A PRACTICAL GUIDE TO SOLID PHASE PEPTIDE SYNTHESIS

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The purpose of this guide is two-fold. First, a brief introduction on the development and most common applications of solid-phase peptide synthesis will enable the user to best apply the two most widely-used synthetic strategies – Boc/Benzyl and Fmoc/*t*Butyl chemistries – to his/her projects. Second, a detailed description of peptide synthesis, cleavage, and purification in the experimental section is given with 'helpful hints' so that newcomers to peptide science will have easy access and avoid some of the obstacles which often lead to expensive mistakes and/or poor synthesis yields.

Introduction

Solid phase synthesis is a process by which chemical transformations can be carried out on solid support in order to prepare a wide range of synthetic compounds. This idea was first developed by Bruce Merrifield to synthesize polypeptides and earned him the Nobel Prize in 1984. Solid phase chemistry offers many advantages over conventional synthesis in terms of efficiency as well as convenient work-up and purification procedures. In solution phase peptide synthesis, particularly in longer sequences, the repetition of coupling and deprotection cycles can become very labor intensive and require the isolation of all peptide intermediates.

I. A Brief Historical Perspective

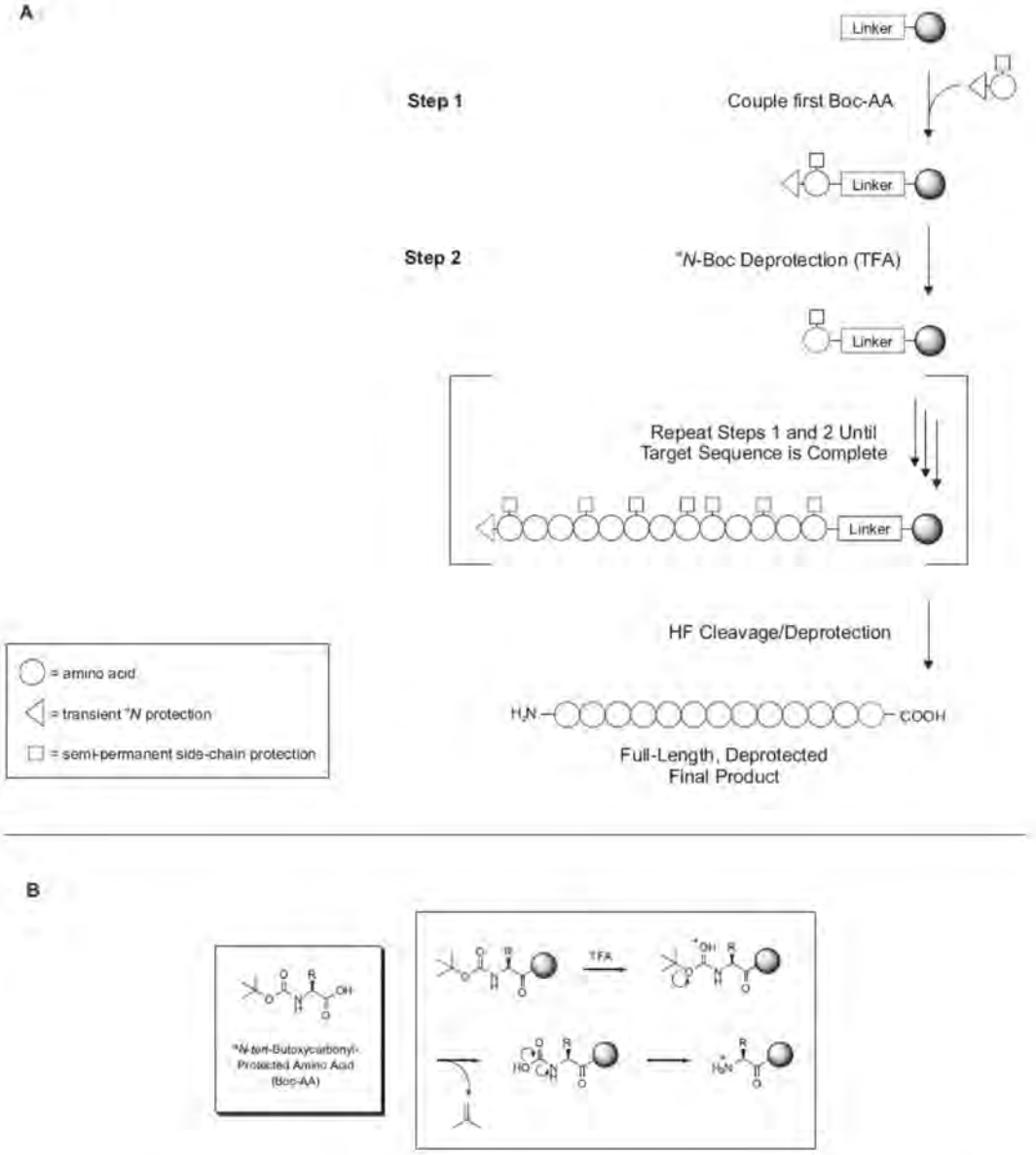
The chemistry of peptide synthesis – first developed in the early 1900's by Emil Fischer – arguably marks the birth of organic synthesis as we know it today. Whereas the lion share of organic synthesis continues to be performed by solution-phase methods, *i.e.,* with each independent

chemical reaction followed by a purification step and characterization of the resulting synthetic intermediate, two peculiarities of peptide chemistry spurred the development of a more efficient synthetic strategy. In contrast to most total synthesis efforts, the synthesis of peptides – at least until the final deprotection step – is an iterative process, with α-amino ($^{\alpha}N$) deprotection and amide couplings performed in succession until the desired full-length target peptide is obtained. In addition, most peptides of biological interest are grossly insoluble in most organic solvents irrespective of side-chain protection tactics. The net result of these two characteristic features of peptides was that the first sixty-odd years of peptide synthesis forged little ground until the landmark work in the late 1950's of R.B. Merrifield at Rockefeller University.

During the course of his Ph.D. work, Merrifield (a biochemist) proposed an entirely new paradigm in organic synthesis. As is often the case when an outsider looks into an insular field and pursues a tack that is anathema to the existing experts in the field, Merrifield's idea of performing all synthetic manipulations using the C-terminus of the target peptide linked to an insoluble solid support was met with much criticism. However, it was not long before the chemistry of solid-phase peptide synthesis (SPPS) was honed to a point where traditional solution-phase methodologies were no match with regard to speed and versatility. The original "Merrifield" version of SPPS[1] – more accurately referred to as Boc/Benzyl chemistry – was roughly finalized in the late 1960s, and employs a *graduated acid lability* system for manipulation of all protecting

[1] In fact, the first variant of SPPS developed by Merrifield employed a different, orthogonal chemistry, which employed extremely harsh acid and base treatments. The merits and general applicability of TFA-mediated $^{\alpha}N$-deblocking and HF-mediated global deprotection/cleavage (Boc/Benzyl chemistry) were early recognized and arose to prominence as the standard "Merrifield" chemistry for SPPS.

groups (Scheme 1). In this strategy, the α-amino *t*-butoxycarbonyl (Boc) protection is removed with TFA, while side-chain protections and the peptide- resin anchorage (the linker) require much harsher acidic conditions for cleavage. This final step is accomplished using liquid HF, a much stronger acid than TFA (acidity functions of -11 and 0.1, respectively).



Scheme 1. **(A)** Generalized scheme for stepwise SPPS *via* Boc/Benzyl chemistry. **(B)** Structure and mechanism for acidolytic $^{\alpha}N$-Boc deprotection.

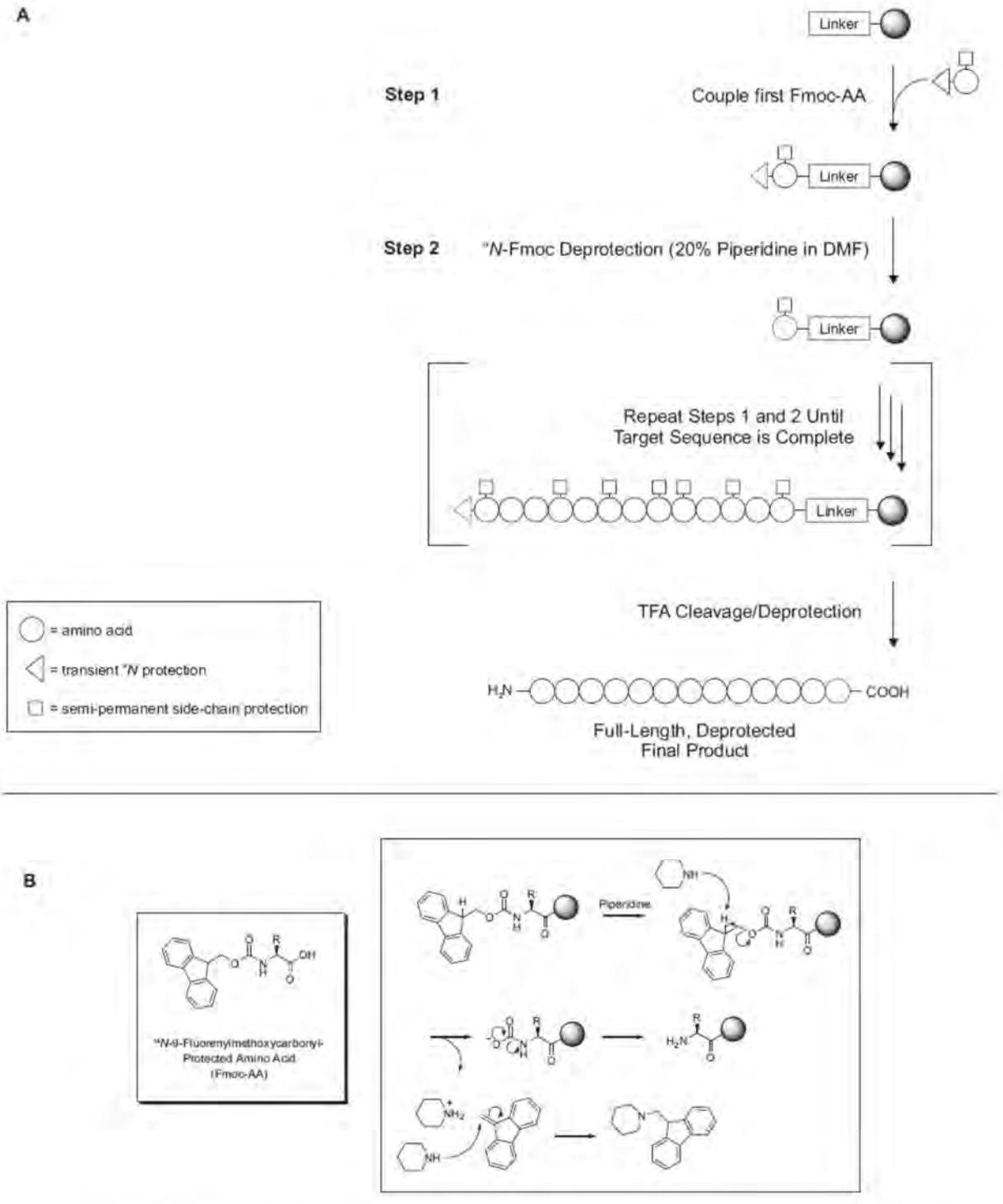
Scheme 1 depicts the manner in which Boc/Benzyl SPPS simplifies all of the reactions involved in peptide synthesis by allowing for purification *via* filtration, so that excess reagents can be

employed and removed by simple washing. It is important to emphasize that the chemistry of SPPS is not fundamentally different from that used in solution-phase peptide synthesis. The sole chemical distinction between solution- and solid-phase peptide synthesis is that the *C-terminal protecting group in the latter is rendered insoluble by virtue of its incorporation into a polymer*. This difference notwithstanding, all side-chain and $^{\alpha}N$ protecting groups, as well as coupling chemistries, employed in solid-phase synthesis have been successfully applied in solution-phase synthesis, and vice-versa, with few exceptions.

During the 1970s several groups were actively developing milder methods for SPPS that avoided the use of liquid HF as for the final deprotection/cleavage reagent. While a variety of milder graduated acid lability systems were devised, the method that rose to general applicability was the *orthogonal* system of Fmoc/*t*Bu chemistry. This strategy – developed by R.C. Sheppard at Cambridge University – differs from Boc/Benzyl chemistry in that the side-chain and $^{\alpha}N$ protecting groups are removed under conditions that leave the other class entirely intact. In Fmoc/tBu chemistry, a mild base – usually piperidine (pKa = 11.1) – is employed for iterative $^{\alpha}N$ 9-fluorenylmethoxycarbonyl (Fmoc) deprotection, while global side-chain deprotection/cleavage is accomplished with TFA (Scheme 2).

It must be emphasized that the more traditional Boc/Benzyl and Fmoc/*t*Bu chemistries, while differing in chemical minutiae, are fundamentally the same process. In both cases, the target peptide chain is assembled in a stepwise fashion from $^{\alpha}N$- and side-chain protected amino acids. In both cases, the 'transient' $^{\alpha}N$ amino protection is employed solely during chain elongation (the coupling reaction) and then removed for the subsequent coupling reaction. Lastly, in both cases, the final step – global side-chain deprotection and cleavage of the peptide-resin anchorage – is

accomplished by acidolysis, and the target peptide isolated by trituration from ether and purified by reversed-phase high performance liquid chromatography (RP-HPLC).



