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The Use of Aryl Hydrazide Linkers for the Solid Phase Synthesis of Chemically Modified Peptides

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Abstract

Since Merrifield introduced the concept of solid phase synthesis in 1963 for the rapid preparation of peptides, a large variety of different supports and resin-linkers have been developed that improve the efficiency of peptide assembly and expand the myriad of synthetically feasible peptides. The aryl hydrazide is one of the most useful resin-linkers for the synthesis of chemically modified peptides. This linker is completely stable during Boc- and Fmoc-based solid phase synthesis and yet it can be cleaved under very mild oxidative conditions. The present article reviews the use of this valuable linker for the rapid and efficient synthesis of C-terminal modified peptides, head-to-tail cyclic peptides and lipidated peptides.

Key words: multi-detachable linker, safety-catch linker, peptide α-thioester, acyl diazene, cyclic peptide, lipidated peptide.

Introduction

The introduction of solid phase peptide synthesis (SPPS) in 1963 by Bruce Merrifield (Merrifield, 1963) opened a door for researchers to prepare structurally diverse peptides as well as biologically relevant non-peptide compounds, such as oligonucleotides and oligosaccharides among others (Marshall and Kaiser, 2004, Gil and Brase, 2004, Larsen et al., 2006). Since then, a variety of research activities examining solid supports, new resin-linkers, peptide coupling chemistry and automated synthesis systems have been conducted to meet the demand for more facile and efficient way to synthesize peptides (Albericio, 2004).

Since the first use of cross-linked chloromethylated-polystyrene for the solid-phase synthesis of peptides, a large variety of different supports and resin-linkers have been developed (Alsina and Albericio, 2003). This paper will review the use of the aryl hydrazide resin-linker in the solid-phase systhesis of chemically modified peptides.

Aryl hydrazides as versatile resin-linkers in SPPS

The hydrazide group has been employed as a carboxyl-protecting group in peptide synthesis for over 30 years (Cheung and Blout, 1965, Wieland et al., 1970). More recently, this group has also been employed as a resin-linker for the solid-phase synthesis of peptide esters (Semenov and Gordeev, 1995, Millington et al., 1998), amides (Millington et al., 1998, Albrecht et al., 2004, Camarero et al., 2004), *p*-nitroanilides (Kwon et al., 2004) and thioesters (Camarero et al., 2004) as well as lipidated (Lumbierres et al., 2005) and head-to-tail cyclized peptides (Rosenbaum and Waldmann, 2001, Shigenaga et al., 2006). The hydrazide linker is oxidized to an acyldiazene, which is then cleaved by a suitable nucleophile (Scheme 1). The oxidation step is usually

performed using atmospheric O_2 in the presence of Cu(II) salts and the nucleophile or using N-bromosuccinimide (NBS) followed by cleavage in a second step with a nucleophile. It has been shown that the NBS oxidation of peptidyl hydrazide resins containing oxidative-sensitive residues (i.e. Trp, Tyr and Cys) can be performed without side reactions if the appropriate protecting groups and reaction conditions are employed (Camarero et al., 2004, Kwon et al., 2004). Met-containing peptides are readily oxidized to the corresponding Met-sulfoxide by NBS. However, Met-sulfoxide can be easily reduced to Met by using 3% EtSH during the trifluoroacetic acid (TFA) deprotection step (Camarero et al., 2004, Kwon et al., 2004).

Typical nucleophiles that can be used for the cleavage of activated acyl diazenes are amines (aliphatic and aromatic) (Millington et al., 1998, Camarero et al., 2004, Kwon et al., 2004), water (Semenov and Gordeev, 1995, Millington et al., 1998) and alcohols (Peters and Wladmann, 2003). This feature allows aryl hydrazide linkers to be used as multi-detachable systems that can yield several C-terminal functionalities depending on the conditions employed during the cleavage (Scheme 2). Furthermore, the hydrazide linker is completely orthogonal to the conditions employed for the deprotection of classical urethane protecting groups, such as Boc, Fmoc and Aloc, which allows the Boc- and Fmoc-based solid phase synthesis of peptides. It has been also demonstrated that racemization does not occur during the oxidative cleavage of this linker (Camarero et al., 2004, Kwon et al., 2004).

Synthesis of C-terminal modified peptides

Although most of the synthetic peptides have either a carboxylic acid or a primary caboxyamide group as the C-terminal functionality, there are other C-terminal modified

peptides of potential interest in the production of therapeutic agents (Camarero et al., 2000, Gazal et al., 2005), enzymatic substrates (Alsina et al., 1999, Kwon et al., 2004), cyclic peptides (Camarero and Muir, 1997, Zhang and Tam, 1997, Camarero et al., 1998b, Shao et al., 1998, Camarero and Muir, 1999) or intermediates for the chemical engineering of proteins (Camarero and Mitchell, 2005, Muralidharan and Muir, 2006).

