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(54) Title: PEPTIDE SYNTHESIS WITH SULFONYL PROTECTING GROUPS

(57) Abstract

Methods and compositions are provided for synthesizing peptides with sulfonyl protecting groups. In the subject methods, a sulfonyl group α-amino protected amino acid is contacted with a second amino acid monomeric unit under coupling conditions so that hte protected amino acid and second amino acid monomeric unit are joined by a peptide bond. The contacting step may be repeated one more additional time to obtain a peptide of desired length. Also provided are methods of site specific modification using the subject sulfonyl protecting groups. The subject methods find particular use in methods of solid phase peptide synthesis.

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PEPTIDE SYNTHESIS WITH SULFONYL PROTECTING GROUPS

INTRODUCTION

Technical Field

The technical field of this invention is peptide synthesis.

Background of the Invention

Peptides (polymers of amino acids) and peptide-like structures can influence a variety of physiological processes, such as the endocrine, neurological, immune and enzymatic processes, with high specificity and potency. As such, peptides are of critical importance to biomedical research and fields related thereto, and have found, or have potential use, in medicine, including the regulation of fertility, the control of pain, the stimulation of growth, the therapy of cancer, cardiovascular applications, treatment of connective tissue diseases, treatment of digestive disorders, treatment of mental illness, treatment of infections by pathogens, and the like.

The demand for peptides is therefore enormous and continues to increase. While natural sources can provide a great variety of peptides, often it is difficult to identify and isolate a particular peptide of interest in useful amounts. Accordingly, peptide synthesis by recombinant DNA or purely chemical means plays an important role.

Modern chemical synthesis of peptides has been summarized as follows: "All modern work in peptide synthesis employs the following approach: the amino group of one amino acid is first stabilized by the introduction of a protecting group R, the carboxyl group is modified so that it is capable of coupling with a second amino acid, the coupling is performed, and finally the group R is removed to produce a finished peptide. The difficulty lies in finding a suitable group R, which can be removed so

gently that the peptide is not significantly attacked." Bergmann & Zervas, Berichte der Deutschen Chemischen Gesellschaft (1932) 65: 1192-1201.

For an "R" group to be suitable for use in peptide synthesis, it must satisfy a number of different criteria. It must suppress the nucleophilic reactivity of the α-amino group, either: (a) by draining the α-amino group's electron density away into an appropriate substituent or (b) concealing the group "behind a screen of gross steric hindrance." It should introduce no problems of its own. It should remain firmly in position while needed. It should be removable under conditions that do not adversely affect the rest of the structure being assembled. It should not affect the chiral integrity of nearby asymmetric centers. It should be orthogonal to other protecting groups that are being used.

A number of different "R" groups have been developed for use in chemical peptide synthesis, and include: the alkoxycarbonyl family of amino-protecting groups, such as the Benzyloxycarbonyl (Z) group, the t-Butoxycarbonyl (Boc) group, the 2-(4-Biphenylyl)-isopropoxycarbonyl (Bpoc) group, the 9-Fluorenylmethoxycarbonyl (Fmoc) group, the 2,2,2-Trichloroethoxycarbonyl (Troc) group; the triphenylmethyl (trityl, Trt) group; the 2-Nitrophenylsulphenyl (Nps) group; the Dithiasuccinoyl (Dts) group; and the Diphenylphosphinyl (DPP) group.

A significant advance in the field of chemical peptide synthesis occurred in 1963 with Merrifield's development of the technique of solid phase peptide synthesis. Merrifield, J. American Chemical Soc. (1963) 85: 2149-2154. Methods of solid phase peptide synthesis are characterized by the iterative coupling of an α-amino protected amino acid monomer to a solid phase bound amino acid monomeric unit, in which the C-terminal amino acid of the desired peptide product is covalently bound to a solid phase, such as polystyrene. In addition to the α-amino protecting group, any reactive side chain functionalities that may be present on the individual amino acid monomeric units of the growing peptide chain are protected. As such, the "temporary α-amino protecting group must be orthogonal in reactivity to the side chain protecting groups." Since its development, different combinations of α-amino protecting groups and side chain protecting groups have been employed in solid phase peptide synthesis. For example, the Boc group has been employed as the α-amino protecting group in combination with benzyl groups as the side chain protecting groups. Of increasing

prevalence is the use of the Fmoc group as the α-amino protecting group in combination with the Boc group, or a similar t-butyl based group, as the side chain protecting group. Although each of the above combinations of protecting groups have been successfully used in the chemical synthesis of a multitude of different properties, no protecting group has been yet been developed that is completely satisfactory.

Accordingly, there is continued interest in the identification of new α-amino protecting groups which are suitable for use in methods of chemical peptide synthesis. Because of its continued growth in popularity in the field of peptide synthesis, of particular interest is the development of new α-amino protecting groups suitable for use in solid phase peptide synthesis.

Relevant Literature

Jones, The Chemical Synthesis of Peptides (Oxford University Press)(1993) provides a review of both solution phase and solid phase peptide synthesis.

SUMMARY OF THE INVENTION

Methods and compositions are provided for peptide synthesis. In the subject methods, a sulfonyl protecting group is used as an α-amino protecting group for at least one amino acid monomer during synthesis. To prepare peptides according to the subject invention, in at least one of the coupling steps in the overall peptide synthesis, an α-amino protected amino acid protected with a sulfonyl protecting group is contacted with a second amino acid monomeric unit under conditions sufficient for coupling to occur through formation of a peptide bond between the α-amino protected amino acid and the second amino acid monomeric unit. The above steps may be repeated one or more additional times with additional α-amino protected amino acids, which may be similarly protected or protected with another α-amino protecting group, to prepare a peptide of desired length. Also provided are methods of N-site specific modification using the subject sulfonyl protecting groups. The subject methods find particular use in solid phase peptide synthesis.

DEFINITIONS

The term "peptide" as used herein refers to any compound produced by amide formation between a carboxyl group of one amino acid and an amino group of another.

The term "oligopeptide" as used herein refers to peptides with fewer than about 10 to 20 residues, *i.e.* amino acid monomeric units.

The term "polypeptide" as used herein refers to peptides with more than 10 to 20 residues.

The term "protein" as used herein refers to polypeptides of specific sequence of more than about 50 residues.

The term "α-amino protected amino acid" is used herein in its broadest sense to refer to a molecule having an α-amino group in which the N is covalently bonded to moiety which can be selectively removed under appropriate conditions. Therefore, specific examples include o-NBS α-amino protected amino acids, such as amino acids in which the α-amino group is covalently bonded to o-NBS and peptides in which the α-amino group of the N-terminal amino acid monomeric unit is covalently bonded to o-NBS.

The term "second amino acid monomeric unit" refers to an amino acid or amino acid residue in which the α-carboxy group is protected. Thus, second amino acid monomeric units may be amino acids free in solution in which the α-carboxy group is protected, either by a protecting group or through a covalent bond to a polymeric support material, or the N-terminal amino acid residue of a peptide, where the α-carboxy group of the C-terminal residue is protected, either by a protecting group or through covalent linkage to a polymeric support material.

The term "amino acids" as used herein, includes α-amino acids, as well as β-, γ-, δ- and non-naturally occurring amino acids.

