhydrous diglyme (500 ml) was added and the mixture was stirred and heated under reflux for 15 minutes, then cooled. Any lumps of solid were broken up and stirring and heating under reflux were continued for a further 30 min.

The mixture was rotary evaporated under high vacuum and the residue was dissolved in water (100 ml), the solution was chilled, and acidified by the addition of concentrated hydrochloric acid and extracted with dichloromethane to give a brown intractable liquid (3.4 g).

The attempted reaction of 3,3-dimethylbutan-2-one (144) with isoamyl nitrite in the presence of sodium ethoxide

A solution of sodium (2.3 g, 0.1 mol) in anhydrous ethanol (100 ml) was treated in one portion with isoamyl nitrite (11.7 g, 0.1 mol) and the mixture was stirred and treated dropwise at room temperature with exclusion of atmospheric moisture with 3,3-dimethylbutan-2-one (10.0 g, 0.1 mol). The resulting mixture was then stirred at room temperature for 3 days.

Rotary evaporation of the mixture yielded an oily solid which when triturated with ethanol and ether gave a high melting solid (1.1 g) which yielded no identifiable material.

The ethereal mother liquor was evaporated leaving a gum (16.5 g) which was dissolved in water, acidified with acetic acid and extracted with dichloromethane but gave no identifiable material.

The attempted reaction of 3,3-dimethylbutan-2-one (144) with methyl nitrite in the presence of anhydrous hydrogen chloride

A solution of 3,3-dimethylbutan-2-one (15.0 g, 0.15 mol) in anhydrous ether (150 ml) was stirred and treated simultaneously at room temperature with slow streams of anhydrous hydrogen chloride and methyl nitrite [generated by dropping a cold aqueous sulphuric acid solution (7.0 ml concentrated sulphuric acid in 13.0 ml water) into a stirred mixture of sodium nitrite (12.4 g), methanol (8.0 ml) and water (8.0 ml)]. After 40 min the flow of methyl nitrite was interrupted and the passage of hydrogen chloride continued for a further 10 min. The mixture was then left stirring at room temperature for 24 h.

The resulting mixture was rotary evaporated to leave a yellow liquid which was treated with 2M aqueous sodium hydroxide solution and extracted with dichloromethane to give an intractable orange liquid which yielded no identifiable material.

The aqueous alkaline mother liquor was acidified to pH1 by the addition of concentrated hydrochloric acid and extracted with dichloromethane to give an intractable oil which could not be characterised.

1-Hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole potassium salt (149a)

1-Hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a) (0.9 g, 5 mmol) was dissolved with warming in anhydrous ethanol (15.0 ml) and the solution was stirred and treated slowly at 60°C (oil bath) with 2M ethanolic potassium hydroxide solution (2.5 ml, 5 mmol) for 1 h.

The mixture was cooled and anhydrous ether (50.0 ml) was added to precipitate a solid which was collected and dried *in vacuo* to give 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole potassium salt (148a) as a colourless solid (0.73 g; 69%) m.p. >270°C which was used without further purification.

1,1,3,3-Tetramethylchloroformamidinium chloride (147)

A solution of tetramethylurea (0.58 ml, 5 mmol) in anhydrous dichloromethane (7.0 ml) was stirred under nitrogen and treated slowly at room temperature with oxalyl chloride (0.63 ml, 5 mmol). The mixture was then stirred and heated under reflux under a continuous flow of nitrogen for 4 h.

Anhydrous ether (35.0 ml) was added to the cooled mixture precipitating the product as a very hygroscopic solid which was washed twice with ether and used immediately without further purification.

1-Hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole 1,1,3,3-tetramethyl-uronium tetrafluoroborate or hexafluorophosphate Salts (150a)

(a) To a stirred solution of 1,1,3,3-tetramethylchloroformamidinium chloride (0.51-0.68 g, 3-4 mmol) in anhydrous acetonitrile (6.0 ml) under nitrogen, was added potassium tetrafluoroborate (0.45 g, 3.5 mmol) followed by a suspension of the potassium salt of 1-hydroxy-5-methyl-4-phenyl-1*H*-1,2,3-triazole (124a) (0.73 g, 3.5 mmol) in anhydrous acetonitrile (6.0 ml). The mixture was stirred under nitrogen at room temperature overnight.

The mixture was filtered to remove a high melting solid and an oil was precipitated from the mother liquor by addition of ether. Trituration of the oil with ethanol gave a solid (0.012 g; 1%) which formed colourless needles (from toluene-acetonitrile), m.p.212-213°C, $\delta_H[(CD_3)_2SO]$ 7.68-7.58 (5H, m, Ar), 3.14 (6H, s, 2 x CH_3), 2.84 (6H, s, 2 x CH_3) and 2.25 (3H, s, CH_3).

Found: C, 46.4; H, 5.5; N, 19.4%; m/z (FAB ms), no parent ion.

$C_{14}H_{20}BF_4N_5O$ requires: C, 46.7; H, 5.5; N, 19.4%; M, 360.