Several resin-linker systems are now available which allow the generation of C-terminal functionalities such as carboxylic acids, carboxyamides, thioesters, esters, hydrazides and hydroxamic acids (Alsina and Albericio, 2003). Multi-detachable resin-linkers offer an attractive alternative to the use of specialized linkers, especially in the creation of combinatorial libraries where the same resin can be used to yield different C-terminal functionalities depending on the conditions employed during the cleavage step (Camarero et al., 2000). The versatility of the aryl hydrazide linker has been demonstrated in the production of different C-terminal modified peptides.

Peptide carboxylates. Semenov et al. showed that peptidyl aryl hydrazides can be efficiently converted into the corresponding C-terminal peptide carboxylates by mild oxidation with air in the presence of aqueous Cu(II)-pyridine complexes (Semenov and Gordeev, 1995). More recently, Millington et al. used an aryl hydrazide linker to obtain peptide carboxylates using catalytic amounts of Cu(II) acetate in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in water (Millington et al., 1998). It was shown that Cu(II) is only required in catalytic amounts due to the rapid aerial oxidation of Cu(I) cations. It also demonstrated the dual function of the nitrogen base being required both for proton abstraction and copper ion complexation. In the absence of a complexing agent, Cu(I) oxide precipitated from the reaction solution.

Oxidation of a peptidyl aryl hydrazide resin is also accomplished with NBS to yield a peptidyl diazene resin, which can be then efficiently cleaved with wet dichloromethane (DCM) containing N,N-diisopropylethyl amine (DIEA) to give the corresponding peptide carboxylate in excellent yield.

Peptide esters. The relatively high reactivity of peptidyl hydrazides to O-nucleophilic attack by water under mild oxidative conditions prompted several groups to explore the ability of this linker to provide a rapid way to obtain C-terminal peptide esters. Work by Millington et al. showed that the aryl hydrazide linker can be also efficiently cleaved under oxidative conditions by aliphatic alcohols such as MeOH, EtOH or ^{i}PrOH to give the corresponding peptide esters (Millington et al., 1998). The reaction was performed using the alcohol as solvent in the presence of Cu(II) acetate and either DBU or pyridine as base. More recently, Peters et al. (Peters and Waldmann, 2003) carried out a more extensive study on the reaction of alcohols with activated peptidyl diazene resins. In this work the two oxidative cleavage methods using Cu(II) acetate or NBS were compared using a variety of different alcohols as nucleophiles. As anticipated, primary and secondary alcohols readily formed the corresponding esters independently of the method employed for the oxidative cleavage. Most notably was the fact that the desired products were obtained with little byproduct formation. This was shown to be particularly true for the oxidative cleavage involving NBS. The acyl diazene formed after NBS oxidation is relatively stable, and all contaminants and byproducts generated during the oxidation step can simply be removed by washing and filtration. When the oxidative cleavage is carried out with copper, the metal can be removed from the crude reaction by using a simple

phase extraction cartridge. In some cases, however, some residual copper may remain bound to the peptide thus requiring extra purification steps.

Analysis of the cleavage efficiency for the different alcohols revealed that the yield for the cleavage step is directly related to the steric hindrance of the nucleophile. Hence, primary alcohols afforded the corresponding peptide esters in relatively high yields (50-60%) while the more sterically impeded secondary alcohols gave lower yields (≈40 %). For sterically hindered secondary nucleophiles, the oxidative cleavage using NBS was shown to give better yields.

The same authors were also able to successfully use allyl and benzyl alcohol to obtain the corresponding peptide esters. These esters are among the most frequently used C-terminal protecting groups employed in solution-phase peptide chemistry as well as other fields of organic synthesis (Greene and Wuts, 1999).

Peptide N-alkyl amides. N-nucleophiles are more reactive than O-nucleophiles and therefore primary and secondary aliphatic amines react almost quantitatively with active peptidyl diazenes to form the corresponding N-alkylated C-terminal carboxyamides (Millington et al., 1998, Camarero et al., 2004). The oxidative cleavage in these cases is usually carried out using Cu(II) acetate in solvents such as N,N-dimethylformamide (DMF) or tetrahydrofuran (THF) in the presence of the nucleophilic amine, which also serves as base and a ligand to complex the copper ions and prevent Cu_2O precipitation. Similarly, NBS is used in the oxidation of aryl hydrazides. Subsequent reaction of the reactive acyl diazene with aliphatic amines affords N-alkyl carboxamides in very good yields (Camarero et al., 2004).

