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The chemistry of Amino Acid and Peptides via Solution-Phase-Peptide Synthesis

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Abstract

In this review, we have presented the different chemical structures of proteinogenic amino acids. Also, we performed a precise reference scan of the proton balance of amino acids. It is worth noting that, the two most important parts in this review are the principles of chemical peptide synthesis in solution and the protection of the amino acid side group, so we focused the reference survey on them, and we reached many old and new references in these two important parts in the field of peptide synthesis. On the other hand, we also performed an important reference survey of peptide-coupling methodologies. Finally, in this review we presented an important part, the use of peptide coupling reagents, in this part; we focused on the most important and most popular methods used in peptide chemistry reactions.

Keywords: Amino Acid; Peptide Chemistry; Solution-Phase; Peptide Synthesis.

1. Amino Acids

Amino acids are the building block of protein and peptide; α-amino acids have both an (NH_3^+) amino group and (COO^-) a carboxylic group. Moiety attached to the same α-carbon atom (figure **1**):

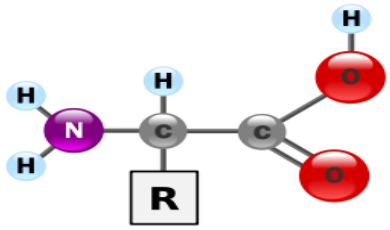

Figure 1: Structure of amino acids

The side chain (R) may be an aromatic, aliphatic, or a heterocyclic residue. A few number of amino acid occurs in mammalian proteins, although there are five hundred amino acids reported as naturally occurring. Complete acid, base, or enzyme-catalyzed hydrolysis of proteins, affords twenty L and α amino acids, namely the proteinogenic amino acids, which are classified in table 1 [1, 2].

2. Protonic Equilibria of Amino Acids:

Amino acids bear, at least, two ionizable groups, namely a carboxyl and an amino function. In solution, two forms of these groups, one charged and one uncharged; exist in a protonic equilibrium A (figure **2**) [3].

Figure 2: Structure of protonic equilibria of amino acids

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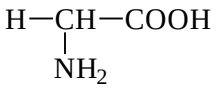
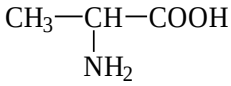
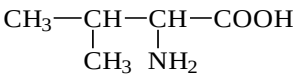
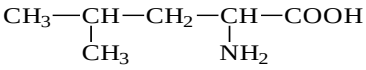
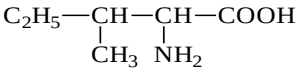
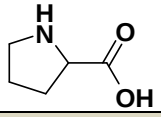
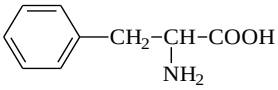
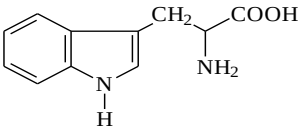
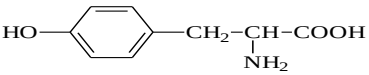
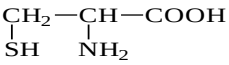
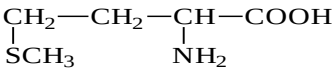
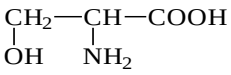
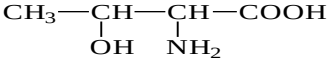
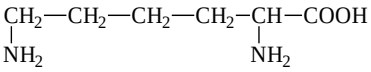
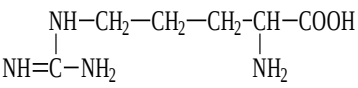
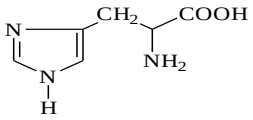
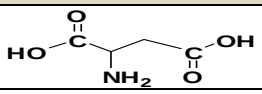
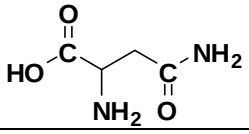
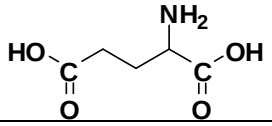
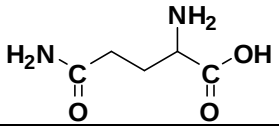
Table 1: Proteinogenic amino acids:

Trivial name	Code		Structural formula
	Three letter code	One Letter code	
1-Aliphatic amino acids			
Glycine	Gly	G	
Alanine	Ala	A	
Valine	Val	V	
Leucine	Leu	L	
Isoleucine	Ile	I	
Proline	Pro	P	
2- Aromatic or heteroaromatic amino acids			
Phenylalanine	Phe	F	
Tryptophan	Try	W	
Tyrosine	Tyr	Y	
3- Sulfur containing amino acids			
Cysteine	Cys	C	
Methionine	Met	M	
4- Hydroxy amino acids			
Serine	Ser	S	
Threonine	Thr	T	
5- Basic amino acids			
Lysine	Lys	K	
Arginine	Arg	R	

Histidine	His	H	
6- Acidic amino acids and their amides			
Aspartic acid	Asp	D	
Asparagine	Asn	N	
Glutamic acid	Glu	E	
Glutamine	Gln	Q	

At the pH of blood plasma (~7.4) or intracellular space, carboxyl groups exist, almost entirely, as carboxylate anion, RCOO-. At such pH value most amino groups are predominantly found in the associated (protonated) form, R-NH_3^+. In terms of the prevalent Ionic species present in blood and most tissues, amino acid structure should be represented as its Zwitter ion (figure **3**) [4].

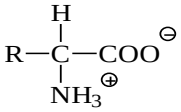


Figure 3: Structure of zwitter ion

2.1.1. Stereochemistry and optical isomerism of amino acids:

A proteinogenic amino acid, with the exception of glycine, has, at least, one asymmetric carbon atom, hence, is optically active. In other words all proteinogenic amino acids are chiral compounds, however, occurring in one of the enantiomeric forms (figure 4), namely, the L and α configuration.