Rotary evaporation of the ethanolic mother liquor gave only an intractable oil (1.2 g) which yielded no identifiable material.

(b) Repetition of the reaction described in (a) but using potassium hexafluorophosphate instead of potassium tetrafluoroborate gave only an intractable oil from which no identifiable material could be obtained.

Ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate potassium salt (149b).

Ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) (4.3g, 0.025mol) was dissolved with warming in anhydrous ethanol (65.0 ml) and the solution was stirred and treated dropwise at 60°C (oil bath) with 2M ethanolic potassium hydroxide solution (12.5 ml, 0.025 mol).

The mixture was cooled and the precipated solid was collected and dried *in vacuo* to give ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (148b) potassium salt as a colourless powder (2.6 g; 50%) m.p. >300° (from ethyl acetate-methanol), ν_{max} 1700 (C=O) cm^{-1}, δ_H ($CDCl_3$) 4.17 (2H, q, J7Hz, CH_2), 2.13 (3H, s, CH_3) and 1.25 (3H, t, J7Hz, CH_3).

Found: C,34.2; H,3.9; N,19.9%; m/z (FABms), 210 $[(M+H)^+]$.

$C_6H_8N_3O_3K^{\pm}$ requires: C,34.4; H,3.8; N,20.0%; (M^+H), 209.

Further extraction of the ethanolic mother liquor yielded no further product.

The attempted preparation of ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate 1,1,3,3-tetramethyluronium tetrafluoroborate or hexafluorophosphate Salts (150b).

(a) To a stirred solution of 1,1,3,3,-tetramethylchloroformamidinium chloride (147) (12.1 g, 0.012 mol) in anhydrous acetonitrile (15.0 ml) under argon, was added

potassium tetrafluoroborate (1.5 g, 0.012 mol) followed by a suspension of the potassium salt of ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (149b) (2.5 g, 0.012 mol) in anhydrous acetonitrile (18.0 ml). The mixture was stirred under argon at room temperature overnight.

The mixture was filtered to remove inorganic material and the filtrate was diluted with ether to precipitate an oil which was shown by t.l.c. in ethyl acetate:petroleum ether (1:1) over silica to be a multicomponent mixture and therefore was not further investigated.

(b) To a stirred solution of 1,1,3,3-tetramethylchloroformamidinium chloride (147) (0.5 g, 3.0 mmol) in anhydrous acetonitrile (10.0 ml) was added the potassium salt of ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) (0.63 g, 3.0 mmol) and then potassium hexafluorophosphate (0.55 g, 3.0 mmol). The mixture was stirred under argon at room temperature overnight.

The mixture was filtered to remove inorganic material and anhydrous ether was added to the filtrate to precipitate an intractable oil from which no identifiable material could be obtained.

The attempted preparation of ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate alkali metal salts (148c).

(a) Ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) (1 mmol) was dissolved with warming in anhydrous ethanol (3.0 ml) and the solution was stirred and treated dropwise at 60°C (oil bath) with 2M ethanolic sodium hydroxide solution (0.5 ml, 1 mmol). The mixture was then cooled and rotary evaporated to give only an intractable oil (0.2 g) which yielded no identifiable material.

(b) Repetition of the reaction described in (a) above but using 2M ethanolic potassium hydroxide solution again gave no identifiable material.

2,5-Dimethyl-1-hydroxy-4-phenyl-1*H*-imidazole (160a).

A solution of 1-phenylpropane-1,2-dione 2-oxime (159a) (8.2 g, 0.05 mol) in ethanol (150 ml) was treated at room temperature with acetaldehyde (4.4 g, 0.1 mol) then concentrated ammonia solution (10.0 ml, 0.2 mol) and the resulting mixture was stirred at room temperature overnight.

The mixture was rotary evaporated to give an oil which was dissolved in water (20.0 ml). 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 (160a) as a colourless solid (17.5 g, 80%), m.p.168-170°C (from acetonitrile-ethanol), ν_{max} 2700-2100 br (OH) cm^{-1}, $\delta_H[(CD_3)_2SO]$ 7.6-7.36 (5H, m, Ar), 3.77 (1H, br, s, OH), 2.27 (3H, s, CH_3) and 2.25 (3H, s, CH_3).

Found: C, 69.9; H, 6.3; N, 14.8%; m/z (EIms), 188 (M^+).

$C_{11}H_{12}N_2O$ requires: C, 70.3; N, 6.4; N, 14.9%; M (188).

1-Phenylethane-1,2-dione 2-oxime (159c)

1-Phenylethane-1,2-dione 2-oxime (159c) was prepared (yield 40%) by the sodium ethoxide catalysed reaction of acetophenone with isoamyl nitrite as described by Claisen and Manasse,[94] and had m.p.123-125°C (lit.,[94] 126-128°C)

1-Hydroxy-2-methyl-4-phenyl-1*H*-imidazole (160c).