Scheme 2. **(A)** Generalized scheme for stepwise SPPS *via* Fmoc/*t*Bu chemistry. **(B)** Structure and mechanism for base-mediated °*N*-Fmoc deprotection.

During the first 20 years or so of SPPS (1970-1990), most laboratories in academia and industry performed Boc/Benzyl chemistry. Only when the comparatively 'younger' Fmoc/tBu chemistry had matured – with appropriate side-chain protections and its unique side reactions circumvented – in the late 1980s did it begin to overtake Boc/Benzyl chemistry for routine SPPS needs. It is our experience that Boc/Benzyl chemistry is *difficult to do, but easy to do well*, while Fmoc/*t*Bu chemistry is *easy to do* (and therefore well-suited to the newcomer to SPPS), *but difficult to do well*.

II. Boc and Fmoc SPPS

There is unfortunately a pervasive mentality that Boc and Fmoc chemistry are fundamentally different, but this is not the case.

Boc and Fmoc SPPS are simply different strategic executions of the same idea.

In both Boc and Fmoc chemistry, $^{\alpha}N$ protection is achieved using a mildly electron-withdrawing carbamate. In both cases, the side-chain protections are based on ethers, esters, carbamates, carboxamides, and sulfonamides. In both cases, carboxyl activation is accomplished using the same activating reagents. And in both cases, the scissile peptide-resin anchorage is cleaved in acid, leaving the desired C-terminal functionality (usually an acid or amide). The principal chemical minutiae differentiating the two strategies lie in the method for iterative $^{\alpha}N$ amino deprotection and the degree of acid stability of the side-chain and peptide-resin linkages. The reader will likely notice this high degree of architectural similarity from Scheme 1a and Scheme 2a.

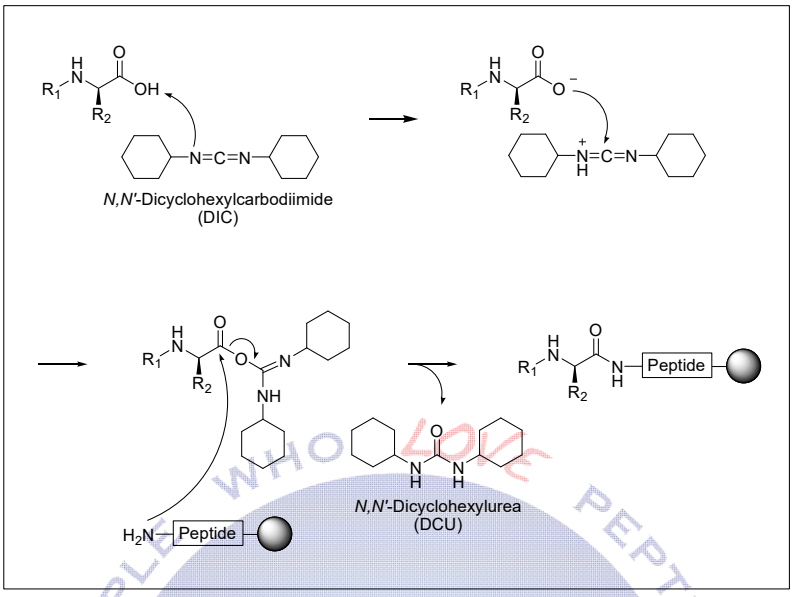
At present, Boc chemistry continues to hold an edge over Fmoc/tBu chemistry from an economic standpoint with regard to the cost of the necessary protected amino acid derivatives and solvents. In addition, longer peptides rich in β-sheet structure tend to more accessible by Boc chemistry

owing to the powerful disaggregating properties of TFA, used during iterative $^{\alpha}N$-deprotection steps. This feature – the lower incidence of failed syntheses – continues to be a potent driving force for the continued popularity of Boc chemistry. Finally, the synthesis of C-terminal α-carboxythioesters is generally regarded to be best accomplished by Boc chemistry. While several methods have been reported to allow the synthesis of peptide thioesters by Fmoc chemistry, such methods have significant drawbacks and have been only sparsely validated. As peptide thioesters are important synthons for the total chemical synthesis of proteins and bioconjugates, the ease with which these can be prepared by Boc chemistry further substantiates its continued popularity.

The convenience of Fmoc/tBu chemistry (vis-a-vis the absence of HF) and its facile amenability to parallel synthesis and cleavage without specialized apparatus are attractive features in industry and academia alike, and are largely responsible for its rise in popularity in recent years. Aside from these practical considerations, Fmoc chemistry is ideally suited to certain side-chain modifications, which are less conveniently installed by Boc chemistry. For instance, the elaboration of synthetic peptide targets with sugars (glycopeptides), sulfates (sulfopeptides), and phosphates (phosphopeptides) is easiest accomplished by Fmoc chemistry. The reason for this admonition is two-fold: first, Fmoc chemistry allows for more dimensions of orthogonality than Boc chemistry, so site-specific modifications of the resin-bound peptide are easier to perform. Second, the milder nature of TFA cleavage/deprotection allows acid-labile modifications (e.g., pendant sugars) to survive intact.

Overall, Boc and Fmoc chemistry can be used equally well for the vast majority of synthetic peptide targets, despite the fact that the chemical peculiarities of specific modified peptides may contraindicate the use of one chemical strategy versus the other. Suffice it to say that the selection of a SPPS strategy for a given peptide target is due as much to the personal experience of the user

and expertise available to him/her than chemical considerations *per se*.



Scheme 3. Mechanism for amide coupling *via O*-acylurea activation

III. Coupling Methods

i. *O*-Acylureas

The oldest form of widely used carboxyl activation in stepwise SPPS is the *O*-acylureas intermediate (Scheme 3). While classical acylation chemistries such as the acid chloride and mixed anhydride are in theory viable for stepwise SPPS, significant levels of side reactions attend the use of such reactive intermediates, and were early recognized to contraindicate their use in the synthesis of targets of any degree of complexity and size. The *O*-acylureas species is readily formed in organic solvent in the absence of base; as this addition reaction is typically faster in nonpolar – rather than polar – solvents, the *O*-acylureas is usually formed in DCM. If desired, solvent can be removed in vacuum so that the acylating species can be re-solubilized in another solvent such as DMF.

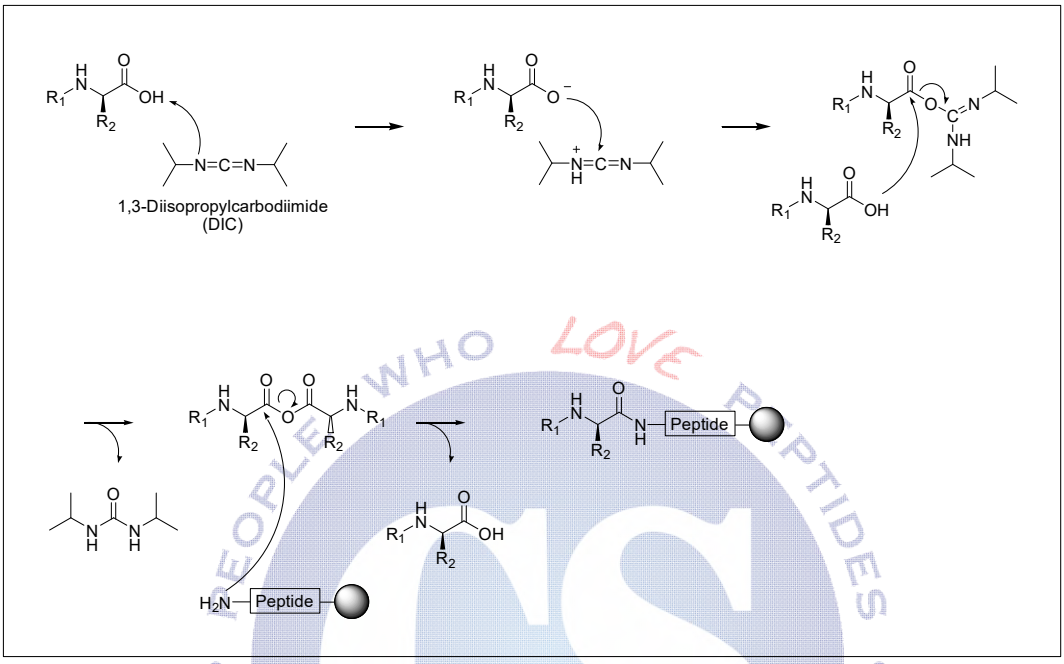
In spite of an improvement over its earlier ancestors with regard to attenuated reactivity, the *O*-

acylureas bears some significant drawbacks. First, an intramolecular *O*→*N* acyl transfer easily allows the reactive *O*-acylureas to rearrange to the inert *N*-acylureas. This species does not pose any specific problems by itself for stepwise SPPS other than the consumption of the active acylating species prior to amide coupling. Second, the high reactivity of these species allows for an unacceptable level of racemization at the $^{\alpha}C$ position, presumably through the formation of an oxazolone intermediate. These considerations – both stemming from the recognized high reactivity of the *O*-acylureas intermediate – early motivated the use of additives which would afford a more stable, albeit still reactive, acylating species.

ii. Symmetric Anhydrides

The use of symmetric anhydrides in place of *O*-acylureas heralded a quantum leap in the efficiency and yields of SPPS. The reader will notice that symmetric anhydrides are formed *via* a two-step reaction sequence in which the initial *O*-acylureas reacts with another molecule of the same $^{\alpha}N$-protected amino acid, liberating a urea by-product along with a symmetric anhydride as the active acylating agent (Scheme 4). It should be noted that the formation of symmetric anhydrides is significantly faster in non-polar solvents such as DCM, which comes with the added benefit of allowing for visual monitoring of the activation process (as the urea of DIC is insoluble in this solvent, whereas it is soluble in DMF).

Symmetric anhydrides have tapered in popularity in recent years owing to the facility with which other chemistries (notably HBTU) can be adapted to automation without the formation of any

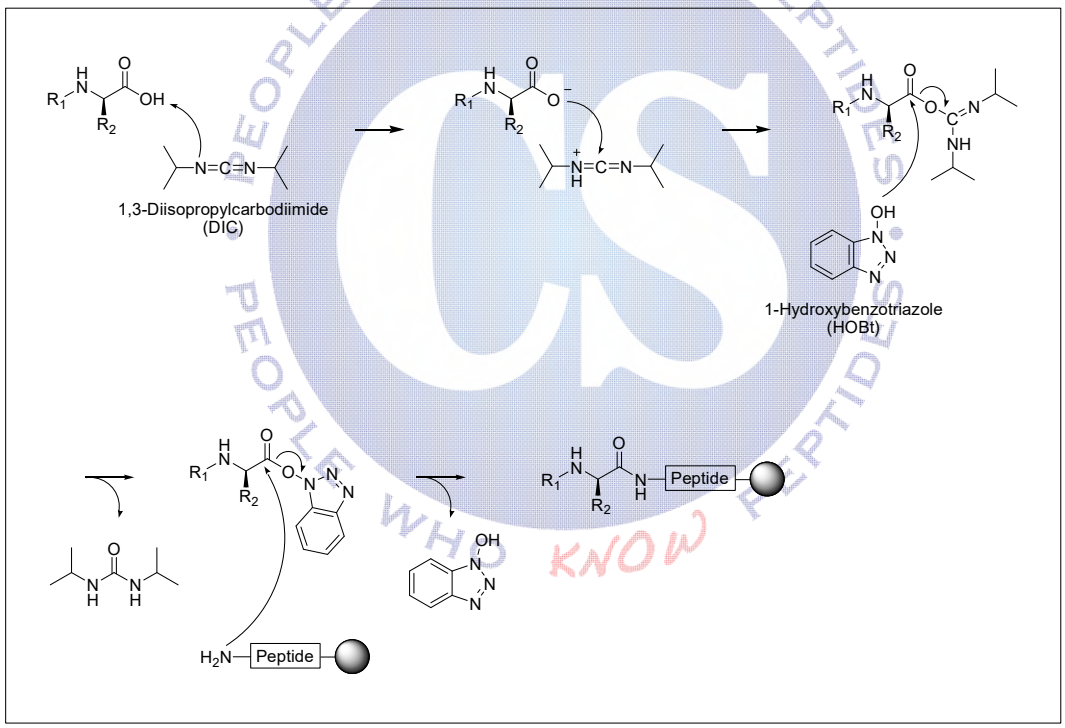


Scheme 4. Mechanism for amide coupling *via* symmetric anhydride activation

troublesome insoluble by-products. However, symmetric anhydrides remain a powerful tool in the peptide chemist's repertoire, as they are exceptionally reactive acylating species – far more so than HOBt esters – while still being nearly impervious to racemization. While early derided as being wasteful of precious protected amino acid derivatives, this is less of a concern today than it was in the early days of SPPS when suitably protected amino acids were rare commodities with correspondingly high unit prices. Furthermore, the high reactivity of symmetric anhydrides makes them the most cost-effective chemistry for difficult acylations, such as those onto α-disubstituted amino acid residues and secondary amines. Symmetric anhydrides also remain the intermediate of choice for DMAP-catalyzed acylation of hydroxyl linkers during the coupling of the C-terminal residue to a linker-functionalized resin.

iii. HOBt esters *via* DIC/HOBt

N-alkyl and *N*-acyl hydroxylamine species were recognized in the mid-1970s as having ideal properties as additives during amide coupling reactions. While several have been popularized to varying extents, 1-hydroxybenzotriazole (HOBt) is far-and-away the most widely used additive in standard amide coupling reactions. HOBt is actually a mild acid at the hydroxyl group (pKa = 4.5), intermediate between citric acid and acetic acids. The acidity of HOBt derives from two sources: (1) the inductive effect of the N1 atom, and (2) aromatic stabilization of the conjugate base. HOBt is a weak nucleophile, but still potent enough to be acylated by an *O*-acylureas



Scheme 5. Mechanism for amide coupling *via* DIC/HOBt activation

intermediate. Of critical importance, the HOBt ester is a stable entity, and prone to far fewer side reactions than more highly activated acylating species (*e.g.*, acid chlorides, anhydrides, *O*-acylureas).