Solid support. A substrate or solid support is a conventional solid support material used in peptide synthesis, including polymeric and spherical beads. Non-limiting examples of such substrates or supports include a variety of support resins and connectors to the support resins such as those that are photo-cleavable, DKP-forming linkers (DKP is diketopiperazine; see WO90\09395, herein incorporated by reference), TFA cleavable, HF cleavable, fluoride ion cleavable, reductively cleavable; Pd(0) cleavable; nucleophilically cleavable; radically cleavable; and base-labile linkers.

Substrate compound: A compound containing at least one primary amine that is modified by the subject methods, and in which there are groups or atoms that could be susceptible to the reactions that modify the target primary amino group.

Compounds of interest include various monomers and polymers, particularly polymers. Polymers include addition polymers and condensation polymers, more particularly polyamides. Polyamides of interest include polypeptides, which as used herein include analogs and mimetics where the amino acid monomers in the polypeptide may have naturally occurring or modified side chains, which may also include polypeptide nucleic acids, or combinations thereof. See, for example, PCT publication US93/09117 (Zuckerman) and US91/04292 (Bartlett). The subject methods find particular use in polypeptides, where modification of specific residues is of interest.

Protecting group. Any group that is capable of preventing the atom to which it is attached, usually oxygen or nitrogen, from participating in an undesired reaction or bonding, usually in a synthesis reaction. Protecting groups are also known to prevent reaction or bonding of carboxylic acids, thiols and the like. Such groups and their preparation and introduction are conventional in the art, and include salts, esters and the like. Protecting groups commonly used in solid phase peptide synthesis include Fmoc and Boc groups, which protect the primary amine of incoming amino acid monomers during formation of the peptide bond. Protecting groups are commonly used to protect amino acid side chains during peptide synthesis, for example TFA-labile tBu, Trt, Pmc and Boc; Pd(0)-labile Alloc, OAll; photolabile groups such as nitro veritryl oxycarbonyl (NVOC); and fluoride labile groups such as trimethylsilyl oxycarbonyl (TEOC).

Activating group: any group that, on reaction of the target primary amine in the substrate compound with an activating agent, renders an amide nitrogen that is more reactive than any other nitrogen in the substrate compound. The activating group renders the target NH group more acidic than other nitrogens in the substrate compound. For the purposes of the subject invention, the activating group is preferable cleavable from the amide under conditions that are compatible with conventional amino acid protecting groups and a polyamide backbone. Exemplary activating agents react with the primary amine to form a sulfonamide, although other structures, such as trifluoroacetamide, may also be formed. Table I provides the structures for exemplary sulfonyl activating groups.

Various sulfonyl halides have been used as activating compounds. The working examples demonstrate the utility of aryl sulfonyl halides, where the aryl moiety is substituted with an electron withdrawing group, *e.g.* a nitro substituent, at the ortho or para position. For alkylation reactions of polypeptides, *ortho*-nitrobenzenesulfonyl chloride and *para*-nitrobenzenesulfonyl chloride are exemplary activating agents, yielding oNBS and pNBS amides, respectively.

Strong base: strong bases useful in the subject methods are capable of selective deprotonation of the activated amide. Hindered guandidinium bases are exemplary, where there is sufficient substitution so as not to act as a nucleophile in the reaction. The pKa of the base will be in the range of about 12 to 15. For ease of use and stability considerations, non-ionic bases are preferred. Examples include MTBD, DBU, DBN, PMG, TMG,low equivalents of BEMP, the ionic base TBAF, TBD and derivatives, and the like.

Modifying agent: an electrophilic agent that will selectively modify the deprotonated, activated amide to add a substituent group. Preferred modifying agents are not strong enough to modify the amide backbone of a polypeptide. Exemplary are alkylating agents such as alkyl nosylates, *e.g.* methyl nosylate; alkyl halides, *e.g.* ethyl iodide; allyl bromide; benzyl bromide, propyl iodide; alkyl sulfates, *e.g.* dimethyl sulfate; Pd-π allyl species; Mitsunobu reactions (Mitsunobu (1981) Synthesis:**1**), particularly for intra-molecular, e.g. cyclization, reactions; and other agents as known in the art.

Substituent, substituted or derivative. substituent describes an atom or radical which is part of a first molecule in that it replaces another atom or radical of the first molecule. When a molecule is substituted, it is a derivative of a molecule bearing one or more substituent. Useful substituents in any of the peptides of the invention include halo, alkyl, alkoxy, aryl, allyl, alkylthio, haloalkyl, haloalkoxy, halothio, and the like, which replace a hydrogen attached to a primary amine.

Alkyl substituents: a hydrocarbon or substituted hydrocarbon group. Alkyls may be linear or branched. Substituted alkyls may have hetero groups, including nitro-, oxy-, hydroxy-, thio-, carbonyl-, halo-, amino, and the like, as known in the art. Preferably, substituent groups are not located on the alpha carbon of the alkyl group.

Aryl substituents: a benzyl or substituted benzyl group. Substituted aryls may have hetero groups, as described for alkyl substituents. These types of modifications may be carried out using reductive alkylation, as known in the art.

Allyl substituents: a hydrocarbon having one or more unsaturated C-C bonds. Allyls may be linear or branched. Substitued allyls having hetero groups as described for alkyl substituents may also be used. Alkynes, particularly terminal alkynes may be used. Alkyne substituents may be modified in a second reaction by various known methods, including Sonogashira reactions, hydrozirconation, *etc.*

Cleavage agent: a compound capable of cleaving the substituted target amide to release the activating group. Some reagents of interest are listed in Table I, including Pd(0), fluoride ion, radical cleavage, bases, photolysis, and nucleophilic aromatic substitution. Cleavage agents of interest for use with nucleophilic aromatic substitution include alkyl and aryl thiols, such as β–mercaptoethanol and thiophenol, dithiothreitol, glutathione, n-propylthiol, ethanedithiol, n-butylthiol, *etc.* Other reagents include hydrazine, pyrrolidine, piperidine, *etc.*

Coupling agent: agents, as known in the art, that activate coupling reactions in polypeptide synthesis. It is known that secondary amines, such as those formed by the subject methods, are more difficult to couple than primary amines. While use of HBTU or BOP as a coupling agent is conventional for SPPS, it may be necessary to use a stronger agent, for example HATU, to achieve a high yield in the synthesis step involving the secondary amine, and/or to drive the coupling reaction to completion by using multiple cycles of coupling reactions.

Solvents: suitable inert solvents include blocked amides, such as dimethylformamide, sulfoxides, such as dimethylsulfoxide ;dimethyl acetamide; methylene chloride; and the like.

Side chains: the substituent groups of naturally occurring amino acids, or analogs and modified versions thereof. Side chains include $-CH_3$ of alanine; $-CH(CH_3)_2$ of valine; $-CH_2CH(CH_3)_2$ of leucine; $-CH(CH_3)CH_2CH_3$ of isoleucine; $-CH_2OH$ of serine; $-CHOHCH_3$ of threonine; $-CH_2SH$ of cysteine; $-CH_2CH_2SCH_3$ of methionine; $-CH_2$-(phenyl) of phenylalanine; $-CH_2$-(phenyl)-OH of tyrosine; $-CH_2$-(indole) of tryptophan; $-CH_2COO-$ of aspartic acid; $CH_2C(O)(NH_2)$ of asparagine; CH_2CH_2COO- of glutamic acid; $-CH_2CH_2C(O)NH_2$ of glutamine;

$-CH_2CH_2CH_2$-N-(H)-$C(NH_2)^+NH_2$ of arginine; -CH2-(imidazole)+ group of histidine; and $-CH_2(CH_2)_3NH_3^+$ of lysine.