A solution of 1-phenylethane-1,2-dione 2-oxime (159c) (1.5 g, 0.01 mol) in ethanol (40.0 ml) was treated with acetaldehyde (0.9 g. 0.02 mol) and concentrated ammonia solution (2.0 ml, S.G. 0.88, 0.04 mol) and the mixture was stirred at room temperature overnight.

The mixture was rotary evaporated to give an oil which was treated with water (16.0 ml) and acidified by the addition of concentrated hydrochloric acid, then extracted with ether to give the starting α-keto-oxime (0.3 g; 20%), m.p.119-122°C, (lit.,[94] 122-123°C) identified by comparison (i.r. spectrum) with an authentic sample. Precipitated solid from the acidic aqueous portion was collected and combined with further material obtained by adjusting the aqueous mother liquor to pH6 by addition of concentrated ammonia solution at 8°C (cold room) to give product (160c) (0.5 g; 26%), m.p.176-178°C [from ethanol-petroleum ether (160-180°C)]; ν_{max} 3100-2300 br (OH) cm^{-1}; δ_H [$(CD_3)_2SO$] 7.75-7.20 (5H, m, Ar) and 2.27 (3H, s, CH_3).

Found: C, 69.1; H 5.8; N; 15.9%; m/z (EIms) 174 (M^+).

$C_{10}H_{10}N_2O$ requires: C, 69.0; H, 5.8; N, 16.1%; M(174).

The attempted synthesis of 1-hydroxy-5-methyl-4-phenyl-1*H*-imidazole (160b).

(a) A solution of 1-phenylpropane-1,2-dione 2-oxime (159b) (1.6 g, 0.01 mol) in ethanol (40.0 ml) was treated with formaldehyde sodium bisulfite addition compound (1.3 g, 0.01 mol) and concentrated ammonia solution (2.0 ml, 0.04 mol) and the resulting mixture was stirred at room temperature overnight.

The mixture was filtered to give a solid (1.0 g; 78%) identical (i.r. spectrum) to an authentic sample of the unreacted bisulfite addition compound. The mother liquor was rotary evaporated, the residue was treated with water and the insoluble solid was collected to give the unreacted keto-oxime (159b) (1.4 g; 88%), m.p.89-108°C, (lit.,[91] 112-113°C) identified by comparison (i.r. spectrum) with an authentic sample.

(b) A solution of 1-phenylpropane-1,2-dione 2-oxime (159b) (1.6 g, 0.01 mol) in ethanol (40.0 ml) was treated at room temperature with 30% w/v aqueous formaldehyde (2.0 ml, 0.02 mol) followed by concentrated ammonia solution (2.0 ml, 0.04 mol) and the mixture was stirred at room temperature overnight.

The mixture was rotary evaporated to give an oil which was triturated with water (10.0 ml) and the resulting suspension was acidified to pH1 by addition of concentrated hydrochloric acid then extracted with ether to give a solid (0.7 g; 44%), m.p.110-113°C identical (m.p. and i.r. spectrum) to an authentic sample of the starting keto-oxime.

(c) To a solution of 1-phenylpropane-1,2-dione 2-oxime (159b) (1.6g, 0.01 mol) in ethanol (25.0 ml) was added ammonium chloride (1.1 g, 0.02 mol) and paraformaldehyde (1.2 g, 0.04 mol). The resulting mixture was heated under reflux overnight. The mixture was filtered to give a little solid (0.14 g), m.p. 150-250°C, the mother liquor was rotary evaporated to give an intractable oil which therefore was not identified.

(d) A solution of 1-phenylpropane-1,2-dione 2-oxime (159b) (8.1 g, 0.05 mol) in concentrated ammonia (180 ml, S.G. 0.88) was treated with paraformaldahyde (3.0 g, 0.1 mol). The resulting mixture was stirred at room temperature overnight. The mixture was rotary evaporated to give an intractable oil which was therefore not identified and no product was obtained.

3.3 Solid Phase Peptide Synthesis.

LeuIlePheAlaGly

The synthesis of the test peptide was achieved using FmocGly-*p*-alkoxybenzyl alcohol resin. The amino acids were double coupled for the indicated time and were activated using DIC/*N*-hydroxytriazole or DIC/*N*-hydroxyimidazole as specified in Figure 3. In each case, the resulting peptide was examined by amino acid analysis, cleaved from the resin and examined by hplc.

AlaGlyResin

Synthesis of the above dipeptide was accomplished using the functionalised resin FmocGly-*p*-alkoxybenzyl alcohol resin (0.5 mmol). The amino acids were added in one equivalent (0.5 mmol) using a double couple cycle and DIC/*N*-hydroxytriazole activation. The dipeptide-resin was dried and weighed and examined by amino acid analysis (Fig. 4).

PheAlaGlyResin

This synthesis was accomplished using the functionalised resin FmocGly-*p*-alkoxybenzyl alcohol resin (0.5 mmol). A single coupling of amino acid (one equivalent) preactivated with DIC/*N*-hydroxytriazole was used (Fig. 5). The synthesis was followed by the deprotection monitoring and the resulting peptide resin was examined by amino acid analysis.