HOBt esters are usually formed through the *O*-acylureas intermediate (Scheme 5). In scheme 5, the carbodiimide N,N'-diisopropylcarbodiimide (DIC) is depicted rather than N,N'-dicyclohexylcarbodiimide (DCC), as in scheme 3. Either carbodiimide may be used in principle, but DIC has superseded DCC in recent years owing to its more facile handling properties, particularly the solubility of the resulting urea by-product. Whichever carbodiimide reagent is used, it is important to recognize that its role in the formation of an HOBt ester is solely as a stoichiometrically equivalent dehydrating reagent.

Several features of HOBt esters substantiate their popularity. First and foremost, the use of HOBt esters for amide couplings is operationally simple. In the case of Fmoc chemistry, there is no particular requirement for a specific order of addition or preactivation phase. This point is of critical importance. It should be mentioned that scheme 5 is an idealized representation of the preparation of HOBt esters *via* DIC activation. In fact, the coupling 'cocktail' is precisely that – a mixture of several acylating species, such as O-acylureas, symmetric anhydrides, and HOBt esters. The exact composition of said intermediates is dependent on the method of preactivation, solvent composition, and reagent stoichiometry. Furthermore, it is difficult to guarantee that preactivation does indeed occur to completion, so in reality a mixture of activated and inactivated species - together with residual carbodiimide - are added to the resin-bound amine. Nonetheless, 'all roads lead to Rome', and the fundamental point from a practical vantage is that provided the resin-bound α-amino functionality is a free base (not a salt), no serious side reactions attends the use of DIC/HOBt to prepare HOBt esters. This is in stark contrast to Boc chemistry, where TFA deprotection of the Boc liberates the α-amine as a trifluoroacetate salt. As ammonium ions react readily with carbodiimides to yield guanidines, DIC/HOBt chemistry is contraindicated in modern Boc SPPS, which is usually performed without pre-neutralization of the resin-bound amine.

The second primary feature of HOBt esters formed *via* DIC/HOBt is that this continues to be the gold-standard for minimal racemization amide couplings using carbamate-protected amino acids, even with preactivation and irrespective of solvent composition. Stereomutation of only a few percent is still unacceptable for stepwise SPPS in light of the cumulative effect of such a side reaction. While carbamate-protected amino acids were early though to be highly resistant to racemization owing to their mildly electron-withdrawing character and resonance stabilization of the carbamate functionality, more recent investigations have shown that the modern side-chain protected forms of certain amino acids – notably Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH – are still prone to racemization by a variety of plausible mechanisms.

There are two shortcomings inherent in the use of HOBt esters formed via DIC/HOBt.. First, the initial nucleophilic addition step – the attack of the carboxylic acid on the electron-poor carbodiimide carbon – is slow at room temperature and in polar aprotic solvents (e.g., DMF) commonly used to achieve high concentrations of activated amino acids in SPPS. Second, the by-product of carbodiimide-mediated couplings is a poorly soluble urea derivative, which can be problematic under certain circumstances. These shortcomings, however, are of little concern when measured against the many advantages afforded by this essentially bulletproof coupling chemistry. With regard to the slowness of this activation chemistry, this need only be put into perspective; with properly-written coupling cycles, DIC/HOBt cycles are at most 50% longer than HBTU cycles (*vide infra*). Furthermore, the insolubility of the urea by-product is essentially a non-issue in modern SPPS practice wherein the classical carbodiimide, DCC, has been all-but-completely supplanted by DIC. The urea formed by DIC activation is quite soluble in DMF, in contrast to the urea formed by DCC activation, which has long been known to clog fritted reaction vessels and contaminate solid supports.

iv. HOBt estes *via* HBTU and BOP

Since the mid-1990s, HBTU has been the coupling method of choice for many laboratories engaged in SPPS. Its merits include (1) convenience (a single reagent for formation of HOBt esters); (2) cost-effectiveness (in recent years); (3) rapid activation chemistry; and (4) the absence of any insoluble by-product. It must be emphasized that HBTU and DIC/HOBt all give rise to the same end product. The most significant difference, however, is that HBTU activation requires the use of an external base (e.g. DIEA) so that the protected amino acid derivative will be deprotonated to the carboxylate form (Scheme 6). This, in turn, is the nucleophilic species, which reacts with the stabilized carbocationic urea salt. Although it has become a staple in many laboratories, base-mediated coupling chemistries such as HBTU must be regarded with due caution. Certain protected amino acids are prone to racemization when activated in the presence of base, and this problem is exacerbated if the base metering is not accurate and precise to solely the stoichiometric equivalent required.

In Boc SPPS, HBTU couplings are commonly used in conjunction with *in situ* neutralization protocols, wherein excess base is added during the preactivation phase. While this is acceptable for some peptides with certain protecting group combinations, His(Dnp) residues are still prone to racemization during activation, and this protection should be used with caution.[2]

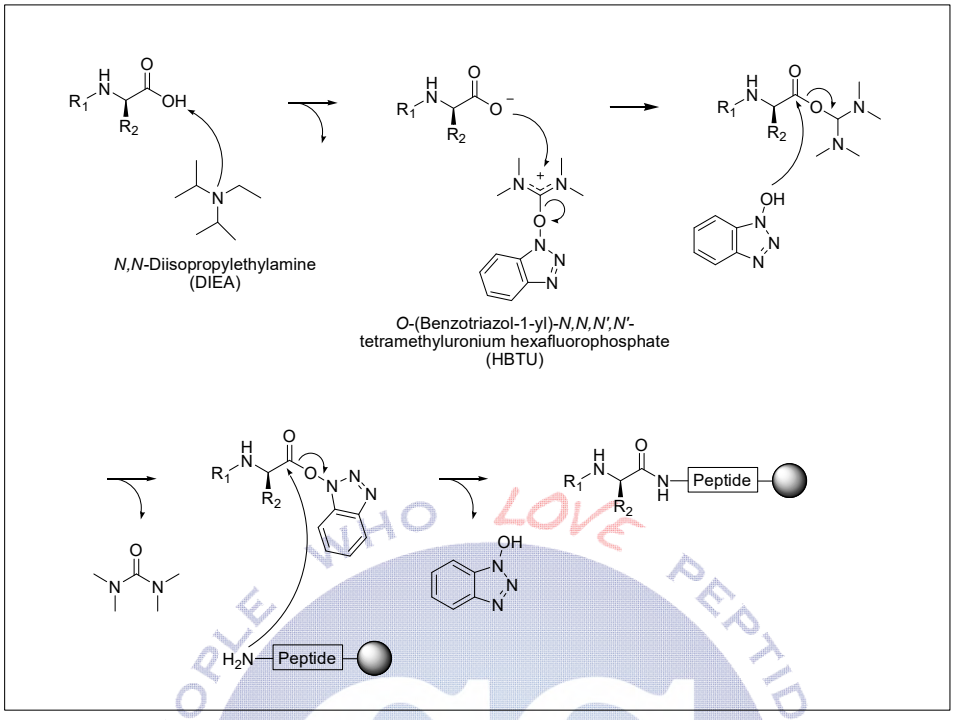
In Fmoc SPPS, HBTU couplings should be performed with even more caution, as the different

[2] Boc-His(Bom)-OH is recommended in virtually every case over Boc-His(Dnp)-OH. Aside from being essentially impervious to racemization during activation, the imN-Bom (benzyloxymethyl) protecting group is inert to nucleophiles, such as the neutralized N-terminus during each coupling. Therefore, with the exception of peptides to be used in native chemical ligation – wherein the formaldehyde generated during Bom deprotection is highly reactive – His(Bom) is the derivative of choice in Boc SPPS.

side-chain protections – notably Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH – are highly prone to racemization. Furthermore, the presence of secondary amine impurities in low-grade DIEA can give rise to a small but consistent percentage of premature Fmoc deprotection during coupling reactions, which is catastrophic for large peptides.

Structurally related to HBTU, BOP was actually the first reagent devised for 'one-pot' activation to an HOBt ester. Several reasons have led to its decline in popularity in recent years relative to its younger chemical sibling. First, BOP activation is considerably slower than HBTU activation, which likely stems from the greater stability of tetravalent phosphorous in comparison to trivalent carbon. BOP activation also liberates a highly toxic by-product, hexamethylphosphoric triamide (HMPA), which can be a significant concern depending on the user's synthesis apparatus and the end-use of the peptide target.[3] Finally, solutions of BOP are not nearly as stable as solutions of HBTU. Whereas 0.5M solutions of HBTU are routinely used up to 1 week old at RT in the dark, BOP solutions show significant decomposition if left overnight.

[3] EMD markets a replacement for BOP – PyBOP® – which incorporates a tri-pyrrolidone replacement for HMPA, and is therefore devoid of a carcinogenic by-product.



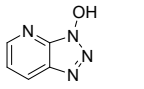
Scheme 6. Mechanism for amide coupling *via* HBTU activation

One significant difference between HBTU and BOP – and which is of fundamental importance in the practice of SPPS, irrespective of the overall chemical strategy employed – is that the high reactivity of HBTU during activation can itself be problematic in certain situations. Shortly after the introduction of HBTU, it was early-recognized that incomplete pre-activation of the incoming $^{\alpha}N$ protected amino acid led to a capped tetramethylguanidinium by-product with a weight-gain of + 100. The resolution to this problem is that pre-activation is an absolute necessity during the use of HBTU, and a 5 minute pre-activation at a concentration of ≥0.2M is adequate. Extended pre-activation is contraindicated on the basis of racemization; almost without exception, racemization of amino acids occurs solely after activation, so limiting this time window to the shortest timeframe that still allows for quantitative consumption of the HBTU reagent is recommended. This is also the reason why a slight excess of incoming $^{\alpha}N$-protected amino acid is usually employed during HBTU couplings, thus ensuring that no un-reacted HBTU may react with the deblocked N-

terminus. The lower electrophilicity of BOP is such that it does not suffer from this shortcoming; in fact, the absence of BOP reactivity with amines has led to its popularity in solid-phase cyclizations *via* lactams.

v. Recent Developments

With the exception of O-acylureas and symmetric anhydrides, the somewhat aged HOBt ester remains the mainstay of modern SPPS, and not without reason. As described above, HOBt esters are quite resistant to racemization for all protected amino acid derivatives commonly used in modern SPPS. Its low cost, virtually infinite shelf life, and high solubility in polar solvents such as DMF are no less-weighty reasons for its popularity. Nonetheless, certain specialized applications have led to variations on this theme. It should be mentioned that the newcomer to SPPS might be bewildered at the choice of available coupling reagents, many of which appear to be redundant and have found varying degrees of independent verification. Described herein is a small selection of this newest generation of coupling reagents; emphasis is placed on those reagents which are commercially available and do not require specialized apparatus to handle.[4]



1-Hydroxy-7-azabenzotriazole (HOAt)

Without doubt the most widely used improvement on the HOBt scaffold has been the introduction of 1-hydroxy-7-azabenzotriazole (HOAt) in 1994 by Han and Carpino. The addition of a nitrogen atom in the 7-position of the benzotriazole skeleton allows for an intramolecular H-bond between the activated carboxyl oxygen and the pyridine nitrogen, which increases the electrophilicity of the former without compromising its resistance to racemization *via* the oxazole intermediate. The HOAt ester is considerably more reactive than the parent HOBt ester; whereas

[4] For additional details and/or inquiries regarding specialized applications, contact the author (*vide supra*, note #1)

secondary amines – such as *N*-methyl and certain *N*-benzyl amino acids residues – are only poorly acylated with HOBt esters, quantitative functionalization using HOAt esters is often achieved.

At present, there are two commonly used reagents for the synthesis of HOAt esters. The simplest method uses DIC and HOAt, in exactly the same fashion as DIC and HOBt. Alternatively, the HOAt analogue of HBTU – termed HATU – can be used for a one-pot activation chemistry. It must be emphasized that the use of HATU vs. HBTU must still be performed with due caution vis-a-viz sufficient preactivation to prevent the formation of a tetramethylguanidinium-capped resin-bound peptide chain. PyAOP – the HOAt analogue of PyBOP – is commercially available as well, but significantly more expensive than HATU. This reagent has found limited application, and it is not entirely clear if the absence of a capping side-reaction in the case BOP (and its structural homologue, PyBOP) translates into PyAOP. In short, we recommend that when HOAt esters are called for, *i.e.*, in the case of a sterically/electronically demanding coupling and/or a highly aggregating peptide chain, they are best formed using DIC/HOAt. Partly this stems from the admonition against the use of an external base whenever possible; however, an economic motive is present as well. As the molecular weight of HOAt (136.1) is approximately 1/3 of that of HATU (380.3), the user can accomplish three times more HOAt acylations by simply using DIC as the intermediate dehydrating reagent rather than HATU alone.