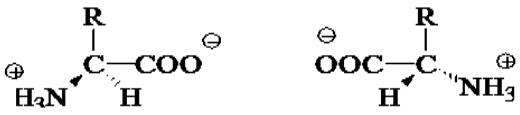


Figure 4: Enantiomeric forms of amino acid

The effect of alkali treatment on the isomerization of amino acids was investigated. The 100 × D/ (D + L) values of amino acids from peptide increased with increase in the number of constituent amino acid residues. Furthermore, the N-terminal amino acid of a dipeptide was isomerized to a greater extent than the C-terminal residue [5].

2.2. Peptides:

The principal reaction in the synthesis or biosynthesis of peptide chains is the acylation of the amino group of an amino acid by the carboxyl group of a second one, with formation of a particular type of amide bond, namely the peptide bond (figure **5**) [6]. A peptide can be considered as polymeric forms of amino acids that are linked together via a peptide bond. Peptides of more than ten amino acid residues are termed oligopeptides. Longer peptide chains (*e.g.* Insulin) are defined as polypeptides [7].

Peptide bond has resonating structure so; its configuration is in a planar geometry. Stereo chemically, the "*trans*" form is more favorable. In addition to be the hydrolytic fragments of proteins, bioactive peptides are naturally occurring as independent and fully functionalized cellular molecular identities. They are of known biological and medicinal properties [8]. Some are herein cited:

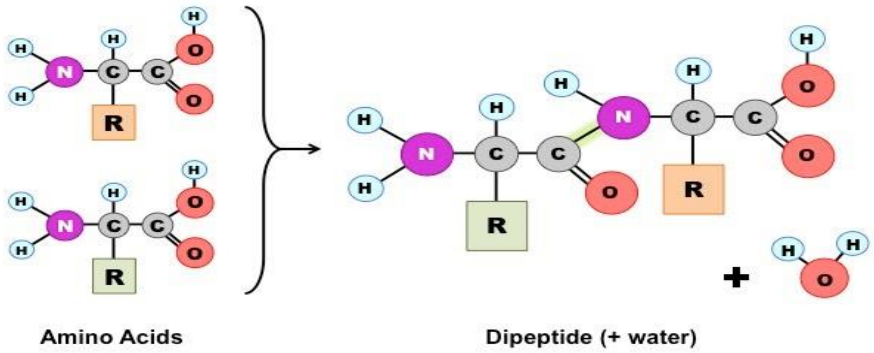


Figure 5: Peptide bond formation

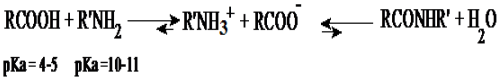
Glutathione: antioxidant and scavenger for natural free radicals, *Enkephalins*: natural brain analgesics, *Oxytocin*: induction of labor during delivery, *Insulin:* hormone carbohydrate metabolism, *Vasopressin*: antidiuretic hormone, *Polymyxins*: antibiotics, *Aspartame*: artificial sweetener, *Cyclosporine*: immunosupressor drug and *Actynomycines:* anticancer. Simple peptides consisting of several amino acids have more favorable pharmacological advantages, as biocompatibility, non-immunogenicity, high tissue permeability, and rapid clearance from the body [9].

Peptide syntheses can be classified according to the hereinafter table 2.

In general, it has been observed during recent references that peptide synthesis and synthetic organic chemistry have a distinct biological activity in all different applied directions [10-43]. Therefore, Herein, we are exclusively concerned with chemical peptide synthesis in solution.

2.2.1. Principles of Chemical Peptide Synthesis in Solution:

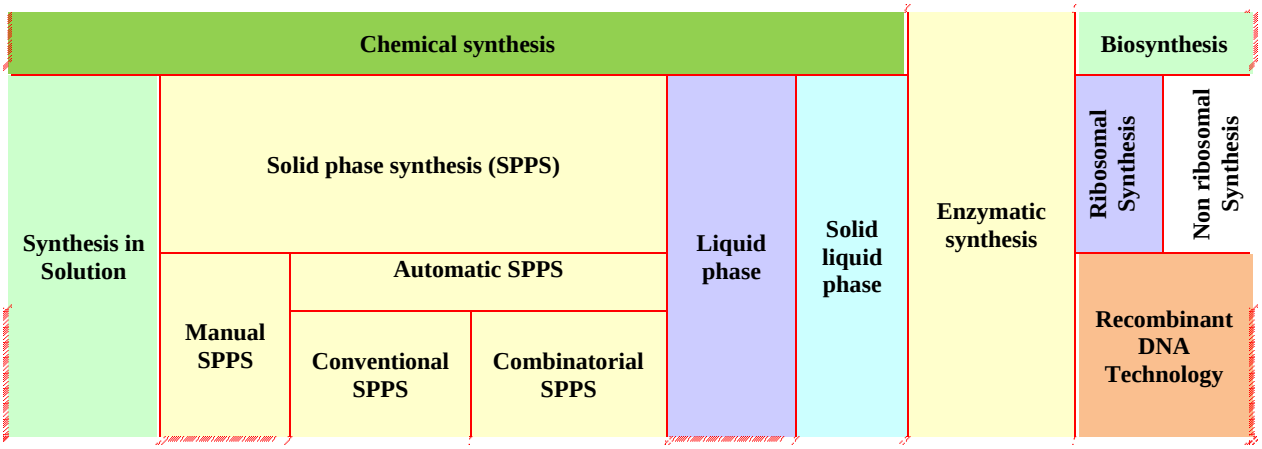
Formation of amide bond between an acid and an amine is formally a condensation reaction, result in water elimination. By mixing an amine with a carboxylic acid, an acid/base reaction initially occurs, forming a stable salt. The amide bond formation must overcome the thermodynamic barriers, since the equilibrium (scheme **I**) lies on the side of hydrolysis, rather than synthesis [44]**.**



