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(54) **Title:** A METHOD FOR PREPARING GLP-1 ANALOGUE BY SOLID-PHASE PEPTIDE SYNTHESIS

(57) **Abstract:** The present invention relates to improved process for manufacturing of glucagon-like peptide-1 (GLP-I) and analogs by utilizing Fmoc protected amino acids and synthesizing peptide by solid phase method. The present invention relates to improved process for manufacturing of GLP-1 and analogs by step-wise process of synthesis comprising a solution of an amino acid or peptide of which the α-amino group is protected by Fmoc; contacting the support with the solution in the presence of reagent for forming a peptide bond between a carboxyl group of the amino acid or peptide and solid support; removal of Fmoc protecting group in presences of a base and organic solvent wherein base is selected form Pyrrolidine, Ethanolamine, Piperazine, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) or mixture of any of these, preferably Pyrrolidine.

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TITLE– **A METHOD FOR PREPARING GLP-1 ANALOGUE BY SOLID-PHASE PEPTIDE SYNTHESIS**

FIELD OF INVENTION:

The present invention relates to improved process for manufacturing of glucagon-like peptide-1 (GLP-I) and analogs by utilizing Fmoc protected amino acids and synthesizing peptide by solid phase method. The present invention relates to improved process for manufacturing of GLP-1 and analogs by step-wise process of synthesis comprising a solution of an amino acid or peptide of which the α-amino group is protected by Fmoc; contacting the support with the solution in the presence of reagent for forming a peptide bond between a carboxyl group of the amino acid or peptide and solid support; removal of Fmoc protecting group in presences of a base and organic solvent wherein base is selected form Pyrrolidine, Ethanolamine, Piperazine, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) or mixture of any of these, preferably Pyrrolidine.

BACKGROUND OF INVENTION:

There are a number of known methods available for peptide synthesis. A classical approach is liquid-phase peptide synthesis (LPPS), which has been a preferred method for producing large quantities of peptides. Another current and commonly used approach for peptide synthesis is solid-phase peptide synthesis (SPPS), wherein the growing peptide chain is covalently attached to a resin on a solid support, until cleaved from it once the desired length and sequence is achieved. In these methods reactive side chains of the incorporated amino acids need to be protected in order to avoid other reactions apart from the desired formation of new peptide bonds in the growing peptide chain. In addition, to avoid side reactions between the added amino acids, as well as incorporation of multiple amino acids in each step, the added amino acids are normally α-amino protected. The synthesis thus becomes one of repeated cycles of deprotection of the α-amine of a solid-phase attached peptide, followed by coupling to a single, α-amino protected amino acid unit.

Solid phase peptide synthesis was introduced in 1963 with the intent to overcome many of the intermediate purification problems associated with solution phase peptide synthesis. Stewart, et

al., Solid Phase Peptide Synthesis (Pierce Chemical Co., 2d ed., 1984). During solid phase synthesis, amino acids are assembled (i.e., coupled) into a peptide of any desired sequence while one end of the chain (i.e., the C-terminus) is anchored to an insoluble support. Once the desired sequence has been linked together on the support, the peptide is then detached (i.e., cleaved) from the support. The two standard protecting groups for α-amino functions of the coupled amino acids are *tert*-butyloxycarbonyl (Boc), which is removed by treatment with a strong acid, and fluoren-9-ylmethyloxycarbonyl (Fmoc), which is removed with a base. The present invention relates to a convenient method of manufacturing peptides using a combination of both of these α-amino protecting groups in a single synthesis on inexpensive polystyrene resin.

In designing a synthesis of a peptide by the solid phase method using either of the above mentioned α-amino protection schemes, it is important that any reactive "side groups" of the constituent amino acids be protected from unwanted chemical reactions throughout the chain assembly. It is also desirable that the chemical groups chosen to protect the various side groups be resistant to removal by the reagents used to remove the α-amino protecting groups. Thirdly, it is important that the linkage of the growing peptide chain to the resin particle be stable to the reagents used to remove either type of α-amino protecting group during chain assembly. In the case of the Fmoc α-amino protection scheme, the side group protection functions should be resistant to the basic reagents used to remove the Fmoc. In practice, these side chain protecting groups are generally removed by acidic reagents after the peptide chain has been assembled. When using the Boc α-amino protection scheme, the side chain protecting groups must be resistant to removal by the acid reagent used to remove the Boc group at every cycle. In practice, these side chain protecting groups for the Boc α-amino protection scheme are generally removed by anhydrous HF after the peptide chain has been assembled. Therefore, in practice, the side chain protecting groups commonly used with the Fmoc α-amino protection are not stable under the conditions used for Boc α-amino deprotection and the two types of α-amino protection schemes are not mixed in the assembly of a peptide chain by solid phase peptide synthesis. In addition, while the least expensive polymeric resin used in peptide synthesis, chloromethylated polystyrene or "Merrifield resin", is widely used with Boc protected amino acids, the literature suggests it is unsuitable for use with Fmoc protection on the α-amino group due to its instability in basic conditions. (see Stewart, et al., Solid Phase Peptide Synthesis (Pierce Chemical Co., 2d

ed., 1984). The present invention is directed to a method for mixed usage of both Boc and Fmoc protected amino acids on "resin" during solid phase synthesis of certain peptides.

Liraglutide is a marketed, chemically modified GLP-1 analog in which, among other modifications, a fatty acid is linked to a lysine in position 20 leading to a prolonged duration of action (Drucker DJ et al, Nature Rev. Drug Disc. 9, 267-268, 2010; Buse, J.B. et al., Lancet, 374:39-47, 2009).