It should be briefly noted here that HOAt is not the only HOBt analogue commercially available. 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt) is commercially available and, owing to the electron-withdrawing chloro- substituent, yields an active ester that is slightly more activated than the parent HOBt ester. Like HATU, 6-Cl-HOBt is available both in native form and as an HBTU-type salt, which has been termed HCTU. We routinely use 6-Cl-HOBt in conjunction with DIC as a general replacement for HOBt, and can roughly approximate the reactivity gradient as HOBt

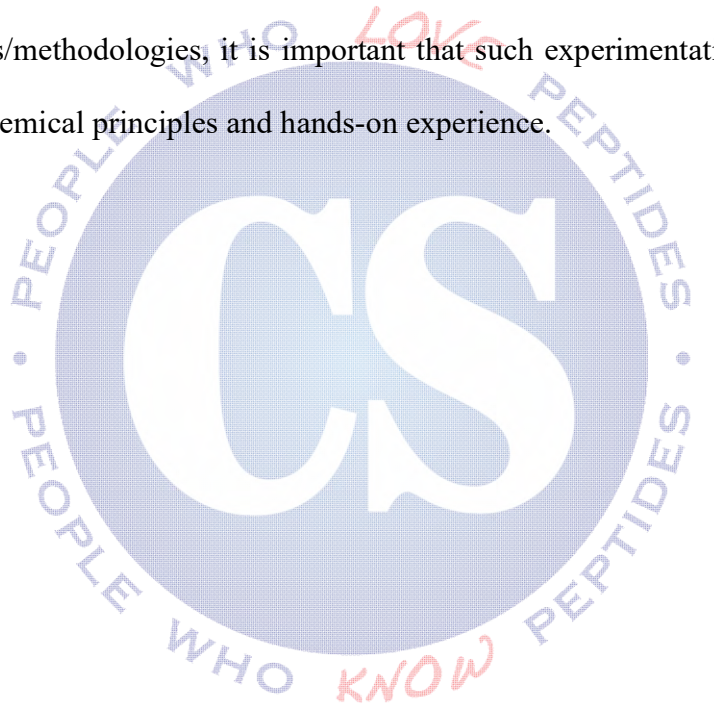
< 6-Cl-HOBt << HOAt. It is noteworthy that the cost gradient for these reagents is the same; thus whereas it is reasonable to use 6-Cl-HOBt in place of HOBt for routine DIC/6-Cl-HOBt protocols, routine replacement of HOBt with HOAt is not economically viable. HOAt is best reserved for special cases (*vide supra*).

Another class of new activating species is called one-pot reagents for the synthesis of acid fluorides and bromides – TFFH and PyBrOP, respectively. It is our experience that TFFH is of limited utility; whether this is due to batch-to-batch irreproducibility in its manufacture or a short shelf life of this salt is uncertain. PyBrOP is of similar utility, and is a useful alternative to active ester couplings during difficult acylations. However, solutions of PyBrOP are not stable, presumably due to adventitious moisture, and it should be dissolved immediately before use in coupling reactions. It is worth noting that these reagents for *in situ* acid halide synthesis are not *a priori* better or worse than HOAt esters or symmetric anhydrides for the acylation of sterically/electronically demanding amines.

We suggest treating each different class of amine on a case-by-case basis and attempt acylation with a variety of coupling chemistries and solvent compositions. This latter point is of special importance; occasionally the choice of solvent for a given acylation will have a significant effect owing to H-bonding disruption by polar solvents (e.g., DMF) rather than nonpolar solvents (e.g., DCM). However, in many cases the solubility of the coupling chemistry does not permit the use of nonpolar solvents. For example, racemization has long been known to be largely suppressed by nonpolar solvents, but the gross insolubility of uranium salt reagents (HBTU, HATU, and HCTU) in solvents other than DMF and DMSO precludes the use of this otherwise desirable expedient. However, symmetric anhydrides and active esters (HOBt, HOAt, and 6-Cl-HOBt esters) can certainly be used in DCM when formed *via* a carbodiimide intermediate, usually after

filtration of the insoluble urea byproduct.

This point cannot be overemphasized – *your CSBio instrument is only as good as the chemistry performed in it*. The flexibility of the CSPEP/TRIPEP software packages is designed to give the user virtually limitless freedom in the customization of existing protocols. However, this is not without an important *caveat emptor*. The successful stepwise synthesis of even a 10-residue peptide embodies the sum of between 30 and 50 independent chemical reactions which, by absolute necessity, proceed to completion. While this should not deter the user from experimenting with new chemistries/methodologies, it is important that such experimentation be performed in accord with sound chemical principles and hands-on experience.



EXPERIMENTAL SECTION

This section has two parts: Peptide Synthesis and Peptide Purification. In the synthesis part, we give detailed procedures of Boc synthesis and cleavage, Fmoc synthesis and cleavage, and peptide modification using CSBio automated synthesizers and equipment. The purification part tells how to deal a target peptide from its crude to a final product with high purity.

Benchtop CSBio II and CS136X Automated Peptide Synthesizers

Commercial Scale CS936X-650L Automated Peptide Synthesizer

Peptide Synthesis

I. Boc Synthesis and Cleavage

Tert-Butyloxycarbonyl (Boc) Strategy: The Boc method has been exclusively used during the first 15 years of SPPS. The Boc protecting group on the alpha amino of the amino acid is removed by Trifluoroacetic acid (TFA) and the final cleavage of the peptide from the resin along with the removal of the amino acid side chain protecting groups requires strong acid, such as hydrogen fluoride (HF) or trifluoromethanesulfonic acid (TFMSA). Dichloromethane (DCM) is the primary solvent used for resin deprotection, coupling, and washing.

In peptide synthesis, t-Butyloxycarbonyl (Boc) protected amino acid residues (in excess millimole quantities) are sequentially bound to a resin support. These residues are assigned individual coupling cycles in accordance to their respective positions within the given peptide chain. Once a residue has been successfully coupled as evidenced by the Ninhydrin test, the compound is deprotected and neutralized for the next coupling cycle.

More or less peptide can be synthesized by varying quantities of reactants and solvents proportionately. Additionally, coupling intervals may be varied from 30 minutes to 72 hours; peptide resin washing intervals may range from 1 to 30 minutes.

Boc Synthesis

i. Resin

Modified polystyrene resins are used for Boc peptide synthesis. In general, the small particle sized resin of low cross-linking is favored. The cross-linked with 1% divinylbenzene (DVB) is most common used: A higher level of cross-linking would reduce the swelling. The old most popular resin is 200-400 mesh (38-75 μm) and these resins allow for rapid diffusion of reagents inside the beads. The 100-200 mesh (75-150 μm) resins are very popular now and these resins

allow for fast draining the reagents. The substitution of resins should approximately 0.5-0.6 meq/gm. The higher substitution resins are available for short and large quality of peptide. The Chloromethyl polystyrene resin (Merrifield resin) is most common for acid peptide. The phenylacetamidomethyl resin (Pam resin) is more stable to TFA deprotection than the benzyl ester linker but HF cleavage may give yields as low as 70%. The 4-methylbenzhydrylamine resin (MBHA resin) is most common for amide peptide. The resins with 100-200 mesh, 1%DVB, 0.5-0.6 meq/gm are most common used at our lab.

ii. Amino Acids

A: Ala	C: Cys(4-Me-Bzl), Cys(4-Me-OBzl), Cys(Acm)
D: Asp(OBzl), Asp(OcHex), Asp(Fmoc)	E: Glu(OBzl), Glu(OcHex)
F: Phe	G: Gly
H: His(Tos), His(Bom), His(Dnp)	I: Ile(1/2H2O)
K: Lys(2-Cl-Z), Lys(Fmoc)	L: Leu(H2O)
M: Met	N: Asn*, Asn(Xan)
P: Pro	Q: Gln*, Gln(Xan)
R: Arg(Tos)	S: Ser(Bzl)
T: Thr(Bzl)	V: Val
W: Trp, Trp(Formyl)	Y: Tyr(2-Br-Z)

Note: Asn & Gln, *: need HOBT. Asn cannot add with HBTU or BOP. Asp: Asp-Gly, Asp-Asn, Asp-Ala, Asp-Ser, and Asp-Leu, use the cyclohexyl ester. Asp(OcHex) instead the Asp(OBzl) to prevent from losing water during HF cleavage. His(Tos): 1 hour deprotection after His. No HOBT should add after His for 2 to 3 amino acids.

iii. Coupling Reagents

The most commonly reagents are DCC, DIC, HBTU, BOP, among which BOP and HBTU (also including TBTU, PyBOP, and HATU) are in situ activating reagents, with which the coupling reaction is very fast. To avoid peptide termination, the ratio of amino acid to coupling reagent such as HBTU should be 1 to 0.9.

iv. Equipment and Materials

Automated CSBio peptide synthesizer

Test tube heating block

Dichloromethane (DCM)

N, N'-Dimethylformamide (DMF)

N-Methyl-2-Pyrrolidione (NMP)

Ethanol (ETOH)

Diisopropylethylamine (DIEA)

Triethylamine (TEA)

Trifluoroacetic acid (TFA)

Indole

Dicyclohexylcarbodiimide (DCC)

Diisopropylcarbodiimide (DIC)

Benzotriazol-1-yl-Oxy-Tris-(Dimethylamino) phosphonium Hexafluorophosphate (BOP)

2-(1H-Benzotriazol-1-yl)-1,1,3,3-Tetramethyluronium Hexafluorophosphate (HBTU)

1-Hydroxybenzotriazole monohydrate (HOBt)

Boc-Amino Acid

Acetic anhydride

Ninhydrin

Phenol

Pyridine (Pyr)

Air 60 psi

Nitrogen 20 psi

v. Synthesis Protocols

t-Boc + DCC / DIC:

Cycle	No	Solvent	Time (min.)
1	2 – 3	DCM	1 – 3
2	1	40% TFA in DCM	1 – 3
3	1	40% TFA in DCM	25 - 30
4	1 – 2	DCM	1 – 3
5	1 – 2	ETOH or DMF	1 – 3
6	1 – 2	DCM	1 – 3
7	2	10% DIEA in DCM	3 – 5
8	2 – 3	DCM	1 – 3
9	1	Coupling	60 - 999
10	2 – 3	DCM	1 – 3

Re-coupling: t-Boc and DCC / DIC:

Cycle	No	Solvent	Time (min.)
1	1 – 2	DCM	1 – 3
2	2	10% DIEA in DCM	3 – 5
3	2 – 3	DCM	1 – 3
4	1	Coupling	60 - 999
5	2 - 3	DCM	1 – 3

t-Boc + BOP / HBTU / TBTU + DIEA:

Cycle	No	Solvent	Time (min.)
1	2	DCM	1
2	1	40% TFA in DCM	1
3	1	40% TFA in DCM	30
4	3	DCM	1
5	2	DMF	1
6	1	10% DIEA in DCM	1
7	1	Coupling	60
9	1	DMF	1
10	2	DCM	1

Re-coupling: t-Boc and BOP / HBTU / TBTU + DIEA:

Cycle	No	Solvent	Time (min.)
1	2	DMF	1
2	1	10% DIEA in DCM	1
3	1	Coupling	60
4	1	DMF	1
5	2	DCM	1

Acetylation protocol

Cycle	No	Solvent	Time (min.)
1	1	DCM	1
2	1	10% DIEA & Ac2O in DCM	10
3	2	DCM	1

Note: DIEA can be substituted with TEA.

Hydrogen Fluoride Cleavage

i. Procedure

The resin bound peptides or side chain protected peptides are treated with liquid hydrogen fluoride. This cleaves the peptide from the resin and protecting groups from the constituent amino acid moieties. An HF cleavage apparatus consists of Kel-F or Teflon reaction vessels, valves and tubing. A high vacuum pump is employed in this process.

The peptide resin is weighed and transferred to the HF reaction vessels. Anisole or P-Cresol is added to the resin, 1 mL per gram of peptide resin. If Cysteine, Methionine, or Tryptophan is present in the peptide, DMS or 1,2 Ethanedithiol is also added, 0.25 mL per gram resin.

The HF reaction vessel is attached to the HF apparatus and placed in a dry ice/acetone batch for approximately 5 minutes. During this time turn on vacuum pump and check the HF apparatus for any vacuum leaks. (IF THERE IS A LEAK, DO NOT PROCEED UNTIL THIS IS FIXED)

The pump valve is then turned off creating a closed system between the HF cylinder and reaction vessel, which is regulated by the HF cylinder valve. This valve is then opened permitting approximately 5 - 10 mL of HF (per gram of peptide resin) to accumulate in the reaction vessel. When adequate HF has collected, the HF cylinder valve is closed, and the reaction vessel is warmed to 0°C in an ice bath. The HF mixture in the reaction vessel is then magnetically stirred at this temperature for 45 - 90 minutes to complete the reaction. HF is evacuated from the reaction vessel into a trap containing calcium oxide and absorbed as calcium fluoride. While stirring at 0ºC, evacuation continues until all HF has been removed. After evacuation, the reaction vessel is removed from the HF apparatus. Approximately 50 - 100 mL of anhydrous ether (per gram of resin) is added and the mixture is agitated. When dispersed, the resin and cleaved peptide are collected by filtration using a sintered glass funnel. This washing procedure is repeated twice

before extracting the peptide with acetic acid or TFA and water. The resulting filtrate contains the crude peptide, ready to lyophilize. After lyophilization, the crude is subject to purification.

HF cleavage apparatus

ii. Equipment and Materials

HF apparatus.

High vacuum pump.

Anisole.

Dimethyl sulfide - DMS.

