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Special features of the synthesis of peptides containing secondary amino acids

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ABSTRACT. Features of the condensation reactions of secondary amino acid residues using various methods for the activation of the carboxyl component are discussed. Problems connected with racemisation and side reactions in the synthesis of peptides containing secondary amino acids are considered.

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I. Introduction

In the present review the term secondary amino acid is taken to mean Pro and *N*-substituted amino acids in which the substituent is an alkyl radical (methyl in the majority of cases).†

Until recently the use of secondary amino acids in peptide chemistry has fluctuated and has been limited primarily to Pro, the amino group of which is the least hindered. However because of the spread of attention to this area of chemistry, other secondary amino acids have become involved in condensations and the experimental data presently available enable certain general conclusions to be drawn on the main features and peculiarities of the use of secondary amino acids in peptide synthesis.

When considering the features of condensation at a secondary amino acid residue it is necessary to consider separately the acylation of the secondary amino group of these amino acids and their derivatives since condensations in which secondary amino acids participate as the carboxy component are no different in practice from those of the usual amino acids. Only the most general features of the latter variant will therefore be given.

The presence of bulky side chains in amino acid carboxy components leads to a decrease in the rate of condensation.[2] An alkyl substituent on the nitrogen atom of a secondary amino acid generates additional steric hindrance to the formation of the peptide bond but this effect is less significant than the influence of the side chain. In the most common cases of activation the presence of a substituent on the nitrogen atom in *N*-methylamino acids and in Pro has practically no effect on the yield of product.[3]

The presence of any protecting group on the *N*-alkylamino group of a carboxyl component begins to show an appreciable influence on the reactivity of the carboxyl group only in cases of the sterically most hindered condensations and the best yields are achieved when using Z and Fmoc rather than a Boc protected amino group.[4]

II. Methods of acylating secondary amino acids and their derivatives

In spite of the increased nucleophilicity of a secondary amino group (the pK of which is 1–1.5 units higher than for an amino group[5]) which must aid acylation, the duration of condensations in these cases is sharply increased and yields of peptides are reduced. This is explained by the shielding of the nucleophilic centre which is displayed to the smallest extent by Pro and increases with the increase in size of the *N*-alkyl substituent. For example in the synthesis of tripeptide fragments of an enkephalin analogue yields are reduced from 70 to 40% on increasing the size of the radical R.[6,7]

$$\text{Boc—D-Ala—Gly} + \text{(N-R)Phe(4-F)—NH}_2 \xrightarrow{\text{DCC/OBT}} \rightarrow \text{Boc—D-Ala—Gly—(N—R)Phe(4-F)—NH}_2$$

R = Me, Et, cyclo-Pr.

The ability of secondary amino acids to be acylated is also reduced with an increase in the bulk of the side chain and by the presence of branching at the α- and β-carbon atoms.[5] Thus steric factors play the chief role in the acylation of secondary amino acid residues and this must be considered when choosing variants of the condensation reaction.

Until recently special methods had not been developed for acylating secondary amino acid residues and traditional acylating reagents were used for this, such as active esters and symmetrical or mixed anhydrides of amino acids. The carbodiimide method was also used for the activation of the carboxyl component.

† Abbreviations used are those recommended by the IUPAC–IUB Commission[1] and also the following: Abu—α-aminobutyric acid, Aib—α-aminoisobutyric acid, BOP—[(benzo-triazol-1-yl)oxy]-tris(dimethylamino)phosphonium hexafluorophosphate, BOP–Cl—bis(2-oxo-3-oxazolidinyl)phosphinic chloride, t-Bu—t-butyl, Lac—lactic acid, (*N*–Me)Bmt—(4*R*)-4[(*E*)-2-butenyl-4,*N*-dimethyl-*L*-threonine, HOBT—*N*-hydroxybenzotriazole, HOSu—*N*-hydroxy-succinimide, Piv–Cl—pivaloyl chloride, SPip—thiopipecolic acid, DKP—diketopiperazine, DCC—dicyclohexylcarbodiimide, THF—tetrahydrofuran.

1. Active ester method

To effect nucleophilic attack on the carbonyl carbon atom of a carboxyl component by a secondary amino acid the carboxyl group must be well activated.

$$R^2{-}NH{-}CHR^1{-}COOX + R^3{-}NH{-}CHR^4{-}COOY \rightarrow$$
$$\rightarrow R^2{-}NH{-}CHR^1{-}CON(R^3){-}CHR^4{-}COOY,$$

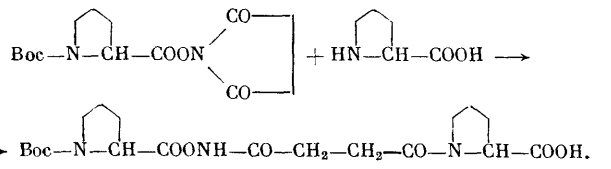
where R^1 is the side chain of the carboxy component, Y and R^2 are protecting groups, R^3 is alkyl, R^4 is the side chain of the amino component and X is an electron-accepting group.

Pentafluorophenyl esters are used fairly successfully for the acylation of secondary amino acids, particularly Pro.[8,9] If the condensation is conducted with the least sterically hindered residues Pro or (*N*-Me)Ala then from 1 h to 1 d is required for complete reaction, although the reaction time is 5–30 min when condensing at a primary amino group by this method which is comparable with the time for acylation with anhydrides.

The electron-accepting properties of pentachloro- and pentafluorophenol are clearly linked with their pK values (5.2 and 5.5 respectively) and determine the reactivity of the corresponding activated esters. However, although pentachlorophenol is a stronger acid as a result of its weaker + *M* effect,[5] the ratio of the aminolysis rates for a primary amino group by pentachloro- and pentafluorophenyl esters is 1:40. This is linked with the steric hindrance of the electrophilic centre by the chlorine atoms in the *ortho* position, since fluorine atoms having a small atomic radius do not cause such hindrance.[5] Thus the application of pentachlorophenyl esters for the acylation of amino acids is of doubtful expedience.

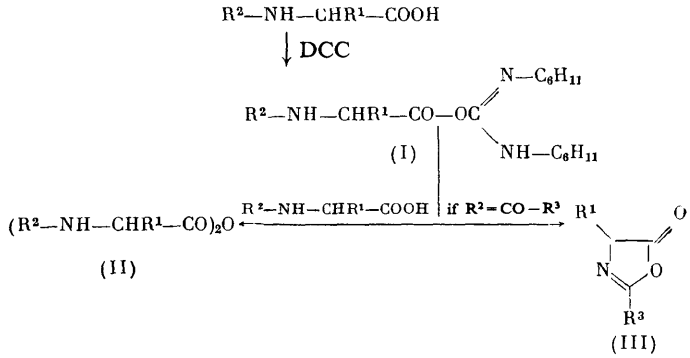
2,4,5-Trichlorophenyl esters of amino acids acylate (*N*-Me)Ala and Pro residues satisfactorily on catalysis by HOBT.[9,10] It is not excluded that HOBT esters are the real acylating reagents in this case.