Il-1-β pre 127-138

H-ArgAspSerGlnGlnLysSerValMetSerGly-OH (155)

Synthesis of the title peptide was achieved using the FmocGly functionalized resin (0.54 mmol g^{-1}; 0.5 mmol). Gln was used without side chain protection and the amino acids (1 mmol) were activated using DIC/1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (1 mmol). The synthesis was completed giving peptide resin (1.4 g). The deprotection traces indicated a 76% yield before the coupling of the last Arg and a final yield of 56%. Monitoring of the acylation was unsuccessful.

The peptide resin (0.5 g) was cleaved in TFA:H_2O):ethanedithiol:thioanisole (95:5:2:4; 15.0 ml) with stirring at room temperature for 2 hours. This mixture was treated as described previously to give crude peptide (0.17 g). Amino acid analysis (acid hydrolysis 18 h) Gly 1.0, Ser_3 2.04, Met 0.96, Val 0.93, Leu 0.94, Lys 0.70, Glx_2 1.52, Asx 0.58, Arg 0.39. Hplc (aquapore C_{18} 10-90% B in 25 min), Rt 7.0 min (214 nm).

Luteimizing Hormone Releasing Hormone (LHRH)

H-PyrHisTrpSerTyrGlyLeuArgProGly-NH_2 (156)

The synthesis of peptide amide LHRH was achieved using amide resin (44)[38] (0.54mmol g^{-1}; 0.27 mmol). His was protected with the Bum group and Z-PyrOH was used. Each amino acid (0.5 mmol) was activated with DIC/ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) (0.5 mmol) except for Gly. A treble couple cycle (3 x 30 minutes) was used throughout except for Pro (2 x 30 minutes) giving peptide-resin (85%; 0.8 g). The deprotection monitoring indicated an overall yield of 60%. The coupling monitoring was unsuccessful due to technical problems.

The peptide-resin (0.3 g) was cleaved in TFA:water:anisole:ethanedithiol (95:5:3:3:3; 10.0 ml) with stirring at room temperature for 3 h. This mixture was treated as described previously to give crude peptide (0.8 g). This peptide (23 mg) was purified by preparative hplc on a C_8 reverse phase Vydac column 10-60% in 40 min, 5 ml min^{-1} (214 nm). The main fraction was lyophilised to give the title

peptide amide (9.0 mg): m/z (FAB) 1181.7 (M^+) [expected 1182.3 (M)]; amino acid analysis (tryptophan method, 18 h) Gly_2 2.40, Pro 1.00, Arg 1.03, Leu 1.00, Tyr 1.14, Ser 0.80, Trp 0.60, His 0.88, Pyr 1.10. Hplc (aquapore C_{18} 10-63% in 16 minutes) Rt 12.0 min (214 nm).

(b) Repetition of (a) above but using a double couple cycle throughout gave the completed peptide resin (0.3 g, 32%). The deprotection monitoring indicated <40% yield at Tyr after which the deprotection wash was too dilute to be recorded. The coupling monitoring was also unsuccessful. Amino acid analysis of peptide resin: (acid hyrolysis; 18 h) Gly_2 2.55, Pro 2.09, Arg 1.00, Leu 0.87, Tyr 0.40, Ser 0.33, Trp /, His 0.11, Pyr 0.18. The peptide amide was cleaved from the resin (0.15 g) as in (a) above and the crude peptide (2 mg) was identified by comparison (hplc) with an authentic sample.

Substance P

H-ArgProLysProGlnGlnPhePheGlyLeuMet-NH_2 (157)

(a) The synthesis of the title peptide amide was achieved using amide resin (44)[38] (0.31 mmol g^{-1}; 0.29 mmol). Gln was used without side chain protection. DIC/HOBt activation was used for Leu and Phe (x2), the remaining amino acids (0.5 mmol) (except Gly) were activated using DIC/ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) (0.5 mmol) with excess triazole (0.5 mmol) present. The synthesis was completed giving peptide resin (1.15 g; 82%). The acylation was monitored for those amino acids activated by the *N*-hydroxytriazole (132). The deprotection monitoring indicated an overall yield of 75%.

The peptide resin (1.1 g) was cleaved in TFA:water:anisole:phenol (100:5:3:2; 10.0 ml) with stirring at room temperature for 2.5 h. The resin was removed by filtration and normal work up gave the crude peptide (0.32 g); m/z (FAB) 1348.2 (MH^+) [expected - 1347.74 (M^+)]. Amino acid analysis: (acid hydrolysis; 18 h) Glx_2 2.40, Pro_2 1.90, Gly 1.10, Met 0.97, Leu 1.0, Phe_2 1.94, Lys 0.83, Arg O.97. Hplc (aquapore C_{18} 10-90% B in 22 min) Rt 15.0 min (214 nm).

(b) Repetition of (a) above but without excess of the *N*-hydroxytriazole (132). The deprotection monitoring indicated a 60% yield of the peptide.