The use of benzylamines as nucleophiles has been reported recently by Albrecht et al. to generate peptide N-benzyl carboxamides in relatively good yields (Albrecht et al., 2004). Several tripeptides containing N- and C-terminal dicathecol ligands were prepared. The dicatechol moieties were used to fix the conformation of the tripeptide via a metallomacrocycle complex with Mo(VI). The solid-phase synthesis of the dicatechol-containing peptides was accomplished on a 4-Fmoc-hydrazinobenzoyl amino resin using standard Fmoc-chemistry. After the deprotection of the N^{α}-group, the N-terminal ligand was introduced by addition of activated 2,3-dimethoxybenzoic acid. Attachment of the C-terminal ligand was carried out by oxidative cleavage of the hydrazide linker with Cu(II)/air in the presence of 2,3-methoxybenzylamine. In all the cases the cleavage was accomplished in relatively good yield (≈70%). Final treatment of the protected peptides with BBr_3 in DCM provided the desired catechol-containing peptides.

Peptide N-aryl amides. Peptide *p*-nitroanilides and peptide 7-amido-4-methylcoumarin (AMC) derivatives are widely used as chromogenic or fluorogenic substrates, respectively, for studying the activity and selectivity of proteolytic enzymes (Kwon et al., 2004, Gosalia et al., 2005). However, the preparation of these useful compounds by SPPS is challenging due to the poor nucleophilicity of aromatic amines.

Our research group recently explored the use of a hydrazide linker to obtain peptide C-terminal *p*-nitroanilides (Kwon et al., 2006). The formation of peptide *p*-nitroanilides is particularly difficult due to the very low nucleophilicity of the aromatic amino group of *p*-nitroaniline (pNA), which is further deactivated by the electron-withdrawing nitro group (pK_a of the conjugated acid of pNA amino group is ≈ 1.0). For this purpose several peptides were synthesized on an Fmoc-hydrazinobenzoyl resin using Fmoc-based

chemistry and then activated by oxidation with 2 equiv of NBS in the presence of anhydrous pyridine for 10 min at room temperature. The resulting peptidyl diazene resin was then washed and immediately reacted with pNA in DCM containing 5% DMF. As anticipated, the cleavage reaction was slower than observed for aliphatic amines (Millington et al., 1998, Camarero et al., 2004) requiring 24 h for completion. Despite the longer reaction time required for the pNA cleavage, the reaction was very clean producing the corresponding peptide *p*-nitroanilides in relatively good yield (≈60%) and high purities (≈95%). It is important to note that no epimerization of the C-terminal residue was observed (Kwon et al., 2004).

The cleavage reaction of several peptidyl diazenes with AMC was also explored (Kwon et al., 2006). In this case the oxidative cleavage only took 6 h for completion with yields around 70%. This result can be attributed to the relatively higher nucleophilicity of the aromatic amino group in AMC (pK_a of the conjugated acid of the AMC amino group is 3.8).

Peptide α-thioesters. C-terminal peptide α-thioesters are key intermediates in the synthesis of small- and medium-sized proteins (Dawson and Kent, 2000, Tam et al., 2001, Muir, 2003) and cyclic peptides (Camarero and Muir, 1997, Zhang and Tam, 1997, Camarero et al., 1998a, Shao et al., 1998) by native chemical ligation (NCL) (Dawson et al., 1994). These mildly activated intermediates can be also used for the construction of topologically altered (Camarero et al., 1998b, Camarero and Muir, 1999, Iwai and Pluckthum, 1999, Evans et al., 1999, Camarero et al., 2001) and backbone engineered proteins (Baca and Kent, 2000, Yang et al., 2004).

C-terminal peptide thioesters have been traditionally prepared by standard SPPS using Boc/Benzyl chemistry (Hojo and Aimoto, 1992, Hackeng et al., 1997, Camarero et al., 1998a, Camarero et al., 2000) or for larger polypeptides using intein-based bacterial expression systems (Perler and Adam, 2000, Muir, 2003). Unfortunately, the Boc/Benzyl approach requires the use of anhydrous HF, which is extremely toxic and not well suited for the synthesis of phospho- or glycopeptides.

The Fmoc-approach, on the other hand, provides an attractive alternative as it does not use HF. However, the poor stability of the thioester functionality to strong nucleophiles such as piperidine, which is usually employed for the deprotection of the N^{α}-Fmoc group during Fmoc-based SPPS, seriously limits the use of thioester linkers, which are employed for the preparation of peptide thioesters through Boc-chemistry (Hojo and Aimoto, 1992, Hackeng et al., 1997, Camarero et al., 1998a, Camarero et al., 2000).