When using HOSu esters, which are insufficiently reactive, a side reaction of succinoylating secondary amino acid residues is observed.[11–13]



2. Carbodiimide method

DCC was introduced into peptide synthesis in the mid-fifties.[14] When reacting equimolar quantities of DCC and a urethane-protected amino acid a mixture is formed of an *O*-acylurea (I) and a symmetrical anhydride (II).



The anhydrides (II) acylate secondary amino acid residues fairly readily but the efficiency of an *O*-acylurea (I) as an acylating reagent for secondary amino acids has not been assessed. When urethane protection is present on the amino group of the carboxy component, racemisation is reduced to a minimum and the use of the DCC method usually leads to a good yield on extended (days or longer) reaction at temperatures from 0 to 20 °C.[3,15–18] Other conditions being equal, the activation of the carboxy component with DCC leads to a higher yield than on using 1-ethyl-3-(dimethylamino)-propylcarbodiimide.[19] When activating fragments not containing *C*-terminal Pro or *N*-acylaminoacids having no urethane protection by the DOC method, an oxazolone (III) may act as acylating agent and its efficiency in relation to secondary amino acids is also unclear.[11]

Recently activation of the carboxy component with DCC has frequently been carried out in the presence of HOBT or HOSu due to numerous side reactions and racemisation. This procedure suppresses the formation of *N*-acylureas and leads to minimum racemisation of the amino acid residue. The duration of acylation is unchanged by this.[20] Many examples of the use of the DCC/HOBT method have been described in the literature for the acylation of secondary amino acids in satisfactory and good yield.[6,7,9,13,21] However for the reaction of Piv–Pro with (*N*-Me)Phe–OMe and Boc-*D*-Ala–(*N*-Me)Leu–(*N*-Me)Leu with (*N*-Me)Val–OBzl the DCC/HOBT method does not lead to the formation of the required product and in the latter case the reaction does not go even in boiling THF.[2,22]

The DCC/HOSu method is not used as frequently as the DCC/HOBT method for the acylation of secondary amino acid residues but there are examples of its fairly successful use. Thus the yield was 96% in the reaction of Boc–Ala with (*N*-Me)Leu–OBzl when using DCC/HOSu and was 82% in the reaction of Z–Pro with Sar–(*N*-Me)Val–OBu^t.[3,23] On the other hand the yield was 10% on condensing Z–Val with (*N*-Me)Leu–OBu^t and 2% overall on using preformed Z–Val–OSu. Under analogous conditions the DCC/HOBT method gave 50% yield.[13] There are numerous data on the acylation of the sterically less hindered Pro residue by this method when yields were 60 to 80% at 20 °C with a reaction time of 20–48 h.[24–26] The use of the DCC/HOSu method turned out to be unsuccessful for the reaction of Z–Arg(Z_2)–Arg($Adoc_2$) with Pro–Pro–Lys(Boc)–Gly.[27] It has also been shown that the DCC/HOBT method gives fewer side products than the DCC/HOSu method.[21]

3. Symmetrical anhydride method

This method is a variant of the DCC method, a double excess of carboxy component being involved in reaction with DCC. The *O*-acylurea (I) formed first reacts with the excess of carboxy component and is converted into a symmetrical anhydride (II). At the present time the symmetrical anhydride method is used mostly in solid phase synthesis and frequently gives good results when making peptides containing secondary amino acids. Thus an analogue of substance P containing (*N*-Me)Phe and Sar was obtained in 60% yield.[28] However in this case a single acylation of the secondary amino acid residue requires no less than 16 h reaction time in place of the more usual 30 min to 1 h.[5,28] An increase in the reaction temperature gives an increase in the reaction rate and in the yield of the desired product. On heating Boc–Pro anhydride with *D*-(*N*-Me)Leu–OMe to 130 °C for 5 min the dipeptide was obtained in 97% yield. The product was contaminated by the epimer according to NMR spectroscopy.[19]

A serious limitation to the carbodiimide method is the impossibility of obtaining anhydrides from peptide fragments and from amino acids without urethane protection although they are formed from other *N*-acyl substituted derivatives of Pro and possibly of other secondary amino acids.[11] The symmetrical anhydride method also gives a whole range of side products linked with the use of DOC in peptide chemistry.

4. Mixed anhydrides with alkylcarbonic acids

The method of mixed anhydrides with alkylcarbonic acids is the most frequently used of the traditional methods of forming peptide bonds for acylating secondary amino acids.[20]

$$Z-NH-CHR^1-CO-O-COOR^2 + R^3-NH-CHR^4-COY \rightarrow$$
(IV) (V)

$$\rightarrow Z-NH-CHR^1-CON(R^3)-CHR^4-COY + R^2-OCON(R^3)-CHR^4-COY$$
(VI) (VII)

In spite of the high reactivity of the anhydrides (IV) the yield of peptide (VI) is frequently less than when the carbodiimide method is used. The main reason for this is the presence in the carboxy component (IV) of two reaction centres and the ambiguous nature of the reaction as a result. The preferred direction of attack depends on the nature of the substituents R^1, R^2 and R^3. The presence of groups R^1 and R^3 hinder the formation of peptide (VI) more strongly the greater their molar volume and degree of branching.[29,30] In some cases the mixed anhydride method is not generally suitable. For example in the reactions of Boc–*D*-Ala–(*N*-Me)Leu–(*N*-Me)Leu with (*N*-Me)Val–OBzl (–20 °C, 26 h),[22] Boc–Phe with a fragment containing *N*-terminal (*N*-Me)Ala [9] and Boc–Ile with Pro–Leu–Ser(Bzl)–Arg(NO_2)–Leu–Phe–OMe [31] it did not lead to the formation of the desired products.

A branched group R^2 increases the electron density on the carbonyl carbon atom of the alkylcarbonic acid residue and hinders it to some extent which reduces the possibility of attack on the latter by the secondary amino group. The acylation of secondary amino acids using mixed anhydrides of isobutylcarbonic acid (IV, R^2 = iso-Bu) is preferred to the use of anhydrides of ethylcarbonic acid (IV, R^2 = Et).[20] Thus when acylating a tripeptide containing an *N*-terminal Pro residue with a mixed anhydride of ethylcarbonic acid the urethane EtOC(O)–Pro–Lys(For)–Asp was obtained in 61% yield.[32] The use of anhydrides of isobutylcarbonic acid is also accompanied by the formation of the urethanes (VII),[33–35] and their yield depends on the choice of solvent in which the condensation is carried out and on the types of base used. For the reaction of Z–Gly–Phe with Pro–OMe the yield of urethane increases from 13 to 33% along the series THF, chloroform, DMF.[36] A minimal quantity of urethane is formed when using *N*-methylmorpholine or *N*-Methylpiperidine on obtaining the anhydrides (IV).[37]

The molecular weight of both the secondary amino and the carboxy component have a significant influence on the yield of product.[20] For example the yields on fragment condensation of Boc–*D*-Ala–Gly and Boc–Tyr–*D*-Ala–Gly with (*N*-Me)Phe–Met-ol were 70 and 50 % respectively.[38]

In spite of the relative stability of pure mixed anhydrides (IV) at temperatures up to 25 °C, they disproportionate with the formation of symmetrical anhydrides in the presence of even traces of base, and the amino component itself may cause this.[22,39] To suppress this process the condensation is carried out at –20 to –15 °C, but to increase the yield it is desirable to carry out the reaction at a higher temperature.

