PATENT REQUEST: STANDARD PATENT/PATENT OF ADDITION

We, being the person(s) identified below as the Applicant, request the grant of a patent to the person identified below as the Nominated Person, for an invention described in the accompanying standard complete specification.

Full application details follow.

[71] Applicant: SYNTAX (U.S.A.) INC.
Address: 3401 HILLVIEW AVENUE
PALO ALTO, CA 94303
USA

[70] Nominated Person: SYNTAX (U.S.A.) INC.
Address: 3401 HILLVIEW AVENUE
PALO ALTO, CA 94303
USA

[54] Invention Title: "TEMPORARY MINIMAL PROTECTION SYNTHESIS OF LH-RH ANALOGS"

[72] Names of actual inventors: John J Nestor, Jr., Natalie L McClure and Humberto Arzeno

[74] Address for service in Australia: c/o WATERMARK PATENT & TRADEMARK ATTORNEYS, of The Atrium, 290 Burwood Road, Hawthorn, Victoria 3122, Australia Attorney Code: WM

BASIC CONVENTION APPLICATION(S) DETAILS

<table>
<thead>
<tr>
<th>Application Number</th>
<th>Country</th>
<th>Country Code</th>
<th>Date of Application</th>
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<tr>
<td>482,428</td>
<td>USA</td>
<td>US</td>
<td>20 February, 1990</td>
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By our Patent Attorneys,

WATERMARK PATENT & TRADEMARK ATTORNEYS

Jan A. Scott
Registered Patent Attorney

23 June 1992
NOTICE OF ENTITLEMENT
(To be filed before acceptance)

We, SYNTEX (U.S.A.) INC.
of: 3401 HILLVIEW AVENUE
PALO ALTO CA 94303
USA

being the applicant in respect of Application No. 71176/91 state the following:-

We have entitlement from the actual inventors and the Applicants of the basic application
listed on the patent request form as we would, on the grant of a patent for the invention to the
inventors, be entitled to have the patent assigned to us.

The basic application listed on the request form:

is the first application made in a Convention country in respect of the invention

By our Patent Attorneys,
WATERMARK PATENT & TRADEMARK ATTORNEYS

[Signature]

Ian A. Scott
Registered Patent Attorney

23 June 1992
1. In a process for the solid phase synthesis of an LH-RH analog containing at least one serine residue, the improvement comprising temporarily protecting only the side chain of the serine residue with a protecting group which is labile to those agents useful for removing α-amino protecting groups.

5. In a process for the solid-phase synthesis of a compound having an amino acid sequence of the formula R¹-R²-R³-Ser-Tyr-R⁴-Leu-R⁵-Pro-R⁶ (I)

wherein

R¹ is selected from (pyro)Glu and N-Ac-D-Nal(2);
R² is selected from His, D-p-Cl-Phe and D-p-F-Phe;
R³ is selected from Trp, D-Trp, D-Nal(2) and D-Pal(3);
R⁴ is selected from D-Nal(2), D-hArg(Et)₂, D-hArg(Bu), D-hArg(CH₂CF₃)₂, D-His(Bzl), D-Leu, D-Pal(3), D-Ser(tBu) and D-Trp;
R⁵ is selected from Arg, L-hArg(Et)₂, L-hArg(Bu), L-hArg(CH₂CF₃)₂ and Lys(iPr); and
R⁶ is selected from Gly-NH₂, NH-NHCONH₂, D-Ala-NH₂ and NHet;
wherein the amino acids are provided with Na protection;
the improvement comprising (a) temporarily protecting only the side
chain of serine at position 4 with a group labile to agents for removing the α-
amino protecting group;
and (b) protecting the side chain of histidine, if present, with a group
liable to an α-amino group deprotection agent, or to aminolysis or ammonolysis.

9. A process of Claim 1 or 5 in which the
deprotecting agent is selected from HCl/CH₂Cl₂,
TFA/CH₂Cl₂, and HCl/(C3-C6)alcohol/CH₂Cl₂.
The following statement is a full description of this invention, including the best method of performing it known to us.
TEMPORARY MINIMAL PROTECTION SYNTHESIS OF LH-RH ANALOGS

FIELD OF THE INVENTION

This invention relates to the solid phase synthesis of LH-RH analog by a minimal protection procedure.

BACKGROUND OF THE INVENTION

LH-RH analogs are nona- or decapetides which are structurally related to LH-RH and exhibit biological activity similar to that of LH-RH. The analogs are the subject of intensive clinical investigation due to their demonstrated ability to alleviate the symptoms of endometriosis, prostate cancer, precocious puberty, and other hormonally mediated disorders. While certain LH-RH analogs are currently available for therapeutic use, their synthesis is a complicated and, consequently, expensive procedure which necessarily increases the cost to those in need of treatment. LH-RH analogs are conventionally described as either agonists or antagonists, depending upon their mode of action.

The LH-RH analogs of interest in this invention are nona- and decapetides, and include both agonists and antagonists. Examples of LH-RH agonists useful in the subject invention are nafarelin, leuprorelin,
buserelin, goserelin, histerelin, triptorelin and deslorelin; these all differ from naturally occurring LH-RH by replacement of a glycine residue at the 6-position with a D-amino acid. The synthetic agonists then have, in common with the naturally occurring hormone, histidine at position 2, serine at position 4, and tyrosine at position 5, all of which have reactive side chains which may present synthetic difficulties.

The LH-RH antagonists differ from the naturally occurring LH-RH generally by the deletion or replacement of the histidyl residue at position 2. From the synthetic perspective, the deletion of histidine reduces the opportunities for undesired side reactions; however, the presence of both serine and tyrosine still requires that special steps be taken to avoid side chain reactions.