Scheme I

The direct condensation of the salt can only be achieved at high temperature (~160-180 ^{o}C), which is, experimentally, impractical and consequently, incompatible with the presence of other functionalities. Therefore, activation of the carboxyl function by attachment of a leaving group to its acyl carbon is indispensable (scheme **II**), where "X" is an electron-withdrawing group (Cl, N_3, OR: R is an electron attracting functional group), which makes the carbon atom of the carboxyl function sufficiently electrophilic to facilitate the nucleophilic attack by the amino group.

Table 2: Classification of current peptide synthesis methodologies



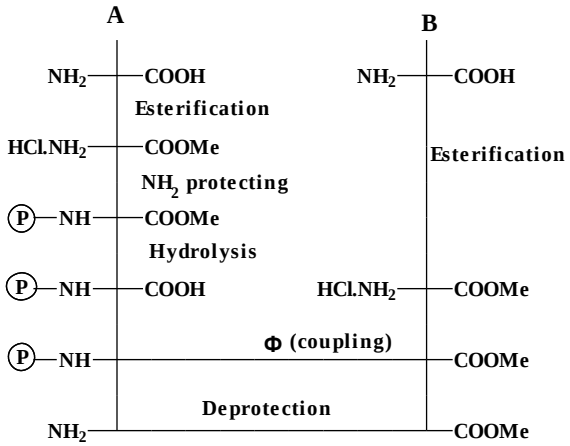
Scheme II

Peptide synthesis have a strategy which requires a specific planning which allows various functional groups to remain blocked, while others are freed for reaction in a subsequent step [45].

This requires an arsenal of protecting groups with a complete spectrum of cleavability to de-blocking agents. Protecting groups for amino and carboxyl functions are of crucial importance. Equally, the side-chain functionalities of polyfunctional amino acids, occasionally, require a special treatment [45].

Most peptide syntheses, in solution, are carried out in the C to N direction, α-amino-protecting groups (temporary protecting groups) are removed several timesduring the synthesis, so removal must be done in mild conditions that do not affect.

The remaining protecting groups (permanent, usually removed in the last step of the synthetic process, and semi-permanent, usually at the C-terminus, removed in the presence of all other protecting groups, when the peptide is to be coupled at its C-terminus) or even the peptide chain [46]. The principles of peptide synthesis could be outlined in four headings, namely: carboxyl group protection, amino group protection, side-chain group protection and peptide bond formation (the coupling reaction). The strategy of peptide synthesis could be represented in scheme **III**:



P- Temporary protecting group, Φ- peptide coupling agent

Scheme III: the strategy of peptide synthesis

2.2.1.1. Carboxyl and amino group's protection:

Some of the protecting groups that are currently used for the carboxyl and amino function are listed in tables **3** and **4**, respectively

Table 3: Temporary protecting groups for carboxyl function

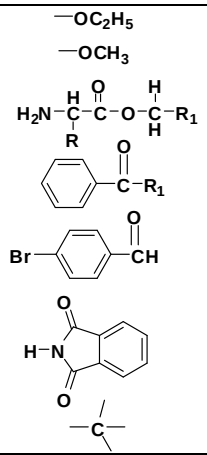
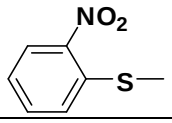
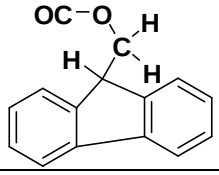
Group	Formula	Introducing reagent	Method of elimination
A. Methyl esters (-OMe), ethyl esters (-OEt) [47] and their substituents, e.g. phenacyl ester [48], 4 bromophenacyl [49], phthalimido methyl [50], and 2, 2, 2-trichloroethylesters [51]		Me OH / HCl, EtOH / HCl	Saponification
B. Tert - butyl ester (-OtBu)	O-C(CH_3)$_3$	isobutylene / sulfuric acid [52]	CF_3COOH , HCL, CH_3COOH [44]
C. Benzyl esters (-OBzl)	-O-$CH_2C_6H_5$	1. BzIOH/P-toluene [53] 2.Reaction of the amino acid cesium carboxylate with benzyl bromide [54]	1. Saponification 2. Catalytic hydrogenation H_2 /Pd [55]

Table 4: Temporary protecting groups for amino function:

Group	Formula	Introducing reagent	Method of elimination
Benzyloxy carbonyl (Z)	C_6H_5-CH_2-O-CO-	Z- CL [56]	H_2/Pd, Na/NH_3 [57], HBr/CF_3COOH/HF [56]
t-butyloxycarbonyl (Boc)	$(CH_3)_3$-C-O-CO-	(Boc) or BocN_3	CF_3COOH [59], HCL CH_3COOH [60],
O- Nitrophenyl sulfenyl (NPS)		NPS-CL or NPS-CN	Pyridine HBr + indole
Fluorenyl methyloxcarbonyl (Fmoc)		Fmoc-Cl or Fmoc-OSu	Piperidine

2.2.1.2. Amino acid

2.2.1.3. side chain group protection:

Some amino acids possess a side chain functional group which requires protection [45]. Since these protecting groups are expected to be intact during the peptide chain building process, they must resist the reagents employed for α -amino or carboxyl group deprotection and the peptide coupling reaction. They should, equally be cleavable, uniquely, at the completion of the scheme [61]. Some of the protecting groups are listed in table **5**.