Of all the above reactants the symmetrical anhydrides are the most acceptable for the acylation of secondary amino acids but for the most sterically hindered condensations of the type of Boc–(*N*-Me)Val with (*N*-Me)Val–OBu^t, even they are ineffective.[2]

5. Mixed anhydrides with pivalic acid and phosphorus acids

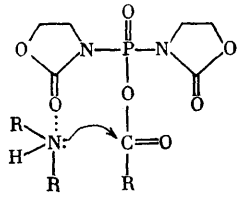
At the present time the mixed anhydrides obtained from Piv–Cl and BOP–Cl are the best known and most widely used reagents for acylating secondary amino acids. In particular they have been used successfully in the synthesis of the cyclic undecapeptide cyclosporin A and its analogues which contain seven secondary amino acids.[22,40–42]

Piv–Cl was first used in peptide chemistry in 1962.[43] However the mixed pivalic anhydride method has not been widespread in usual condensations seemingly due to the possibility of forming *N*-pivaloyl peptides.[20] For example in the reaction of the acetonide of (*N*-Me)Bmt with Abu–Sar–(*N*-Me)Leu–Val–(*N*-Me)Leu–Ala–OBzl a mixture was obtained of heptapeptide and *N*-pivaloyl hexapeptide in a 1 : 1 ratio.[42] It was later shown that the formation of *N*-pivaloyl peptides does not occur when acylating secondary amino acids with mixed pivalic anhydrides.[22] Other virtues of this method are the high acylating ability of the anhydride and its thermal stability up to 50 °C even in the presence of triethylamine and *N*-methylmorpholine which enables the most sterically hindered condensations to be effected.[2,22]

Some authors[2,44] regard the significant duration of the stage of forming the mixed pivalic anhydride in chloroform a disadvantage of this method. It is recorded that the yield of the desired peptide depends markedly on the duration of the activation. Thus for the reaction of Boc–*D*-Ala–(*N*-Me)Leu–(*N*-Me)Leu with (*N*-Me)Val–OBzl the yield of tetrapeptide is 60% when activating the carboxyl component with Piv–Cl for 2 h and 88% for 4 h.[22] However on carrying out the activation in THF the formation of the mixed pivalic anhydride is complete after 5–20 min at –20 °C.[13,45,46]

Activation of secondary amino acids with urethane protection with Piv–Cl is not accompanied by racemisation even at elevated temperature. Only in the case of the extremely sterically hindered condensation of Z–(*N*-Me)Val with (*N*-Me)Val–OBu^t for 24 h at 50 °C was 6% epimer detected in the dipeptide.[2] Fragment condensations using Piv–Cl are accompanied by racemisation, the extent of which depends on a large number of factors.[2,22,40]

In 1980 BOP–Cl was proposed as a reagent for the synthesis of esters and other derivatives of carboxylic acids.[47] Its high efficiency when forming peptide bonds at residues of secondary amino acids was shown for the first time in 1985.[4] A mechanism of electrophilic catalysis at the condensation stage has been proposed to explain this effect.[2]



It has been recorded that BOP–Cl is a more effective reagent than Piv–Cl for carrying out sterically very hindered condensations.[2,4] Thus in the reaction of the mixed anhydride of Z–(*N*-Me)Phe and pivalic acid with (*N*-Me)Val–OBu^t a positive result is achieved only at enhanced temperature while BOP–Cl

enabled preparation of the product in 92% yield after 48 h at 20 °C in this condensation.[2] The difference in yields of product when using BOP–Cl or Piv–Cl is reduced with a reduction in steric limitations. Thus in the reaction of Z–Pro–(*N*-Me)Phe with Ala–Bu^t both reagents lead to the formation of tripeptide in 85% yield but in this condensation racemisation was 38% with Piv–Cl and on using BOP–Cl it was 64%.[2] This ratio is less in other examples. Usually the use of BOP–Cl is accompanied by less racemisation than the use of Piv–Cl in fragment condensations.[2] This is possibly explained by the high reactivity of the mixed anhydride of the carboxyl component with the phosphoric diamide compared with its mixed pivalic anhydride, consequently the long condensation time in the second case leads to an increase in the extent of racemisation of the carboxy component.[48]

One further advantage of BOP–Cl is the possibility of using it as a condensing reagent in a one-pot synthesis without preactivating the carboxy component as a separate stage. The analogous method using Piv–Cl leads to the formation of up to 40% *N*-pivaloyl peptide,[22] which is explained by the lower reactivity of BOP–Cl towards an amino group compared with carboxylate anion.[49] However the best results of all were achieved by carrying out the preparation of the mixed anhydride of the carboxy component and the diamidophosphoric acid as a separate stage since otherwise some contamination by the BOP-amino component is formed.[2]

Carrying out sterically hindered condensations using BOP–Cl, as for Piv–Cl, may be accompanied by a little racemisation of the carboxy component even in the presence of urethane protection of its amino group. For example 4% epimer was obtained in the reaction of Z–(*N*-Me)Val with (*N*-Me)Val–OBu^t.[8] In this case it is possible to suppress the racemisation by the addition of HOBT but not HOSu.[2] However on adding HOBT the overall yield of dipeptide was sharply reduced (from 92 to 21%). This fact is readily explained by the formation of an HOBT ester of the carboxy component caused by steric factors. This ester is a weak acylating agent in such difficult condensations.