1, 2 Ethanedithiol.

P-Cresol

Ether, anhydrous.

Acetic acid - AcOH.

Trifluoroacetic acid - TFA.

Water - Distilled water/deionized water.

Ice.

Dry Ice.

Acetone.

Filter funnel.

Filtering flask.

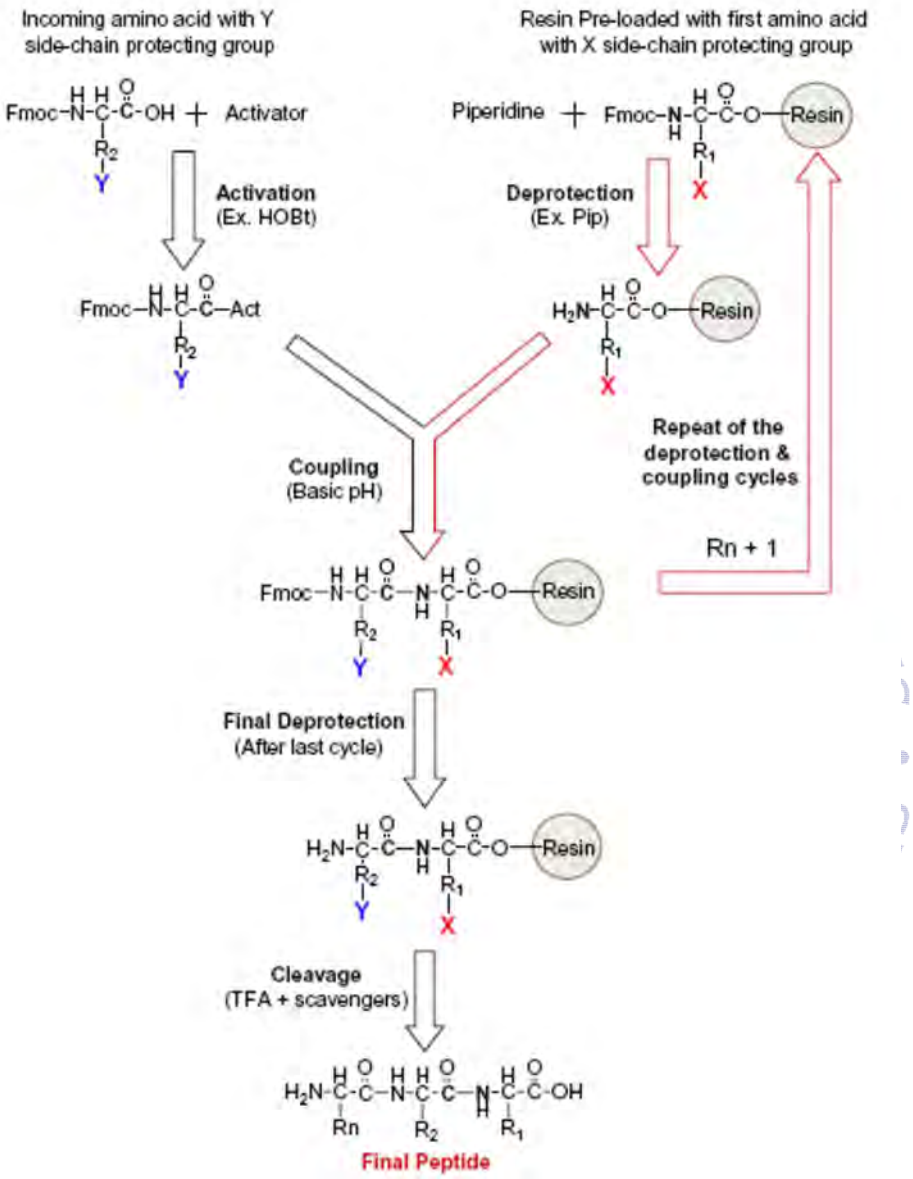
II. Fmoc Synthesis and Cleavage

9-Fluorenylmethyloxycarbonyl (Fmoc) Strategy: The Fmoc protecting group is deprotected by mild base treatment of 20% piperidine in N, N'-Dimethylformamide (DMF) and the final cleavage of the peptidyl resin and side chain groups deprotection by Trifluoroacetic acid (TFA). N, N'-Dimethylformamide (DMF) is the primary solvent used for resin deprotection, coupling, and washing. N,N-Dimethylacetamide (DMA) or N-Methyl-2-Pyrrolidione (NMP) may also be used.

In peptide synthesis, Fmoc protected amino acid residues (in excess millimole quantities) are sequentially bound to a resin support. These residues are assigned individual coupling cycles in accordance to their respective positions within the given peptide chain. Once a residue has been successfully coupled as evidenced by the Ninhydrin test, the compound is deprotected for the next coupling cycle.

By the methods employed, material of equivalent quality in larger or smaller amounts can be obtained while varying quantities of reactants and solvents proportionately. Additionally, coupling intervals may be varied from 30 minutes to 72 hours, and peptide resin washing intervals may range from 1 to 30 minutes.

Basic Steps of Fmoc peptide synthesis



Fmoc Synthesis

i. Resin

The hydroxymethyl-based resins (Wang, HMPA, HMPB resins) are the most common used except the Cys, His and Pro residues at the C-terminus, in this case Trityl-based resins such as 2-

chlorotrityl resin should be used. The Rink amide MBHA resin, consisting of 4-methylbenzydrylamine (100-200 mesh, 1% DVB) polystyrene, derivatized sequentially with norleucine and the Fmoc-protected modified form of the Rink amide linker, which incorporates an acetic acid spacer, is not degraded by TFA, and is therefore compatible with the standard 95% TFA cleavage reaction.

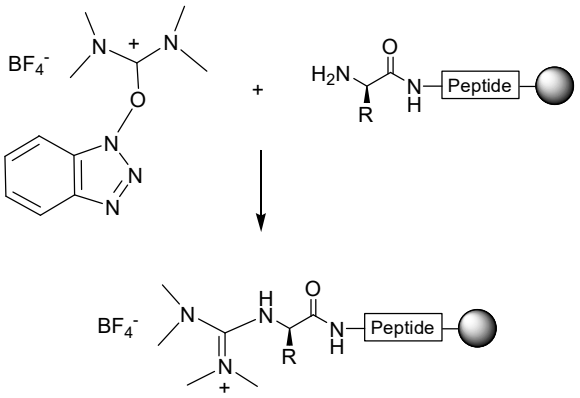
The resins with 100-200 mesh, 1%DVB, 0.5-0.6 meq/gm are most common used at our lab.

ii. Amino Acids

A: Ala	C: Cys(Trt), Cys(Mmt), Cys(Acm)
D: Asp(OtBu)	E: Glu(OtBu)
F: Phe	G: Gly
H: His(Trt)	I: Ile
K: Lys(Boc), Lys(Fmoc)	L: Leu
M: Met	N: Asn(Trt)
P: Pro	Q: Gln(Trt)
R: Arg(Pbf), Arg(Pmc)	S: Ser(tBu)
T: Thr(tBu), Thr(Trt)	V: Val
W: Trp, Trp(Boc)	Y: Tyr(tBu)

iii. Coupling Reagents

DCC and DIC are traditional. The most commonly reagents are DCC, DIC, HBTU, and BOP, among which BOP and HBTU (also including TBTU, PyBOP, and HATU) are in situ activating reagents, with which the coupling reaction is very fast. To avoid peptide termination, the ratio of amino acid to coupling reagent such as HBTU should be 1 to 0.9.



Guanylation: this reaction occurs during a coupling induced by uronium/arniniurn reagents

iv. Equipment and Materials

Automated CSBio peptide synthesizer.

Test tube heating block.

Dichloromethane (DCM).

N,N'-Dimethylformamide (DMF).

N-Methyl-2-Pyrrolidione (NMP).

N,N-Dimethylacetamide (DMA)

4-Dimethylaminopyridine (DMAP)

Ethanol (ETOH).

Diisopropylethylamine (DIEA).

Triethylamine (TEA).

Trifluoroacetic acid (TFA).

Piperidine.

Dicyclohexylcarbodiimide (DCC).

Diisopropylcarbodiimide (DIC).

Benzotriazol-1-yl-Oxy-Tris-(Dimethylamino) phosphonium Hexafluorophosphate (BOP).

2-(1H-Benzotriazol-1-yl)-1,1,3,3-Tetramethyluronium Hexafluorophosphate (HBTU).

1-Hydroxybenzotriazole monohydrate (HOBt).

Fmoc-Amino Acids.

Acetic anhydride (AcOH)

Ninhydrin.

Phenol.

Pyridine (Pyr).

Air 60 psi.

Nitrogen 20 psi.

v. Synthesis Protocols

Fmoc amino acid and DCC or DIC:

Cycle	No	Solvent	Time (min.)
1	2 – 3	DMF	1 - 3
2	1	20% Piperidine in DMF	5 - 10
3	1	20% Piperidine in DMF	5 - 20
4	2 – 3	DMF	1 - 3
5	1 – 2	DCM	1 - 3
6	2	DMF	1 - 3
7	1	Coupling	60 - 999
8	2 – 3	DMF	1 - 3
9	2 – 3	DCM	1 - 3

Doubling coupling: Fmoc AA + DCC or DIC:

Cycle	No	Solvent	Time (min.)
1	2 – 3	DMF	1 - 3
2	1	Coupling	60 - 999
3	2 – 3	DMF	1 - 3
4	2 – 3	DCM	1 – 3

Fmoc AA + BOP or HBTU or TBTU + DIEA (AA: HBTU:DIEA = 1:0.95:1.2)

Cycle	No	Solvent	Time (min.)
1	2 - 3	DMF	1 - 3
2	1	20% Piperidine in DMF	5 - 10
3	1	20% Piperidine in DMF	5 - 20
4	2 - 3	DMF	1 - 3
5	1 - 2	DCM	1 - 3
6	2	DMF	1 - 3
7	1	Coupling	60 - 999
8	2 – 3	DMF	1 - 3
9	2 – 3	DCM	1 - 3

Double coupling: Fmoc AA+ BOP or HBTU or TBTU + DIEA:

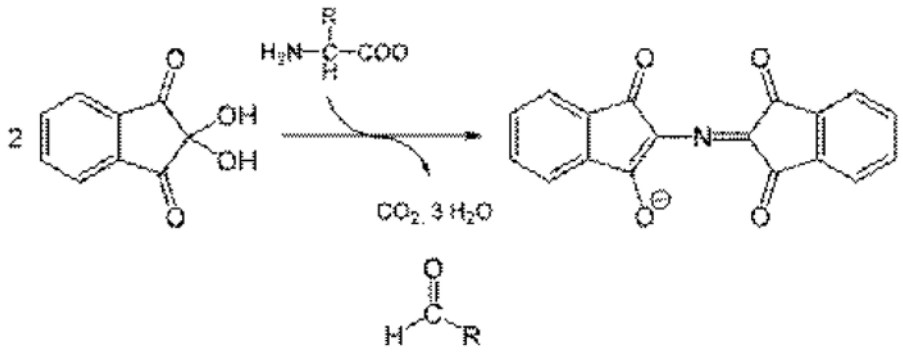
Cycle	No	Solvent	Time (min.)
1	2 – 3	DMF	1 – 3
2	1	Coupling	60 – 999
3	2 – 3	DMF	1 – 3
4	2 – 3	DCM	1 – 3

Acetylation protocol.

Cycle	No	Solvent	Time (min.)
1	1 - 2	DMF	1 - 3
2	1	10% DIEA & AcOH in DMF	5 - 30
3	2 - 3	DMF	1 - 3

Note: DIEA can be substituted by TEA.

vi. Ninhydrin Test



A small sample of resin (Approx. 3-10 mg) is removed from the synthesizer's reaction vessel and placed in a test tube. The resin is washed with 3-4 mL 10% DIEA/DCM solution one time and 2 times with 3-4 ml ETOH. Washing is accomplished by addition of solution, mixing, and decanted. One drop from each of the following reagents is added to the test tube:

Ninhydrin Solution (5.0 gm Ninhydrin in 100 mL ETOH).

Phenol Solution (80 gm Phenol in 20 mL ETOH).

Pyridine.

The contents in each tube are then mixed thoroughly. All tubes are placed in a heating block and heated at 120º C for 3 min. The tubes are then promptly removed. Hold each tube against a white background and evaluate the solution color to interpret test results. Results for the sample-containing tubes are compared with that of the standard. A blue, purple, or red color is indicative of free amine (positive result). The absence of color - as exhibited by the clear polystyrene blank solution - is indicative of no free amine (negative result). Samples are re-tested when variable results are obtained.

A negative test result means that the synthesis can continue to the next cycle. A positive test result means a determination will be made by the chemist to either re-couple the same amino acid using a double coupling program or to cap the resin using the acetylation program.

vii. Acetaldehyde/Chloranil Test (Option)

This sensitive test has been developed for reliable detection of secondary amino groups, but it will also detect primary amines. The stock solutions should be kept in the refrigerator and for one month maximum.

Solution 1: 2% acetaldehyde in DMF

Solution 2: 2% chloranil in DMF

Add a small spatula-tip-size sample of resin to a micro centrifuge tube, and 2-5 drops of each solution are added. Vortex the mixture and allow to stand at room temperature for 5 minutes. Acetone is used for the detection of secondary amines, where acetaldehyde is used for primary amines.

Results: dark blue to green beads for positive, and colorless to yellowish beads for negative.