LH-RH analogs may be synthesized by various methods, such as are taught by J.M. Stewart and J.D. Young, *Solid Phase Peptide Synthesis*, W.H. Freeman Co., San Francisco, 1969; J. Meinenhofer, *Hormonal Proteins and Peptides*, Vol. 2, page 46, Academic Press (New York), 1973; and E. Schroder and K. Lubke, *The Peptides*, Vol. 1, Academic Press (New York), 1965. The methods may be broadly characterized as either solution phase or solid phase techniques. Both methods involve the sequential addition of amino acids to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid is protected by a suitable protecting group. The protected amino acid can then be either attached to an inert solid support or utilized in solution by adding the next protected amino acid in the
sequence under conditions suitable for forming the amide linkage. The protecting group is then removed from this newly added amino acid residue, and the next amino acid is then added and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining protecting groups, and any solid support, are removed to afford the final polypeptide. By simple modification of this general procedure, it is possible to add more than one amino acid at a time to a growing chain, for example, by coupling a protected tripeptide with a protected dipeptide to form a pentapeptide.

The more rigorous conditions of solid phase synthesis, however, generally require that any reactive side chains on the amino acids be protected during formation of the amide linkage. The side chain protecting groups are usually removed in a separate step after cleavage of the completed polypeptide from the inert support on which it is made, or concurrently therewith.

One particularly useful solid phase synthetic method for preparing LH-RH analogs is disclosed in Nestor et al., U.S. Patent No. 4,234,571, the disclosure of which is incorporated by reference herein. In this commonly used approach, the α-amino (N\(^\alpha\)) function of each amino acid is protected by an acid or base sensitive group, such as t-butyloxy carbonyl (Boc); any reactive side chains, as are present on serine, histidine and tyrosine, are also protected with strongly bound groups which require treatment with hydrogen fluoride (HF) or similarly drastic procedures for their removal. Also, the removal of the α-amino protecting
groups and of the side-chain protecting groups are commonly performed in separate steps.

This approach is adequate for the preparation of research quantities of peptides, but when large scale production of peptides is contemplated, these methods are not satisfactory. Amino acids with fully protected side chains are expensive, and these costs can be significant in a commercial-scale production of peptides. Also, the use of hydrogen fluoride, in addition to posing serious environmental hazards, contributes to commercially unacceptable yield losses. What is more, because a separate production step is required to remove the side chain protecting groups, this involves additional time and cost in the synthetic process.

Alternate protocols are no more appealing. Tien et al., Pept. Chem. 375 - 379, T. Shiba and S. Sakakibara (Ed.), Protein Research Foundation, Osaka (1988), have reported the synthesis of LH-RH using tosyl protection on histidine and benzyl protection on tyrosine and serine. This approach, while avoiding the use of hydrogen fluoride, still requires a separate dehydrogenation step to remove the benzyl protecting groups, with some reduction of tryptophan occurring.

D.H. Coy et al., Int. J. Peptide Protein Res., 14, 339 - 343 (1979) report the synthesis of the LH-RH antagonist, [D-Phe², D-Trp³, D-Phe⁶]-LH-RH using a variety of side chain protection protocols, all of which require HF deprotection: providing benzyl side chain protection of serine only, tosyl side chain protection of arginine only, both serine and arginine side chain protection, and serine, arginine and tyrosine (with
2-bromobenzyl-oxycarbonyl) side chain protection. Salt protection of arginine (as Arg HCl) was also used in the "serine only" synthesis. Hydrogen fluoride was used to cleave the crude peptide from its support and to remove the side chain protecting groups. Only when the peptide is "fully" unprotected (and only salt protection for arginine) did Coy avoid hydrogen fluoride treatment. All of the protected syntheses gave poorer yields than that of the unprotected side chain synthesis.

Coy et al. further reported the synthesis of the LH-RH agonist [D-Leu₆, desGly-NH₂¹⁰]-LH-RH ethylamide with dinitrophenyl side chain protection of histidine only, salt protection of arginine, and no HF treatment. The dinitrophenyl side chain protecting group was removed during cleavage of the peptide from its support with a solution of ethylamine in dimethylformamide. The yield from the histidine-only protected synthesis was only 34% versus 24% for a fully protected, HF cleavage synthesis. No comparison with an unprotected synthesis was made.

The art suggests that, of the various minimal protection strategies, histidine-only protection may provide some improvement in yield over fully protected syntheses for certain LH-RH antagonists; however, no particular benefit is associated with any of the reported side chain protection approaches for LH-RH agonists.

While the ideal approach for eliminating the HF deprotection step may be to conduct an unprotected synthesis, lack of protection for histidine leads to excessive racemization. Following Coy et al., however,
we have found that the use of histidine-only protection also results in high levels of a bis-serine impurity, due to the acylation of the serine residue. Significant improvement over the teachings of the art is needed in order to obtain a practicable minimal protection synthesis for LHRH analogs that does not require an HF deprotection step, yet provides protection for those groups which, if unprotected, will adversely affect the purity and yield of peptide.

**SUMMARY OF THE INVENTION**

It is an object of this invention to provide a process for the synthesis of LH-RH analogs in which the side chains of only an essential minimal number of amino acid residues are protected.

It is a further object of this invention to provide a process for the synthesis of LH-RH analogs which obviates the need for an HF deprotection step, and thus also in avoiding the use of the toxic HF reagent, decreasing the toxic waste stream often encountered in conventional processes.

The above noted aspects of the present invention offer the additional advantages of decreasing the costs of preparing the LHRH compounds, as well as avoiding an additional process step to remove the side chain protecting groups.
The objects of this invention are achieved for LH-RH analogs by a temporary minimal protection process in which only the hydroxy side chain of the amino acid residue serine is protected with a group which is removed immediately following the coupling of the serine to the peptide chain. The side chain protecting group is one which is labile under the same conditions useful for removing the \( \alpha \)-amino protecting group. For those LH-RH analogs which contain a histidine residue, the imidazole side chain may also be protected with a group labile during the coupling cycle, suitably, labile to an \( \alpha \)-amino group deprotecting agent, but optionally it may also be protected with a group removable by aminolysis or ammonolysis.