It is also interesting to record the BOP–Cl proved to be ineffective in condensations of *C*-α-methylamino acids. For example after carrying out the reaction of Boc–Aib with Aib–OBzl none of the desired product was detected.[4]

It is possible to regard the possibility of disproportionation of such mixed anhydrides as being a drawback of the use of BOP–Cl for the activation of both secondary and primary amino acids, particularly at long reaction times.[2,4] The relatively poor solubility of BOP–Cl in certain organic solvents, especially those used in peptide synthesis, should also be noted.[41]

It is also possible to use BOP–azide, which is a fairly stable compound, in place of BOP–Cl for the activation of the carboxy component. When carrying out preactivation with BOP–azide (10 min, 0 °C) in the condensations of Boc–Ala with (*N*-Me)Phe and Z–Phe with (*N*-Me)Ala–OMe the yield of dipeptide was 60% after 4 h.[50]

The use of diethyl phosphorocyanidate as an activating reagent has been described. In the condensation of Boc–Pro with *D*-(*N*-Me)Phe–*D*-Ala–Val–Ant–OMe (2 h, 0 °C, then 24 h, 20 °C) the pentapeptide was formed in 89% yield.[51]

Data have appeared recently on the use of diphenyl phosphinic chloride as a highly efficient activation reagent for the acylation of secondary amino acid residues.[13,52–55] The advantage of this reagent over BOP–Cl was shown in the activation of both primary and secondary amino acids in the synthesis of cyclosporin fragments.[54] The use of diphenyl phosphinic chloride for the activation of a carboxy component with a urethane protected amino group was not accompanied by racemisation of the latter but the process of forming the mixed anhydride was analogous to activation using Piv–Cl.[53,54] An advantage of diphenyl phosphinic chloride is the speed of formation and the stability towards disproportionation of mixed anhydrides made from it.[48,56]

Of the new condensing reagents mention must be made of benzotriazolyloxy-tris(dimethylamino)phosphonium hexafluorophosphate with which a fragment condensation at an (*N*-Me)Bmt residue has been effected without racemisation of the carboxyl component.[40,57]

Other condensation methods have occasionally been used for secondary amino acid residues. Thus a new reagent was proposed recently, viz. 1,2-dihydro-4,6-dimethyl-2-thiooxo-3-pyridinecarbonitrile, and was used in the synthesis of a protected (8–11)cyclosporin fragment without racemisation and with yields at the condensation stages of 80–87%.[58] It is interesting that this reagent may be used for building up a chain both by traditional activation of a carboxyl group or by activation of the amino group of an amino component. To effect condensation in this case it is also necessary to have an equimolar quantity of triphenylphosphine present.[59]

Occasionally the azide method is used for fragment condensations at a Pro residue.[59] The *N*-carboxyanhydride method gives poor results for similar condensations.[60] Good results were recorded using Woodward's reagent K in solid phase synthesis for the acylation of secondary amino acids.[61]

III. Side reactions caused by the presence of secondary amino acids in peptides

In addition to the peculiarities described above, complications also arise when obtaining esters and amides of secondary amino acids and on removing protecting groups during peptide synthesis. All these processes proceed with significantly more difficulty or are accompanied by side reactions which are absent when working with unsubstituted amino acids.

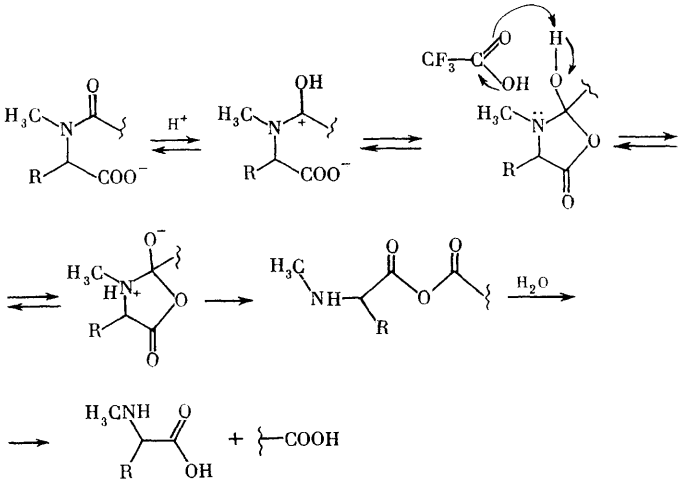
For example Sar–OMe is obtained in only low yield when using a solution of thionyl chloride in methanol while both Gly and many other amino acids give good yields of methyl esters under these conditions.[62] The t-butyl ester of Val is obtained in quantitative yield after 4 h at 20 °C when using isobutene in the presence of sulphuric acid. Under the same conditions the reaction of (*N*-Me)Val is incomplete even after 96 h.[41]

The saponification of alkyl esters of secondary amino acids proceeds with significantly more difficulty than in the case of the corresponding derivatives of unsubstituted amino acids. For example under identical conditions the rate of saponification of Sar–OMe is five times slower than for Gly–OMe,[62] and the hydrolysis of Lac–Pro-*D*-(*N*-Me)–Leu–OMe even with a three-fold excess of sodium hydroxide proceeds only to 90% after 2 h.[19] It has been reported that it is impossible to obtain the hydrazide of Bz–(*N*-Me)Val from its methyl ester by reaction with hydrazine hydrate.[21] Similarly the aminolysis of alkyl esters of secondary amino acids is difficult, consequently their amides are obtained by treating an activated ester or mixed anhydride of the protected secondary amino acid with an amine.[6,7,63] Occasionally the roundabout method of obtaining the amide of an amino acid with subsequent alkylation of its amino group is used.[6,7] A similar approach is applied to the synthesis of fragments consisting only of

secondary amino acids. For example on treating the tripeptide Boc–Leu–Leu–Val–OBzl with methyl iodide in the presence of silver oxide, methylation of the nitrogen atoms of all three amino acid residues occurs.[44] Milder conditions are used to prevent methylation of the urethane nitrogen atom (CH_3I, K_2CO_3, 18-crown-6, DMF, 20 °C).[64] Such a method of synthesis of tripeptide sequences of secondary amino acids enables prevention of the formation of cyclic dipeptides (DKP) in a 1 + 2 cocondensation.[44]

Data have recently appeared on the ready fission of the peptide bond on acid treatment of peptides containing sequences of two or more secondary amino acids.[65,66] Two mechanisms have been proposed by which such fission may occur. The first of these, in which the hydrolysis process does not depend on acid strength in the medium in which it occurs, has acquired the name exopeptolysis. The second, which depends on acid strength, is called endopeptolysis. Both mechanisms assume a ready transition of the peptide bond into the *cis* form and the spatial approach of the appropriate portions of the molecule.

