Industrial application of coupling reagents in peptides

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ABSTRACT

Peptides play a key role in the post-genomic and proteomic era. From an industrial basis, the formation of the amide bond is crucial for obtaining an efficient and economic process. Mechanisms associated with the different methods are reviewed. Cl-HOBt as additive and HCTU/TCTU are excellent alternatives to the most classic coupling methods.

INTRODUCTION

There is no doubt that the last years are recognised as the renaissance of the peptide world. The important advances in molecular biology and gene technology are bringing significant changes in biomedical sciences. The elucidation of the human genome, followed by advancements in functional genomics and proteomics, will revolutionize our understanding of the detailed molecular mechanisms, underlying a broad spectrum of diseases. These developments will also identify new therapeutic targets and suggest novel mechanism-based therapeutic paradigms. Peptides will play a key role in all these processes.

There has been a notable increase in research institutions and pharmaceutical companies involved in the development of peptide-based drugs, resulting in an increase in the number of university core facilities and spin-offs, commercial peptide suppliers, contract peptide manufacturers, and other outsourcing institutions.

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In the actual pharmaceutical market, peptides are not only considered as hormones, as in the past, but also as active pharmaceutical ingredients (API), in antibiotics, antiviral and in other therapeutic areas such as cancer as immunomodulators and anti-angiogenesis agents, CNS and neurogogical disorders as analgetics and anti-obesity drugs, immune disorders for the treatment of allergy, asthma and autoimmune disease (1).

Furthermore, over 200 new peptide-based drugs are under different stages of development with 50% of them under clinical trials and prior to approval (2). Nowadays, peptides represent 1% of total API with a market of US$ 300-500 M per year and a growth rate of 15-25% annually (1), with the expectation of a 100% increase in the next two years when generic and recently approved new chemical entities enter the market (3). This is reflected by the fact that over ten known bulk peptide producers and over twenty companies are offering custom peptides synthesis and these numbers, as well as capacity are growing (4). This trend has subsequently affected all raw material manufacturers and the entire peptide industry supply chain.

Today, manufacturing companies face the unprecedented challenge of production of hundred kilograms to tons quantities of complex peptides involving modern technologies. Thus, the customer supplier relationships are becoming more complex, involving basic producers with their extensive knowledge and experience in the reagents field and increasingly into a process development at different stages of production.

A key step in the peptide production process is the formation of the peptide bond. This requires the activation of a carboxylic acid, which is usually carried out using the so-called **peptide coupling reagents** (5,6). In addition to peptides, amide bonds are present in a huge array of other organic compounds of biological interest such as peptoids, oligocarbamates, oligoamides, β-lactams, polyenamides, benzodiazepines, diketopiperazines, and hydantoins. Furthermore, the ester group is another important functionality present in many organic compounds and can also be prepared directly from the carboxylic acid using **peptide coupling reagents**.