Temporary side chain protection of serine and side chain protection of histidine, if present, minimizes formation of impurities and maximizes yields without requiring an HF or alternative separate deprotection step.
DETAILED DESCRIPTION OF THE INVENTION

Description of the Process and LHRH Analogs

The temporary minimal protection process of this invention is expected to be applicable to the solid phase synthesis of any serine-containing polypeptide having a few to several dozen residues, regardless of the remainder of the sequence. LH-RH analogs, and other nona- and decapetides, are preferred synthetic targets. While the invention is described with reference to the sequential addition of individual amino acids, those skilled in the art will recognize that the process is equally applicable to syntheses in which blocks of smaller polypeptides are coupled to form a larger polypeptide, e.g., by adding a tetrapeptide to a pentapeptide, provided that the side chain of any serine residues are temporarily protected during the serine coupling cycle.

Temporary protection means that the serine side chain is protected for a relatively short period of the synthetic cycle. The side chain protecting group and the α-amino or carboxyl protecting group are removed simultaneously, after the serine coupling is effected. Generally, the critical criterion for selecting the serine side chain protecting group is that the group be stable to coupling conditions but labile to α-amino deprotecting conditions. In one aspect of this invention, employing α-amino protection, the serine side chain is preferably protected by a group selected from t-butyl, t-butyldimethylsilyl, trimethylsilyl, trityl, pivalyl, and tetrahydropyran-2-yl.
For those LH-RH analogs which have histidine residues it is generally desirable to protect the imidazole side chain. This protection may also be of the temporary variety, i.e. labile during the coupling cycle, or may remain in place until the peptide is removed from its support. Preferably, aminolysis or ammonolysis is used to cleave the resin from its support and simultaneously remove the histidine protecting group.

In another aspect of this invention, the deprotecting agent is selected from solutions of hydrogen chloride in C₃ to C₆ alcohols and dichloromethane. Preferably, the ratio of alcohol to dichloromethane is from 0.1 to 10.0 (v/v) and the acid concentration is 2N to 9N. Most preferably, the alcohol is i-propanol.

Abbreviations and Definitions

For purposes of this invention, the expression "LH-RH" refers to luteinizing hormone releasing hormone, and "LH-RH analogs" is meant to encompass LH-RH itself as well as other polypeptides that are structurally related to LH-RH or derived from it and that exhibit biological activity similar to that of LH-RH.

The abbreviations for the various common amino acids are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, *Biochemistry, 11*, 1726 (1972). All peptide sequences mentioned herein are written according to the generally accepted convention whereby the N-terminal amino acid is on the left and the C-terminal amino acid is on the right.
The abbreviations herein represent L-amino acids, with the exception of the achiral amino-acid glycine, and with the further exception of any unnatural amino acids which are achiral, or are otherwise designated as D- or D,L-. Et is ethyl, Bu is butyl, and iPr is iso-propyl.

Other abbreviations useful in describing the invention involve replacements of the amino acids in the natural LH-RH peptide by the following:

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Abbreviation</th>
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<tr>
<td>3-(2-naphthyl)-alanyl</td>
<td>Nal(2)</td>
</tr>
<tr>
<td>3-(p-fluorophenyl)-alanyl</td>
<td>p-F-Phe</td>
</tr>
<tr>
<td>3-(p-chlorophenyl)-alanyl</td>
<td>p-Cl-Phe</td>
</tr>
<tr>
<td>3-(3-pyridyl)-alanyl</td>
<td>Pal(3)</td>
</tr>
<tr>
<td>N(^{\text{a}}),N(^{\text{a}})'-bis(ethyl)-homoarginyl</td>
<td>hArg(Et)(^{2})</td>
</tr>
<tr>
<td>N(^{\text{a}}),N(^{\text{a}})'-bis(2,2,2-trifluoroethyl)-homoarginyl</td>
<td>hArg(CH(_2)CF(_3))(^{2})</td>
</tr>
<tr>
<td>N(^{\text{a}})-butyl-homoarginyl</td>
<td>hArg(Bu)</td>
</tr>
<tr>
<td>N(^{\text{e}})-Isopropyl-lysly (benzyl)-histidyl</td>
<td>Lys(iPr)</td>
</tr>
<tr>
<td>(benzyl)-histidyl</td>
<td>His(Bzl)</td>
</tr>
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As used herein, the term "pharmaceutically acceptable salts" refers to salts that retain the desired biological activity of the parent compound without toxicological side effects. Examples of such salts are acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the
like; and salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acids, naphthalenedisulfonic acids, polygalacturonic acid, and the like.

The abbreviation "N-Ac" refers specifically to the N-acetyl protecting group, i.e., an acetyl group attached to a terminal amino acid residue on the amine nitrogen, in conformance with generally accepted nomenclature.

**Preferred Embodiments**

In one embodiment of the invention there is provided an improved minimal protection process for the solid-phase synthesis of a compound having an amino acid sequence of the formula

\[
R^1-R^2-R^3-Ser-Tyr-R^4-Leu-R^5-Pro-R^6 \quad (I)
\]

wherein

- \(R^1\) is selected from (pyro)Glu and N-Ac-D-Nal(2);
- \(R^2\) is selected from His, D-p-Cl-Phe and D-p-F-Phe;
- \(R^3\) is selected from Trp, D-Trp, D-Nal(2) and D-Pal(3);
- \(R^4\) is selected from D-Nal(2), D-hArg(Et)_2, D-hArg(Bu), D-hArg(CH_2CF_3)_2, D-His(Bzl), D-Leu, D-Pal(3), D-Ser(tBu) and D-Trp;
R5 is selected from Arg, L-hArg(Et), L-hArg(Bu), L-hArg(CH₃CF₃)₂ and Lys(iPr); and
R6 is selected from Gly-NH₂, NH-NHCONH₂, D-Ala-NH₂ and NHEt;
wherein the amino acids are provided with N° protection; in which the improvement comprises (a) temporarily protecting the side chain of serine at position 4, suitably with a group labile to those agents useful for removing α-amino protecting groups without inducing racemization, side reactions, or cleavage of the growing peptide from its resin support and (b) protecting the side chain of histidine, if present, with a group labile to an α-amino group deprotection agent, or to aminolysis or ammonolysis.