Exopeptolysis may be illustrated by the example of the dipeptide Tfa–(*N*-Me)Phe–(*N*-Me)Ala which is split 29% at the peptide bond in a medium of formic acid after 120 h at 20 °C and 21% in trifluoroacetic acid under the same conditions.[65] At the same time the alkyl esters of this dipeptide are stable in trifluoroacetic acid.[65,66] The following mechanism has been proposed for exopeptolysis.[65]



Endopeptolysis is characteristic of tripeptides made up of secondary amino acids through the *N*-terminal position may be occupied by a Gly residue. The peptide bond between the second and third secondary amino acids is readily split in acid medium in such tripeptides, particularly in cases when the *N*-terminal secondary amino acid is Pro.[66] The rate of hydrolysis of such tripeptides depends sharply on the strength of the acid in which the reaction is effected, i.e. trifluoroacetic ≫ formic ≫ acetic acid. The sensitivity of tripeptides towards acidic fission is proportional to the steric strain about the amide bond between the second and third secondary amino acids, with the exception of the case when the first two positions of the triad are occupied by Pro residues.[66]

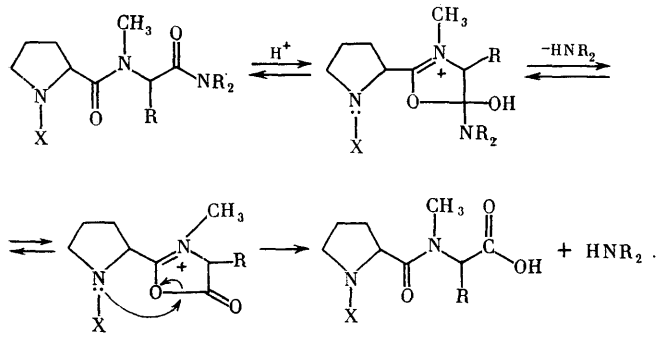
Z–Pro–(N-Me)Phe–SPip > Z–Pro–(N-Me)Phe–(N-Me)Ala,
Z–Pro–(N-Me)Phe–(N-Me)Ala–OMe > Z–Val–(N-Me)Phe–(N-Me)Ala–OMe, Z–Val–(N-Me)Phe–(N-Me)Ala,
Z–Pro–(N-Me)Phe–Ala–OMe ≫ Pro–(N-Me)Phe–NR_2 > > Pro–Pro–Pro, Pro–Pro–SPip, Pro–Phe–NR_2.

In certain cases the hydrolysis goes so easily that when deblocking the tripeptide Z–Abu–Pro–(*N*-Me)Phe–SPip–OBu^t fission of the *C*-terminal secondary amino acid is effected more rapidly than removal of the t-butyl protection. Endopeptolysis is difficult if the *N*-terminus in the triad is an unsubstituted secndary amino acid or the dipeptide Pro–Pro. If the sequence consists of alternating *L* and *D* isomers of secondary amino acids (*L*, *D*, *L*) then the rate of endopeptolysis is two orders of magnitude lower than for sequences containing only *L* isomers. Thus the tripeptide Z–Pro–*D*-(*N*-Me)Phe–(*N*–Me)Ala–OBu^t does not decompose in the presence of trifluoroacetic acid for several days but its *L*–(*N*-Me)Phe analogue is completely decomposed under the same conditions in 15 min.[2] The dipeptide Z–Pro–*D*-(*N*-Me)Phe–NMe_2 remains unchanged in trifluoroacetic acid for days while its *L* analogue loses the NMe_2 group completely under the same conditions after 14 h.[66] This may be explained by the fact that the energy barrier for the transition of a *trans* amide bond into the *cis* configuration for an *L*-, *D*-pair of amino acids in the triad is greater than for an *L*-, *L*-pair. It may therefore by considered quite definite that conformational factors plays a most important role in the stability of the peptide bond between secondary amino acids.

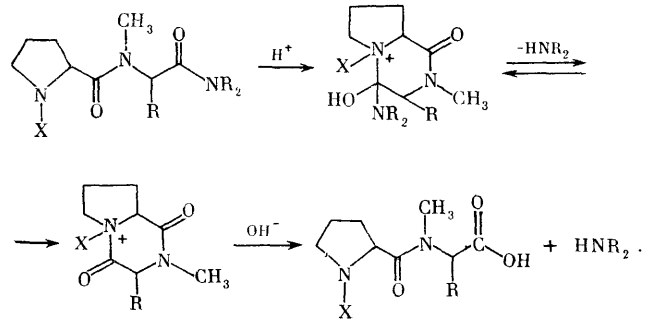
Two possible mechanisms have been proposed for the endopeptolysis reaction (the second is preferred) (see Scheme).[66]

Scheme

Oxazolonium



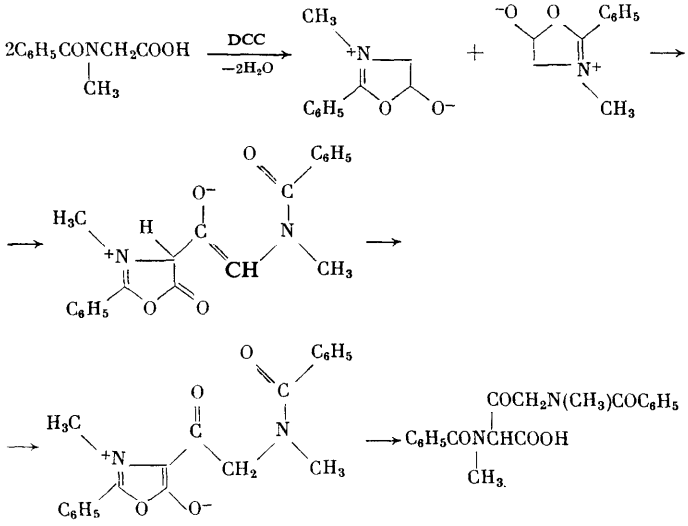
Diketopiperazine



Similar processes were observed in the synthesis of cyclosporin when for example the deblocking of the undecapeptide Boc–*D*-Ala–(*N*-Me)Leu–(*N*-Me)Leu–(*N*-Me)Val–(*N*-Me)Bmt–Abu–Sar–(*N*-Me)Leu–Val–(*N*-Me)Leu–Ala with a solution of trifluoroacetic acid in chloroform at 0 °C with subsequent neutralisation of the excess of acid led to a complex mixture of products among which were peptides of shortened sequence. The

avoidance of side reactions in this case was successful if the deblocking temperature was reduced to −20 °C.[40,42]

The side reaction of *C*-α-acylation of the Sar residue, observed on using the carbodiimide method, is also effected by an oxazolonium mechanism.[67]



The ease of forming DKPs from certain peptide derivatives is an extremely pressing problem when using secondary amino acids in peptide synthesis. This concerns primarily condensation reactions where a dipeptide ester containing a *C*-terminal secondary amino acid acts as the amino component. In this case the energies of the *cis* and *trans* configurations of the peptide bond are close and for the formation of DKPs it is necessary to overcome a significantly lower energy barrier for the *trans*−*cis* transition (since DKPs exist only as the *cis,cis* isomers) than in the case of a dipeptide containing no secondary amino acids.[3,5,20,68] The formation of DKPs in the case under consideration is most frequently catalysed by bases, especially by an excess of tertiary amine, frequently present in the reaction medium.[5,68,69] The rate of formation of a DKP depends on several factors except in the presence of a catalyst. The presence of side chains and radicals on the nitrogen atom of the secondary amino acids forming part of the dipeptide gives an energy advantage to the formation of the six-membered DKP ring due to the entropy factor. Steric hindrance has great importance in the nucleophilic attack on the carbonyl carbon atom by the amino group, and arises particularly in the presence of a side chain on the *N*-terminal amino acid.[68,69]

The rate of cyclisation of dipeptide esters consisting of two secondary amino acids is fairly high seemingly due to the enhanced nucleophilicity of the secondary amino group.[11] Thus the formation of DKP from the dipeptide (*N*-Me)Leu−(*N*-Me)Val−OBzl was the main obstacle in the synthesis of the (7−11) cyclosporin fragment when extending the peptide chain from the *C*-terminus.[22] The process of forming DKPs may not only compete with peptide synthesis but may completely exclude it when using a dipeptide ester as the amino component. This process is obviously particularly dangerous when using esters of dipeptides consisting of two secondary amino acids as the amino component since in this case the condensation time grows and there is a fairly high rate of DKP formation.