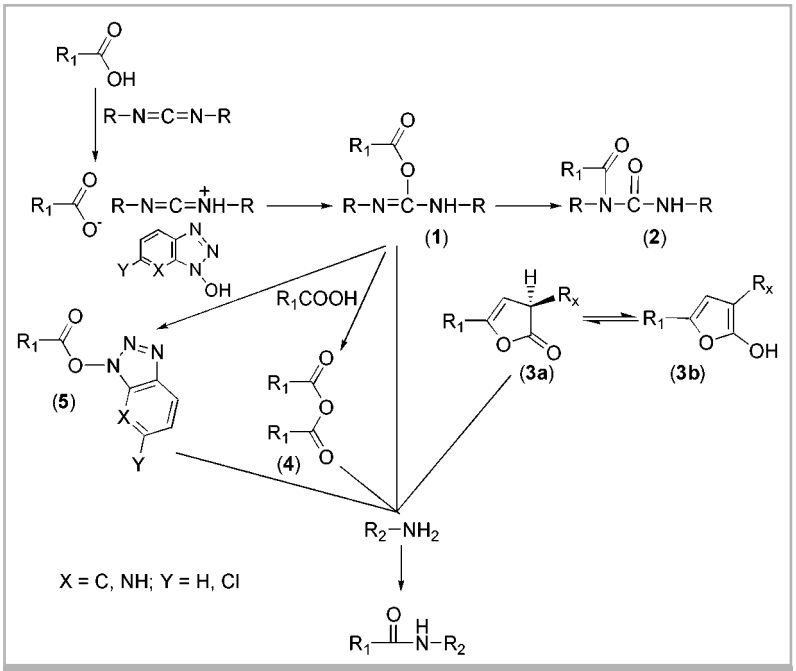

Figure 1 - Mechanism of peptide bond formation through carbodiimide activation

Although the synthesis of medium-large peptide for basic research is a well established procedure, the combination of the 20 proteinogenic amino acids and growing number of unnatural amino acids makes each peptide synthesis at the industrial level unique, requiring closer attention to each amino acid coupling. Some of the rules for coupling reagents validated in the research scale can be applied at industrial level, but the results are still hardly predictable. Therefore, although peptides are produced in the industry in hundred kilograms to tons scale, there is still a need to search for the ultimate **coupling reagent**.

The **peptide coupling reagent** field has clearly evolved in the last decade from carbodiimides to onium (phosphonium and uronium) salts. The era of industrial coupling reagents began in 1955 with the introduction of dicyclohexylcarbodiimide (DCC) (7), which at that time was already known and well studied, as a reagent for formation of amide bond (8). Unfortunately, carbodiimides did not comply with the concept of ultimate coupling reagents because its high reactivity provokes racemization and side reactions during the coupling reaction (Figure 1).

The mechanism of the carbodiimide activation, which is complex and depending on the solvent, starts by a proton transfer, followed by addition of the carboxylate to form the *O*-acylisourea (**1**). This is the most reactive specie that can attack the amino component to give the corresponding amide. However, the *O*-acylisourea (**1**) can undergo a rearrangement to give the *N*-acylurea (**2**), which is not reactive, or sustain an intramolecular cyclization to give a 5(4*H*)-oxazolone (**3**), which is less reactive than **1** and can tautomarize with the corresponding loss of chirality. If activation is carried out in a solvent of low dielectric constant such as $CHCl_3$ or CH_2Cl_2, the formation of **1** occurs instantaneously, which is absent of a nucleophile or base and can be stable for many hours. However, if the activation is carried out in a more polar solvent such as DMF, no immediate reaction can be detected, and a complex mixture of starting amino acid, symmetrical anhydride (**4**), and **2** is formed. If the activation is carried out in the presence of an extra equivalent of acid, **4** is formed, which is also very reactive.

At the beginning of the 70's, 1-hydroxybenzotriazole (HOBt) (9) was proposed as an additive to DCC to reduce racemization and from then on other benzotriazole derivatives such as 1-hydroxy-5-chlorobenzotriazole (Cl-HOBt) (10) or 1-hydroxy-7-azabenzotriazole (HOAt) (11) have also been used. The OBt active esters (**5**) are less reactive than **1**, but are more stable and less prone to racemize. All these factors make the addition of benzotriazole derivatives almost mandatory to preserve the peptide bond formation by carbodiimide activation of low yields and undesired side reactions.

In the last decade onium (phosphonium and aminium/uronium) salts of hydroxybenzotriazole derivatives have been introduced. Although, they have been rapidly adapted for research purposes, only a few of them have been found compatible with current industrial requirements and synthetic strategies and therefore adopted by the industry.

The specie that reacts with onium salts is the carboxylate (Figure 2) and therefore the presence