The peptide resin (0.8 g) was cleaved as in (a) above to give the crude peptide (0.3 g) m/z (FAB) 1349 (MH^+) [expected - 1347.7 (M)]. Amino acid analysis (acid hydrolysis; 18 hours) Glx_2 2.16, Pro_2 1.34, Gly 1.15, Met 0.86, Leu 1.00, Phe_2 2.14, Lys 0.70, Arg, 0.62. The crude peptide (170 mg) was purified by preparative hplc on a C_{18} reverse phase column 10-90% B in 40 min, 5 ml min^{-1} (230 nm) to give the title peptide (46 mg); hplc (aquapore C_{18} 10-90% B in 22 min) Rt 13.0 min (214 nm).

(c) Repetition of (a) above but using DIC /ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) (0.75 mmol) activation for all amino acids (0.75 mmol,) (except Gly), Gln was used with trityl protection. The deprotection monitoring indicated an 87% yield of the peptide resin (0.4 g).

The peptide resin (0.32 g) was cleaved in TFA:water:anisole:thioanisole:EDT (100:5:3:3:3; 10.0 ml) and normal work up gave the crude peptide (70 mg) m/z (FAB) 1347 (M^+) [expected - 1347.74 (M^+)]. Amino acid analysis: (acid hydrolysis; 18 h) Glx_2 2.30, Pro_2 2.09 Gly 1.03, Met 0.98, Leu 1.00, Phe_2 2.10, Lys 0.86, Arg 0.78. Hplc (aquapore C_{18} 10-90% B in 22 min) Rt 13.0 min (214 nm). The crude peptide (60 mg) was purified by preparative hplc on a Vydac column 10-90% B in 45 min, 7 ml min^{-1} (230 nm) to give the title peptide (37 mg) m/z (FAB), 1347.7 (M^+) [expected 1347.74 (M^+). Amino acid analysis: (acid hydrolysis; 18 h) Glx_2 2.26, Pro_2 0.50, Gly 1.06, Met 0.98, Leu 1.00, Phe_2 2.02, Lys 0.79, Arg 1.01. Hplc (aquapore C_{18} 10-90% B in 25 min) Rt 13.0 min (214 nm).

(d) Repetition of (a) above but using DIC/ethyl 1-hydroxy-5-methyl-1*H*-1,2,3-triazole-4-carboxylate (127) (0.5 mmol) activation with excess of the *N*-hydroxytriazole (127) (0.25 mmol). Met, Leu and Phe(x2) were added as their HOBt esters (0.5 mmol) and Gln was used without side chain protection. The deprotection monitoring indicated a 23% yield of peptide resin (0.3g).

The peptide resin (0.15 g) was cleaved in TFA:water:anisole:thioanisole (100:5:3:3; 10.0 ml) and the normal work up gave the crude peptide (30 mg); m/z (FAB) 1347.4 (M^+) [expected 1347.74 (M^+)]. Amino acid analysis: (acid hydrolysis; 18 h) Glx_2 1.74, Pro_2 0.86, Gly 1.10, Met 1.00, Leu 1.00, Phe_2 1.98, Lys 0.37, Arg 0.23. The crude peptide (23 mg) was purified by preparative hplc on a vydac column 10-90% B in 45 min, 7 ml min^{-1} (230 nm) to give the title peptide (1.4 mg), hplc (aquapore C_{18} 10-90% B in 22 minutes) Rt 15.0 min (214 nm).

(e) Repetition of (a) above but using DIC/1-hydroxy-5-methyl-1*H*-1,2,3- triazole-4-carboxylate (127) (0.5 mmol) for activation of Gln^1 to Arg. Monitoring of the coupling and deprotection failed due to a blocked sinter in the synthesiser, however, manual deprotection of Fmoc-Arg-peptide-resin indicated a 36% yield of peptide resin (0.3 g).

The peptide resin (0.13 g) was cleaved as in (d) above to give the crude peptide (0.03 g): m/z (FAB) 1346.7 (M^+-1) [expected 1347.74 (M^+). Amino acid analysis (acid hydrolysis; 18 h) Glx_2 1.98, Pro_2 1.43, Gly 1.09, Met 1.00, Leu 1.00, Phe_2 2.00, Lys 0.60, Arg 0.41. Hplc (aquapore C_{18} 10-90% in 25 min) Rt 13.0 min (214 nm).

Nerve Growth Factor (NGF) 100-114.

H-ArgPheIleArgIleAspThrAlaCysValCysValLeuSerArg-OH (158)

(a) The synthesis of NGF 100-114 was achieved using FmocArgPmc-resin (0.34 mmol g^{-1}; 0.25 mmol). Amino acids 109-113 were coupled *via* symmetrical anhydride (1st coupling) and DIC/HOBt (2nd coupling) with extended coupling times. Amino acids 100-108 (0.5 mmol) were coupled using DIC/ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) (0.5 mmol) activation in the presence of 2,6-lutidine (2.0 mmol). The synthesis was completed giving peptide-resin (0.99 g; 72%). Manual deprotection of FmocArg-peptide-resin indicated a 70% yield by ultraviolet spectrometry.