The presence of a side chain on the *C*-terminal secondary amino acid seemingly does not have an appreciable effect on the rate of DKP formation while the nature of the tertiary amine and the solvent in which the condensation is carried out influence it appreciably.[68,69]

The problem of DKP formation from dipeptide esters with a *C*-terminal secondary amino acid is a characteristic not only of synthesis in solution but also for the solid phase method of peptide synthesis based on chloromethylated resin. In the case of such a polymeric support DKPs are readily formed from the protected dipeptide benzyl esters at the stage of neutralising the dipeptidylpolymers with tertiary amine after deblocking.[70] In a similar situation, when using Sephadex LH-20 as the polymeric support, formation was also recorded.[71]

Furthermore it was observed that on using the DCC condensation method in solid phase synthesis on chloromethylated resin, DKPs were also formed at the stage of equilibrating the dipeptidylpolymer with a solution of the next amino acid.[72] In this case acid catalysis of DKP formation occurs and has been confirmed on numerous occasions in the classical synthesis of peptides. DKPs are formed most frequently when removing protecting groups from dipeptide esters with acids having pK_a 3−5. This effect is observed in the removal of a Z group by hydrogenation in solutions containing acetic acid or, for example, on removing 2-(4-biphenylyl)propyl-2-hydroxycarbonyl protection with formic acid.[18,73] The mechanism of such catalysis is obviously similar to the general acid catalysis mechanism.

It must also be noted that there is a danger of forming DKPs from dipeptide esters with a *C*-terminal secondary amino acid when removing Fmoc protection by the action of such strong organic bases as piperidine or diethylamine.[74,75] When using 4-(aminomethyl)piperidine in this situation 10−20% DKP is formed even from benzyl esters of dipeptides containing no secondary amino acid.[74]

If a phenacyl ester of a dipeptide is introduced into the condensation reaction instead of an alkyl or benzyl ester then the probability of DKP formation is increased.[5] Consequently to prevent this process it is recommended to use tertiary butyl esters of dipeptides.[3,22]

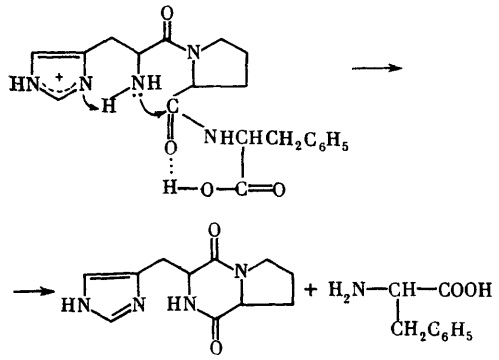
In certain cases cyclisation to a DKP may occur even with *N*-protected peptides. For example the 4-nitrophenyl ester of the dipeptide Z−Gly−Pro forms an (*N*−Z)-substituted DKP and in the course of condensing Z−(*N*-Me)Val with (*N*-Me)Val−OBu^t when using BOP−Cl, and particularly when using Piv−Cl at increased temperature, up to 10% of the corresponding DKP was obtained the mechanism of forming which is unclear.[2,75]

The DKP cyclo[(*N*-Me)Phe−(*N*-Me)Ala] is formed from the tripeptide cinnamyloxycarbonyl−Pro−(*N*-Me)Phe−(*N*-Me)Ala−OMe during column chromatography on silicagel and substituted Pro is split off.[76]

Although it is considered that trifluoroacetates and especially hydrochlorides of dipeptide esters consisting of any amino acids are completely stable,[72] the trifluoroacetate of the dipeptide (*N*-Me)Phe−(*N*-Me)Ala−OMe is converted into the DKP on storage in chloroform solution.[76]

It is also known that amides of dipeptides containing a *C*-terminal secondary amino acid are inclined to DKP formation.[77,78] For example, the dipeptide Phe(4-NO_2)−Pro−NH_2 forms the DKP during recrystallisation.[78] Individual cases of similar transamidations have been recorded when obtaining certain tripeptides. When treating the tripeptide His−Pro−Phe with 50% acetic acid fission of Phe occurs and the DKP (VIII) is formed after 24 h at 20 °C. Under the same conditions the tripeptide

Trp–Pro–Phe is stable and no traces of decomposition were detected after 24 h. The methyl ester of the first tripeptide is between these compounds in stability. In the opinion of the authors[79] the instability of the tripeptide His–Pro–Phe is explained by a combination of conformational factors favouring DKP formation and a so-called intramolecular catalysis.[79]



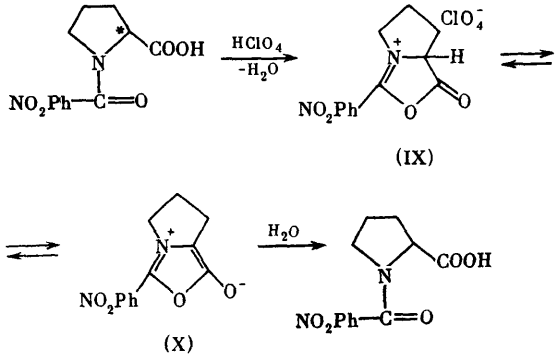
(VIII)

Intramolecular transamidation has also been described in the tripeptide *D*–Val–Pro–Sar which is 50% decomposed on storage for 3 weeks at 20 °C. It was reported that no sign of decomposition was detected for the hydrochloride of this peptide under the same conditions.[80]

The tripeptide Aib–Pro–Trp also shows an increased inclination towards intramolecular transamidation.[81]

IV. Racemisation of secondary amino acids in peptide synthesis

Proline is a unique secondary amino acid, widely distributed in nature, and has been used in peptide synthesis for a long time. Equally with Gly, Pro plays an important role as a *C*-terminal residue in fragment condensations which is linked with its high stability towards racemisation in activation processes and removal of protecting groups.[3,82] This was explained previously by the impossibility of effecting an oxazolonium mechanism of racemisation for its *N*-acyl derivatives because of the absence of a hydrogen atom on the tertiary nitrogen atom. However it was found in 1972 that *N*-acyl substituted Pro and other secondary amino acids may give the stable oxazolonium salts (IX) on treatment with perchloric acid.[67,83]