Table 5: Temporary protecting groups for amino acid side chain protection:

Amino Acid	Functional Group	Protecting Group	Deprotection
Lysine Ornithine	ε - NH_2 δ - NH_2	1-Z and derivatives [62] (2-ClZ, 3-ClZ, 4-ClZ, 2, 4-Cl_2Z, 2, 6-Cl_2Z, 3, 4-Cl_2Z)	Catalytic hydrogenation and strong acids such as HF
		2- Boc	TFA
Cysteine	β - mercapto SH	1- S-Benzyl [63]	Na/liq NH_3 or HF (20° C for 30 min) seldom used recently
		2-S-acetamidomethyl [64] (SAcm)	Hg $(AcO)_2$ or I_2
		3- S-3-nitro-2-pyridylsulphenyl (Snpys)	Tri-n-butylphosphine or β-mercaptoethanol
Aspartic acid Glutamic acid	β - carboxyl γ - carboxyl	1- Benzyl ester (OBzl) [65]	Catalytic hydrogenation or HF
		2- t-butyl ester (OtBu) [66]	TFA, HCl in organic solvent, HBr/AcOH

Table 5: Temporary protecting groups for amino acid side chain protection (continued):

Amino Acid	Functional Group	Protecting Group	Deprotection
Histidine	Imidazole nitrogen	1- N-tosylhistidine (His (Tos)) [67]	HF
		2-N-dinitrophenyl histidine (His(Dnp)) [68]	Thiolysis
Arginine	δ-guanidino	1- Nitroarginine (Arg (NO_2)) [69]	HF (0 °C, 30 min)
		2-Benzyloxy carbonyl arginine (Arg (Z)) [70]	Catalytic hydrogenolysis
		3- p-toluene sulfonylarginine (Arg (Tos)) [71]	HF (0 °C, 30 min)
Tyrosine	Phenolic OH	1- O-Benzyltyrosine (Tyr (OBzl)) and O-2, 6 dichloro Benzyltyrosine [72]	Na/liq NH_3 or catalytic hydrogenolysis
		2- O-2-Bromo benzyloxycarbonyltyrosine (Tyr(O-2 BrZ)) [73]	HF or catalytic hydrogenolysis
Serine, Threonine	Aliphatic OH	1- Benzyl ester (OBzl) [74]	Catalytic hydrogenolysis, HBr/dioxane, HF
		2- t-butyl ester (OtBu) [75]	TFA, HCl/TFA and HF
Tryptophan	Indole nitrogen	Formyl (For) [76]	Piperidine 0.1M hydrazine or hydroxylamine

2.2.2. Amide bond formation and peptide coupling: [77]

There are different ways of coupling the carboxyl and amine components:

A) Forming and isolation of an intermediate acylating agent then subjected to amylolysis. B) Forming of amore reactive acylating agent from the acid in separate steps, then occur coupling with the amine. C) The acylating agent is produced from the acid in the presence of the amine, by adding an activating or coupling agent.

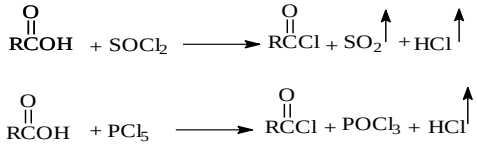
Peptide coupling methodologies, consequently:

2.2.2.1. Acid chloride method:

The chlorine atom is an obvious choice for the role of an electron withdrawing moiety (X). The powerful activation presented by an acid chloride was described by Emil Fisher (1903) [78]. Acid chlorides are regarded an easy method to activate an acid and more acid chlorides are commercially available. A two-step process usually occurs, first conversion of the acid into its halide then the coupling reaction.

2.2.2.1.1. Acyl chloride formation:

Thionyl chloride $SOCl_2$ [79], phosphorus trichloride PCl_3 and phosphorus pentachloride PCl_5 [80] are commonly used to generate acid chlorides from their corresponding acids (equation **1**):

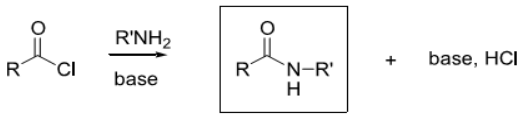


Equation 1

These reactions are often promoted by the addition of a trace of (DMF) [81]. One of the major disadvantages of the previously cited chlorinating agents is the undesirable production of HCl.

2.2.2.1.2. Coupling reactions with acyl chlorides:

The amide bond is formed by reacting the acyl chloride with the desired amine (equation **2**). An extra base is needed to trap the formed HCl and to avert the conversion of the amine into its unreactive HCl salt. Couplings are usually occurred in inert dry solvents, in the presence of a non-nucleophilic tertiary amine (*e.g.* TEA or NMM) [82].



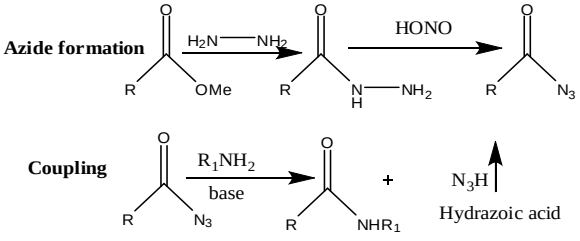
Equation 2

2.2.2.1.3. Limitations of acyl chlorides:

Due to the danger of hydrolysis, racemizations, breaking of protecting groups and other side reactions, acyl chlorides have limited value in peptide coupling

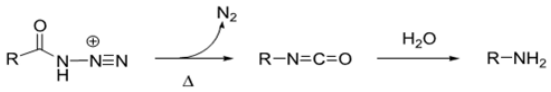
2.2.2.2. Azide method:

The acyl azide route [83] is one of the first developed methods for peptide coupling by Curtius (1902) [84]. Acyl azides can be prepared from the corresponding methyl esters via a two-step synthesis. The methoxy group is displaced with hydrazine to generate the acyl hydrazide, which then undergoes a nitrosation reaction to yield the final acyl azide (scheme **IV**):



Scheme IV

This is usually an efficient coupling method with almost no appreciable racemization, but an occasional side reaction is a Curtius rearrangement, leading to the formation of the unwanted corresponding isocyanate [85] (scheme **V):**



Scheme V

2.2.2.3. Anhydride methods:

Treatment of amines with anhydrides of carboxylic acids is regarded one of the simplest and more efficient methods of acylation so peptide bond formation could take place.