The peptide resin (0.2 g) was cleaved in TFA:water:anisole:EDT (90:4:2:4; 10.0 ml) for 3 h and gave the crude Cys protected peptide after normal work up. A solution of this peptide (0.8 g) in trifluoroethanol:water (95:5; 15.0 ml) was treated with tributylphosphine (80 μl) and stirred at room temperature for 3 h. The mixture was then rotary evaporated under high vacuum, ether was added and the precipitated peptide (0.63 g) was collected. The peptide (0.3 g) was then stirred in TFA (9.0 ml) and DMSO (1.0 ml) at room temperature for 1 h. The mixture was concentrated to remove most of the TFA, 30% acetic acid was added and the oxidised peptide was purified by preparative hplc on a C_{18} reverse phase column 10-50% in 25 min, 5 ml min^{-1} (214 nm). Three fractions were obtained, each contained some product as indicated by mass spectrometry. The first fraction was further purified by preparative hplc using a C_{18} reverse phase Vydac analytical column isocratically at 28%B, 1.0ml min^{-1} (214 nm). This gave the desired product (1.0 mg; 3.2%) m/z FAB 1751.7 (M^+) $C_{75}H_{13}N_{24}O_{20}S_2$ requires 1751.94122, Found : 1751.94115. Hplc (vydac C_{18} 5-95% B in 30 minutes) Rt 13 min (214 nm).

(b) Repetition of (a) above but using excess ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) (1.0 mmol). Manual deprotection of FmocArg-peptide-resin indicated a 63% yield by ultraviolet spectroscopy of peptide resin.

(c) Repetition of (a) above except that amino acids 100-108 (0.5 mmol) were single coupled using DIC/ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (132) (1.0 mmol) manual deprotection of FmocArg-peptide-resin indicated a 44% yield of peptide resin.

The Determination of pK_a Values

Potassium hydroxide (@ 0.1M) was standardized by titration against potassium hydrogen phthalate (KHP) (25.0 ml; 0.05 mol dm^{-3}) using phenolphthalein indicator. The pH meter was standardized using 0.05 mol dm^{-3} KHP (pH 4.0 at 20°C). A 0.01 mol dm^{-3} solution of the compound to be tested (50.0 ml) was titrated against the potassium hydroxide solution standardized above. Readings were taken at 0.5 ml intervals.

The pH meter was restandardized and the procedure repeated for each of the compounds. Throughout the experiment the solution being treated was stirred with a stream of carbon dioxide free air.

To calculate the pK_a:
b = Potassium hydroxide = 0.0936M
V = initial volume of weak acid solution = 50.0 ml
v = vol of KOH added at the end point
a_o = concentration of the weak acid solution calculated from the end point ie a_o x 50.0 = 0.0936 x v.
From a graph of pH versus volume four experimental points were selected from the initial flat portion of the curve and pK_a was calculated for each point:

1 Calculate H^+ = antilog (-pH)
2 Calculate $K^+ = bv/(v+V)$
3 Calculate $[A^-] = [K^+] + [H^+]$
4 Calculate $a = a_o (V/v+V)$
5 Calculate $[HA] = a - [A^-]$
6 Calculate $\log_{10} ([A^-] / [HA])$
7. Calculate $I^{0.5} = [K^+]^{0.5}$
8 Calculate $0.5I^{0.5} / (1+I^{0.5})$

pKa=pH-6+8

REFERENCES

1. E. Fischer and E. Fourneau, *Chem. Ber.*, 1901, *34*, 2868.
2. M. Bergmann and L. Zervas, *Chem. Ber.*, 1932, *65*, 1192.
3. H. Bechtolsheimer and H. Kunz, *Angew. Chem., Int. Ed. Engl.*, 1982, *21*, 630.
4. H. Kunz, *Angew. Chem., Int. Ed. Engl.*, 1978, *17*, 67.
5. L.A. Carpino, B.J. Cohen, K.E. Stephens, S.Y. Sadat-Aalall, J.H. Tien and D.C. Langridge, *J. Org Chem.*, 1986, *51*, 3732.
6. T. Curtius, *Chem. Ber.*,1902, *35*, 3226.
7. T. Shioiri, K. Ninomiya, S. Yamada, *J. Am. Chem. Soc.*, 1972, *94*, 6203.
8. E. Fischer, *Chem. Ber.*, 1902, *35*, 1095.
9. T. Curtius, *Chem. Ber.*, 1904, *37*, 1284.
10. R. Schwyzer, B. Iselin and M. Feurer, *Helv.Chim.Acta*, 1955, *38*, 69.
11. M. Bodansky, *Nature*, 1955, *175*, 685.
12. W. Konig, R. Geiger, *Chem. Ber.*, 1973, *106*, 3626.
13. G.W. Anderson, J.E. Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, 1964, *86*, 1839.
14. J. Jones, *Chem. Ind.*, 1974, 724.
15. J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, 1955, *77*,1067.
16. H.G. Khorana, *Chem. Ind.*, 1955, 1087.
17. M. Smith, J.G. Moffat and H.G. Khorana, *J. Am. Chem. Soc.*, 1958, *80*, 6204.
18. D.F. De Tar and R. Silverstein, *J. Am. Chem. Soc.*, 1966, *88*, 1013,1020.
    D.F. De Tar, R. Silverstein and F.F. Rodgers, *ibid.*, 1966, *88*, 1024.
19. E. Wunsch and F. Drees, *Chem.Ber.*, 1966, *99*,110.
20. F. Weygand, D. Hofmann and E. Wunsch, *Z. Naturforsch.*, 1966, *216* 426; (Chem. Abs., *65*:72696).
21. W. Konig and R. Geiger, *Chem.Ber.*, 1970, *103*, 788.
22. R.B. Merrifield, *J. Am. Chem. Soc.*, 1963, *85*, 2149.
23. R.B. Merrifield, *Angew. Chem., Int. Ed. Engl.*, 1985, *24*, 799.
24. M. Bodansky, 'Peptide Chemistry'. Springer-Verlag, Berlin, 1988.
25. A.R. Mitchell, B.W. Erickson, M.N. Ryabster, R.S. Hodges and R.B. Merrifield, *J. Am. Chem. Soc.*, 1976, *98*, 7357.
26. S.S. Wang, *J. Am. Chem. Soc.*, 1973, *95*, 1328.