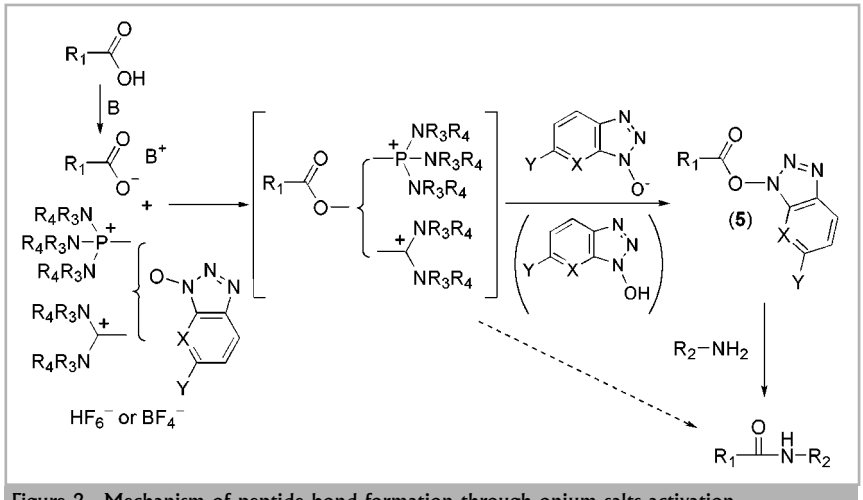

Figure 2 - Mechanism of peptide bond formation through onium salts activation

of at least one equivalent base is essential. The intermediate species acyloxy-phosphonium or amidinium salts have not been detected and react immediately with the benzotriazole derivative (an extra equivalent of it is added in some synthetic protocols) to give **5**, which react with the amino component to give the corresponding amide.

Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977–983.

The following additional abbreviations are used:

Cl-HOBt, 6-chloro-1-hydroxybenzotriazole;

DCC, *N*,*N*'-dicyclohexylcarbodiimide;

DIPCDI, *N*,*N*'-diisopropylcarbodiimide;

DMF, *N*,*N*-dimethylformamide;

EDC, 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide;

Fmoc, 9-fluorenylmethoxycarbonyl;

HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo [4,5-*b*]pyridino-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate;

HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide;

HCTU, *N*-[(1*H*-6-chlorobenzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide;

HOAt, 7-aza-1-hydroxybenzotriazole;

HOBt, 1-hydroxybenzotriazole;

PyAOP, 7-azabenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino) phosphonium hexafluorophosphate;

TNBSA, trinitrobenzenesulfonic acid;

TBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino) methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide;

TCTU, *N*-[(1*H*-6-chlorobenzotriazol-1-yl) (dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide;

TFA, trifluoroacetic acid;

Amino acid symbols denote the L-configuration unless stated otherwise.

WHAT IS A GOOD COUPLER FROM THE INDUSTRIAL POINT OF VIEW?

Effective Coupling Reagent

- Works with high efficiency for a wide variety of peptide sequences.
- Works in steochimetric quantities.
- Has a high conversion rate at room temperature.
- Works for both solution and solid-phase peptide synthesis.
- Is soluble in all the currently used solvents and can be used at high concentrations.
- Solutions are stable for several days at room temperature.
- Allows monitoring of coupling reagents.
- Shows few side reactions.
- Its secondary products after coupling can be completely removed by solvent extraction.

Cost-Effective Reagent

Can Be Produced in Large Quantities

- Conventional raw material available.
- Technology is known and does not require installation of sophisticated equipment.
- Chemistry is adaptable for up-scaling.
- Raw material solvents and catalysts are safe for the producer.
- Effective waste management.
- Has a prolonged shelf-life at ambient conditions.

Safe for Producer, User, and Environment

- Material is not toxic, corrosive or self-reactive.
- The use of material does not result in formation of toxic by-products.
- Does not generate hazardous waste.
- Can be disposed with low pollution risk.

THERE ARE MORE THAN 80 REAGENTS KNOWN TODAY, HOWEVER ONLY A FEW HAVE FOUND THEIR WAY TO THE INDUSTRY

Analyzing the bulk coupling reagent market, we can see that is shared between three main groups of coupling reagents in addition to the additives (Figure 3).

Additives

As was outlined in the introduction, the addition of benzotriazoles [HOBt (**6**) and Cl-HOBt (**7**)] to the carbodiimides based coupling reagents leads to the formation of the benzotriazole active esters that are less reactive than the *O*-acylisourea (**5**), reducing racemization of the protected amino acid and avoiding the formation of other derivatives less reactive. Cl-HOBt (**7**) performs at least as well as HOBt (**6**), but since it is more acidic (pK_a: 3.35 for Cl-HOBt and 4.60 for HOBt) it is a better leaving group and its active esters are more reactive than OBt esters. As discussed in section 5, another advantage of Cl-HOBt is that the chlorine atom stabilized the structure, making