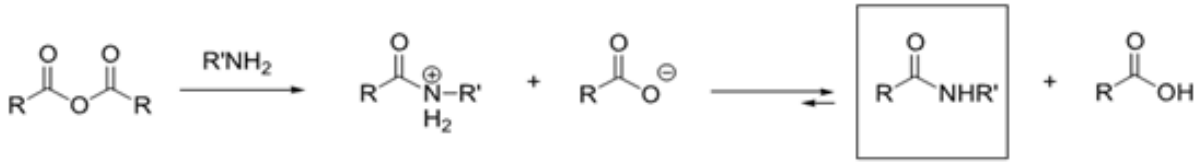
2.2.2.3.1. Symmetric anhydrides:

Symmetric anhydrides are formed either by treating the corresponding acid or, in milder conditions, by reacting two molecules of acid in the presence of one equivalent of dicyclohexylcarbodiimide (DCC) [86]. The anhydride is then reacted, in a second step, with the selected amine. In theory, no additional base is required as the addition generates a carboxylate anion *in situ* (scheme **VI**). This mild and efficient coupling method is compatible with peptide formation. The main limitation is that only half of the acid is, effectively, coupled and the other half is wasted. This could be a problem, if the acid is valuable.

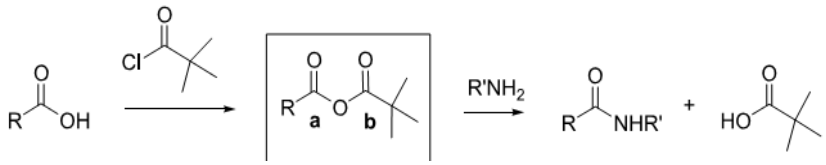
1.1.1.1.1. Mixed anhydrides:

1.1.1.1.1.1. Mixed carboxylic anhydrides:

Long chain fatty acids such as isovaleric acid [87] and pivalic acid [88] are used in these mixed anhydrides. The difficulty is to get regioselectivity in the nucleophilic addition for position an over position b (scheme **VII**) [65]. Reactivity of the activating part of the anhydride molecule (b) is decreased by both electron releasing factors and steric hindrance.



Scheme VI



R= isovaleryl: $(CH_3)_2CHCH_2$, Pivaloyl: $(CH_3)_3C$

Scheme VII

1.1.1.1.1.1. Mixed carbonic anhydrides:

Mixed carbonic anhydrides can be generated using ethyl chloroformate [79] or isobutylchloroformate [89].

Another strategy is to differentiate both reactive centres by their chemical nature. Excellent selectivity is observed with mixed carbonic anhydrides. The carbonate electrophilic Centre is more reactive than the carboxylic site **b** as the reactive centre **at** is less stabilized by resonance (scheme **VIII**) [90]:

Ethoxycarbonyl anhydride can also be generated using1-ethoxycarbonyl -2-ethoxy-1, 2-dihydroquinoline (EEDQ) [91] (scheme **IX**). The driving force of this reaction is the generation of quinoline.

The displacement of ethanol leads, via an intermolecular attack, to the formation of an ethoxy carbonyl anhydride. The major advantage of EEDQ lies in the absence of the tertiary base that is required to neutralize the liberated HCl in the conventional mixed anhydride method; consequently, it minimizes racemization or the other side reactions originated by proton abstraction.

The most important factors influencing the formation of mixed **anhydrides are:**

i.Nature of the coupling agent:

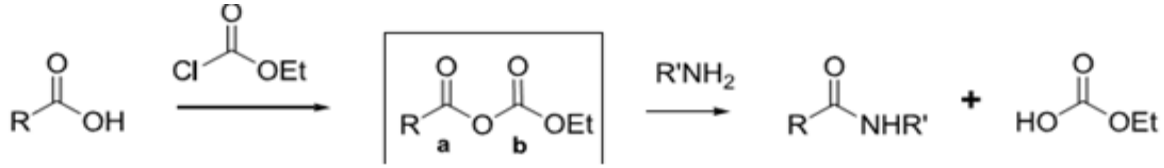
ECF, frequently employed in early works, is used with acceptable results. Isobutylchloroformate is a more favorable reagent since it provides a higher yield. The branched alkyl group increases the electron density at the adjacent carbonyl in the mixed anhydride and minimizes the attack by the nucleophiles (amino component). The carbonyl of the amino acid becomes, consequently, the predominant point of attack [92].

ii. Nature of the used tertiary amine:

NMM is the preferred tertiary base, since it minimizes the racemization. The tertiary amine reacts with the chlorocarbonate to form an alkoxycarbonyl ammonium ion complex [t-Bu-O-CO-N(R) $_3$] $^+$Cl, which subsequently reacts with the carboxylate [93].