27. D.G. Mullen and G. Barany, *J. Org. Chem.*, 1988, *53*, 5240.
28. R. Ramage, C.A. Barron, S. Bielecki and C.W. Thomas, *Tetrahedron Lett.*, 1987, *28*, 4105.
29. D.H. Rich and S.K. Gurwara, *J. Am. Chem. Soc.*, 1975, *97*, 1575.
30. F.S. Tjoeng and G.A. Heavner, *J. Org. Chem.*, 1983, *48*, 355.
31. J.P. Tam, R.P. Di Marchi and R.B. Merrifield, *Tetrahedron Lett.*, 1981, *22*, 2851.
32. J.P. Tam, *J. Org. Chem.*, 1985, *50*, 5291.
33. G. Breipohl, J. Knolle and W. Stuber, *Tetrahedron Lett.*, 1987, *28*, 5651.
34. F. Albericio, N. Kneib-Cordonier, S. Biancalana, L. Gera, R.I. Masada, D. Hudson and G. Barany, *J. Org. Chem.*, 1990, *55*, 3730.
35. S. Funakoshi, E. Murayama, L. Guo, N. Fujii and H. Yajima, *J. Chem. Soc., Chem. Commun.*, 1988, 382.
36. A. Ajayaghosh and V.N. Pillai, *Proc. Indian Acad. Sci.*, 1988, *100*, 389, (Chem. Abs., *111*:174635m).
37. C. McInnes, PhD Thesis, University of Edinburgh, 1990.
38. I. Irving, personal communication.
39. F.C. McKay and N.F. Albertson, *J. Am. Chem. Soc.*, 1957, *79*, 4686.
40. G.W. Anderson and A.C. McGregor, *J. Am. Chem. Soc.*, 1957, *79*, 6180.
41. R.B. Merrifield, *J. Am. Chem. Soc.*, 1964, *86*, 304.
42. L.A. Carpino and G.Y. Han, *J. Am. Chem. Soc.*, 1970, *92*, 5748.
43. E. Atherton, C.J. Logan and R.C. Sheppard, *J. Chem. Soc., Perkin. Trans. 1*, 1981, 538
44. M. Florence, PhD Thesis, University of Edinburgh, 1987.
45. P. Roach, PhD Thesis, University of Edinburgh, 1990.
46. J. Green, O.M. Ogunjobi, R. Ramage and A.S.J. Stewart, *Tetrahedron Lett.*, 1988, *29*, 4341.
47. B. Castro, J.R. Dormoy, G. Evin and C. Selve, *Tetrahedron Lett.*, 1975, 1219.
48. D. Hudson, *J. Org. Chem.*, 1988, *53*, 617.
49. J. Coste, D. Le-Nguyen and B. Castro, *Tetrahedron Lett.*, 1990, *31*, 205.
50. V. Dourtoglou and B. Gross, *Synthesis*, 1984, 572.
51. R. Knorr, A. Trezciak, W. Bannwarth and D. Gillesen, *Tetrahedron Lett.*, 1989, *30*, 1927.