Library: library and mixture are used herein to describe three or more substrate compounds together, where each of the substrate compounds comprise at least one primary amine. Preferably the mixture includes 10 or more, 100 or more, 1,000 or more, 10,000 or more, 100,000 or more or 1,000,000 or more distinct and different compounds, e.g. different polypeptides with different amino acid sequences.

ABBREVIATIONS

Chemical abbreviations used herein are as follows: 9-fluorenylmethyloxycarbonyl (Fmoc); *tert*-butyl-oxycarbonyl (Boc); solid-phase peptide synthesis (SPPS); 7-methyl-1,5,7-triazobicyclo[4.4.0]dec-5-ene (MTBD); methyl *p*-nitrobenzene sulfonate (methyl nosylate); 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU); dimethylformamide (DMF); dimethylacetamide (DMA); *o*- and *p*-nitrobenzenesulfonyl (oNBS and pNBS); trifluoracetic acid (TFA); o(7-azabenzotriazol-1-yl-n,n,n',n'-tetramethyluronium hexafluoro phosphate (HATU); polypeptide nucleic acids (PNA); hydroxybenzotriazole tetramethyl uronium hexafluoro phosphate (HBTU); benzotriazole-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP); N-methyl morpholine (NMM); 4-methyl benzhydrylamine (MBHA); pentamethylguanidine (PMG); tetramethylguanidine (TMG); 1,5-diazabicyclo[4.3.0]non-5-ene (DBN); tetrabutyl ammonium fluoride (TBAF).

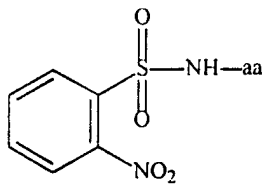
DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are provided for the chemical synthesis of peptides. In the subject methods, a sulfonyl protecting group is used as an α-amino protecting group. To synthesize peptides according to the subject methods, in at least one of the coupling steps of the peptide synthesis protocol a sulfonyl group α-amino protected amino acid is contacted with a second amino acid monomeric unit under coupling conditions, whereby a peptide bond is produced between the α-amino protected amino acid and the second amino acid monomeric unit. The above process may be repeated one or more times to generate a peptide of desired length, where the α-amino protected

amino acid monomers may be similarly protected or protected with a different protecting group, *e.g.* BOC, FMOC and the like. Also provided is a method of N-site specific modification using the subject sulfonyl protecting groups. Of particular interest is the use of the subject methods for solid phase peptide synthesis.

Critical to the subject invention is the use of a sulfonyl protecting group to protect the α-amino acid. By sulfonyl group is meant a group of atoms that comprises a sulfonyl moiety and, generally, is capable of covalently bonding with a primary amine to produce a sulfonamide. Sulfonyl groups that find use in the subject invention typically comprise a sulfonyl group linked to an electron withdrawing group, where the linking moiety may be alkyl or aryl, and will preferably be aryl. Representative sulfonyl protecting groups that find use in the subject invention are shown in Table 1.

TABLE 1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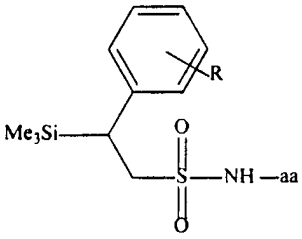
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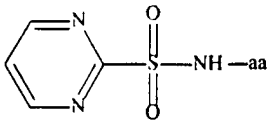
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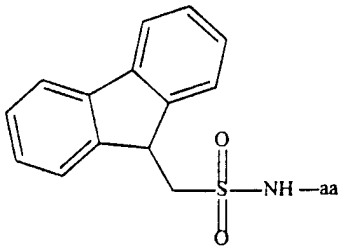
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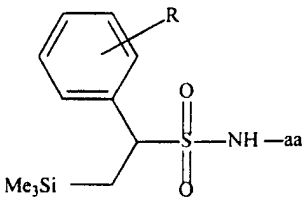
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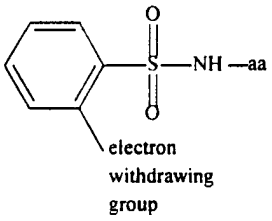
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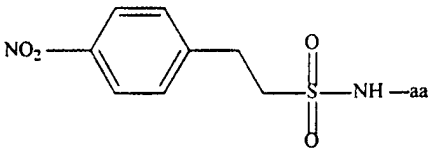
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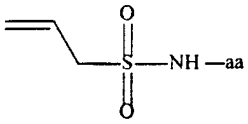
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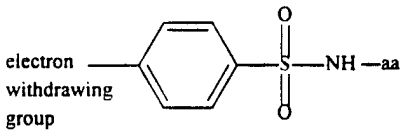
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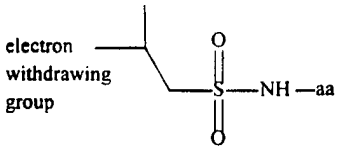
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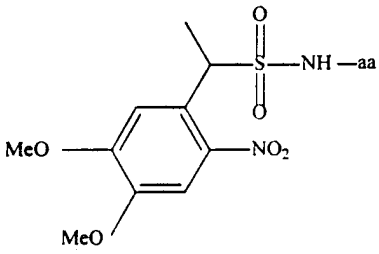
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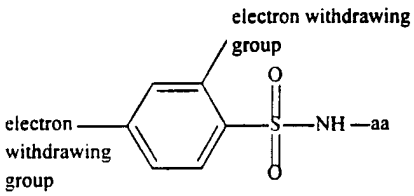
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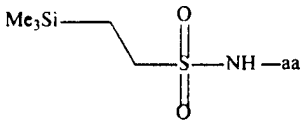
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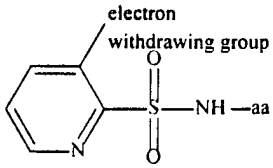
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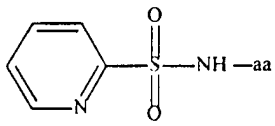
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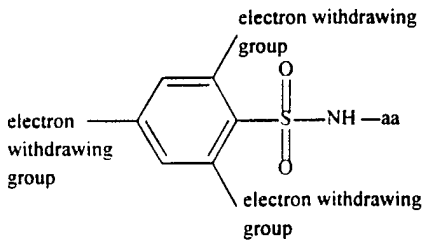
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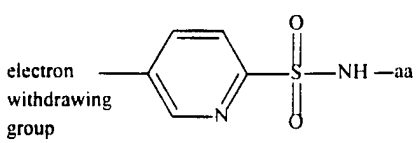
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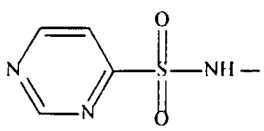
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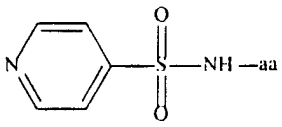
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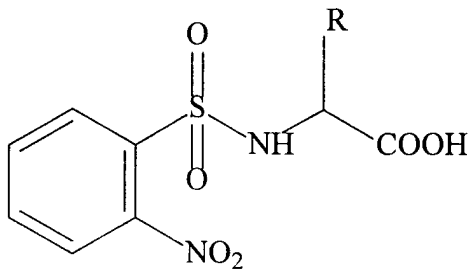
Preferably, the linking group is a nitrobenzene sulfonyl group, the nitro will preferably be ortho or -para to the sulfonyl group. Mor preferably, o-nitrobenzyl sulfonyl (o-NBS) is the protecting group of the α-amino moeity of the amino acid monomer. In the o-NBS group, a sulfonyl group which may be covalently bonded to the α-amino group of an amino acid is ortho to the nitro group of nitrobenzene. The structure of an o-NBS α- amino protected amino acid which finds use in the preferred embodiments of subject invention may be represented by the generic formula:



where R is a side chain, such as the side chain found on the naturally occurring amino acids or any one of the variety of non-naturally occurring synthetic amino acids that are known in the art.