Our research group has overcome this limitation by using the hydrazize safety-catch resin-linker for the synthesis of C-terminal peptide thioesters using Fmoc chemistry (Camarero et al., 2004). In this work, the Fmoc-assembled peptidyl hydrazide resin was oxidized with NBS in DCM for 10 min and then efficiently cleaved (<30 min) with an amino acid alkyl thioester (H-Gly-SEt or H-Ala-SEt) to give the fully protected peptide α-thioester. Subsequent side-chain deprotection with TFA in the presence of the appropriate scavengers provided the desired peptide thioester.

It should be noted that when thiols such as EtSH, BnSH or PhSH were used to cleave the peptidyl diazene resin, only minor amounts (<2%) of the corresponding peptide thioester were detected. This is chiefly due to the reductive character of the thiol group, which

reacts with the acyl diazene through a redox reaction to give the original acyl hydrazide and the corresponding disulfide (unpublished results).

A number of model peptide thioesters were synthesized in good yield (70-90%) using this approach. These included a 13-mer N-terminal Cys-containing peptide thioester derived from the 10^{th} type III module of Fibronectin that was used to generate a backbone (head-to-tail) cyclized peptide through intramolecular NCL, and a 22-mer peptide thioester derived from the N-terminal SH3 domain of the Crk protein that was used to generate a fully active variant of this protein domain by NCL (Camarero et al., 2004).

Synthesis of backbone cyclic peptides

Peptidyl aryl hydrazides have been also used for the production of backbone cyclic peptides. In 1970 Wieland et al. investigated the use of the aryl hydrazide group for the backbone cyclization of peptides in liquid phase (Wieland et al., 1970). The authors used linear N^{α}-Boc protected C-terminal peptide aryl hydrazides as precursors. The first step involved the deprotection of the N^{α}-Boc group followed by oxidation of the C-terminal aryl hydrazide with NBS to give the reactive peptidyl diazene. After neutralization, the N^{α}-amino group reacted intramolecularly with the acyl diazene to yield the corresponding cyclic peptide. In this work several cyclic peptides were synthesized with rather modest yields. More recently, Rosenbaum et al. reported the use of the aryl hydrazide resin linker for the solid-phase of cyclic peptides via an intramolecular oxidative cleavage approach (Scheme 3) (Rosenbaum and Waldmann, 2001). In this work the peptides were assembled on a commercially available 4-Fmoc-hydrazinobenzoyl AM resin using Boc chemistry. After N-terminal deprotection, the hydrazide was oxidized to the corresponding acyl diazene, which subsequently was attacked by the α-amino group thus

resulting in cyclization and concomitant cleavage from the solid support (Scheme 3). This approach was first tested with the synthesis of a model cyclic peptapeptide that was obtained with an overall yield of 19% after two days of reaction. This rather modest yield was significantly higher than the yields obtained for the cyclization of the same peptide in solution phase (Schmidt and Langner, 1997). The naturally occurring cycloheptapeptide Stylostatin 1 was also cyclized following this approach with 7% overall yield. This peptide was used to investigate the sequence dependence of the cyclization via the hydrazide linker, showing that the cyclization yields depend strongly on the steric bulk of the residues located at the cyclization site.

Shigenaga et al. have also used this approach for the solid-phase synthesis of several cyclodepesipeptide analogs of autoinducing peptides from different *Staphyloccocus aureus* subgroups as panning reagents for *in vitro* antibody selection (Shigenaga et al., 2006). In this work the linear precursors were assembled on an Fmoc-4-hydrazinobenzoyl-Rink amide resin. The Rink amide linker served as an internal reference control, allowing for rapid, small-scale test cleavage to be performed at various stages of the synthesis for monitoring. The assembly of the linear precursors was started from the penultimate residue. The conserved fourth Ser residue (in place of the native Cys, numbering from the penultimate residue) was incorporated as the side-chain-unprotected derivative to facilitate post-assembly side-chain esterification with the C-terminal amino acid (Scheme 4). After the synthesis of the O-acyl branched linear precursor was accomplished, the N^{α}-Fmoc group of the Nle residue was removed and the resin was allowed to cyclize in pyridine–*o*-dichlorobenzene containing a saturated solution of Cu(II) acetate for two days. After removal of the remaining protecting groups

with TFA, the desired cyclodepsipeptides were obtained with yields ranging from 30 to 60% and moderate to good purities (15-70%).

Synthesis of lipidated peptides

Lipidated proteins play extremely important roles in biological processes, specially serving as pivotal molecular switches in human oncogenic signaling cascades (Gelb, 1997). Prominent examples of this can be found in many cancer cells in which mutated Ras-type GTPase proteins are arrested in the active state thus resulting in uncontrolled cell growth (Shaw and Cantley, 2006).