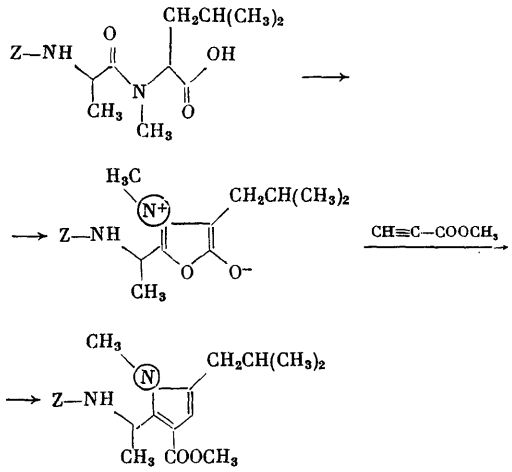


(IX)

(X)

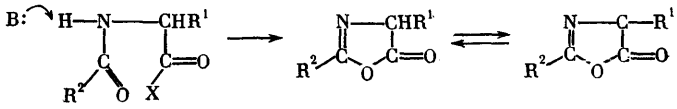
Complete racemisation of the Pro residue took place in 1 h at 20 °C on storing the salt (IX) in the presence of anhydrous perchloric acid. This process is connected with the equilibrium in the oxazolinium ion (IX)–mesoionic oxazolone (X) system, since it was shown that on precipitating salt (IX) from the reaction medium with dry ether no racemisation occurred on storing it in the dry state.[67] These facts serve to confirm the possibility of racemising *N*-acyl-Pro according to the oxazolonium mechanism but the formation from it of the mesoionic oxazolone (X) is difficult due to conformational restrictions and occurs only in the presence of strongly acidic reagents (perchloric acid). The extent of the structural limitations for other secondary amino acids is far lower than for Pro and the formation of the mesoionic oxazolone and consequent isomerisation is observed under the mild conditions of peptide synthesis. In addition their susceptibility towards racemisation according to the oxazolonium mechanism is extremely high.

The formation of the mesoionic oxazolone (X) is accompanied by the appearance of a rapidly disappearing bright yellow colouration of the reaction mixture (absorption λ_{max} = 400 nm). Subsequently its formation was successfully confirmed by reaction with the 1,3-dipolarophile acetylenecarboxylic acid.[3]



In 1981 NMR signals for the mesoionic oxazolone (X) were recorded on reacting Z–(*N*-Me)Val and Z–(*N*-Me)Ala with DCC and it was established that this ion is stable in deuterochloroform at −20 to −5 °C for 3 h.[21]

The formation of 5(4H)-oxazolones on activation of *N*-acylamino acids is explained by base catalysis but in the case of *N*-acyl secondary amino acids acid catalysis of the formation of the mesoionic oxazolone (X) takes place.[65,67,83] Consequently the presence of salts of strong acids and tertiary amines, normally accompanying almost any peptide condensation, is undesirable in the reaction mixture.[3,28] An excess of tertiary amine reduces the racemisation of secondary amino acid residues when using DCC and isobutyl chloroformate as condensing reagents.[3] A stronger base such as triethylamine is more effective than *N*-methylmorpholine.



On treating derivatives of *N*-acyl secondary amino acids with strong acids, such as when decarbobenzoxylating the dipeptide Z–Ala–(*N*-Me)Leu with a solution of 5.6 N hydrobromic acid in acetic acid, significant racemisation takes place and was linked with

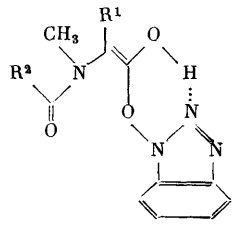
acid catalysed enolisation.[82] However the fact that the addition of water in this case sharply reduced the extent of racemisation indicates that in the above case a mesoionic oxazolone was formed in the anhydrous medium and no acid-catalysed enolisation took place.

Racemisation of secondary amino acid derivatives by enolisation may be catalysed both by alkali and by acid and its mechanism is evidently the same as for derivatives of the usual amino acids.[20,83]

It must be noted that the racemisation of derivatives of secondary amino acids rarely proceeds according to one mechanism. Frequently, depending on many factors, it is possible that either the oxazolonium or the enolisation mechanism may predominate.

The factors affecting the mechanism of racemisation include the method of activation, the nature of the substituents in the secondary amino acid residue, the polarity of the solvent used for the condensation, the pH of the medium, etc. Definite rules for this problem have now become apparent.

In particular the use of such well known antiracemisation additives as HOBT or HOSu in the activation of *N*-acyl secondary amino acids with DCC suppresses their racemisation by the oxazolonium mechanism.[3,21] HOSu proved to be more effective than HOBT which, while suppressing the formation of the oxazolonium ion, aids racemisation by the enolisation mechanism by being a stabiliser of the enolised form of *N*-alkylamino acid HOBT esters.[21]



In the condensation of Boc–Abu–Sar with (*N*-Me)Leu–Val–(*N*-Me)Leu–Val–(*N*-Me)Leu–Ala–OBzl by the mixed pivalic anhydride method no *C*-α-acylation side reaction, which proceeds through the formation of a mesoionic oxazolone [of the type of (X), see previous section], was observed.[40] The racemisation process in this case, as for DCC activation, is suppressed by an excess of triethylamine more efficiently than by *N*-methylmorpholine. This may serve as indirect confirmation of the fact that the racemisation of secondary amino acids when activated by Piv–Cl is explained by acid catalysed enolisation and not by the formation of an oxazolonium ion.[40]

It has been established that racemisation when using BOP, BOP–Cl and diphenylphosphinic chloride as activating reagents for *N*-acyl secondary amino acids is chiefly explained by the formation of an oxazolonium ion.[2,40]

The addition of HOBT or HOSu when using BOP–Cl has little effect.[84] According to other data HOBT has a weak influence on the suppression of racemisation but HOSu has no effect at all.[2,85] It has been recorded that on cyclising a hexapeptide by activation of an (*N*-Me)Phe residue with BOP–Cl the addition of HOBT increased the racemisation by even twofold.[86]

As in the case of the usual amino acids, urethane protection of an *N*-alkylamino group reliably prevents racemisation of a secondary amino acid.[3,21,22,40] For example, on carrying out the reaction of Boc–(*N*-Me)Leu with (*N*-Me)Val–OBzl using Piv–Cl the product contained no diastereomeric contamination even at 50 °C.[22] Only in cases of the most sterically hindered condensations when the reaction time was huge was a small degree of racemisation observed for a secondary amino acid with urethane protection. For example, the epimerisation of the (*N*-Me)Val residue activated by BOP–Cl in the reaction of Z–(*N*-Me)Val with (*N*-Me)–OBu^t was 4% after 2 d at 20 °C but less than 1% after 4 d at 0 °C.[2]