TFA Cleavage for Fmoc Synthesis

Before acid cleavage of the peptidyl resin can be performed, the N-terminal Fmoc group must be removed using piperidine. Having successfully synthesized a protected peptide, one is confronted with a difficult task of having to simultaneously detach the peptide from the resin support and remove all the side chain protecting groups of the amino acid residues to yield the desired peptide. In Fmoc SPPS, this step is normally carried out by treating the peptidyl resin with TFA.

i. Procedure

a. If Cysteine (Cys), Methionine (Met), or Tryptophan (Trp) is present in the peptide, Reagent K (TFA / water / phenol / thioanisole / EDT = 82.5 : 5 : 5 : 5: 2.5) will be applied. Otherwise TFA / water / TIS = 95 : 2.5 : 2.5 will be used.

b. The dry peptide resin is weighed and transferred to the reaction vessels. TFA solution containing appropriate scavengers (10 - 15 mL / gm of resin) is added. Stopper the reaction vessel and leave to stand at room temperature with stirring or shaking. Make sure the resin beads are dispersed throughout the TFA solution. (No aggregation occurs)

c. Agitate the solution at rt for 2-4 hours. Remove the resin by filtration under pressure. Wash the resin twice with TFA and collect the filtrates. Make sure the suction is not on so TFA wash can be slow and thorough.

d. Reduce the filtrate volume (if necessary > 20 mL) by rotary evaporator until the peptide almost precipitates, and add a 10 fold volume of cold ether to precipitate the crude peptide. Sometimes it is necessary to evaporate most of the TFA to achieve a good precipitation of the crude peptide. The ether can be cooled in an ice bath to further assist precipitation.

e. Filter the precipitated peptide through hardened filter paper in a Hirsch funnel or fritted funnel under a light vacuum. Wash the precipitate further with cold ether, dry the crude peptide by nitrogen blow or air. In some cases, the crude peptide can be dissolved in a suitable aqueous buffer and lyophilized.

OR: the precipitated crude peptide can be washed by centrifugation. After precipitation, transfer the suspension to a centrifuge tube, seal, and centrifuge. More ethyl ether is added to the residue, and the tube is sealed, shaken, and centrifuged. Make sure a spark-free centrifuge is used for this process. Carefully decant the ether from the tube. Repeat the ether wash 5 times as necessary, and dry the crude peptide by nitrogen blow or air overnight.

ii. Equipment and Materials

Trifluoroacetic acid - TFA.

Phenol.

Thioanisole.

Triisopropylsilane - TIS.

3.6-Dioxa-1.8-Octanedithiol

1, 2 Ethanedithiol - EDT.

Ether, anhydrous (or tert-Butyl methyl ether, MTBE)

Ice.

Dry Ice.

Acetone.

Acetic acid (AcOH).

Rotary evaporator.

Filter funnel.

Stirrer or stir bar.

Filtering flask.

Round bottom flask.

III. Peptide modification

A lactamized peptide with three disulfide bridges

Cyclization (Oxidation)

Cyclization is accomplished by oxidizing free sulfhydryl groups among constituent amino acids within the peptide chain to form disulfide bridges. To detect the presence of sulfhydryl groups, a sample of the uncyclized peptide is evaluated with Ellman's test.[5]

5. Ellman's test: Reagent 1: Ellman reagent - 40.0 mg of 5,5'-dithio-bis (2-nitrobenzoic acid), DTNB, are dissolved in 10 mL of pH 8 phosphate buffer. Make up fresh before use; Reagent 2: 0.1 N Sodium phosphate Na2PO4 buffer, pH 8. Make 4.60 gm sodium phosphate monohydrate NaH2PO4-H2O and 307.4 mL of 0.1 N NaOH to 1 L distilled water. Standard sulfhydril compound: 0.24 mg of Cysteine is dissolved in 10 mL of distilled water. Peptide sample: 0.24 mg of peptide is dissolved in 10 mL of distilled water. Test solutions are prepared in glass tubes by combining

i. Cyclization (Oxidation) Methods

Method 1: Disulfide bridge formation with potassium ferricyanide.

The bulk cleaved peptide is transferred to an appropriate size container and dissolved / diluted to a concentration of per gram of peptide in 1 L – 5 L of water. After thoroughly mixing the solution, the pH is adjusted to 7.4 - 7.6 with ammonium hydroxide. From a separatory funnel, 0.01 M K3Fe(CN)6 is added to the peptide solution at a rate of approximately 5 drops per minute. Upon addition of the oxidizing agent, the reaction mixture will exhibit a yellow color that dissipates. The reaction is complete when this color persists for 30 minutes. The pH of the reaction mixture is adjusted to 4.5 with acetic acid. If Lysine or Arginine residues are present in the peptide, Bio-Rex 70 resin may be used. Add the Bio-Rex 70 resin (80 - 150 gm/m mole peptide) into solution and stir overnight. Or pass the solution through C-18 column. Extract the peptide from Bio-Rex 70 resin or C-18 column.

Method 2: Disulfide bridge formation with air pump or without air pump:

The bulk cleaved peptide is transferred to an appropriate size container and dissolved in water to a concentration 1 L - 5 L per gram of peptide. After thoroughly mixing the solution, the pH is adjusted to 8.0 - 8.3 with ammonium hydroxide. By using the air pump, pump the air into the solution for 20 to 60 hours. The pH of the reaction mixture is adjusted to 4.5 with acetic acid. If Lysine or Arginine residues are present in the peptide Bio-Rex 70 resin may be used. Add the Bio-Rex 70 resin (80 - 150 gm/m mole peptide) into solution and stir overnight. Or pass the solution through C-18 column. Extract the peptide from Bio-Rex 70 resin or C-18 column.

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the following: 0.1 mL of standard or peptide solution, 0.1 mL of reagent 1, and 5 mL of reagent 2. A blank solution of 0.1 ml reagent 1 and 5 ml reagent 2 is compared to the test solution. More yellow in the test solution is indicative of free S-H.

Method 3: Disulfide bridge formation with Iodine:

The bulk cleaved peptide is transferred to an appropriate size container and dissolved in water to a concentration of 1 L – 5 L per gram of peptide. After thoroughly mixing the solution, drop a drop of the solution of 10 gm Iodine in 100 mL methanol slowly. Upon addition of the oxidizing agent, the reaction mixture will exhibit a yellow color that dissipates. The reaction is complete when this color persists for 30 minutes then adds the absorbic acid until the solution color is clear. If Lysine or Arginine residues are present in the peptide, Bio-Rex 70 resin may be used. Add the Bio-Rex 70 resin (80 - 150 gm/m mole peptide) into solution and stir overnight. Or pass the solution through C-18 column. Extract the peptide from Bio-Rex 70 resin or C-18 column.

Method 4: Disulfide bridge formation with DMSO:

The bulk cleaved peptide is transferred to an appropriate size container and dissolved in water with 10% - 20% DMSO (depending on peptide hydrophobicity) in TRIS or sodium bicarbonate buffer to a concentration of per gram of peptide in 1 L of water. After thoroughly mixing the solution, let the solution stand for several hours to overnight with stirring, and drop a drop of the solution to Ellman's test. If Lysine or Arginine residues are present in the peptide, Bio-Rex 70 resin may be used. Add the Bio-Rex 70 resin (80 - 150 gm/m mole peptide) into solution and stir overnight. Or pass the solution through C-18 column. Extract the peptide from Bio-Rex 70 resin or C-18 column.

ii. Equipment and materials

Container (2 L to 100 L).

Stirrer & Stirrer bar.

pH meter or pH paper.

Glass filter funnel (1 L to 3L).

Vacuum filtration flask.

Air pump.

Separatory funnel.

Ammonium hydroxide - NH4OH.

Acetic acid - AcOH.

Trifluoroacetic acid (TFA).

Potassium ferricyanide - K3Fe(CN)6.

Iodine.

Methanol

Bio Rex 70 resin.

Reverse phase resin - C-18.

Water – Distilled water / deionized water.

<u>ALLOC/ALLYL DEPROTECTION</u>

i. Materials/Reagents

Peptide-resin with Alloc and/or Allyl functionalities

Tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$, FW 1155.6)

1,3-Dimethylbarbituric Acid (DMBA, 156,14)

DMF

DCM

ii. Equipment

Fritted polypropylene or glass reaction vessel with stopcock and cap.

iii. Procedure

a. Relative to each Alloc/Allyl group, weigh out:

0.25 eq. $Pd(PPh_3)_4$ (the catalyst)

10 eq. DMBA

So for instance, if you have 1.0 mmol of peptide-resin and you have a Lys(Alloc) and Glu(OAll), then you have 2.0 mmol of Alloc/Allyl groups present. So you need 0.5 mmol $Pd(PPh_3)_4$ and 20 mmol DMBA.

b. Dissolve the $Pd(PPh_3)_4$ and DMBA in an appropriate amount of 1:1 DMF:DCM to swell the resin while shaking. This is the 'Pd cocktail'

c. Wash the peptide-resin with sufficient DMF to ensure that no traces of TFA, $Pd(PPh_4)_3$, or other reagents remain in the resin. Two 20 second flow washes are sufficient for this purpose. If the resin was previously subjected to an Mmt, Mtt, Alloc, or Boc deprotection, free-base the deprotected amine(s) by batch treatment with 5% Et_3N in DMF for 5 mins (and then wash again with DMF)

d. Add the Pd cocktail to the peptide-resin, cap, wrap with aluminum foil, and shake overnight for this deprotection reaction. After this reaction is complete, the Alloc and Allyl groups will be removed, yielding free amine and carboxylic acid functionalities, respectively, ready for cyclization (Lactam).

Notes:

1) $Pd(PPh_3)_4$ is sensitive to light, temperature, and air. Unnecessary exposure to any of them will oxidize the catalyst and decrease its potency. When we buy $Pd(PPh_3)_4$, it comes in Ar-filled glass ampules; these should be opened and stored in separate vials and used serially so that no one portion receives unnecessary exposure to air and light. Only open the vials when necessary, cap them immediately, and keep in the refrigerator.

2) When you remove the $Pd(PPh_3)_4$ from the refrigerator, let the vial warm to room temperature so water does not condense on the catalyst.

3) After the reaction, wash with DMF extensively as the precipitated salts will accumulate in the peptide-resin. The orange color in the resin can be removed by performing a standard Fmoc deprotection cycle – whenever possible this should be done so as to minimize contamination of the cleaved peptide product with Pd salts. However, if the Alloc/Allyl deprotection is to be followed by a lactamization, treat the resin with 2 x 5 min treatments with DIEA in DMF instead of piperidine for this purpose, as piperidine would interfere with the subsequent lactamization step.

<u>DIC/HOBt MEDIATED LACTAMIZATION</u>

i. Materials/Reagents

Peptide-resin with free acid and amine functionalities

DIC

HOBt

DMF

ii. Equipment

Fritted polypropylene or glass reaction vessel with stopcock and cap.

iii. Procedure

a. Wash the peptide-resin with sufficient DMF to ensure that no traces of TFA, $Pd(PPh_4)_3$, or other reagents remain in the resin. Two 20 second flow washes are sufficient for this purpose. If the resin was previously subjected to an Mmt, Mtt, Alloc, or Boc deprotection, free-base the deprotected amine(s) by batch treatment with 5% Et_3N in DMF for 5 mins (and then wash again

with DMF).

b. Prepare DIC/HOBt cocktail for cyclization – this is DMF containing:

0.25 M DIC (400 μL per 10 mL)

0.30 M HOBt (400 mg per 10 mL)

Make a sufficient quantity of this cocktail to fully solvate the peptide-resin while mixing.

c. Add the DIC/HOBt cocktail to the peptide-resin, cap, and shake overnight for cyclization.

Notes:

1) The DIC/HOBt cocktail should be prepared immediately before use.

2) After overnight cyclization, do not wash the resin with DCM. Wash with DMF first and then with DCM. This is because the byproduct from the cyclization reaction is insoluble in DCM and so will contaminate the resin and cleaved peptide if not first washed away in DMF.

<u>BOP MEDIATED LACTAMIZATION</u>

i. Materials/Reagents

Peptide-resin with free acid and amine functionalities

BOP (FW 442.3)

DIEA

DMF

ii. Equipment

Fritted polypropylene or glass reaction vessel, with stopcock and cap.

iii. Procedure

a. Prepare BOP/DIEA cocktail for cyclization – make a 0.25 M (1.1 g per 10 mL) solution of BOP in DMF containing 5 vole% DIEA. Make a sufficient quantity of this cocktail to fully solvate

the peptide-resin while mixing.

b. Wash the peptide-resin with sufficient DMF to ensure that no traces of TFA, $Pd(PPh_4)_3$, or other reagents remain in the resin. Two 20 second flow washes are sufficient for this purpose. If the resin was previously subjected to an Mmt, Mtt, Alloc, or Boc deprotection, free-base the deprotected amine(s) by batch treatment with 5% Et_3N in DMF for 5 mins (and then wash again with DMF)

c. Add the BOP/DIEA cocktail to the peptide-resin, cap, and shake overnight for lactamization.

Note:

It is better to perform this lactamization with the N-terminus blocked in either Boc-protected or acetylated form, since the Fmoc group will be slowly deprotected during long-term exposure to base during this reaction. So if this chemistry is to be used for lactamization, it is better to either:

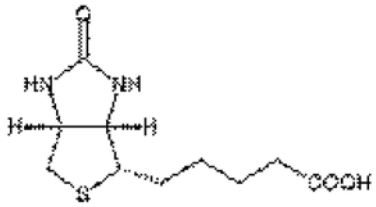
1) Synthesize the target sequence with an $^{\alpha}$*N*-Boc protected amino acid in the N-terminal position;

2) Before performing any chemistry on the side chains, remove the N-terminal Fmoc group and execute Boc protection.