Members of the Ras superfamily are highly posttranslationally modified at their C-termini through prenylation and in some cases acylation. The three most common posttranslational lipid modifications in this family are N-myristoylation, S-palmitoylation and S-isoprenylation. Additionally, some of the Ras proteins are also posttranslationally converted into C-terminal methyl esters. The lipidation of the C-termini of the Ras proteins results in membrane association of the modified proteins and it is required for proper protein function.

The Waldmann group has recently reported the semi-synthesis of fully functional lipid-modified Ras proteins by ligating synthetic lipidated peptides to the corresponding soluble protein domains (Reuther et al., 2006, Brunsveld et al., 2006, Goody et al., 2005). The synthesis of lipidated peptides, however, is extremely challenging because of the high acid- and base-sensitivity of the isoprenyl thioethers and the palmitoyl thioesters, respectively. The solid-phase synthesis of these compounds requires the use of orthogonal resin-linkers that at the same time can be cleaved under very mild conditions. The hydrazide linker has been shown to be ideal for this task (Ludolph et al., 2002,

Ludolph and Waldmann, 2003, Kragol et al., 2004, Lumbierres et al., 2005). A typical lipidated peptide sequence synthesized on the hydrazide linker using lipidated building blocks is shown in Scheme 5. The target peptide contains a farnesyl (Far) thioether, a palmitoyl thioester, a fluorophore attached at the ε-amino group of the Lys residue and C-terminal methyl ester. Peptide assembly on this resin employs standard amino acid coupling conditions. One important precaution must be taken, however, to avoid S,N-acyl shift of the palmitoyl group during the Fmoc-deprotection of palmitoylated Cys and subsequent amino acid coupling in order. The best conditions to minimize this reaction involve the deprotection of the N^{α}-Fmoc group with 1% DBU in DMF for 2 x 30 seconds followed by immediate coupling of the next O-(7-azabenzotriazole-1-yl)-N, N,N'N'-tetramethyluronium hexafluorophosphate (HATU) preactivated amino acid (Kragol et al., 2004). The final peptide cleavage is accomplished with Cu(II) acetate/pyridine in DCM containing MeOH to afford the corresponding C-terminal peptide methyl ester (28% overall yield).

The synthesis of lipidated peptides using an on-resin lipidation approach has been also explored by the same group (Scheme 6) (Ludolph and Waldmann, 2003, Ludolph et al., 2002). It relies on the combined use of the base labile Fmoc-group for N-terminal deprotection of the peptide chain, the application of different acid-labile protecting groups for the cysteine side chain, as well as S-farnesylation and S-palmitoylation of the growing peptide chain on the solid support. As shown in Schme 6, the S-trityl (Trt) group of the first Cys residue was removed by treatment with 50% TFA in DCM and then S-farnesylation was carried out by treatment of the liberated thiols with farnesyl bromide in the presence of a tertiary amine. The thiol group of the second cysteine required in the

sequence was protected as monomethoxytrityl (Mmt) thioether. After completion of peptide assembly, the Mmt group was removed by treatment with 1% TFA in DCM and in the presence of triethylsilane. The farnesyl group was stable under these mild, weakly acidic conditions. S-palmitoylation was then carried out by treatment with palmitoyl chloride in the presence of 1-hydroxybenzotriazole (HOBt) and triethylamine to yield a doubly lipidated polymer-bound peptide (Scheme 6). The peptide cleavage was performed with Cu(II)/air in DCM containing MeOH to afford the desired C-terminal peptide methyl ester (42% overall yield) (Ludolph et al., 2002).

Miscellaneous reactions

The aryl hydrazide linker is compatible with a variety of different transformations and reaction conditions, which extends its utility beyond the solid-phase synthesis of modified peptides. Several different solid-phase approaches for the synthesis of non-peptidic biologically active compounds are now possible (Stieber et al., 1999).

For example, Stieber et al. have reported the use of the hydrazide linker for the multistep solid-phase synthesis of several biologically active compounds, including an antibiotic against *Mycobacterium tuberculosis* and several 2-aminothiazole-based libraries with inhibitory activity against different receptor tyrosine kinases (Stieber et al., 2003).

More recently, Rodenko et al. used the same linker for the solid phase synthesis of several di- and trisubstituted 5'-carboxamidoadenosine analogues in very good yields (Rodenko et al., 2006). It is interesting to note that the authors initially used Kenner's benzenesulfonamide linker for the production of the 5'-carboxyadenosine libraries. However, the results obtained with this safety-catch linker were extremely poor due to the intrinsically low nucleophilicity of the benzenesulfonamide linker, especially with

sterically demanding carboxylic acids. In contrast, the beneficial sterical and electronic properties of the aryl hydrazide linker provided excellent results.