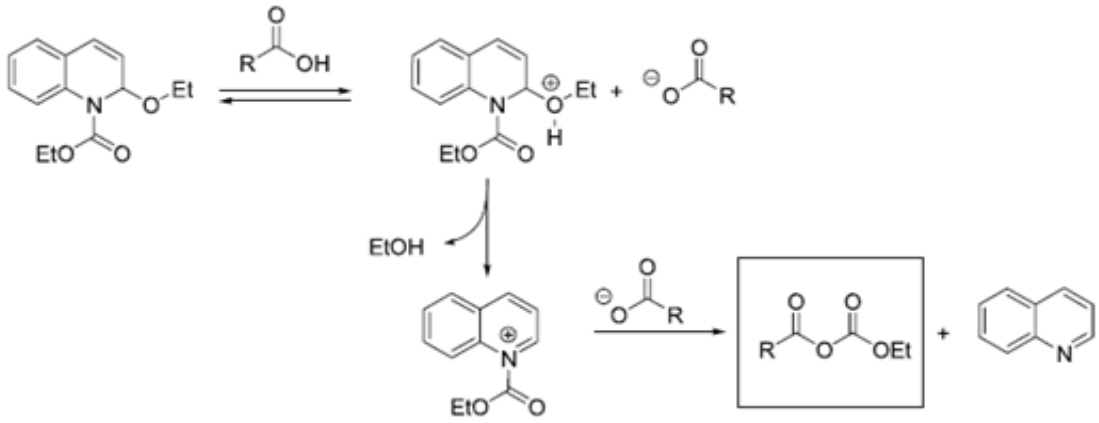
iii. Acyl substituents and side chain of the carboxyl component:

Generally, the more sterically hindered the acyl substituent, the lower the mixed anhydride yield [94]. Side chain of the carboxyl component plays reverse role in coupling activation process, bulky groups as in Ile, Leu or Valine may slow down anhydride formation [94]. Similarly, the side chain (OH, COOH, NH_2 …) should preferably be protected to avoid undesirable side reactions.



R= ethyl: CH_3CH_2, Isobutyl: $(CH_3)_2CHCH_2$

Scheme VIII



Scheme IX

iv. Solvent effects:

Anhydrous solvents of high purity are essential for successful mixed anhydride formation [95]. Ethyl acetate, THF, toluene, *t*-butanol, acetonitrile are examples for recommended solvents for such formation, to provide optimum overall yields, while chloroform or methylene chloride results in considerably lower yield and purity.

v. Temperature and activation time:

An activation of 1-2 min at -15 to -5 ^{o}C, is generally recommended [92]. A few minutes longer time for the anhydride formation may be required for carboxylic components which have bulky side chains such for Ile, Leu, Val or Arg. Temperatures below (-20 ^{o}C) are not recommended, because of the marked decrease in the overall yield. On the other hand, the temperature must be kept below zero because the decomposition of mixed anhydride *via* disproportionation becomes a considerable drawback. Low temperature and/or short activation time suppress side reactions and racemization compared with mixed anhydride method.

1.1.1.2. Use of peptide coupling reagents:

1.1.1.2.1. Carbodiimides:

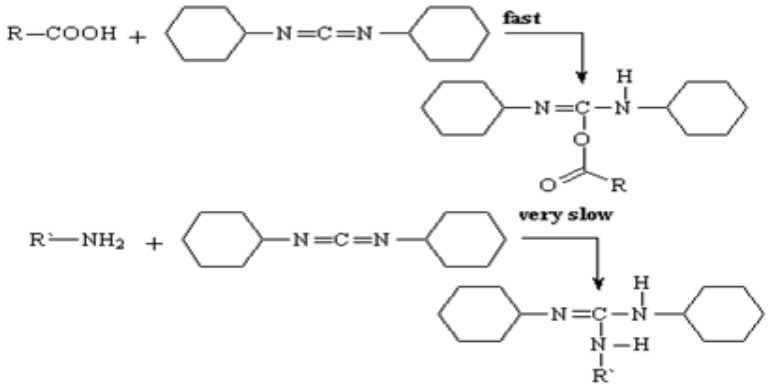
In 1955, Sheehan and Hess used dicyclohexylcarbodiimide (DCC) as a reagent to be used in peptide bond formation [96]. The procedure involves the addition of the coupling reagent to a mixture of both amino and carboxyl components. Thus, activation and coupling proceed concurrently while amines do react with carbodiimide to give guanidine derivatives. The rate of the reaction, however, is negligible, when compared with the rapid rate observed by the addition of carboxyl group to one of the double bonds of the carbodiimide (scheme **X**).

A reaction mechanism for DCC coupling is summarized in (scheme **XI**) [31]. Addition of the carboxyl group of the N-protected amino acid to one of N = C bonds of DCC gives the O - acylisourea mixed anhydride intermediate A, the first active species formed in the coupling reaction [97].