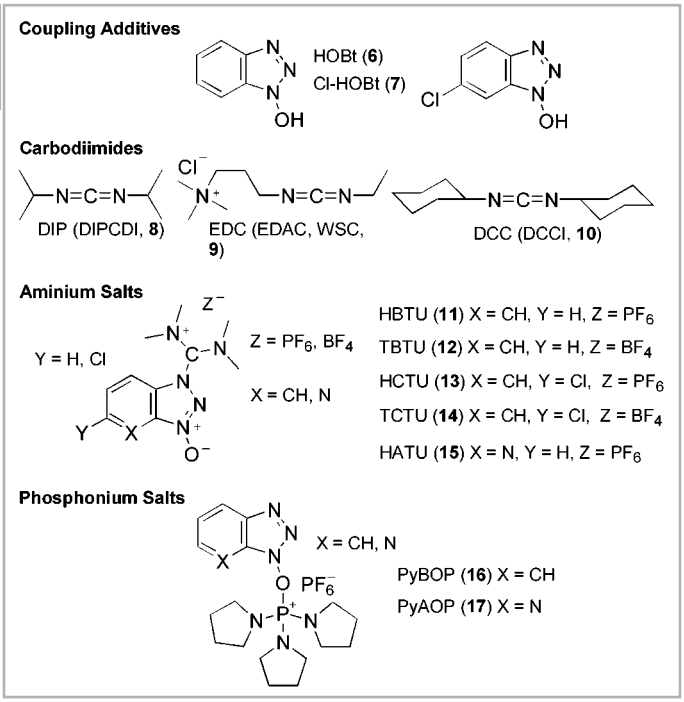

Figure 3 - The most common coupling reagents and additives used in the industry

Cl-HOBt a less hazardous reagent. Although its concourse is not strictly necessary as shown in Figure 2, HOBt and Cl-HOBt are also added to the aminium salts mediated coupling reactions, with the purpose of favouring the active ester formation. Thus, Cl-HOBt is used as an additive together with HBTU to suppress racemization during fragment condensation assay in the industrial synthesis of Fuzeon (T-20) (12).

The pyridine derivative of HOBt (HOAt) is not for industrial use, because the extra nitrogen in the phenyl ring makes the structure unstable (see section 5b).

Coupling Reagents

1. Carbodiimide Coupling Reagents [DIPCDI (8), EDC (9), DCC (10)]

Carbodiimides are the cheapest coupling reagents and their primary active species [*O*-acylisourea (**5**)] one of the most reactive. Problems associated with their use are racemization and low yields due to the formation of the poorly active *N*-acylurea (**2**). Use of solvents of low dielectric constant such as $CHCl_3$ or CH_2Cl_2 minimize both reactions. Although, the use of these solvents is precluded in the automatic solid-phase mode at the research level due to specific characteristics of the synthesizers, they can be used in manual or semi-automatic modes. Addition of benzotriazole derivatives also minimizes side-reactions. DCC (**10**) is incompatible with Fmoc/*t*Bu solid-phase chemistry, because the dicyclohexylurea is not soluble is common solvents. In solution chemistry, ureas are always difficult to be removed and water-soluble carboddiimide [EDC (**9**)] is a useful alternative.

When carbodiimides are used with two equivalents of protected amino acids, the symmetrical anhydride (**4**) is formed, which is a very reactive, active specie. This method has some economical consequences, because a double amount of protected amino acids are required.

2. Aminium Salts of Benzotriazoles

The use of the most reactive aminium salt, HATU (**15**) (11), is inconvenient because of the price, which makes its use detrimental for industry. HCTU (**13**)/TCTU(**14**) (13) are a good alternative to HBTU (**11**)/TBTU (**12**) (14), because the presence of the Cl-HOBt makes those reagents more reactive.

During the activation of hindered carboxylic components, such as those involved in cyclization reactions of coupling of hindered amino acids, the aminium salts can react with the amino component, leading to a guanidine derivative, a process that terminates the peptide chain. Recently, it has been discovered that aminium salts can contain traces of dimethylamine, which can also react with the carboxylic component to give the corresponding dimethylamide.

Aminum salts having the counterion tetrafluoroborate are more soluble than hexafluoroborate salts, which allow preparation of more concentrated solutions.

3. Phosphonium Salts of Benzotriazoles

As occurs with the aminium salts, the use of PyAOP (**16**) (15), which is derived from HOAt, is the most reactive phosphonium salt, but it is prohibitive for industrial purposes due to its extremely high price. The benzotriazole derivative, PyBOP (**15**) (16), which is also more expensive than its aminium analogue, is specially useful for cyclization steps or for the activation of hindered amino acids, where the use of aminium salts can lead to the formation of guanidine derivatives. For the same reason, PyBOP can be used in excess and added during the coupling step, because it will not terminate the peptide chain.