Summary and perspectives

In summary, we have highlighted the extreme versatility of the hydrazide linker for the solid phase synthesis of chemically modified peptides. This versatility is due to the orthogonality of this linker to most of the protecting groups employed in SPPS and, equally important, it can be cleaved under very mild conditions. These two features have allowed its use in the synthesis of a wide variety of C-terminal modified peptides, head-to-tail cyclic peptides as well as lipidated peptides.

It should be also noted that the stability of this linker to the conditions employed in a number of reactions widely used in combinatorial chemistry (Heck, Suzuki and Sonogashira couplings, reductive aminations, etc) favors the use of this linker in solid-phase and combinatorial chemistry in general.

Acknowledgements

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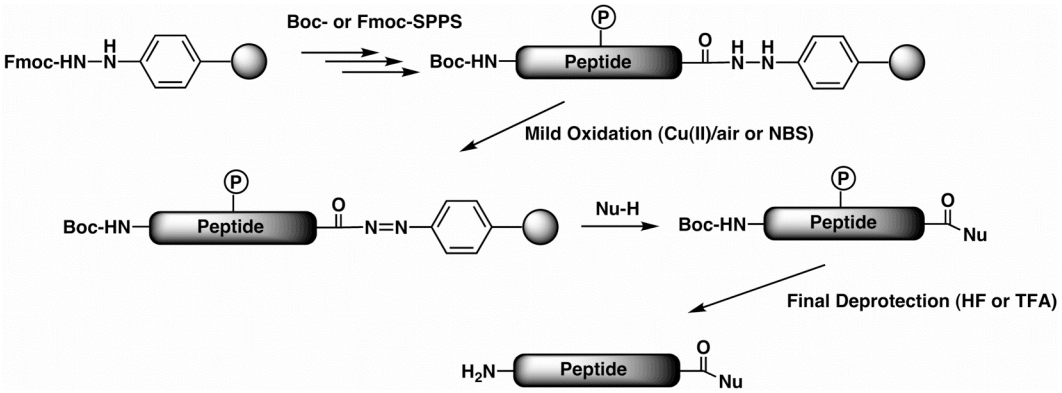
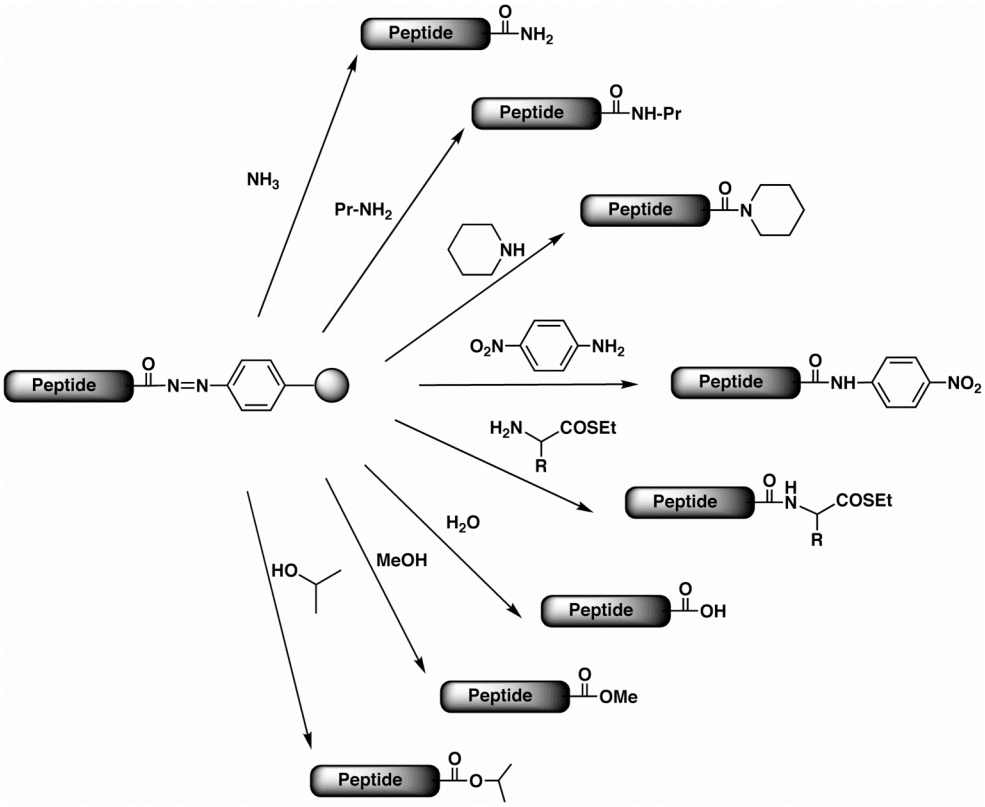
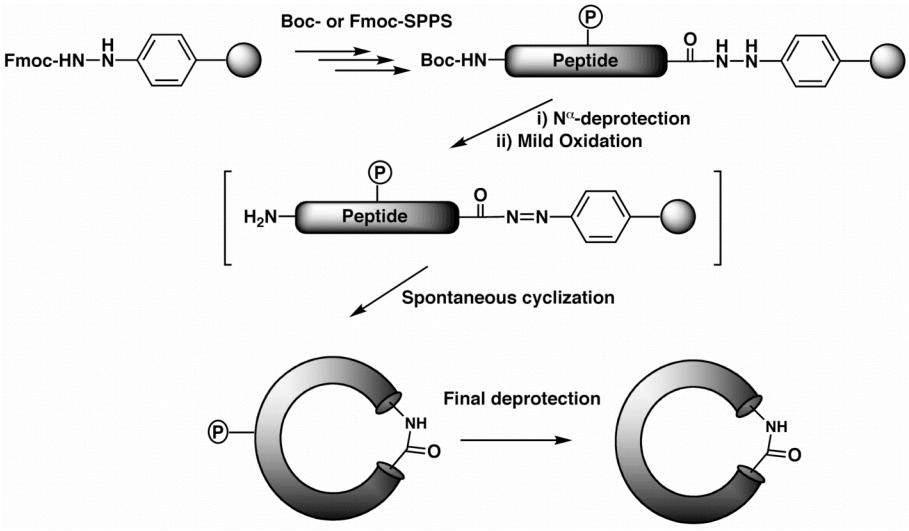
Figure Captions