It was considered previously that the presence of urethane protection prevents racemisation of amino acids by the oxazolone mechanism.[20] It was established more recently that urethane protection may not be a reliable barrier to the formation of 5(4H)-oxazolones. Under certain conditions, for example with an excess of DCC in the reaction mixture, oxazolones are also formed from such amino acids, but they are extremely stable towards the tautomeric conversion which causes the racemisation.[11,87] However the presence of an excess of tertiary amine may in this case lead to marked racemisation.[11]

Racemisation proves to be extremely significant when activating *N*-acyl secondary amino acids by the DCC method if acetyl or benzoyl is used as the acyl group. If the same acyl groups are used on amino acids then racemisation is displayed to a lesser degree.[21]

Among the factors affecting the racemisation of amino acids the so-called asymmetric induction, which has been little considered until recently, may be mentioned.[88,89] This phenomenon explains the high extent of racemisation of *C*-terminal amino acid residues on activation or deblocking when a thermodynamically more favoured epimer is formed in the racemisation process.[22] This process may lead to the preferential formation of a product with reversed configuration, consequently the term 'racemisation' is unsuitable in this case and one should speak of a high degree of epimerisation.

On the other hand asymmetric induction may be caused by the different reactivites of *L* and *D* activated amino acids towards the asymmetric amino component. Thus an *L* isomer is activated, but the *D* isomer reacts more readily, then as a result of epimerisation occurring in parallel with the condensation it is possible to obtain a product containing residues chiefly of the *D* amino acid.[11,89] Occasionally this process leads to the complete inversion of the configuration of the amino acid being introduced.[40]

In certain cases the phenomenon of asymmetric induction may be a very valuable method of synthesis for individual epimers which are difficult to obtain by classical means. Thus when making a protected undecapeptide intermediate in the synthesis of cyclosporin by a 4 + 7 fragment condensation from identical amino and carboxy components, two different pure epimers are obtained depending on the reaction conditions.[40]

Boc-*D*-Ala—(N-Me)Leu—(N-Me)Leu—(N-Me)Val +
+ (N-Me)Bmt—Abu—Sar—(N-Me)Leu—Val—(N-Me)Leu—OBzl

BOP, 20 °C	Piv—Cl, −25 °C
Boc-*D*-Ala—(N-Me)Leu— —(N-Me)Leu—(N-Me)Val— —(N-Me)Bmt—Abu—Sar— —(N-Me)Leu—Val—(N-Me)Leu— —OBzl	Boc-*D*-Ala—(N-Me)Leu— —(N-Me)Leu—*D*-(N-Me)Val— —(N-Me)Bmt—Abu—Sar— —(N-Me)Leu—Val—(N-Me)Leu— —OBzl
(XI), 73%	(XII)

Piv—Cl, 20 °C

Mixture of (XI) and (XII) in a 4 : 1 ratio

When activating the tetrapeptide fragment with Piv–Cl racemisation of the (*N*-Me)Val residue occurs through the enol and because of the absence of steric constraints for this process the thermodynamically more favoured sequence of *L* and *D* amino acids

is formed. When activating the same tetrapeptide with BOP on oxazolonium ion is formed which due to steric constraints may react only in the *L* form.[40]

The increased tendency of derivatives of secondary amino acids apart from Pro to racemise on alkaline or acid treatment was explained previously by the ease of enolisation of these compounds under the conditions indicated.[82] Facilitated enolisation is linked with the absence of a hydrogen atom on the nitrogen atom or tertiary amides which *N*-acyl secondary amino acids are.

The hydrogen atom of *N*-acyl amino acids is significantly more mobile than a hydrogen atom of a carbon atom. By ionising first, the amide hydrogen atom prevents deprotonation of the hydrocarbon and provides the higher stability of an amino acid towards racemisation compared with secondary amino acids.

Experimental confirmation of the above is provided by the fact that treatment of (*N*-Me)Ile–OMe with alkali gives 1% epimer while Z–(*N*-Me)Ile–OMe having no hydrogen on the nitrogen atom gives 9% epimer under the same conditions. Analogously under alkaline conditions the ionisation of the carboxyl group prevents enol formation, and consequently racemisation, of Z–(*N*-Me)Ile.[82]

It is also reported that the electron-donating effect of the *N*-alkyl group is an additional factor facilitating the enolisation of secondary amino acids. However the enolisation mechanism presented may not explain the high stability towards racemisation of the Pro residue in the dipeptide Boc–Ala–Pro–OMe [less than 0.1% epimer in 1 N sodium hydroxide after 3 h).[82]

There are hints after the enhanced tendency towards racemisation of derivatives of secondary amino acids is impossible to explain fully by the absence of a hydrogen atom on a completely protected nitrogen atom preventing enolisation.[20] In order that this mechanism can be sufficiently justified it would be necessary for more than 90% of the amide groups in a peptide consisting of the usual amino acids to be converted under saponification conditions into the conjugate bases, which is improbable since the p*K* for monosubstituted amides lies in the 14–15 range. Furthermore these mechanisms do not explain the increased racemisation linked with the formation of a mesoionic oxazolone. Since disubstituted amides, which *N*-acyl secondary amino acids are, exist as *cis* and *trans* conformers which are close in energy, and monosubstituted amides have predominantly a *trans* conformation of the amide bond, it has been suggested that the *cis*-conformation existing more readily in *N*-acyl secondary amino acids facilitates the racemisation process.[20] Indirect confirmation of this hypothesis is the known ease of racemisation of residues of secondary amino acids in cyclic dipeptides on treatment with alkali. Both amide bonds in DKP has only the *cis* configuration.[90] It is evident that steric factors make a decisive contribution to the easing of racemisation of derivatives of secondary amino acids.

* *
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It may be concluded from the material considered above that no single traditional condensation method is able to guarantee success when acylating residues of secondary amino acids with the exception of the easiest cases. Such reagents as diphenyl phosphine chloride, BOP–Cl and Piv–Cl are capable of effecting the most sterically hindered condensations. Recently BOP–Cl has been used most widely for these purposes. In the synthesis of peptides containing secondary amino acids and especially for sequences of several secondary amino acids the risk of obtaining unstable compounds, which may decompose according to a different scheme, is sharply increased. The danger of decomposing such peptides is particularly high in the acidolysis process when removing protecting groups.

The side reaction of DKP formation is extremely acute and in certain cases requires a change in the synthesis strategy for peptides containing secondary amino acids.

The prevention or reduction of the extent of racemisation of secondary amino acid residues during peptide synthesis may be achieved mainly by known methods such as urethane protection or by adding agents to prevent racemisation. However the efficiency of these methods depends strongly on the carboxy and amino components reacting and on the condensing agent used. When using BOP and BOP–Cl the racemisation of secondary amino acid residues is caused mainly by the formation of the oxazolonium ion and when using Piv–Cl by acid catalysed enolisation.

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