In another embodiment, there is provided a temporary minimal protection process for the solid-phase synthesis of a compound of Formula (I), which comprises the following steps: (a) protecting the α-amino groups of the amino acids in the polypeptide, (b) protecting the side chain of serine with a group labile to those agents useful for removing the α-amino protecting group, (c) protecting the side chain of histidine, if present, with a group labile to a basic deprotection agents or a group removable by aminolysis or ammonolysis, (d) bonding the C-terminal amino acid to an inert solid support, (e) sequentially coupling, with a suitable coupling agent, one or more selected amino acids to each other in successive cycles, starting from the C-terminal end, (f) eliminating, at the end of each cycle, the protecting groups by treatment with a deprotecting agent, said deprotecting agent selected from those agents capable of removing both the α-amino protecting group and the side chain protecting group without inducing racemization, side reactions, or cleavage of the growing peptide from the resin, (f) repeating the coupling and eliminating steps as needed to form a nona- or decapeptide, (g) cleaving the polypeptide from the support by aminolysis.
or ammonolysis, and (h) isolating and purifying the resulting polypeptide.

In yet another embodiment, there is provided a process for the solid-phase synthesis of a compound of Formula (I) which comprises the steps of:

(a) coupling by solid phase synthesis appropriate Boc-protected and t-butyl protected serine in successive cycles and in the order from right to left of the amino acid sequence of the compound of Formula (I), starting with Boc-R6-O- covalently bound to an inert solid support, (b) eliminating, at the end of each cycle, the Boc-protecting group and simultaneously the t-butyl group from serine or D-serine by treatment with a deprotecting agent selected from HCl/CH₂Cl₂ and HCl/lower alkanol/CH₂Cl₂ to form a polypeptide bound to said solid support, (c) cleaving the polypeptide from the support by ammonolysis, and (d) isolating the resulting polypeptide.

In a preferred embodiment, there is provided a process as described above for the production of a polypeptide having the formula above wherein,

R¹ is (pyro)Glu or N-Ac-D-Nal(2);
R² is His or D-p-Cl-Phe;
R³ is Trp and D-Pal(3);
R⁴ is D-Nal(2), D-Leu, D-Trp, D-Ser(tBu),
R⁵ is D-His(Bzl) or D-hArg(Et)₂;
R⁶ is Gly-NH₂, NHEt or D-Ala-NH₂.

Most preferably, the invention provides a process for the production of the LH-RH antagonist of Formula (I), i.e., nafarelin, wherein,

R¹ is (pyro)Glu,
R² is His
R³ is Trp
R⁴ is D-Nal(2),
R⁵ is Arg, and
R⁶ is Gly-NH₂,
or for the LH-RH antagonist of Formula (I) wherein

\[ R^1 \text{ is } Ac-D-Nal(2); \]
\[ R^2 \text{ is } D-p-\text{Cl-Phe}; \]
\[ R^3 \text{ is } D-Pal(3); \]
\[ R^4 \text{ is } D-h\text{Arg(Et)}_2; \]
\[ R^5 \text{ is } L-h\text{Arg(Et)}_2; \] and
\[ R^6 \text{ is } D-\text{Ala-NH}_2. \]

In the preferred embodiment the \( \alpha \)-amino (N\(^\alpha \)) function of the amino acids is protected by an acid or base sensitive group. The protecting group is stable to the conditions of peptide bond formation, while being readily removable without destruction of the growing peptide chain or racemization of any of the chiral centers contained therein. Suitable protecting groups are t-butoxycarbonyl (Boc), biphenylisopropoxycarbonyl, t-amyloxy carbonyl, isobornyloxycarbonyl, \( \alpha,\alpha \)-dimethyl-3,5-dimethoxybenzyloxycarbonyl, \( \alpha \)-nitrophenylsulfenyl, 2-cyano-\( t \)-butyloxy carbonyl, 9-fluorenlymethoxycarbonyl (Fmoc) and the like. Preferably the \( \alpha \)-amino protecting group is t-butoxycarbonyl (Boc). When it is desired to prepare a peptide such as buserelin or goserelin, in which \( R^4 \) in Formula (I) above, is D-Ser(t-Bu), Fmoc is preferred for N\(^\alpha \) protection in the coupling cycles including and following the addition of the D-Ser(tBu). Fmoc is labile to basic agents (pH \( \geq 8.5 \)), such as piperidine, which will not remove tBu from the D-Ser(tBu). In later cycles, following the addition of the D-Ser(tBu), it is required to use base sensitive N\(^\alpha \) protection. The side chain of the serine at position 4 is protected with a group which may be removed with for example, a mild fluoride treatment. A preferred serine side chain protecting group is t-butyldimethylsilyl.

The hydroxy side chain of the serine residue is protected during the coupling of serine to the growing peptide, as described for the generic embodiment of this invention. The side chain protecting group is removed after the coupling is effected and prior to adding the next amino
acid. The serine side chain protecting group is removed with the same agent used to remove the N^α protecting group. Preferred side chain protecting groups for serine are t-butyl, t-butyldimethylsilyl, trimethylsilyl, trityl, pivalyl and tetrahydropyran-2-yl.

Further, the imidazole side chain of histidine, generally present in LH-RH agonists, is also protected. The histidine side chain protecting group may also be labile during the coupling cycle, for example, labile to an N^α group deprotecting agent, but, optionally, its removal may be completed when the peptide is cleaved from its support. Preferred side chain protecting groups for histidine are p-toluenesulfonyl and 2,4-dinitrophenyl.

To initiate the synthesis, the first amino acid, which will generally be the C-terminal amino acid in the final product, is attached to a suitable solid support. Suitable solid supports useful for the above synthesis are those materials which are inert to the reagents and reaction conditions of the stepwise condensation-deprotection reactions, as well as being insoluble in the media used. Examples of commercially available resins include styrene/divinylbenzene resins modified with a reactive group, e.g., chloromethylated styrene/divinylbenzene copolymer, hydroxymethylated styrene/divinylbenzene copolymer, and the like. Merrifield resin (1% crosslinked chloromethylated styrene/divinylbenzene copolymer) is preferred.