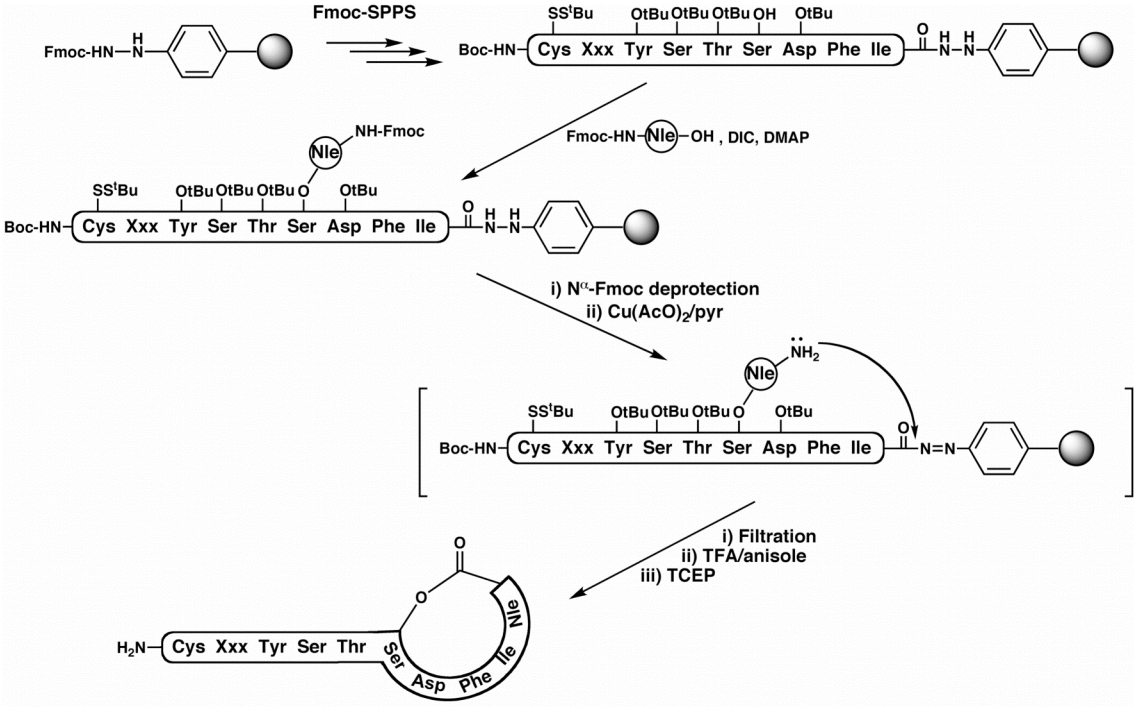
Scheme 1. Cleavage of the aryl hydrazide linker under mild oxidative conditions.