These derivatives can contain pyrrolidine, which can also react with the carboxylic acid giving the corresponding pyrrolidide derivative.

WHY CLASSIC ACTIVATORS WILL REMAIN FOR DECADES IN THE MARKET?

Despite the presence of novel, highly effective coupling reagents, the classical activators have remained in the market for years. We could point out a few reasons to define this fact:

- Influence of the human factor, which includes inertness or unavailability of human recourses to test and evaluate new coupling systems.
- Marketing problem, when a manufacturing company or distributor could not identify and localize its potential target group and end users.
- Supply chain when the information about new products and application provided by developer or supplier does not always can find its addressee.
- Legal and secrecy issues do not always give the opportunity of knowledge exchange between peptide contract manufacturer and raw material producer.
- Role of existing regulation requirements. List of the reagents used in a synthesis and synthetic process and methods used in a synthesis, including the reagents and synthesis condition should come as a part of an Investigational New Drug application (IND) for Phase I study (17), of a NDA (New Drug Application) (18) submitted to FDA at the completion of phase II clinical trial, and of a DMF (Drug Master File). The changes in manufacturing process and reagents used should be introduced before submission of NDA. The post approval changes in manufacturing process can be submitted and approved by FDA (Food and Drug Administration), together with additional tests confirming that such changes had no effect on identity, quality, bioavailability, toxicity and even efficiency of new drug substance. That would cause a delay in product approval and will tremendously affect cost of development of new drug substance. Process development chemist should present final manufacturing process that should be up-scalable and cost effective at an early stage of drug development. The close connection of development chemist with manufacturer of raw and source materials at an

early pre-clinical stage would help to avoid any post-approval changes in manufacturing process.

USING HIGH QUALITY, SAFE AND COST EFFECTIVE COUPLERS AT THE EARLY STAGE OF PROCESS DEVELOPMENT

The use of efficient coupling reagents is an effective way of cost saving at production stage and de-bottlenecking at purification step.

a. Contaminant and Impurities During Peptide Synthesis

Epimerised (Racemised) Peptides

The presence of these side products is strongly tied to the coupling method. Thus, efficient coupling reagents in combination with racemization suppressor additives and solvents of low dielectric constant should minimize the formation of epimers. Furthermore, reducing the time of preactivation of the carboxylic acid could also reduce the racemization. The presence of these peptides is very often difficult to detect, because their chromatographic behavior is similar to the target peptide.

Deletion Peptides

As above, the use of the most efficient coupling reagents should minimize the deletion of peptide formation. In solid-phase, the use of more than one analytical method to detect the presence of free amines is advisable. Although, ninhydrine test is the broader method used (19), very often it gives a false negative. Trinitrobenzenesulfonic Acid (TNBSA) (20) and especially NF-31 (21) , are excellent alternatives to the ninhydrine test.

Truncated Peptides

In solid-phase mode, truncated peptides are often formed by precipitation of the peptide. This phenomenon occurs when the resin is overdried. For this reason, it is advisable to leave the resin wet with solvent between the different synthetic steps.

Terminated Peptides

The presence of acetic and even of trifluoroacetic acid traces can terminate the peptide chain. Furthermore, as discussed above, aminium salts can also guanilidate free amine functions.

Modified Peptides

Aspartiimides that can lead to both α and β peptides are difficult to detect. The formation of aspartiimides, which can take place during basic treatments (*e.g.* during piperidine treatment in a Fmoc/*t*Bu strategy) can be reduced by adding HOBt.

Other important modifications can take place during the final deprotection/cleavage step. This can be minimized by the using of the appropriate scavengers.

Reaction By-Products

Ureas from carbodiimides and aminium salts, phosphotriamides from phosphonium salts, coupling additives, and scavengers used in the last step are the most important reaction by-products.