The attachment to the resin, for example, a chloromethylated styrene divinylbenzene resin is made by means of the reaction of the N^α protected C-terminal amino acid, especially the N^α-Boc amino acid, as its cesium, tetramethylammonium, triethylammonium, 1,5-diazabicyclo [5.4.0] undec-5-ene, or similar salt in ethanol, acetonitrile, N,N-dimethylformamide (DMF), and the like, especially the cesium salt in DMF, with the chloromethylated resin at an elevated temperature, for example between about 40° and 60°C., preferably about 50° C., for from about 12 to
72 hours, preferably about 48 hours.

The coupling of successive protected amino acids is carried out by methods well-known in the art, typically in an automated polypeptide synthesizer. Each protected amino acid is introduced in from about 1.5- to about 2.5-fold molar excess and the coupling is carried out in an inert, non-aqueous, polar solvent such as dichloromethane, DMF or mixtures thereof, preferably in dichloromethane at about ambient temperature. The coupling agent is selected from N,N'-dicyclohexylcarbodiimide (DCC), N,N'-di-iso-propyl-carbodiimide (DIC) or other carbodiimide either alone or in the presence of 1-hydroxybenzotriazole (HBt), O-acyl ureas, benzotriazole-∆-yl- oxy-tris(pyrrolidino phosphonium) hexafluorophosphate (PyBop), N-hydroxysuccinimide, other N-hydroxyimides or oximes. Alternately, protected amino acid active esters (e.g., p-nitrophenyl, pentafluorophenyl and the like) or symmetrical anhydrides may be used.


If the completion test(s) suggest that the reaction is not complete, the coupling is repeated using additional amino acid but omitting additional acid deprotection. When the last coupling is completed, the resin is washed with methanol or methanol containing...
dichloromethane and dried at a maximum of 60°C.

At the end of each cycle, i.e., after each successive N\textsuperscript{2}-protected amino acid is added to the growing polypeptide chain, the protecting group is removed by treatment with a deprotecting agent. When serine is added, the deprotecting agent removes both the N\textsuperscript{2}-Boc protecting group and the serine protecting group. Among the preferred deprotecting agents are hydrogen chloride in dichloromethane (HCl/CH\textsubscript{2}Cl\textsubscript{2}), trifluoroacetic acid in dichloromethane (TFA/CH\textsubscript{2}Cl\textsubscript{2}), and hydrogen chloride dissolved in a C3-C6 alcohol, preferably isopropanol, mixed with dichloromethane. Generally, the concentration of the HCl will be 2N to 9N, preferably 4N to 5N. The ratio of CH\textsubscript{2}Cl\textsubscript{2} to the C3-C6 alcohol is 0.1 to 10 (v/v), preferably about 1:1. A particularly preferred deprotecting agent is 4.5N HCl in i-PrOH:CH\textsubscript{2}Cl\textsubscript{2} (1:1). The deprotection step generally takes place at temperatures of 0°C to 45°C, preferably at ambient temperatures (20°C to 27°C).

Those skilled in the art will appreciate that selection of a coupling/deprotection protocol utilizing agents other than those described above is entirely appropriate provided that the serine residue is deprotected with an agent which accomplishes the objectives of this invention. A protocol which uses HCl/iPrOH/CH\textsubscript{2}Cl\textsubscript{2} for each deprotecting cycle may be employed. Alternatively, a mixed protocol in which TFA/CH\textsubscript{2}Cl\textsubscript{2} is used for certain cycles and HCl/iPrOH/CH\textsubscript{2}Cl\textsubscript{2} for others is also useful. Other cycles will be readily apparent to the skilled artisan.

At the end of the solid phase synthesis the
polypeptide is cleaved from the resin. Cleavage is by ammonolysis with a saturated solution of ammonia in a suitable solvent for peptides with an alanine or glycine C-terminus; for those peptides having a proline C-terminus cleavage is by means of aminolysis with an alkylamine or fluoroalkylamine. The cleavage is conducted at a temperature between about 10° and 50° C., preferably about 25° C., for between about 12 and 24 hours, preferably about 18 hours. Suitable solvents include methanol, ethanol, isopropanol, dimethylformamide, tetrahydrofuran, N,N-dimethylethanolamine, hexanes and mixtures thereof. Preferably, a saturated solution of ammonia in methanol is used. Alternatively, the peptide may be removed from the resin by transesterification with a base, followed by aminolysis.

The polypeptide is then purified by a sequence of chromatographic steps employing any or all of the following types: ion exchange on a weakly basic resin in the acetate form; hydrophobic adsorption chromatography or underivatized polystyrene-divinylbenzene (e.g., Amberlite® XAD); silica gel adsorption chromatography; ion exchange chromatography on carboxymethylcellulose; partition chromatography (e.g., on Sephadex® G-25), or countercurrent distribution; high performance liquid chromatography (HPLC), especially reversed-phase HPLC on octyl- or octadecylsilyl-silica bonded phase column packing.

If a racemic amino acid is used in one or more of the 1, 2, 3 or 6 positions and individual isomeric
products are desired, the diastereomeric nonapeptide or
decapeptide final products are separated, and the
desired peptide containing a D-amino acid in the
appropriate position is isolated and purified,
preferably during the above-described chromatographic
process.

Optionally, the isolated and purified polypeptide
is converted to a pharmaceutically acceptable salt.

The following Examples compare the temporary
protection process of this invention with an unprotected
process for both an LH-RH agonist and an LH-RH
antagonist. These Examples are presented for purposes
of specificity only and should not be construed so as to
place any undue limitations on the scope of the claimed
invention.

In both the products of Examples 1 and 3, using
the temporary minimal protection process of this
invention, there are significantly fewer impurities
compared to the products obtained in Examples 2 and 4
using unprotected syntheses.