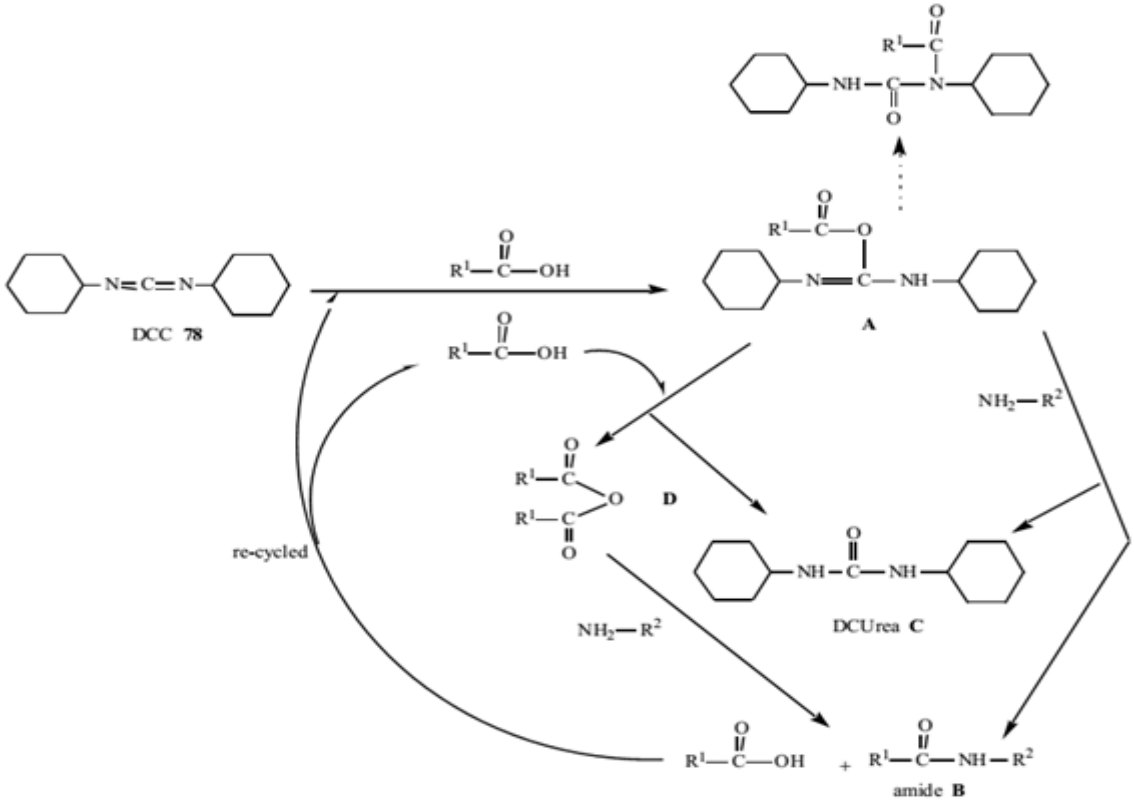
The highly reactive (A) can then undergo amino lysis by the amino component leading to the formation of an amide (B), in addition to dicyclohexylurea as a by-product, C. Alternatively, another molecule of carboxylic acid can react with the O-acylisourea, forming the amino acid symmetrical anhydride D. This is another potent acylating species that can also react with the amino component, again leading to amide bond formation [65].

The nucleophilic Centre on O-acylisoureas competes with the amino component for acyl residue and this competition leads to the formation of the unreactive byproducts, namely, the N-acyl urea.

The speedy execution of activation and coupling in a single operation and the simple removal, however, occasionally incomplete of the insoluble byproduct “dicyclohexylurea” (DCHU) by filtration, all contribute to the persistent popularity of the DCC method. It is noteworthy that DCHU is generally insoluble in most organic solvents, however may be partially soluble, particularly in the presence of other dissolved materials. A remedy for this imperfection could be in the use of water soluble carbodiimides (WSC) that are extractable with water, for example 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC) (figure **6**) [98]. Diisopropyl carbodiimide (DIC) [96] is also, equally, effective as a coupling reagent, and forms a urea by product soluble in dichloromethane (figure **7**).



Scheme X



Scheme XI

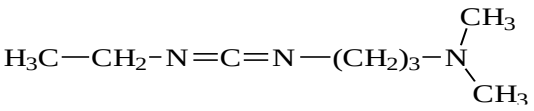


Figure 6: EDC

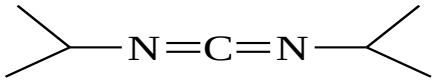
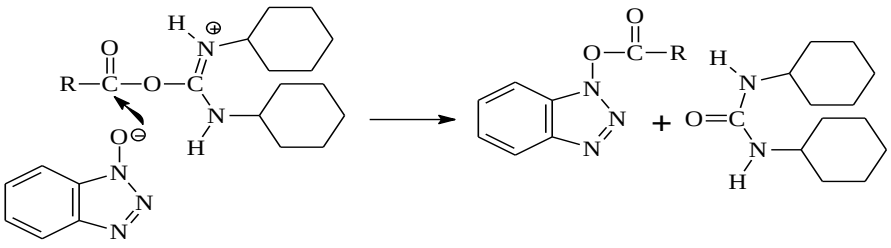


Figure 7: DIC

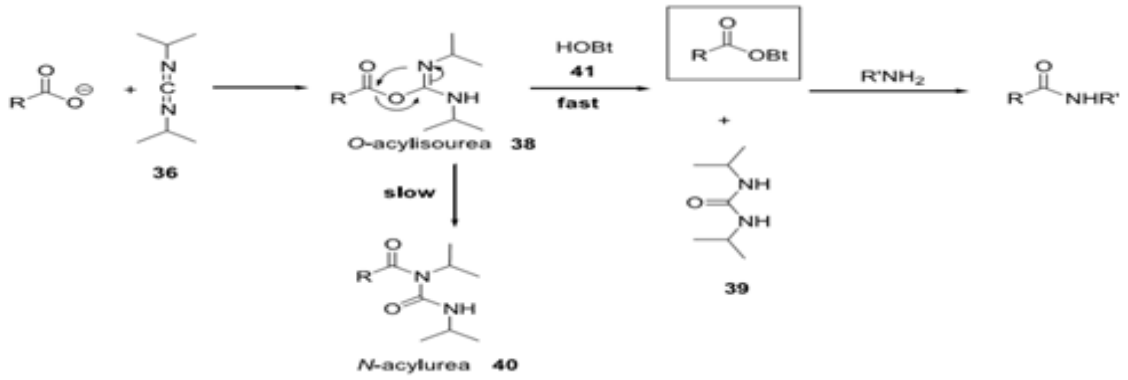
1.1.1.2.2. **Carbodiimide coupling with additives:**