In the subject invention, the sulfonyl group is used as an α-amino protecting group for amino acids during peptide synthesis. Amino acids having side chain functional groups may be further side-chain protected, as is known in the art, where conventional side chain protecting groups may be used. Preferably the sulfonyl protecting group is orthogonal in reactivity with respect to the side chain protecting groups. For example: for lysine residues, Boc and Z protecting groups may be employed; for arginine residues, PMC, Pbf and nitro groups may be employed; for aspartic and glutamic acids, benzyl, t-butyl ester, esters of secondary alcohols, *e.g.* cyclohexyl esters and the like, may be employed; for cysteine, thioacetal groups, TRITYL and t-butyl groups may be employed; for serine, threonine and tyrosine, benzyl and t-butyl groups may be employed; for asparginine and glutamine, Gln(Mbh) and trityl groups may find use; for histidine, Bom, Bum, and trityl groups may find use; for tryptophan, when side chain proteciton is indicated, carbamoyl (BOC) protection may find use. The particular reactive side chain protecting group employed will be chosen with respect to a number of parameters, including: overall conditions to

which the α-amino acid and peptide produced therefrom are subjected during synthesis, the nature of the amino acid; and the like. In a preferred embodiment of the subject invention in which solid phase synthesis is employed, the reactive side chains of the α- amino protected amino acids will be protected with t-butyl groups.

The α-amino protected amino acids employed in the inventive method may be prepared from commercially available side chain protected amino acids, where sources of such side chain protected amino acids include: Advanced Chemtech, Bachem, Sigma, Novabiochem, and the like; and the appropriate sulfonyl halide, e.g. o-nitrobenzenesulfonyl chloride, which is readily available from Aldrich, Fluka and the like. For example, in preparing the o-NBS α-amino protected amino acids from the above reagents, the reagents will be contacted with each other under conditions sufficient for the α-amino group to become covalently bonded to the sulphonyl group with the concomitant production of HCl, where such conditions are known to those of skill in the art and include: Schotten-Baumann conditions (aq. base (Na_2CO_3 or NaOH)/dioxane) *see e.g.* Bodanszky & Bodanszky, Practice of Peptide Synthesis.

In certain embodiments according to the subject invention, *i.e.* where it is desired to couple the α- amino protected amino acid to a N-substituted amino acid monomeric unit, *e.g.* an N-allyl substituted amino acid monomeric unit, as described in greater detail below, the α-amino protected amino acid described above may be converted to a highly reactive acid halide, *e.g.* chloride, where such reactive acid chlorides may be prepared according to methods known to those of skill in the art and described in Feiser & Feiser, Reagents for Organic Synthesis (Wiley), the disclosure of which is herein incorporated by reference.

The second amino acid monomeric unit to which the α- amino protected amino acid covalently bonds through production of a peptide bond is an α-carboxy protected amino acid monomeric unit, where the monomeric unit may be free in solution, at the N-terminal of peptide, where the peptide may be free in solution or stably associated with the surface of, *e.g.* covalently bonded to, a supporting polymeric material, where preferably the second amino acid monomeric unit (*i.e.* the α-carboxy protected monomeric unit) will be covalently bound to a solid support, either directly through the α-carboxy group or through an intervening linking chain, *e.g.* a linking chain of amino acid residues. Where the α-carboxy protected amino acid is free in solution, for

example in methods of solution phase peptide synthesis, a variety of α-carboxy groups may be employed, where a plurality of such protecting groups are known in the art and the include: esters, *e.g.* methyl, ethyl, benzyl, t-butyl, trimethylsilylethyl, phenyl, allyl, phenacyl, and the like. For the preferred solid phase synthesis method embodiments of the subject invention, the α-carboxy group of the second amino acid monomeric unit will be bonded, either directly or through an intervening linking group such as a stretch of one or more amino acid residues, to a polymeric support material, where such polymeric support materials include: cross-linked polystyrene and derivatives thereof; polyamides and derivatives thereof, *e.g.* Tentagel, PEG, and the like, where preferably the resin will be Tentagel or polystyrene.

In preparing peptides according to the subject invention, the α-amino protected monomer protected with the sulfonyl group will be contacted with the second amino acid monomeric unit in the presence of a coupling agent under conditions sufficient for a peptide bond to form between the α-carboxy group of the α-amino protected amino acid and the unprotected amino group (*i.e.* primary amine) of the second amino acid monomeric unit. A number of coupling agents are known in the art and may be employed, where such agents include: carbodiimide reagents, such as DCCI, PriN=C=NPri, EtN=C=N$(CH_2)_3NMe_2$,HCl; isoxazolium reagents, such as those of Woodward and Kemp; acyloxyphosphonium reagents, such as BOP, PyBOP; acyloxyuronium reagents, e.g. TBTU, HBTU, HATU; acid fluorides; and the like. Coupling is carried out the presence of an appropriate solvent which does not adversely affect the coupling reaction, where suitable solvents include inert solvents such as blocked amides, *e.g.* DMF, DMA, NMP, sulfoxides, *e.g.* DMSO; methylene chloride (especially for acid chlorides); and the like. Other components which may be present in the coupling reaction mixture include: HOBt, HOAt, and the like.

Following coupling of the α-amino protected amino acid to the second amino acid monomeric unit, the resultant peptide will be separated from the remaining components of the coupling reaction mixture, where particular components from which the resultant peptide will be separated include reaction byproducts, unreacted amino acid monomers and other reagents, and the like. Separation may be accomplished using any convenient protocol, including one or more washings, and the like.

Following coupling and usually also following washing, at least the sulfonyl N-terminal amino protecting group of the resultant peptide will be removed to yield an unprotected N-teminal amino group or primary amine moiety at the N-terminal residue of the resultant peptide. The sulfonyl protecting group can be removed with thiol nucleophiles, such as thiophenol, mercaptobenzothiazole, 2-mercaptoethanol (particularly where the AA is N-alkylated), thiocresols, and the like. Deprotection will typically take place by contacting the peptide with the thiol nucleophile in the presence of base, e.g. DBU, piperidine, Et_3N, DIEM, BEMP, K_2CO_3, MTBD, and the like, where bases with a pKa >9, particularly ranging from 11-12, are preferred, in a suitable solvent, where suitable solvents include those described *supra* suitable for use in the coupling step, and the like, where DMF is preferred.