In addition to fewer impurities, the process of
this invention offers the additional advantages of
providing higher yields, and employing less hazardous
reagents, over a shorter time period and with lower
energy expenditures in the isolation and purification of
LH-RH analogs. A further advantage is the generation of
smaller amounts of a considerably less toxic waste
stream.
PREPARATION A
Preparation of Boc-Gly-O-Resin

4.9 g of N\textsuperscript{2}-Boc-glycine was dissolved in a mixture of 50 ml. methanol and 50 ml. distilled water. The pH of the solution was brought to 7.5 with aqueous cesium bicarbonate. The solvent was then removed under vacuum. After 18 hours of drying under high vacuum, the residue was dissolved in 150 ml. dry DMF. 25 g 1% chloromethylated polystyrene/divinylbenzene (Merrifield) resin (corresponding to 25 mmole chloride) was added. The mixture was shaken at 50°C for 24 hours, filtered, and the resin was then washed sequentially with DMF, water, and ethanol. The resin was dried under vacuum for 3 days to yield 28.34g of Boc-Gly-O-Resin.

PREPARATION B
Preparation of Boc-Ala-O-Resin

Following the procedures of Preparation A, N\textsuperscript{2}-Boc-D-alanine was added to 1% Merrifield resin to provide N\textsuperscript{2}-Boc-D-Ala-O-resin.

EXAMPLE 1
SYNTHESIS OF NAFARELIN WITH TEMPORARY SERINE PROTECTION

In this Example, nafarelin was prepared using the following side chain protection protocol: salt protection for arginine (as the chloride), tosyl
protection for histidine, and t-butyl protection for serine.

N\textsuperscript{\textcircled{2}}-Boc amino acids were obtained from Bachem (Torrance, CA) (Leu, Tyr, His(Tos), Arg, Trp and Gly), Star Biochemicals (Torrance, CA) (Pro and Ser(tBu)), Synthe Tech (Albany, OR) (D-Nal(2)).

Solutions of 4-4.5N HCl in i-PrOH/CH\textsubscript{2}Cl\textsubscript{2}(1/1) were prepared by bubbling HCl into cooled i-PrOH. Once the solution became saturated (determined by titration, approximately 9N), the solution was kept at room temperature for no more than 3 days and diluted with an equal volume of CH\textsubscript{2}Cl\textsubscript{2} before use.

1.0 mmol of N\textsuperscript{\textcircled{2}}-Boc-Gly-O-resin from Preparation A was placed in the reaction vessel of a 5.0 L Vega 296 automated solid phase peptide synthesizer fitted with accessory bottles and flasks for addition of reagents and for pressurization, depressurization and maintenance of an inert atmosphere of nitrogen.

The following amino acids were added to the N\textsuperscript{\textcircled{2}}-Boc-Gly-O-resin by DIC or Hbt-assisted DIC coupling for 3 hours:

- N\textsuperscript{\textcircled{2}}-Boc-Pro 2.0 equiv.
- N\textsuperscript{\textcircled{2}}-Boc-Arg.HCl 2.0 equiv.
- N\textsuperscript{\textcircled{2}}-Boc-Leu.H\textsubscript{2}O 2.0 equiv.
- N\textsuperscript{\textcircled{2}}-Boc-D-Nal(2) 1.5 equiv./Hbt
- N\textsuperscript{\textcircled{2}}-Boc-Tyr 1.5 equiv./Hbt
- N\textsuperscript{\textcircled{2}}-Boc-Ser(tBu) 2.0 equiv./Hbt
- N\textsuperscript{\textcircled{2}}-Boc-Trp 1.75 equiv./Hbt
- N\textsuperscript{\textcircled{2}}-Boc-His(Tos) 1.75 equiv./Hbt
- (pyro)Glu 2.5 equiv./Hbt
The following protocols were used to remove the N° protecting group following each addition.

**Program A:** The resin was first washed with \( \text{CH}_2\text{Cl}_2 \) 1x1min., TFA-\( \text{CH}_2\text{Cl}_2 \) (40/60) 1x1min., TFA-\( \text{CH}_2\text{Cl}_2 \) (40/60) 1x30min., \( \text{CH}_2\text{Cl}_2 \) 5x1min., Et\(_3\)N-\( \text{CH}_2\text{Cl}_2 \) (5/95) 3x1min., \( \text{CH}_2\text{Cl}_2 \) 4x1min.

**Program B:** The resin was first washed with \( \text{CH}_2\text{Cl}_2 \) 1x1min., 4-4.5N HCl in \( \text{CH}_2\text{Cl}_2/i\text{-PrOH} \) (1/1) 1x1min., 4-4.5N HCl in \( \text{CH}_2\text{Cl}_2/i\text{-PrOH} \) (1/1) 1x30min., \( \text{CH}_2\text{Cl}_2 \) 3x1min., DMF 1x1min., Et\(_3\)N-\( \text{CH}_2\text{Cl}_2 \) (5/95) 3x1min., DMF 1x1min., \( \text{CH}_2\text{Cl}_2 \) 4x1min.

Program A was used to remove the N° protecting groups on Gly, Pro, Arg, Leu, D-Nal(2) and Tyr. Program B was used for the removal of the N° protecting groups on Ser, Trp, and His and for the removal of the serine side chain protecting group.

After each deprotecting and washing step, following protocol A or B, the next amino acid in sequence was added and the resin washed with \( \text{CH}_2\text{Cl}_2 \) 3x1min., MeOH 4x1min., DMF 2x1min. and \( \text{CH}_2\text{Cl}_2 \) 4x1min. When the sequence was completed, the peptide was cleaved from the resin by treatment with a saturated solution of ammonia in methanol for about 18 hours at about 25°C.

The crude peptide was dissolved in 2M acetic acid and converted to the acetate salt by passage through a column of AG3-X4A resin (Bio-Rad). The acetate was dissolved in a minimal amount of methanol and acetone added to reprecipitate the peptide. Reversed phase HPLC (Partisil ODS-3, 40μ, acetonitrile with 0.5% acetic acid was used to remove polar and non-polar impurities. Fractions containing at least 57% nafarelin acetate were combined and diluted with water and reloaded on a reversed phase HPLC column, and washed with 1% acetic acid in water. The residue was precipitated, filtered, washed and then dried under vacuum.
Amino acid analyses were performed on a Beckman 119CL amino acid analyzer. Samples for amino acid analyses were hydrolyzed with 4N CH₃SO₃H (0.2% 3-(2-aminomethyl indole) HCl) for 20 hours at 110°C.