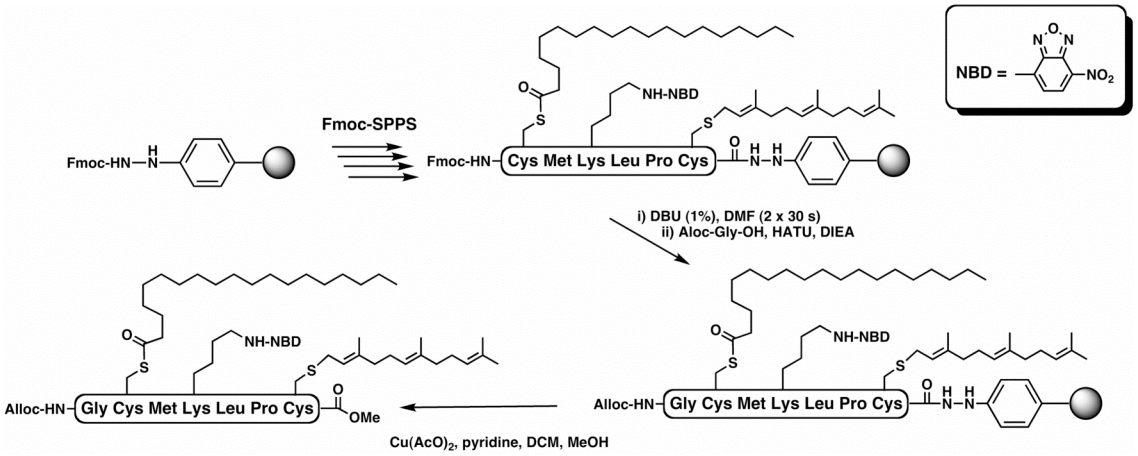
Scheme 2. *In situ* generated acyl diazenes, obtained from the corresponding hydrazide resins by mild oxidation, can be used as a multi-detachable system for the introduction of several C-terminal modifications into peptides.



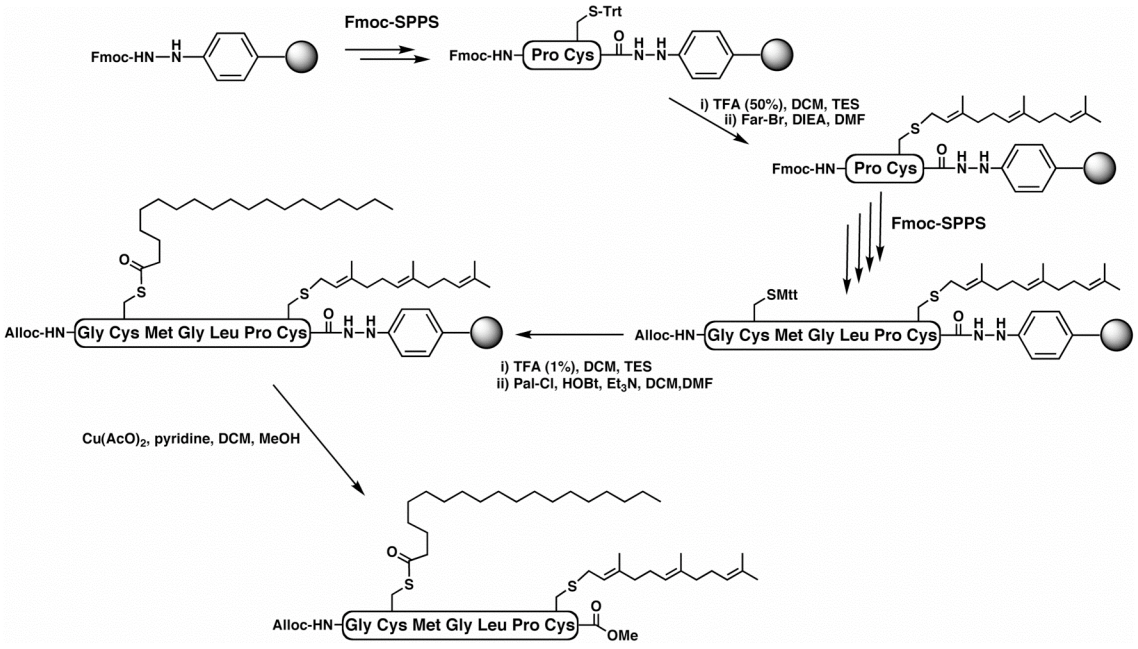
Scheme 3. Principle for the solid-phase of head-to-tail cyclized peptides employing an oxidation-labile aryl hydrazide linker.



Scheme 4. Solid-phase assembly and cyclization/cleavage approach for the synthesis of cyclic depsipeptides analogs derived from *S. aureus* autoinducing peptides (Shigenaga et al., 2006).



Scheme 5. Solid-phase synthesis of an N-Ras lipopeptide on a hydrazide resin using lipidated building blocks (Kragol et al., 2004).



Scheme 6. Solid-phase synthesis protocol for the preparation of lipidated peptides by using an on-resin lipidation approach (Ludolph et al., 2002).

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