Where desired, the above steps may be reiterated or repeated one or more additional times, where a new α- amino protected amino acid is contacted with the peptide, where the now unprotected N-terminal amino group (*i.e.* primary amine) of the peptide of the just added residue of the peptide is now the second amino acid monomeric unit. The α-amino protected amino acid may be similarly protected or protected with another protecting group, such as FMOC or BOC (when its the final residue), where a variety of alternative protecting groups are known to those of skill in the art. Preferably, the protecting group will be o-NBS or FMOC. The above synthesis steps of coupling, washing and deprotecting may be reiterated one or more additional times to produce a peptide of desired length and residue sequence specificity.

At one or more steps in the overall peptide synthesis process, N-subtituent groups may be selectively introduced into the growing peptide chain at the α-amino moiety by the site specific modification process according to the subject invention and as described in U.S. application serial no. 08/761,023, the disclosure of which is herein incorporated by reference. Where this methodology is employed to introduce N-modifcation, the following summarized steps will be performed prior to removal of the sulfonyl protecting group from the α- amino group of the N-terminal amino acid monomeric unit, as the sulfonyl group serves as the activating group as described in greater detail below.

As mentioned above, the subject invention also provides a method for site-selective addition reactions of primary amines that are present on compounds,

polymers, or mixtures and libraries thereof, in which there are groups or atoms that could be susceptible to the reactions that modify the target primary amino group. A compound, *e.g.* a polymer such as a polyamide, having a free amine is treated with an activating agent that reacts with the nitrogen of the amine to create a reactive amide. The resulting amide is selectively deprotonated with a strong base, and then modified by the addition of a substituent group. The activating group is then cleaved off to leave the substituted, secondary amine.

The method is compatible with conventional solid-phase peptide synthesis, including those that utilize Fmoc protecting groups. The procedure does not cause detectable racemization of polypeptides. The method provides a powerful tool for site-specific N-modification of polymers, particularly peptides and peptide analogs synthesized on a solid support, using commercially available chemicals. This method can easily be automated, and provides a general procedure for N-alpha-modification of bioactive peptides. The method can be used in a number of combinatorial strategies, for producing large libraries of N-modified peptides and other amines.

Site-selective addition reactions of substrate compounds having primary amines are achieved by the subject methods, using substrate compounds as described above. For brevity, the reactions will be primarily described in terms of modifications of polypeptides. It should be understood, however, that the subject methods are not limited to such uses. The reaction steps may be performed in solution, or with the substrate compound coupled to a solid support resin. In general, solid phase reactions are preferred for their convenience, but the use of a support is not necessary for the reaction chemistry.

The N-alpha modification of polypeptides is most conveniently combined with conventional SPPS, where amino acids are added in a step-wise manner while the chain is anchored on a solid phase resin. A benefit of the subject invention is the compatibility with Fmoc and Boc chemistry, allowing commonly available methods and reagents to be used. The modification reaction is performed at the point in the synthesis when the desired target residue has been added to the nascent chain and has been deprotected, leaving a free primary amine. After the nitrogen has been modified, the peptide synthesis is resumed. In order to achieve high yields, the coupling reaction

to the modified, secondary, amine may be repeated, and/or performed with a strong coupling agent.

The substrate compound is treated with an activating agent that reacts with the nitrogen of the amine to create a reactive amide. For reactions involving polypeptides, aryl sulfonyl halides are found to be useful, including *o*- and *p*-nitrobenzene sulfonyl chloride. The polypeptide is treated with an excess of activating agent, usually from about 1 to 5 molar equivalents. The choice of solvent is not critical, for example, methylene chloride, toluene, *etc.* may be used. The reaction is allowed to go to completion, usually from about 10 minutes to 24 hours, more usually from about 0.5 to 2 hours when performed at room temperature.

The resulting amide is selectively deprotonated by addition of a molar excess of strong base, conveniently a non-ionic base, in combination with a modifying agent. Any convenient solvent that is compatible with the base and modifying agent may be used, for example DMA, DMF, *etc.*

A number of modifying agents can be used at this point in the reaction. Examples include alkylating agents such as methyl nosylate, ethyl iodide, dimethyl sulfate, *etc.* Allyl groups may be introduced through the use of allyl carbonates, cinnamyl carbonates, vinyl epoxides, e.g. butadiene monoxide and analogs, *etc.* activated with palladium (0), where the allyls may be substituted with various hetero groups of interest.

After washing, the modified polypeptide is treated to cleave the activating group. Typically a large excess of the reagent is added, at least about 2-3 molar excess, and may be as much as a ten-fold molar excess. For nucleophilic aromatic substitutions, any nucleophile that has eletronic properties similar to $R\text{-}S^-$ may be used, *i.e.* soft nucleophiles. Thiophenol and 2-mercaptoethanol have been used successfully with DMF as a solvent.

On completion of the modification step, polypeptide synthesis may be resumed. As previously noted, double coupling may be desirable.

A number of schemes for generating combinatorial libraries have been described in the scientific and patent literature. U.S. patents include U.S. 5,573,905 (Lerner *et al.*); 5,565,324 (Still *et al.*); 5,556,762 (Pinilla *et al.*); and 5,545,568 (Ellman), herein incorporated by reference. The modification methods of the subject

invention can readily be included in such prior art combinatorial syntheses, because of the compatibility with conventional solid phase synthesis reactions.

The individual modified compounds and peptides of the invention are useful in a variety of ways similar to that of unsubstituted peptides and oligomers. For example, they can have one or more properties in binding to various moieties, including proteins, such as enzymes, receptors, antibodies and the like, nucleic acids, carbohydrates, lipids, they can react with enzymes to form products or have other properties such as antigenic compounds for vaccines or diagnostic reagents, including as probes. Compounds can also be used as enzyme inhibitors and in connection with affinity chromatography.

The chemical synthesis methodology of the present invention has utility with respect to producing a wide range of modified compounds and in particular modified peptides. The invention is particularly useful with respect to the modification of a mixture or library of compounds. For example, the synthesis methodology can be simultaneously applied to a library of peptides in a manner so as to modify different peptides in the library and thereby greatly increase the number of different compounds and the diversity of compounds contained with in the library. The resulting library is then useful in carrying out assay methodology whereby the modified library is screened against another library compound or receptor.

As an example of the utility of the claimed invention, it is possible to create a mixture of peptides containing thousands, tens of thousands, millions and even tens of millions of different peptides. Within the peptide library one can include a large number of different amino acid sequences which sequences correspond to variants of a naturally occurring peptide, protein or portion thereof which bind to a naturally occurring receptor. The library of peptides is then modified using the methodology of the present invention. After modification the library is screened against the same receptor. A series of screening tests can be carried out where the conditions such as concentration or binding affinity are changed so as to find compounds which are particularly selective for and/or bind to the receptor with a relatively high degree of affinity as compared to the naturally occurring peptide or protein. In view of such, the invention includes libraries of compounds which compounds are produced by first providing a diverse library of compounds (e.g., peptides) and then subjecting those

compounds to the site-specific modification methodology of the present invention. Preferably, the library will include at least one biologically active compound.