Both racemization and N-acyl urea formation can be suppressed by the addition of auxiliary nucleophiles, such as 1-hydroxybenzotriazole (HOBt) to the reaction mixture. Attack of the additive on the reactive intermediate yields an O-acyl-1-hydroxybenzotriazole [99], which is a powerful acylating agent (scheme XII):

The presence of a second nucleophile in the reaction mixture reduces the extent of racemization. HOBt, as a weak acid, prevents proton abstraction from the chiral carbon and thus contributes to the conservation of the chiral purity. Additionally, the availability of the auxiliary nucleophile (HOBt) efficiently shortens the life time of the overactivated O-acyl-isourea intermediate and thus reduces the extent of O→N acyl migration that leads to N-acylureas (scheme **XIII**):



Scheme XII



Scheme XIII

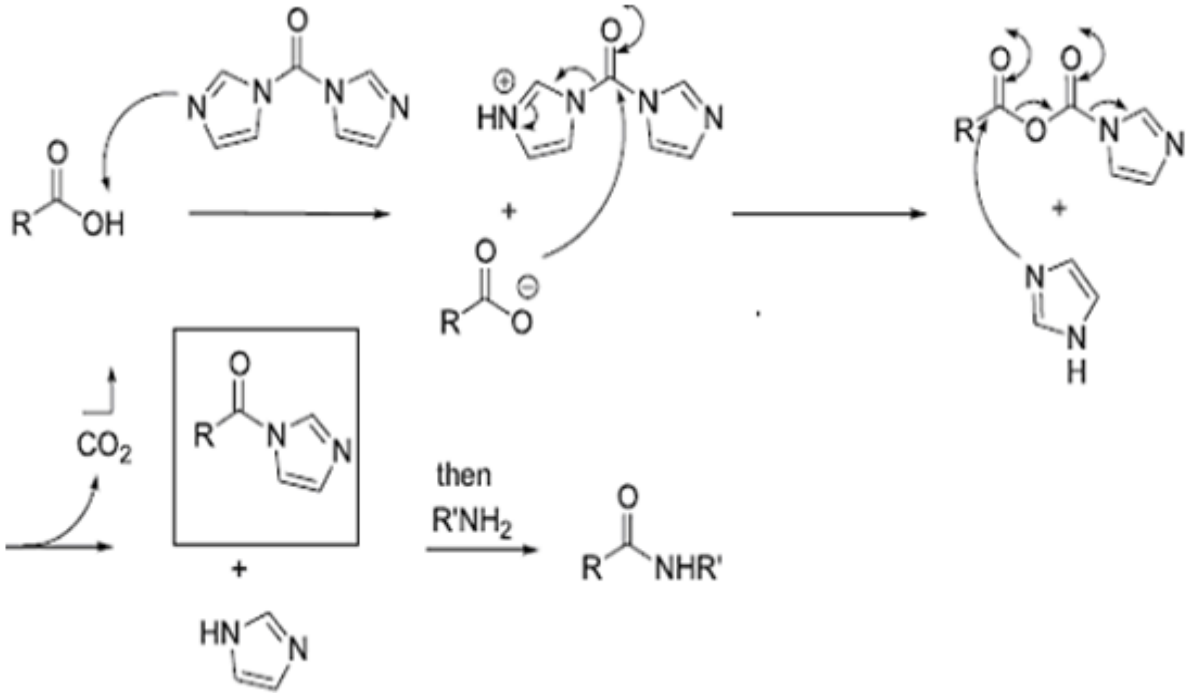
1.1.1.2.3. Carbonyldiimidazole (CDI):

Carbonyldiimidazole (CDI) [100] is a useful coupling reagent that allows one-pot amide formation. Acyl carboxy imidazole and imidazole are initially formed but readily react together to yield the activated species as the acyl imidazole (scheme **XIII**): Practically, the acyl imidazole is preformed for one hour and then the amine is added. This reaction, which generates imidazole *in situ*, does not need an additional base and is, even, compatible with Hydrochloride salts of the amine [100]. This reagent is commonly used on a large scale [101] in synthetic peptide chemistry.

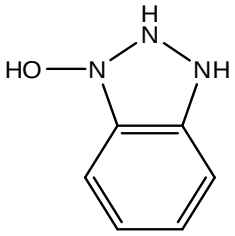
1.1.1.1. Active ester method:

Active esters are prone to react with a wide range of nucleophiles. They, practically, clearly react with amines under mild conditions with usually reduced racemization.

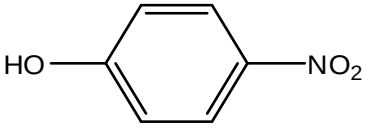
Figure **8** presents a selection of different alcohols that are commonly used. The increased electrophilicity of the carbonyl centre results from the electron-withdrawing character of the selected alcohols.



Scheme XIV

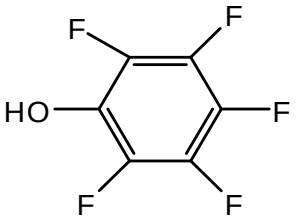


1-Hydroxybenzotriazole, HOBt

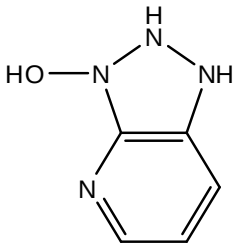


P-nitrophenol, PNP

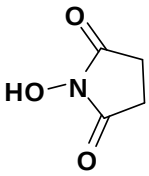
[10, 95]



Pentafluorophenol



1-Hydroxy-7-azabenzotriazole, HOBt



N-hydroxysuccinimide, HOSu

Figure 8: Alcohols that are commonly used in active ester method [102-105]

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