Oxidized Peptides

These kinds of peptides are mainly encountered in sulfur-based peptides and are unrelated to the coupling reagent used.

b. Toxic and Hazardous Reagents Used in Synthesis

Up-scaling from the bench and beyond requires from the manufacturer reevaluation of very critical parameters such as transportation, storage, user safety and waste management of raw materials and in particular coupling reagents. There are situations where reagents, convenient and handy in laboratory, become unmanageable by contract manufacturer when it comes to bulk peptide manufacturing. This may lead to production downscaling and reevaluation of synthetic procedure. The transportation and subsequently storage and use issue, becomes critical for the reagents containing the imidazole ring benzotriazoles (HOBt), as well as for reagents with an extra nitrogen in the phenyl ring (HOAt), which according to recent studies make the structure unstable with relatively high sensitivity to friction, spark, and electrostatic discharge resulting in burning or explosion. However the onium salt component (HBTU, TBTU, HATU, PyBOP), and derivatization of the phenyl ring with halogen ion (HCTU, TCTU) stabilize the structure (13). It is shown that addition of water or solvents can also make the compound less sensitive. Reagents can still be transported and stored to comply with international transportation regulations for hazardous substances, but the bulk use of some of them is in question, since the additional safety and logistic measures should be considered. This may include the safety revaluation of existing equipment following installation of an additional gear in production site, antistatic flooring in storage facilities and production site and personnel training. Logistic and transportation department control material packed in appropriate containers should not exceed weight restrictions and appropriate labeling and documentation.

Another important issue is the toxic properties of substances used in bulk peptide synthesis. The modern chemical laboratories are usually well equipped to provide environmental and personal protection to technicians and researchers from possible toxic effect of raw material. In general, chemical fume hood, laboratory coat, gloves and facemask usually gives necessary protection, yet a number of researchers have reported the adverse effect of certain coupling reagents and additives on their physical condition.

Carboxydiimides for example is well known for their skin irritating properties while prolonged use some of benzotriazole based coupling reagents and additives (HOBt, HBTU, TBTU) may not only cause skin irritation and contact dermatitis, but also sensitization and allergic reaction of respiratory tract (22). The situation becomes more complicated when it comes to industry. To protect the worker and the environment, special precautions should be taken by manufacturer. This includes installation of HEPA filters, special equipment for preventing dusty conditions at the time of reactor loading, personal protection which usually consists of protective suit, gloves, boots and full face mask with air breathing apparatus and requires additional training of personnel (23).

The above factors can be overcome if users

Table I - Examples of peptide drugs and manufacturing methods

Peptide	Length	Quantities	Status	Synthetic strategy / Coupling method
Eptifibatide	7	>200 kg	commercial	solution
Leuprolide	9	25-50 kgs	commercial	solution / solid
Pramlintide	37	>10 kgs	III	solution / solid
Exendine	39	NA	III	solid
Zinconotide	25	1-5 kgs	III	solid
Fuzeon (T-20)	36	up to 4 MT	approved	solid/fragment condensation HBTU/HOBt or / Cl-HOBt
Autosiban	8	NA	approved	solution DCC/HOBt
Theratope	43	NA	III	solution/block coupling DCC/HOBt
Thymalfasin	28	NA	III/approval	solid phase
Desmopressin	9	NA	approved	solution DCC/HOBt

comply to transportation and storage regulations and increase their alertness to safety precautions, installation of special protective equipment, as well as working in close contact with the supplier of raw materials and learning from his experience. However, this may affect the manufacturing schedule and production price.

c. Yield of Coupling

As it is obvious, adopting highly effective coupling reagents will result in avoiding double coupling, deletion and modified peptides, will cut the solvent and protected amino acid use, favour the purification step and therefore, decrease production cost.

Today, majority of peptide drugs up to 13-15 amino acids are synthesized using solution approach, Boc chemistry and conventional DCC/HOBt method. Recently we observe a shift of industry to solid phase Fmoc chemistry, increasing in use of modern coupling techniques (HBTU/TBTU and HCTU/TCTU) together with fragment condensation approach of the 12-15 amino acid sequences (1,3,24-26).

CONCLUSIONS

The formation of the amide bond together with chirality of the molecules plays a key role in the preparation of a broad range of organic compounds. It begins with the small molecules through the peptides to fully synthetic proteins. Today, the synthesis of peptide or protein based pharmaceutical drug requires up to 100 steps, the name of the game is "production cost" and the modern synthetic technologies play a central role in this battle. A thorough study of the mechanism in each method involving basic producer for logistic and technical support is a necessary term for the optimization of the coupling step of a new peptide drug and further up-scaling towards bulk manufacturing. Today, Cl-HOBt as an additive and HCTU/TCTU are excellent alternatives to the most classic methods for the large scale manufacturing of peptide and proteins.

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