3) Before performing any chemistry on the side chains, remove the N-terminal Fmoc group and acetylate it with acetic anhydride.

4) Use *N*-methylmorpholine (NMM) instead of DIEA if the Fmoc group must be left present.

BIOTINYLATION



i. Materials/Reagents:

Peptide-resin with free amine functionality

Biotin (FW 244.3)

HBTU (FW 379.25)

DMF

DMSO

ii. Equipment:

Fritted polypropylene or glass reaction vessel, with stopcock and cap.

iii. Procedure

For 1 mmol amine-functionalized resin, prepare the following Biotin cocktail:

4.5 mmol Biotin DIC (1.10 g)

4.0 mmol HBTU (1.52 g)

all in 16 mL 1:1 DMF:DMSO

It will not be homogeneous. Shake for 10 seconds, and then add 1 mL DIEA. And shake/vortex for 10 minutes to activate. The solution should become colored, but clear.

Wash the peptide-resin with sufficient DMF to ensure that no traces of TFA, $Pd(PPh_4)_3$, or other reagents remain in the resin. Two 20 second flow washes are sufficient for this purpose. If the resin was previously subjected to an Mmt, Mtt, Alloc, or Boc deprotection, free-base the

deprotected amine(s) by batch treatment with 5% Et_3N in DMF for 5 mins (and then wash again with DMF)

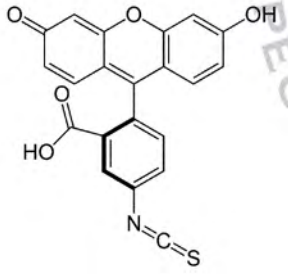
Add the Biotin cocktail to the peptide-resin, cap, and shake. Remove a small aliquot of resin to check by Ninhydrin – the coupling should be complete in 1 hour.

Note:

1) It is critically important that the preactivation be complete – do not add the Biotin cocktail to the resin if it is not clear (colored is ok).

2) After the reaction is complete, do not wash the resin with DCM. Wash with DMF first and then with DCM. This is because the precipitated unreacted biotin is insoluble in DCM and so will contaminate the resin and cleaved peptide if not first washed away in DMF.

FITC COUPLING



i. Materials/Reagents

Peptide-resin with free amine functionality

FITC (FW 389.4)

DMF

DIEA

ii. Equipment

Fritted polypropylene or glass reaction vessel, with stopcock and cap.

iii. Procedure

a. For 1 mmol amine-functionalized resin, dissolve 3.0 mmol FITC (1.25 g) in 8 mL DMF. Add 1 mL DIEA. Shake/vortex for a few minutes until dissolved.

b. Wash the peptide-resin with sufficient DMF to ensure that no traces of TFA, $Pd(PPh_4)_3$, or other reagents remain in the resin. Two 20 second flow washes are sufficient for this purpose.

c. Add the FITC cocktail to the peptide-resin, cap, and shake. Remove a small aliquot of resin to check by Ninhydrin – the coupling should be complete in a few hours.

Notes:

If the FITC is to be coupled to a side chain (e.g., Lysine, Ornithine, etc.), the N-terminus should be blocked in either Boc-protected or acetylated form, since the Fmoc group will be slowly deprotected during long-term exposure to base during this reaction. So the best possible options are: 1) Synthesize the target sequence with an $^{\alpha}$*N*-Boc protected amino acid in the N-terminal position; 2) Before performing the FITC conjugation, remove the N-terminal Fmoc group and acetylate it with acetic anhydride. 3) Before performing the FITC conjugation, remove the N-terminal Fmoc group and protect it with Boc anhydride. 4) Use *N*-methylmorpholine (NMM) instead of DIEA if the Fmoc group must be left present.

Unreacted FITC will contaminate the completed peptide-resin and complicate purification and analysis. The best way to remove unreacted FITC from the peptide-resin is by treatment with 20% piperidine/DMF (a standard Fmoc deprotection protocol).

ACETYLATION

i. Materials/Reagents

Peptide-resin with free amine functionality

Acetic Anhydride (Ac_2O)

DMF

ii. Equipment

Fritted polypropylene or glass reaction vessel with stopcock and cap.

iii. Procedure

a. Wash the peptide-resin with sufficient DMF to ensure that no traces of TFA, $Pd(PPh_4)_3$, or other reagents remain in the resin. Two 20 second flow washes are sufficient for this purpose. If the resin was previously subjected to an Mmt, Mtt, Alloc, or Boc deprotection, free-base the deprotected amine(s) by batch treatment with 5% Et_3N in DMF for 5 mins (and then wash again with DMF)

b. Prepare Ac_2O cocktail (5% Ac_2O in DMF) immediately before use. Make a sufficient quantity of this cocktail to fully solvate the peptide-resin while mixing.

c. Add the Ac_2O cocktail to the peptide-resin, cap, and shake. Remove a small aliquot of resin to check by Ninhydrin – the reaction should be complete in 30 minutes.

Note: Add 5% DIEA or Pyridine into the cocktail if acid labile protecting group on the peptide.

S-BENZOYLMERCAPTOACETYLATION

i. Materials/Reagents

Peptide-resin with free amine functionality

Bromoacetic Acid (FW 139.0)

Thiobenzoic Acid (FW 138.2, d 1.174)

DIC

DIEA

DMF

ii. Equipment

Fritted polypropylene or glass reaction vessel, with stopcock and cap.

iii. Procedure

a. Make the symmetrical anhydride of Bromoacetic acid. For 1 mmol of amine-functionalized resin, dissolve 10 mmol Bromoacetic acid (1.39 g) in 10 mL DCM in a scintillation vial, and then add 5 mmol (782 µL) DIC, cap, and shake for 10 minutes. The solution will become warm and cloudy.

b. Add 10 mL DMF to the Bromoacetic anhydride just prepared, shake and add to the peptide-resin with free amine. Add additional DMF as necessary to fully solvate the resin while shaking, cap the vessel, and shake. This reaction should be complete in 30 min (monitor by Ninhydrin).

c. Dissolve 4 mmol Thiobenzoic acid in 8 mL DMF, then add 1 mL DIEA. The solution will turn dark green. Add this cocktail to the Bromoacetylated peptide-resin and add DMF as necessary to fully solvate the resin while shaking. Cap the vessel and shake for 4 hours to overnight. This reaction cannot be monitored by Ninhydrin.

d. Drain, wash with DMF and DCM, and cleave.

Notes:

1) Thiobenzoic acid smells bad – keep it in the hood.

2) The S-benzoylmercaptoacetamide is labile to base and nucleophiles, so always handle this peptide at acidic pH and do not expose it to piperidine.

3) Do not use thiol scavengers during TFA cleavage – so no DODT, EDT, etc.

BOC PROTECTION

i. Materials/Reagents

Peptide-resin with free amine functionality

Boc Anhydride (Di-tert-butyl dicarbonate, $(Boc)_2O$, FW 218.25, d 0.95)

DMF

ii. Equipment

Fritted polypropylene or glass reaction vessel with stopcock and cap.

iii. Procedure

a. Wash the peptide-resin with sufficient DMF to ensure that no traces of TFA, $Pd(PPh_4)_3$, or other reagents remain in the resin. Two 20 second flow washes are sufficient for this purpose. If the resin was previously subjected to an Mmt, Mtt, Alloc, or Boc deprotection, free-base the deprotected amine(s) by batch treatment with 5% Et_3N in DMF for 5 mins (and then wash again with DMF).

b. Prepare $(Boc)_2O$ cocktail (5% $(Boc)_2O$ in DMF) immediately before use. Make a sufficient quantity of this cocktail to fully solvate the peptide-resin while mixing.

c. Add the $(Boc)_2O$ cocktail to the peptide-resin, cap, and shake. Remove a small aliquot of resin to check by Ninhydrin – the reactions should be complete in 1 hour.

Note: Add 5% DIEA or Pyridine into the cocktail if acid labile protecting group on the peptide.

FORMYLATION

i. Materials/Reagents

Peptide-resin with free amine functionality

p-Nitrophenyl Formate (NPF, FW 167.12)

DMF

DIEA

ii. Equipment

Fritted polypropylene or glass reaction vessel with stopcock and cap.

iii. Procedure

a. Wash the peptide-resin with sufficient DMF to ensure that no traces of TFA, $Pd(PPh_4)_3$, or other reagents remain in the resin. Two 20 second flow washes are sufficient for this purpose. If the resin was previously subjected to an Mmt, Mtt, Alloc, or Boc deprotection, free-base the deprotected amine(s) by batch treatment with 5% Et_3N in DMF for 5 mins (and then wash again with DMF)

b. For 1 mmol amine-functionalized resin, dissolve 4.0 mmol NPF (0.50 g) in 8 mL DMF. Add 1 mL DIEA. Shake/vortex for a minute or so until dissolved.

c. Add the NPF cocktail to the peptide-resin, cap, and shake. Remove a small aliquot of resin to check by Ninhydrin – the coupling should be complete in 1 hour.

Notes:

1) Do not expose the formylated peptide-resin to piperidine; this may partially remove the formyl group from the peptide.

2) The yellow residue contaminating the resin after the formyl group should be washed away with DMF and DCM so it does not contaminate the crude, cleaved product and complicated purification.

FMOC PROTECTION

i. Materials/Reagents

Peptide-resin with free amine functionality

9-Fluorenylmethyl N-succinimidyl carbonate (Fmoc-OSu, FW 337.3)

DMF

HOAt

ii. Equipment

Fritted polypropylene or glass reaction vessel with stopcock and cap.

iii. Procedure

a. Wash the peptide-resin with sufficient DMF to ensure that no traces of TFA, $Pd(PPh_4)_3$, or other reagents remain in the resin. Two 20 second flow washes are sufficient for this purpose. If the resin was previously subjected to an Mmt, Mtt, Alloc, or Boc deprotection, free-base the deprotected amine(s) by batch treatment with 5% Et_3N in DMF for 5 mins (and then wash again with DMF).

b. For 1 mmol amine-functionalized resin, dissolve 4 mmol Fmoc-OSu (1.35 g) in 12 mL DMF immediately before use. Add a catalytic amount of HOAt (0.8 mmol, 109 mg).

c. Add the Fmoc-OSu cocktail to the peptide-resin, cap, and shake. Remove a small aliquot of resin to check by Ninhydrin – the reaction should be complete in 1 hour.

Peptide Purification

A revolution in purification techniques for many peptides and proteins is high performance liquid chromatography (HPLC). It is highly cost effective in that it is able to replace multiple steps in conventional purification processes, thus eliminating handling, labors costs, and also able to perform purifications which cannot be carried out by any other process.

I. METHODS

i. ION EXCHANGE CHROMATOGRAPHY

Method 1: Ion Exchange Chromatography (CM-52 or DE-52).

Cation exchanger CM-52 or anion exchanger DE-52 as appropriate may treat the crude peptide.

The mobile phase of cation exchange chromatography consists of:

Buffer A: 0.05 M - 0.1 M Ammonium acetate.

Buffer B: 0.1 M - 1 M ammonium acetate.

The mobile phase of anion exchange chromatography consists of:

Buffer A: 0.05 M - 0.1 M Ammonium bicarbonate.

Buffer B: 0.1 M - 1 M ammonium bicarbonate.

In either, the buffer molarity may be varied to accommodate the particular peptide in maximizing yield and purity. Also 0 - 6 M urea can be used in buffer A or Buffer B depending on the solubility of the peptide.

Stir and pour ion exchanger into Buffer A. Stir the slurry with a magnetic stirrer in a stopper flask connected to a pump until no more bubbles appear. Set up the glass column vertically containing about 3 - 4 cm Buffer A at the bottom. The resin slurry in buffer A is then poured into the column to a depth of 20 cm. Additional buffer A is passed through the column and then eluent is monitored until its pH is comparable with that of the buffer. The peptide in buffer A solution is

then applied to the column and eluted with a gradient of 0 - 100 percent buffer B by gravity. Fractions of the sample are collected in appropriately sized tubes. TLC or analytical HPLC analyzes these fractions for purity. Pure fractions are lyophilized and forwarded to QC; impure fractions are subjected to further purification steps.

Method 2: Ion Exchange Chromatography (Bio-Rex 70).

The Bio Rex 70 resin used in the extraction must be in the sodium free form. 1 kg of resin first washed with 1 L of 1 N HCl then washed with D.I. water until pH of washing solution equal to D.I. water pH should be used. The crude peptide (after cyclization contains Lys, Arg, Orn, or positive charged group) may be absorbed by hydrogen chloride form Bio-Rex 70 after stirring overnight. The resin is filtered and packed into a column. Elute with 70% AcOH isocratically by gravity. It takes approximately 10 times of column volume until the peptide is completely eluted out from the column. Fractions of the sample are collected in appropriately sized tubes. These fractions are analyzed for purity by analytical HPLC. Pure fractions are lyophilized and forwarded to QC; impure fractions are subjected to further purification steps.

Method 3: Ion Exchange Chromatography (AG1-X8).