Turning back to the subject method of synthesizing peptides with the sulfonyl protecting groups, where the sulfonyl group serves as the activating group, to introduce a substituent onto the α-amino moiety, the first step is to deprotonate the α-amino moiety. Deprotonation is accomplished by producing a reaction mixture comprising the peptide to be modified and a molar excess of a strong base, conveniently a non-ionic base having a pKa in the range of about 12 to 15, in combination with a modifying agent in a suitable solvent. Strong bases that find use include: hindered guanidinium bases, MTBD, DBU, DBN, PMG, TMG, low equivalents of BEMP, TBAF, TBD and derivatives, and the like. The modifying agent used to introduce an N-substituent is an electrophilic agent that selectively modifies the deprotonated, sulfonyl group "activated" amide to add the substituent group of interest but will not be strong enough to modify the amide backbone of a polypeptide. Exemplary modifying agents that find use include: alkylating agents such as alkyl nosylates, *e.g.* methyl nosylate; alkyl halides, *e.g.* ethyl iodide; allyl bromide; benzyl bromide; propyl iodide; alkyl sulfates, *e.g.* dimethyl sulfate; Pd-π allyl species; Mitsunobu reactions (Mitsunobu (1981) Synthesis:1), particularly intra-molecular, *e.g.* cyclization reactions; and other agents known in the art. Substituents that may be selectively introduced onto the sulfonyl protected N-terminal amino group by this method include: alkyl, including linear and branched of from 1 to n carbon atoms, where the alkyl group may be substituted with hetero groups, such as nitro-, oxy-, hydroxy-, thio-, carbonyl-, halo-, amino- groups, etc., where when substituted, the substituent is preferably not located on the α-carbon of the alkyl group, where methyl groups are preferred alkyl groups in many embodiments of the invention; alkoxy; aryl, such as benyzl and substituted benzyl groups, *e.g.* heterosubstituted benzyl; allyl (*i.e.* hydrocarbons having one or more sites of unsaturation carbon carbon bonds, where allyls be straight or branched, substituted with one or more hetero groups; alkylthio; haloalkyl; haloalkoxy; halothio; and the like. Solvents in which the selective N-substitution reaction may be carried out include those solvents listed above as suitable for use in the coupling reaction, where DMF is preferred for alkylation and THF is preferred for Pd-π-allyl species. The above selective N-modification reaction will

typically be conducted at about ambient temperature of 20°C and pressure of one atmosphere. Following selective N-modification as described above, the sulfonyl group can be removed as described above.

Where the above steps are employed to introduce selective α-amino N-substitution at a non-N-terminal residue of the final peptide to be produced, in the next coupling step, use of an sulfonyl protected amino acid chloride, as described above, is preferred for groups larger than methyl, e.g. allyl, while for methyl, HATU & acid fluorides are generally employed.

The final step of the subject methods is to deprotect the protected reactive side chains of the residues of the peptide. In the preferred solid phase peptide synthesis method of the subject invention, the final step of the subject methods further comprises cleavage of the peptide from the polymeric support material. This final deprotection step is typically accomplished by acid treatment by methods known to those of skill in the art, which methods include: HF treatment, TFA treatment, TMSOTf, TMSBr and the like, where TFA treatment is preferred.

All of the above reactions can be carried out over a wide range of temperatures of from about -78 °C to about 150 °C, where the temperature at which the reactions are carried out will depend, at least in part, on the solvent used. Depending on the temperature, the time of the reaction can vary between about 5 minutes to about 24 hours.

In the preferred solid phase synthesis embodiment of the subject invention, one or more of the above discussed steps may be automated. A variety of automated peptide synthesis devices are known, commercially available and suitable for use in carrying out the subject methods, where such devices include those devices described U.S. Patent Nos.: 5,614,608; 5,612,002; 5,593,642; 5,582,801; 5,567,391; 5,565,552; 5,565,173; 5,347,979; 5,362,447; 5,240,680; 5,186,898; 4,816,513; and 4,668,476, the disclosures of which are herein incorporated by reference.

Kits are also provided for carrying out the above described methods. The kits according to the subject invention will at least comprise one or more o-NBS protected amino acids, where the kits may further comprise additional reagents suitable for use in the subject methods, such as coupling reagents, solvents, polymeric support materials and the like. The kits will further comprise instructions for carrying out the

above methods with the reagent components of the kit, where the instructions may be present on an enclosed package insert and/or association with the packaging of and/or any containers present in the kit.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to insure accuracy with respect to the numbers used (*e.g.* amounts, temperature, concentrations, *etc.*) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

A. *o*NBS Protected Amino Acid Synthesis

The synthesis of *o*NBS-amino acids was carried out using the standard Schotten-Baumann procedure. Briefly, the amino acid was dissolved in a mixture of dioxane and 1M sodium hydroxide and cooled in an ice bath. *o*-Nitrobenzenesulfonyl chloride in dioxane was added portionwise with concomitant addition of 1M NaOH. After 2h, the reaction mixture was worked up in the usual manner.

B. Peptide Synthesis with *o*NBS-Amino Acids

Peptide synthesis with *o*NBS-AAs was performed in analogy to routine Fmoc-SPPS; the *o*NBS-amino acid was activated for coupling with HBTU in 0.4M N-methyl morpholine/DMF, and coupled to Rink Amide MBHA resin for 20min. After washing the resin with DMF, the *o*NBS amino-protecting group was removed with a 5%

solution of thiophenol in DMF containing an equivalent amount of base. The best results have been obtained with potassium carbonate, which allows virtually complete deprotection in less than 10 minutes. It is essential that the deprotection step be performed under inert atmosphere (e.g., nitrogen or argon) to prevent extensive oxidation of the thiol. The deprotection was conveniently followed by the release of a yellow *o*-nitrobenzyl chromophore. Following deprotection, the resin was again washed with DMF and coupling of the next amino acid was performed.

C. Synthesis of the Peptide SFLLRN

In order to demonstrate the viability of this approach, the thrombin receptor activating peptide SFLLRN was synthesized according the above protocol on an automated peptide synthesizer using the following *o*NBS-protected amino acids: oNBS-Ser(OtBu)-OH, oNBS-Phe-OH, oNBS-Leu-OH, oNBS-Arg(Pmc)-OH, and oNBS-Asn(Trt)-OH. Analysis of the synthesized peptide by mass spectrometry and HPLC demonstrated that it was identical to SFLLRN synthesized by Fmoc-SPPS, and was obtained in 85% purity.

D. Selective Alkylation of the Terminal Nitrogen

One great advantage of oNBS-protected amino acids over Fmoc-amino acids is the ability to selectively alkylate the terminal nitrogen. For example, allylation of oNBS-NH-LLRN on Rink Amide MBHA resin was performed with 10 eq allyl methyl carbonate, 10 mol% tris(dibenzylideneacetone)dipalladium(0)-chloroform adduct, 80mol% triphenylphosphine, and 50 eq water in THF for 2 h at rt, to selectively yield the desired allylated peptide in 98% purity by RP-HPLC. Deprotection of the oNBS group was carried out with 10 eq BME/DBU in DMF for 30 min.

Subsequent coupling of Fmoc-amino acids to the highly hindered N-allyl leucine residue has proven problematic, however. The peptide coupling reagent HATU, which is suitable for coupling to N-methyl amino acids, gave ~5% coupling.

Acid fluorides, PyBroP, symmetric anhydrides, and other methods proved similarly disappointing.