Analytical HPLC was performed on a Spectra Physics 8800 chromatograph, using an ODS-II column from Alltech, 5μ, 4.6 x 250mm, 10 μl inj., flow - 1.5 ml/min., 2.5% CH₃CN, 72.5% 0.16M KH₂PO₄ pH=5.1, temp. =40°C.

HPLC analysis of the crude peptide showed a main peak with a retention time of 18 min. corresponding to nafarelin and no impurity over 1% at rt 14 min.

**EXAMPLE 2**

**SYNTHESIS OF NAFARELIN WITHOUT SERINE PROTECTION**

The procedure of Example 1 was followed except that N°-Boc-Ser was substituted for N°-Boc-Ser(tBu).

HPLC analysis showed a main peak at 18 min. corresponding to nafarelin and 8.1 to 11.5% of an impurity at a retention time (rt) of 14 min.

Also, the yield from this "unprotected" synthesis was approximately the same as that obtained from a fully protected synthesis with an HF treatment; in the latter instance, the yield was significantly lower than that achieved with the temporary protection synthesis of Example 1.
EXAMPLE 3
SYNTHESIS OF AN LH-RH ANTAGONIST USING TEMPORARY SERINE PROTECTION

In this Example an LH-RH antagonist, N-Ac-D-Nal(2)-D-pCl-Phe-D-Pal(3)-Ser-Tyr-
D-hArg(Et)₂-Leu-hArg(Et)₂-Pro-D-AlaNH₂, was prepared using the following side chain protection protocol: salt protection for L- and D-hArg(Et)₂ (as the chloride) and t-butyl protection for serine.

Nα-Boc amino acids were obtained from Bachem (Torrance, CA) (D-Ala, Arg and Leu); Star Biochemicals (Torrance, CA) (Pro); Synthe Tech (Albany, OR) (D-Nal(2)), Incell (Milwaukee, WI) (D-Pal(3)) and UCB Bioproducts (Belgium)(p-Cl-Phe).

Amino acids were added to the Nα-Boc-D-Ala-0-Resin of Preparation B in the following sequence:

Nα-Boc-Pro 2.3 equiv.
Nα-Boc-hArg(Et)₂.HCl 1 equiv./HBT
Nα-Boc-Leu.H₂O 2.3 equiv.
Nα-Boc-D-hArg(Et)₂.HCl 1.6 equiv./HBT
Nα-Boc-Tyr 2.1 equiv./HBT
Nα-Boc-Ser(tBu) 2.0 equiv.
Nα-Boc-D-Pal(3) 1.8 equiv./HBT
Nα-Boc-D-p-Cl-Phe 2.0 equiv.
Nα-Boc-D-Nal(2) 2.1 equiv./HBT

Acetic anhydride

An acetylation (capping) was done after Ala, Pro and Leu. Excess HBT (2 equiv.) was used for the
coupling of the basic amino acids, hArg(Et)₂ and Pal(3).

The amino acids were attached by DIC or HBT-assisted DIC coupling for 3 hours and the resin was subsequently washed with CH₂Cl₂ 3x1min., MeOH 4x1min., DMF 2x1min. and CH₂Cl₂ 4x1min.

The following protocols were used to remove the Na⁺ protecting group following each addition:

Program A: The resin was first washed with CH₂Cl₂ 1x1min., TFA-CH₂Cl₂ (40/60) 1x1min., TFA-CH₂Cl₂ (40/60) 1x30 min., CH₂Cl₂ 5x1min., Et₃N-CH₂Cl₂ (5/95) 3x1min., CH₂Cl₂ 4x1min.

Program B: The resin was first washed with CH₂Cl₂ 1x1min., 4-4.5N HCl in CH₂Cl₂/i-PrOH (1/1) 1x1min., 4-4.5N HCl in CH₂Cl₂/i-PrOH (1/1) 1x30min., CH₂Cl₂ 3x1min., DMF 1x1min., Et₃N-CH₂Cl₂ (5/95) 3x1min., DMF 1x1min., CH₂Cl₂ 4x1min.

Program A was used for the removal of the protecting groups on Ala, Pro, L-hArg(Et)₂, Leu and D-Nal(2); Program B was used for the removal of the protecting groups on D-hArg(Et)₂, Tyr, Ser, D-Pal(3) and p-Cl-Phe.

After each deprotecting and washing step, following protocol A or B, the next amino acid in sequence was added and the resin washed with CH₂Cl₂ 3x1min., MeOH 4x1min., DMF 2x1min. and CH₂Cl₂ 4x1min.

When the sequence was completed, the peptide was cleaved from the resin by treatment with a saturated solution of ammonia in methanol for about 18 hours at about 25°C.

The crude peptide was first dissolved in 2M acetic acid and converted to its acetate salt by passage through a column of AG3-X4A resin (Bio-Rad). The
acetate was subjected to chromatography on a silica gel column (CH$_2$Cl$_2$/i-PrOH/MeOH/H$_2$O/HOAc solvent); the acetate fractions dissolved in water and loaded onto a reversed-phase column (Vydec C-18, 15-20μ) and purified using acetonitrile/TEAP (pH 3). Fractions of the desired purity were combined and diluted with water and reloaded on a reversed-phase HPLC column, then washed with 1% acetic acid in water. The peptide was stripped with a mixture of MeOH/CH$_3$CN/HOAc/H$_2$O (44/50/1/5). The residue was dissolved in methanol or acetic acid and precipitated over ether, filtered, washed with ether and dried under vacuum.

Amino acid analyses were performed on a Beckman 119CL amino acid analyzer. Samples for amino acid analyses were hydrolyzed with 6N HCl at 110°C for 20 hrs.

Analytical HPLC was performed on a Spectra Physics 8800 chromatograph, using a Spherisorb C-8 (Alltech), 5μ, 4.6 x 250 mm, 10μl inj., flow =1.5 ml/min., 30% CH$_3$CN, 70% NH$_4$H$_2$PO$_4$ 0.04M, dimethyloctylamine 4.3 x 10$^{-3}$, temp. 40°C. Synthesis of the antagonist was confirmed by the presence of a main peak at rt 18 min.; no other peak over 1% was noted, at rt 16 min.
EXAMPLE 4
SYNTHESIS OF LH-RH ANTAGONIST WITHOUT
TEMPORARY SERINE PROTECTION

Example 3 was repeated using N*-Boc-Ser instead of
N*-Boc-Ser(tBu).