52. P. Henklein, M. Beyermann, M. Bienert and R. Knorr, 'Peptides 1990', Proceedings of the Twenty First European Peptide Symposium, ed. E. Giralt and D. Andreu, Escom, Leiden, p67.
53. Y. Kiso, T. Kimura, Y. Fujiwara, H. Sakikawa and K. Akaji, *Chem. Pharm. Bull.*, 1990, *38*, 270.
54. R.B. Merrifield, *J. Am. Chem. Soc.*, 1964, *86*, 304.
55. F.C. Westall, J. Scotchler and A.B. Robinson, *J. Org. Chem.*, 1972, *37*, 3363.
56. K. Esko, S. Karlsson and J. Porath, *Acta Chem. Scand.*, 1968, *22*, 3343.
57. L.C. Dorman, *Tetrahedron Lett.*, 1969, 2319.
58. B.F. Gisin, *Anal. Chim. Acta*, 1972, *58*, 248.
59. W.S. Hancock, J.E. Barrersby and D.R.K. Harding, *Anal. Biochem.*, 1975, *69*, 497.
60. G. Losse and R. Ulbrich, *Chem. Abs.*, 1973, *78*, 84822.
61. E. Atherton and R.C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1985,165.
62. W. Konig and R. Geiger, *Chem. Ber.*, 1970, *103*, 2034.
63. E. Atherton, L. Cameron, M. Meldal and R.C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1986, 1763.
64. L. Cameron, M. Meldal and R.C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1987, 270.
65. M.H. Jakobsen, O. Buchardt, M. Meldal and A. Holm, 'Peptides 1990', Proceedings of the Twenty First European Peptide Symposium, ed. E. Giralt and D. Andreu, Escom, Leiden, p192.
66. S.C. Young, P.D. White, J.W. Davies, D.E.I.A. Owen, S.A. Salisbury and E.J. Tremeer, 'Peptides 1990', Proceedings of the Twenty First European Peptide Symposium, ed. E. Giralt and D. Andreu, Escom, Leiden, p313.
67. M. Bodansky and J.T. Sheehan, *Chem. Ind.*, 1966, 1597.
68. A. Dryland and R.C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1986, 125.
69. N. McFerran and B. Walker, *Innovations and Perspectives in Solid Phase Synthesis,* 1990, 261, 1st Int. Symp. Proc. (Epton,R., ed.) SPPC, Birmingham, England.
70. L. Wolff, *Liebigs Ann. Chem.*, 1902, *325*, 152, 162.
71. G.M.F. Jenkins, PhD Thesis, University of Edinburgh, 1983.

72. R. Huisgen and V. Weberndorfer, *Chem. Ber.*, 1967, *100*, 71.
73. G. L'abbé, E. Vanderstede, W. Dehaen, P. Delbeke and S. Toppet, *J. Chem. Soc., Perkin Trans. 1*, 1991, 607.
74. R.K. Bartlett and T.S. Stevens, *J. Chem. Soc., (C)*, 1967, 1964.
75. J.H. Boyer, T. Manimaran and V.I. Ramakrishnan, *J. Chem. Soc. Perkin Trans. 1*, 1987, 2163.
76. A.B. Theocharis, A.J. Maroulis, C.P. Hadjiantoniou-Maroulis and N.E. Alexandrou, *J. Chem. Soc., Perkin Trans.1*, 1989, 619.
77. B.H. Lipshutz and M.C. Morey, *Tetrahedron Lett.*, 1984, *25*, 1319.
78. M.L. Scheinbaum and M.B. Dines, *Tetrahedron Lett.*, 1971, 2205.
79. G. Laus, J. Stadlwieser and W. Kloetzer, *Synthesis*, 1989, 773.
80. K. Volkamer and H.W. Zimmermann, *Chem. Ber.*, 1969, *102*, 4177.
81. F.J. Allan and G.G. Allan, *Chem. Ind.*, 1964, 1837.
82. I.S. Bennett, G. Brooks, N.J.P. Broom, S.H. Calvert, K. Coleman and I. Francois, *J. Antibiotics*, 1991, *44*, 969.
83. F.M. Stojanovic and Z. Arnold, *Collect. Czech. Chem. Commun.*, 1967, *32*, 2155.
84. N.E. Searle, *Org. Synth.*, 1955, *36*, 25.
85. Z. Arnold and A. Holy, *Collect. Czech. Chem. Commun.*, 1962, *27*, 2886.
86. L. Jiang, personal communication.
87. D. Maclean, PhD Thesis, University of Edinburgh, 1991.
88. A. Kelly, personal communication.
89. K. Shaw, personal communication.
90. D.D. Perrin, W.L.F. Armarego and D.R. Perrin, 'Purification of Laboratory Chemicals,' Pergamon Press, Oxford, 2nd Edition.
91. W.H. Hartung and F. Crossley, *Org. Synth.*, 1936, *16*, 44.
92. H.H. Bosshard, R. Mory, M. Schmid and H. Zollinger, *Helv. Chim. Acta,* 1959, 1653.
93. H. Metzger, 'The Preparation and Transformation of Oximes ' in Houben-Weyl, ed. E. Muller, Springer-Verlag, Stuttgart, 1968, *10/4*, 35.
94. L. Claisen and O. Manasse, *Chem. Ber.*, 1887, *20*, 2194.

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Discovery, Development and Pharmacology of Zoladex for Treatment of Prostate Cancer
(various speakers, ICI Pharmaceuticals).

Peptide and Protein Group of the Biochemical Society and Royal Society of Chemistry
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