Fmoc-amino acid chlorides are stable and highly reactive, but tend to be of limited use for solid-phase synthesis due to competing cyclization to the oxazolone. Like Fmoc-amino acids, oNBS-amino acids can be converted to stable amino acid chlorides. However, oNBS-amino acid chlorides cannot form oxazolones, although they will decompose in the presence of base. The relative rates of these processes should determine their usefulness as acylating agents in SPPS. Fmoc-Phe-OH and oNBS-Phe-OH were converted quantitatively to their respective acid chlorides by refluxing in 2M thionyl chloride/dichloromethane for 2h. Coupling to the N-allyl peptide in dichloromethane with collidine as base gave 16.5% coupling of Fmoc-Phe-Cl versus 68% for oNBS-Phe-Cl. Thus, oNBS-amino acid chlorides are at present the best reagents for coupling to such hindered amino acids on solid support.

E. N-Methylation of Peptides

N-alkylation was initially attempted via a three-step procedure involving trifluoroacetylation, selective alkylation via the Mitsunobu reaction (as described in Mitsunobu (1981) Synthesis **1**; Hughes (1992) Org. Reactions **42**:335-656), and deprotection to afford the N-alkyl amine. No reaction was achieved using Mitsunobu conditions on solid support, nor from "enhanced" Mitsunobu conditions (described in Tsunoda *et al.* (1995) Tetrahedron Lett. **36**:2529-2530). N-tosyl and N-mesyl peptides also failed to alkylate under these conditions.

The treatment of N-tosyl-LLONSF on Tentagel resin with alkylating agents and base was subsequently investigated. Methylation of the sulfonamide in DMF with dimethyl sulfate in the presence of the hindered, non-ionic guanidium base MTBD, was selective and complete in less than two hours. The use of MTBD as base was important for achieving high yields, as weaker bases gave poor or no yield of product, and stronger bases were not selective.

Attempts to alkylate N-trifluoroacetyl-LLONSF using 10 eq Me_2SO_4 and MTBD gave only 50% product after 24 h. This result prompted exploration for a sulfonamide activating group that could be selectively removed after alkylation. The

cleavable alkyl sulfonyl chlorides that were synthesized formed sulfonamides with amines in solution, but failed to yield the sulfonamide when added to the resin bound peptide free amine in methylene chloride.

The failure of alkyl sulfonyl chlorides to couple to the resin led to the the use of aryl sulfonyl chlorides, which cannot form sulfenes. Initially the deprotection of aryl sulfonamides using photochemical or free-radical mechanisms was contemplated. Instead, the methods described by Fukuyama *et al.* (1995) Tetrahedron Lett. **36**:6373-6374 were used to cleave nitrobenzenesulfonamides via nucleophilic aromatic substitution with thiophenol.

Both *o*- and *p*-nitrobenzenesulfonyl (oNBS and pNBS) peptides were readily methylated using the conditions described above, but the peptidyl 2,4-dinitrobenzenesulfonamide was not fully methylated even after 48 h. The Mitsunobu reaction failed with all three sulfonamides. Attempted cleavage of oNBS and pNBS with hydrazine and piperidine failed, but was effected with thiophenol and DBU in DMF. The oNBS group cleaved more readily than pNBS, and was used for all subsequent reactions. β-mercaptoethanol was substituted for thiophenol, as it cleaved the sulfonamides faster and is less toxic, less noxious, and more commonly found. Finally, methyl nosylate, a commercially available crystalline solid, was substituted for dimethyl sulfate on the basis of handling and toxicity considerations.

The procedure employs the protection of the resin-bound peptide free amine as the o-nitrobenzenesulfonamide by treatment with the corresponding sulfonyl chloride in CH_2Cl_2 containing collidine for two hours. The sulfonamide is then selectively deprotonated with strong, non-ionic base MTBD and alkylated with methyl nosylate in DMF for 30 minutes. Finally, the oNBS group is removed quantitatively with β-mercaptoethanol and DBU in DMF for 30 minutes. The deprotection is easily followed via the formation of a bright yellow color, presumably due to the release of o-nitrobenzenethioethanol. This three step procedure did not cause any detectable racemization.

The N-methyl scanning of SFLLRN-NH_2 was performed on 0.1 mmol scale using Rink Amide MBHA resin. The yields are shown in Table II. Standard Fmoc solid-phase peptide synthesis was carried out to the position where N-methylation was desired, the Fmoc group was then removed and the resin bound peptide was treated.

The next amino acid residue was introduced using double coupling with HATU (see Carpino *et al.* (1993) J. Am. Chem. Soc. **115**:4397-4398), and standard Fmoc SPPS was continued.

Table II

Crude and Purified Peptide Amide Yields

Peptide Amide	Crude yield[a]	Purified Yield[b]
(N-Me)SFLLRN	102.4 mg (85.5%)	66.5% of theory
S(N-Me)FLLRN	79.5 mg (63.6%)	32.7%[c]
SF(N-Me)LLRN	95.4 mg (83.7%)	64.6%
SFL(N-Me)LRN	93.8 mg (88.7%)	58.7%
SFLL(N-Me)RN	102.8 mg (88.7%)	63.7%
SFLLR(N-Me)N	84 mg (89.1%)	48.0%

(a) crude purity in parentheses assessed by RP-HPLC at 215 nm (10-50% CH3CN in H_2O containing 0.1% TFA). (b) Purified by preparative RP-HPLC (same solvent system) to >99% by HPLC (215 nm). Yield based on manufacturer's stated resin substitution. (c) Only 91% pure. The lower yield of the (N-Me)Phe peptide was primarily due to poor coupling of the final serine residue. Purification was difficult due to a closely-eluting impurity arising from this coupling step.

The introduction of the oNBS group was quantitative, and the methylation was >95% complete in all cases, however some side-product was formed in the arginine-containing peptide fragments from modification of the Arg(Pmc) residue. This posed no problem for the N-methyl scanning of SFLLRN, as the small amount of side product is easily separated by reverse-phase HPLC. Its formation could also be eliminated entirely by the substitution of arginine with an orthagonally-protected ornithine, which can be guanylated at the completion of the synthesis. Removal of the oNBS group by treatment with β-mercaptoethanol and DBU in DMF proceeded rapidly and quantitatively. The most difficult aspect of the synthesis was the expected difficulty in subsequent coupling of the N-methyl peptides.

The six N-methylated peptides synthesized have identical masses, differing only in the location of the N-methyl group. However, high energy collision-induced dissociation (CID) analysis with tandem mass spectrometer and matrix- assisted laser desorption ionization post-source decay (MALDI-PSD) mass spectometry both allowed unequivocal assignment of the site of methylation in each peptide. The N-

methylated residue is readily identified by its often prominent N-methyl immonium ion peak. Analysis of the peptide fragments was used to corroborate the assignment and to confirm its location in the peptide sequence.

Detailed Materials and Methods

Fmoc-amino acids and HBTU were purchased from Advanced Chemtech. Rink Amide MBHA resin was purchased from Novabiochem. Collidine, 2-nitrobenzenesulfonyl chloride, methyl 4-nitrobenzenesulfonate, 1,3,4,6,7,8-hexahydro-1-methyl-2H-pyrimido[1,2a]pyrimidine, piperidine, ethanedithiol, thioanisole, TFA and HATU were purchased from Aldrich. DMF and NMM were purchased from Fisher. β-mercaptoethanol was purchased from SIgma.