HPLC analysis showed the presence of a main peak at
18 min. corresponding to the antagonist and the presence of
an impurity of 6.5% at rt 16 min.

The following claims particularly point out and
distinctly claim the subject matter which applicants regard
as their invention. These claims are entitled to the full
range of equivalents recognizable by those skilled in the
art of solid phase peptide synthesis.
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. In a process for the solid phase synthesis of an LH-RH analog containing at least one serine residue, the improvement comprising temporarily protecting only the side chain of the serine residue with a protecting group which is labile to those agents useful for removing α-amino protecting groups.

2. A process of claim 1 in which the serine side chain is protected with a group selected from t-butyl, trityl, pivalyl, t-butylidimethylsilyl, trimethylsilyl, and tetrahydropyran-2-ol.

3. A process of claim 2 in which the serine side chain protecting group is removed by treatment with hydrogen chloride in a C3-C6 alcohol / dichloromethane solution.

4. A process of claim 3 in which the C3-C6 alcohol is isopropanol.

5. In a process for the solid-phase synthesis of a compound having an amino acid sequence of the formula

   \[ R^1-R^2-R^3-Ser-Tyr-L^1-Leu-R^5-Pro-R^6 \]  

   (I) 

   wherein 

   \( R^1 \) is selected from (pyro)Glu and N-Ac-D-Nal(2);  
   \( R^2 \) is selected from His, D-p-Cl-Phe and D-p-F-Phe;  
   \( R^3 \) is selected from Trp, D-Trp, D-Nal(2) and D-Pal(3);  
   \( R^4 \) is selected from D-Nal(2), D-hArg(Et), D-hArg(Bu), D-hArg(CH\_2CF\_3), D-His(Bzl), D-Leu, D-Pal(3), D-Ser(tBu) and D-Trp;  
   \( R^5 \) is selected from Arg, L-hArg(Et), L-hArg(Bu), L-hArg(CH\_2CF\_3) and Lys(iPr); and  
   \( R^6 \) is selected from Gly-NH\_2, NH-NHCONH\_2, D-Ala-NH\_2 and NHet;
wherein the amino acids are provided with Nα-protection;
the improvement comprising (a) temporarily protecting only the side
chain of serine at position 4 with a group labile to agents for removing the α-
amino protecting group;
and (b) protecting the side chain of histidine, if present, with a group
liable to an α-amino group deprotection agent, or to aminolysis or ammonolysis.

6. A process of claim 5 in which the serine side chain protecting
group is selected from t-butyl, trityl, pivalyl, tetrahydropyran-2-yl, trimethylsilyl
and t-butyldimethylsilyl, preferably, t-butyl.

7. A process of claim 5 in which the α-amino protecting group is
selected from t-butyloxy carbonyl, t-amyl oxycarbonyl, isobornyl oxycarbonyl, α,
α-dimethyl-3,5-dimethoxybenzoyl oxycarbonyl, α-nitrophenylsulfenyl, 2-cyano-t-
butyloxy carbonyl, and 9-fluoren l methyl-oxycarbonyl.
A process of claim 5 in which the α-amino protecting group is t-butyloxycarbonyl, the serine side chain protecting group is t-butyl, and the histidine side chain protecting group is p-toluenesulfonyl.

9. A process of Claim 1 or 5 in which the deprotecting agent is selected from HCl/CH₂Cl₂, TFA/CH₂Cl₂, and HCl/(C₃-C₆)alcohol/CH₂Cl₂.

10. A process of Claim 9 in which the deprotecting agent is HCl/iPrOH/CH₂Cl₂.

11. A process of Claim 5 in which
R¹ is Ac-D-Nal(2);
R² is D-p-Cl-Phe;
R³ is D-Pal(3);
R⁴ is D-hArg(Et)₂;
R⁵ is L-hArg(Et)₂; and
R⁶ is D-Ala-NH₂.

13. A process of Claim 5 in which
R¹ is (pyro)Glu,
R² is His,
R³ is Trp,
R⁴ is D-Nal(2),
R⁵ is Arg, and
R⁶ is Gly-NH₂.
A process for the solid-phase synthesis of a compound having an amino acid sequence of the formula

\[ R^1-R^2-R^3-Ser-Tyr-R^4-Leu-R^5-Pro-R^6 \] (I)

wherein
- \( R^1 \) is selected from (pyro)Glu and N-Ac-D-Nal(2);
- \( R^2 \) is selected from His, D-p-Cl-Phe and D-p-F-Phe;
- \( R^3 \) is selected from Trp, D-Trp, D-Nal(2) and D-Pal(3);
- \( R^4 \) is selected from D-Nal(2), D-hArg(Et)_2, D-hArg(Bu), D-hArg(CH_2CF_3)_2, D-His(Bzl), D-Leu, D-Pal(3), D-Ser(tBu) and D-Trp;
- \( R^5 \) is selected from Arg, L-hArg(Et)_2, L-hArg(Bu), L-hArg(CH_2CF_3)_2 and Lys(iPr); and
- \( R^6 \) is selected from Gly-NH_2, NH-NHCONH_2, D-Ala-NH_2 and NHEt;

which process comprises the steps of:

(a) coupling by solid phase synthesis appropriate Boc-protected and t-butyl protected serine in successive cycles and in the order from right to left of the amino acid sequence of the compound of Formula (I), starting with Boc-R6-O- covalently bound to an inert solid support.

(b) eliminating, at the end of each cycle, the Boc-protecting group and simultaneously the t-butyl group from serine or D-serine by treatment with a deprotecting agent selected from HCl/CH_2Cl_2 and HCl/lower alkanol/CH_2Cl_2 to form a polypeptide bound to said solid support,
(c) cleaving the polypeptide from the support by ammonolysis, and
(d) isolating the resulting polypeptide.

DATED this 18th day of February, 1991.

SYNTEX (USA) INC.