An anion exchange resin AG1-X8 is used for the conversion of Trifluoroacetic or fluoride to acetate as the counter anion for peptides. Pack a column with AG1-X8 (size of column is dependent on the quantity of peptide. Peptide : AG1X8 = 1 gm : 25 gm). Solubilize peptide in elution buffer (10% to 70% AcOH) and load onto column. Elute isocratically by gravity with the same buffer. It takes approximately 10 times of column volume until the peptide is completely eluted out from the column. Fractions of the sample are collected in appropriately sized tubes. These fractions are analyzed for purity by analytical HPLC. Pure fractions are lyophilized and forwarded to QC; impure fractions are subjected to further purification steps.

ii. Gel Filtration Chromatography

Bio-gel P series resin P-2, P-4, P-6 and Sephadex G-series - G-25, G-50 serve as matrices in this technique. The mobile phase consists of a 5 - 70% acetic acid buffer. Prior to use, this buffer is vacuum filtered through a membrane of 0.45 micron pore size and degassed.

The column matrix is prepared by adding the resin to the acetic acid buffer and allowing it to stand 20 - 30 minutes. A glass column (size of column is dependent on the quantity of peptide) is then primed with additional buffer before filling 50 - 70% of glass column capacity with the swollen resin. Once packed, the column is equilibrated with acetic acid buffer (about 5 times of column volume). The crude or partial pure peptide in solution is then applied to the column and eluted isocratically by gravity. Fractions of the sample are collected in appropriately sized tubes. These fractions are analyzed for purity by analytical HPLC. Pure fractions are lyophilized and forwarded to QC; impure fractions are subjected to further purification steps.

iii. Reverse phase Chromatography (Low pressure).

The silane or polystyrene reverse phase (C-18, C-8, or C-4) resin (60 Å to 300 Å) is used as matrices in this procedure. The resin is slurry with MeOH and packed into glass column (size of column is dependent on the quantity of peptide. Peptide : resin = 1 gm : 50 gm). Typically, the mobile phase may consist of buffer A and B as follows:

Buffer A1 - 0.1% TFA in water.

Buffer A2 - 0.05 M NH4OAc in water.

Buffer A3 - 1% AcOH in water.

Buffer A4 - 0.05 M NaH2PO4 (Sodium Phosphate monobasic) pH 4.5 in water.

Buffer A5 - 1% H3PO4 (Phosphoric acid)

Buffer A6 - TEAP pH 2.5 or pH 4.5 or pH 6.5

Buffer A7 - 0.1% HCl in water

Buffer B - 5% to 99% CH3CN with same ion pairing of Buffer A

The ion pairing concentration and pH changes reversed phase selectivity for peptide purification.

The buffer molarity may be varied to accommodate the particular peptide in maximizing yield purity. The column is equilibrated with buffer A. The peptide is dissolved in buffer A, and if necessary small quantities of buffer B, AcOH or TFA can be added. The clear peptide solution is loaded onto the column and eluted linearly on a gradient from 0 -100% buffer B. Fractions of the sample are collected in appropriately sized tubes. These fractions are analyzed for purity by analytical HPLC. Pure fractions are lyophilized and forwarded to QC; impure fractions are subjected to further purification steps.

iv. Preparative Reverse phase Chromatography (High pressure)

The silane or polystyrene reverse phase (C-18, C-8, or C-4) resin (60Å to 300Å) is used as matrices in this procedure. The resin is slurry with MeOH, IPA or other suitable solvents and packed into steel column (size of column is 1" to 8" diameter with 10" to 40" long). Typically, the mobile phase may consist of buffer A and B as follows:

Buffer A1 - 0.1% TFA in water.

Buffer A2 - 0.05 M NH4OAc in water.

Buffer A3 - 1% AcOH in water.

Buffer A4 - 0.05 M NaH2PO4 (Sodium Phosphate monobasic) pH 4.5 in water.

Buffer A5 - 1% H3PO4 (Phosphoric acid)

Buffer A6 - TEAP pH 2.5 or pH 4.5 or pH 6.5

Buffer A7 - 0.1% HCl in water

Buffer B – 0% to 100% CH3CN in buffer Ax (x = 1 or 2 or 3).

The buffers are vacuum filtered through a membrane of 0.45 micron pore size or degassed prior to use. The buffer molarity may be varied to accommodate the particular peptide in maximizing yield purity. The column of choice is configured to an HPLC instrument having dual pumps and equilibrated with buffer A. The peptide is dissolved in buffer A, and if necessary small quantities of buffer B, ion can be added. The clear peptide solution is loaded onto the column and eluted linearly using a gradient that is varied to accommodate to particular peptide. Fractions of the sample are collected in appropriately sized tubes. These fractions are analyzed for purity by analytical HPLC. Pure fractions are lyophilized and forwarded to QC; impure fractions are subjected to further purification steps.

II. EQUIPMENT & MATERIALS

Glass columns with coarse (60 u) filters. The selection of the column and filter size is based on the quantity of the peptide to be purified.

Preparative columns.

Solvent reservoirs.

Peristaltic pumps for delivering the solvents.

HPLC system.

Magnetic stirrer & stir bar.

Lab jack.

Fraction collector.

Rotary evaporator.

Beakers & flask.

Ammonium acetate - NH4OAc.

Ammonium bicarbonate - NH_4HCO_3.

Acetic acid - AcOH.

Hydrogen chloride - HCl.

Acetonitrile -CH_3CN.

Trifluoroacetic acid - TFA.

Methanol – MeOH

Hydroxymethyl Aminomethane - Tris.

Phosphoric Acid – H_3PO_4

Triethylamine – TEA

Water: Deionized water (D. I. water) / Distilled water.

Whatman diethylmethyl cellulose DE-52.

Whatman carboxymethyl cellulose CM-52.

Bio-Rad 70.

AG1-X8.

Bio-gel, P-series - P-2, P-4, P-6.

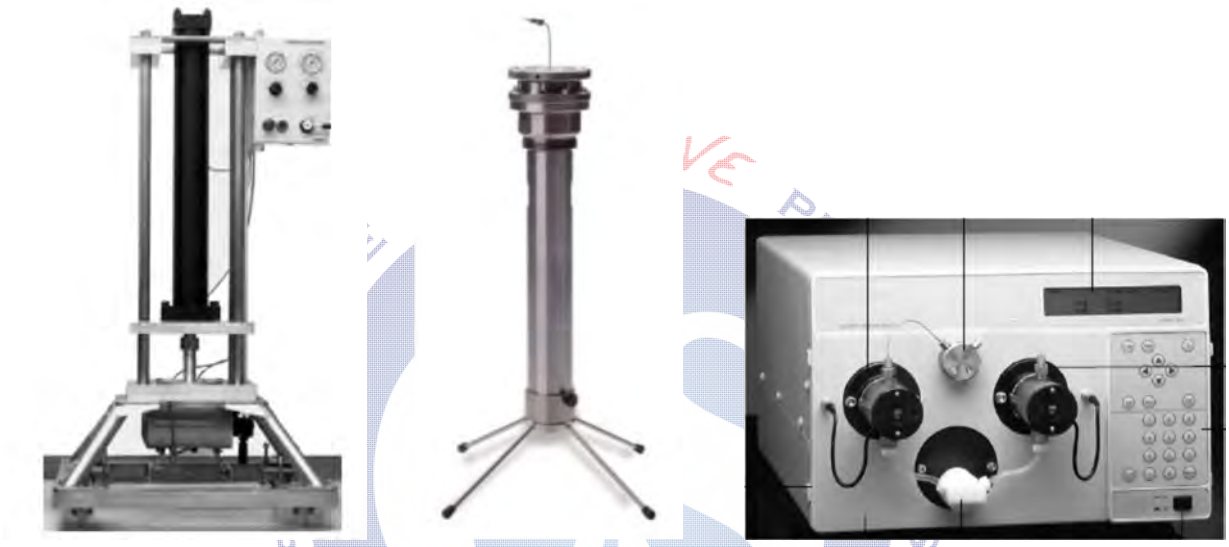
Sephadex G-series - G-25, G-50.

Silica gel with octadecyl boned phase resin - C-18 (from 5 μm to 40 μm): 60 Å to 300 Å.

Silica gel with octyl boned phase resin - C-8 (from 5 μm to 40 μm): 60 Å to 300 Å.

Silica gel with butyl boned phase resin - C-4 (from 5 μm to 40 μm): 60 Å to 300 Å.

III. A PRACTICAL PROCEDURE FOR PEPTIDE PURIFICATION USING PREPARATIVE HPLC Reverse phase HPLC is applied and C-18, C8, C-4 Silica gel resins are served as matrices in this procedure.



Preparative HPLC columns and pump systems

i. Crude analysis

a. Weigh the crude

In general, MW x synthetic mole =< experimental amount =< W (final Rx) – W (original Rx)

b. Test Solubility

Solubility guide:

Pure water: try dissolving an aliquot of peptide in a small volume of pure water. Dissolved?

If Yes, dilute and buffer to a clean solution;

If No, add acetonitrile with gentle sonication;

If No, try dissolving in a small volume of organic solvent such as acetonitrile, DMF, methanol

or isopropanol, and then water with sonication;

If No, try dissolving in a small volume of DMSO OR TFA OR FA with sonication;

If No, try guanidinium-HCL or other denaturing salts with sonication;

c. Perform Mass Spectrum Analysis to verify the mass

In general, signal should be above ~10E4.

ii. Perform analytical HPLC to calculate the retention percentage of B

In general, B percentage of the sample = (Retention Time - Injection Delay Time) x B percentage Increased Rate + Initial B percentage.

iii. Definition of bad crude

a. Insufficient Crude Amount (judge by MW x Synthetic mole, and purification yield, 10-30 %)

b. Poor Result of Analytical HPLC (Abs >=0.1)

c. Wrong mass

iv. System preparation.

Make sure the preparative HPLC system is ready, collection tubes are clean, and HPLC Column is equilibrated.

a. Buffer preparation.

Prepare appropriate buffer according to the analytical HPLC buffer system.

General Buffer System:

Buffer A: TFA/H_2O (0.1%), Buffer B: TFA/MeCN (0.1 %);

Buffer A: 10 -50 mM NH_4Ac aq (pH 5, 6.5, 7.5-8, 9), Buffer B: MeCN;

Buffer A: 60 mM NH_4OH aq (pH =< 10), Buffer B: MeCN;

Buffer A: 20 mM TEAP (pH 2-3, 6.5, 8), Buffer B: MeCN;

Buffer A: HOAc/H_2O (1%), Buffer B: MeCN;

Buffer A: H_2O, Buffer B: MeCN;

Buffer A: TFA/H_2O (0.1%), Buffer B: MeOH OR MeOH/IPA (1/1).

b. Column Selection

For a very hydrophobic peptide, pick a C-4 column. In general, a C-18 column is recommended as follows,

for an ½ inch column (250 mm in length), 5 mg - 100 mg crude

for a 2 inch column (250 mm in length), 80 mg - 3 g crude

for a 3 inch column (250 mm in length), 1 g -12 g crude

c. Column Preparation

Wash the column by 2 void-column-volumes with 90% Buffer B.

Balance the column by 2.5 void-column-volumes with the starting buffer system, normally 10% less than the retention percentage or 1% for very hydrophilic peptides.

v. Sample preparation

a. Weigh out the set amount of crude, and dissolve it according to the result in crude analysis. Use minimum amount of solvent and make sure the content of MeCN is less than the retention percentage.

b. Gradient/flow setup. Use (retention percentage –10%) to (retention percentage +30%) in 120 minutes.

c. Flow rate is set to: 4-5 ml/ minute for an ½ inch column

15-30 ml/ minute for a 2 inch column

50-90 ml/ minute for a 3 inch column

90-120 ml/minute for a 4 inch column

vi. Start the purification.

a. For DMSO/DMF/TFA/AcOH dissolved peptides, wait for a flat baseline to start the gradient, otherwise start the gradient after loading crude sample right away.

b. For air oxidation peptides, add till 0.5% MeCN to the huge amount of aqueous solution, and watch out the system pressure (< 1000 psi).

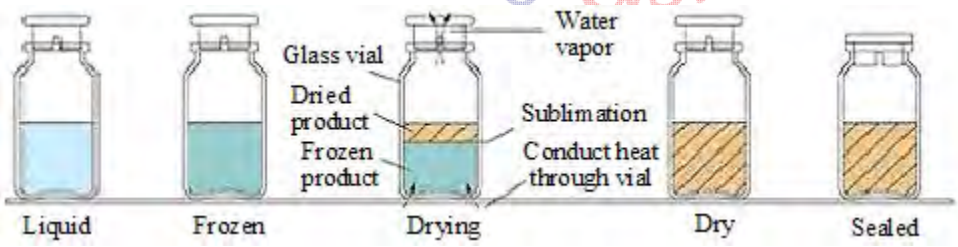
c. During the purification. Monitor the process every 5-10 minutes. Check MW if any major peak appears. Pay attention to early eluted peaks w/ low Buffer B

vii. Stop the run and wash column.

As soon as the correct MW is found, and the right Mass becomes weakening gradually in the tubes, stop the run and wash the column with 90% MeCN according to 'iv. c'. Be ready for a next run. Collection of wash maybe required in some special cases.

viii. Fraction Analysis

Check each fraction's purity by appropriate Analytical Gradient, combine all fractions with desired purity into a clean lyophilization jar, and freeze-dry the combination *via* acetone/dry ice. After the frozen jar attached to the lyophilizer, make sure the vacuum works properly. Otherwise, check leakage.



Peptide Lyophilization

Pure fractions are lyophilized and forwarded to QC; impure fractions are subjected to further purification steps.