Peptide synthesis was conducted on a Protein Technologies PS3 peptide synthesizer on 0.1 mmol scale using standard Fmoc SPPS. The following side chain protected amino acids were used: Arg(Pmc), Asn(Trt), Orn(Boc), Ser(OtBu). Each amino acid was coupled sequentially to the Rink Amide MBHA resin (0.55 mmol/g substitution) in four-fold excess using 3.8 equivalents of HBTU in 0.4 M NMM/DMF for 20 minutes. Fmoc deprotection was accomplished with 20% piperidine in DMF (2 x 5 min). When the desired site of methylation was reached, the Fmoc-deprotected peptide resin was removed from the synthesizer, washed with CH_2Cl_2 and treated with five equivalents of collidine and three equivalents of 2-nitrobenzenesulfonyl chloride in CH_2Cl_2 for 2 h at room temp. The resin was then washed with and resuspended in DMF and treated with 2-4 equivalents of MTBD followed by 3-5 equivalents of methyl 4-nitrobenzenesulfonate for 30 min at room temp. After washing, the resin was again suspended in DMF and treated with 5 eq DBU and 10 eq 2-mercaptoethanol for 30 min at room temp. A bright yellow solution was indicative of oNBS cleavage. Following cleavage, the resin was extensively washed with DMF, and returned to the synthesizer. The next amino acid in the peptide sequence was double coupled to the resin using HATU in place of HBTU (2 x 20 min couplings). The remaining amino acids in the peptide were coupled with HBTU, as described. At each step in the N-methylation sequence, a small sample of the resin was removed and cleaved for HPLC and MALDI analysis.

The peptides were cleaved from the resin using a cocktail consisting of 90% TFA, 5% H_2O, 2.5% ethanedithiol, and 2.5% thioanisole for 90 minutes, followed by filtering and washing of the resin with TFA. The volume of the TFA solution was reduced by rotary evaporation, and the crude peptide precipitated with ethyl ether. The crude peptide was sedimented and the ether discarded. This was repeated 2-3 times. The crude was dried *in vacuo*.

The crude peptide was dissolved in 0.1% aqueous TFA and purified on a Dynamax C_{18} preparative HPLC column using a linear gradient of 10-50% acetonitrile in water containing 0.1% TFA. Fractions containing only the desired peptide were pooled and lyophilized, yielding 32-66 mg of purified peptide, >99% pure in all cases except for S(N-Me)FLLRN, which was approximately 91% pure.

Peptide structures were verified using collision induced dissociation (CID) tandem mass spectrometry. The LSIMS molecular ion peak for all six peptides was 762.4. Immonium ion peaks for all amino acids in each peptide were present, and the site of N-methylation evident from the +14 increase in mass. MALDI-PSD fragmentation occurred at the peptide bond, making sequence analysis straightforward.

The above results demonstrate a powerful new tool for site-specific N-methylation of peptides on solid support using commercially available chemicals. This method can easily be automated, and provides a general procedure for N-methyl scanning of bioactive peptides. It also opens the doors to other modifications of the amide backbone, such as N-allylation using Pd(0) chemistry and cyclization of peptides, and for combinatorial strategies for producing large libraries of N-modified peptides.

It is further evident from the above results and discussion that the subject methods have several advantages over previous peptide synthesis methods, particularly solid phase peptide synthesis methods. The subject methods allow for backbone modifications and are better suited for use in difficult reactions, such as coupling of an amino acid to an N-substituted amino group. Furthermore, the o-NBS groups employed in the subject methods are less expensive than other protecting groups such as FMOC. The subject methods are compatible with other methods of peptide synthesis, such as FMOC synthesis, providing for the possibility of using already available FMOC automated peptide synthesis devices and/or using both

FMOC and o-NBS protected amino acids together. Furthermore, removal of the o-NBS group during the deprotection step yields a yellow chromophore (an o-nitrobenzene derivative) providing for the possibility of quantitatively monitoring the reaction, *e.g.* with UV/VIS spectroscopy.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of synthesizing a peptide, said method comprising:

contacting an sulfonyl group α-amino protected first amino acid with a second amino acid monomeric unit under coupling conditions;

whereby said peptide is synthesized.

2. The method according to Claim 1, wherein said contacting step is repeated at least one additional time with an additional α-amino protected amino acid.

3. The method according to Claims 1 or 2, wherein said second amino acid monomeric unit is stably associated with the surface of a polymeric support material.

4. In a method of chemical peptide synthesis, the improvement comprising:

using a sulfonyl comprising group as an α-amino protecting group.

5. The method according to any of the preceding claims, wherein said sulfonyl group is a nitrobenzene sulfonyl group.

6. The method according to any of the previous claims, wherein said nitrobenzene sulfonyl group is o-NBS.

7. A method of modifying a substrate compound comprising a target primary amine to generate a secondary amine, wherein said substrate compound comprises groups susceptible to reactions modifying said target primary amine, the method comprising:

treating said substrate compound with an activating agent that, on reaction with said primary amine, results in an activated amide;

deprotonating said activated amide with base and selectively modifying the resulting deprotonated amide with a modifying agent to provide a substituted amide;

cleaving the activating group from said substituted amide to provide a substituted, secondary amine.

8. A method according to Claim 7, wherein said substrate compound is attached to a solid support.

9. A method according to Claims 7 or 8, wherein said substrate compound is a polyamide.

10. A method according to Claims 7, 8 or 9, wherein said activating agent is an aryl sulfonyl halide substituted with an electron withdrawing group at the ortho or para position.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/22184

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : A61K 38/00, C07K 5/00
US CL : 530/334
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/334

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Advance Organic Chemistry, Jerry March, 4th edition, John Wiley and Sons 1992

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog, APS
Sulfonyl, tosyl, peptide, amino acid, synthesis, synthesize, amine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	MILLER, S. C. Site selective N-methylation of Peptides on Solid Support. Journal of the American Chemical Society. 05 March 1997, Vol. 119, No. 2, pages 2301-2302, see entire document.	1-10
X	FUKUYAMA, T. et al. 2- and 4-Nitrobenzenesulfonamides: Exceptionally Versatile Means for Preparation of Secondary Amines: And Protection of Amines. Tetrahedron Letters. 04 September 1995, Vol. 36, No. 36, pages 6373-6374, see entire document.	7-10
X	ZWIERZAK, A. et al. Phase-Transfer Catalyzed Alkylation of Diphenylphosphinic Amide-A new Versatile Synthesis of Primary and Secondary Amines. Angew. Chem. Int. Ed. Engl. 1977, Vol. 16, No. 10 , pages 702-704, see entire document.	7-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/22184

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	BOWMAN, W. R. et al. A Facile Method for the N-Alkylation of α-Amino Esters. Tetrahedron. 1997, Vol. 53, No. 46, pages 15787-16798, see entire document.	7-10
X	MARCH, J. Reactions, Mechanisms, and Structure. In: Advanced Organic Chemistry, 4th edition. New York: Published by, John Wiley and Sons. 1992, pages 425-427 especially page 427.	7-10
Y	US 4,219,497 A (PLATTNER et al.) 26 August 1980, column 5, lines 28-34.	1-6
Y	MERRIFIELD, R. B. Solid-Phase Peptide Synthesis. Advances in Enzymology: And Related Areas of Molecular Biology. 1969, Vol. 32, pages 221-296, especially pages 221-225 (general method) and 240-243 (amino group protection).	1-6
Y	GREENE, T. W. N-sulfonamide Derivatives. In: Protective Groups in Organic Synthesis. New York: Published by, Wiley-Interscience Publication. 1981 see pages 284-287, see entire document.	1-6