Liraglutide, peptide sequence: H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(N-ε-(Nα-Palmitoyl-L-γ-glutamyl))-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH. Liraglutide, a glucagon-like peptide-1 (GLP-1) receptor agonist, developed by Novo Nordisk as a subcutaneous injection formulation, can play a good role in lowering blood glucose.

US6458924B2 reported the synthesis of solid-liquid liraglutide, intermediate GLP-l (7-37) –OH purified by reverse phase HPLC to require, under liquid phase conditions and then reacted with Nα-alkanoyl-Glu(ONSu)-OtBu, and since GLP-l (7-37)-OH N terminal unprotected side chain protecting groups and the removal of all, can result in a number of impurities, purification is difficult, complicated operation. Conventional synthetic methods Liraglutide presence complicated operation required two-step purification, synthesis cycle is long, multiple wastes is not environmentally friendly and not conducive to high cost mass production disadvantages.

Semaglutide is a chemically modified glucagon-like peptide-1 (GLP-1) analog. Semaglutide is used for treating type 2 diabetes. Semaglutide peptide sequence has a structure of:

H-His-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Ase-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys (PEG-PEG-γ-Glu-Octadeca-nedioicAcid)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH

Present invention is an improved process for preparation of glucagon-like peptide-1 (GLP-I) and its analog such as Semaglutide and liraglutide, which are prepared by solid phase peptide synthesis comprises stepwise coupling of amino acid or peptide. The process for preparation of

peptide-1 (GLP-I) by stepwise coupling of peptide wherein the peptide has a sequence more hydrophobic amino acids, coupled stepwise.

The process disclosed in prior art for the preparation peptide-1 (GLP-I) has drawback wherein the incomplete reaction, results in low yield, and impurities due to the unreacted crude peptide. Further the prior art for the preparation peptide-1 (GLP-I) requires advanced purification for the removal of unreacted crude peptide and impurities.

SUMMARY OF INVENTION:

The present invention is a stepwise synthesis of glucagon-like peptide-1 (GLP-I) comprising Nα-protected amino acids by Fmoc used as raw material, and de-protection of Fmoc protecting group in presences of base and organic solvent wherein base is selected form Pyrrolidine, Ethanolamine, Piperazine, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) or mixture of any of these, further followed by protection and de-protection of peptide to get glucagon-like peptide-1 (GLP-I).

According to the one of the aspect of the invention is process for preparation of glucagon-like peptide-1 (GLP-I) by solid phase peptide synthesis comprising:

(a) attaching a first amino acid to a solid support resin through an ester bond to form a first-coupled-product,

(b) removing the Fmoc from the first-coupled-product in presences of a base (bases) and organic solvent to form a first-deprotected-coupled-product;

(c) optionally coupling a next-amino-acid to the first-deprotected-coupled-product, which comprises reacting the next-amino-acid with the first- deprotected-coupled-product in an organic solvent comprising a peptide coupling reagent to form a next-protected-coupled-product, wherein the next-amino-acid has an alpha (non-side chain) amino group protected by Fmoc and if the next-amino-acid has one or more side chain functionalities then the side chain functionalities do not require protection or the side chain functionalities have protecting groups that are stable to acid and base reagents used to remove Boc or Fmoc;

(d) removing the Fmoc from the next-protected-coupled-product which comprises reacting the next-protected-coupled-product with an organic base to yield a next-deprotected-coupled-product;

(e) optionally repeating steps (b) and (d), each cycle forming an (X+1)-next-deprotected-coupled-product where X is the number of desired cycle repetitions;

(f) coupling a next-amino-acid to the first-unprotected-coupled-product from (b), or optionally to the (X+1)-next-unprotected-coupled-product from (e), which comprises reacting the next-amino-acid with said first-deprotected-coupled-product or said (X+1)-next-deprotected-coupled-product in an organic solvent comprising a peptide coupling reagent to form a next-protected-coupled-product, wherein the next-amino-acid has a non-side chain amino group protected by Fmoc, provided that if the next-amino-acid has one or more side chain functionalities then the side chain functionalities do not require protection or the side chain functionalities have protecting groups that are stable to base reagents used to remove Fmoc;

(g) cleaving the peptide from the solid support resin of the completed-peptide-resin-product or the unprotected-completed-peptide-resin-product to yield the peptide, which comprises reacting the completed-peptide-resin-product or the unprotected-completed-peptide-resin-product with scavengers until the cleavage of the peptide from the resin is substantially complete.

According to the present aspect of the invention a method for preparing a glucagon-like peptide-1 (GLP-I) comprises: a solution of an amino acid or peptide of which the α-amino group is protected by Fmoc; contacting the support with the solution in the presence of reagent for forming a peptide bond between a carboxyl group of the amino acid or peptide and solid support; removal of Fmoc protecting group in presences of a base and organic solvent wherein base is selected form Pyrrolidine, Ethanolamine, Piperazine, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) or mixture of any of these, preferably Pyrrolidine.

According to the present aspect of the invention a method for preparing a glucagon-like peptide-1 (GLP-I) comprising as first step, Fmoc α-amino-protected amino acid is coupled to the resin and then to one another amino acids in a step- wise, cyclic and sequence-dependent manner. Each step of coupling of an amino acid is followed by a step of removal of the Fmoc protection group

and allow for the next N-protected amino acid to be coupled. Removal of Fmoc is achieved by base such as Pyrrolidine, Ethanolamine, Piperazine, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) or mixture of any of these, preferably Pyrrolidine. Pyrrolidine, also known as tetrahydropyrrole, is an organic compound with the molecular formula $(CH_2)_4NH$. It is a cyclic secondary amine, also classified as a saturated heterocycle.

According to the present aspect, the invention is related to synthesis of GLP-1 analogue by standard solid phase peptide synthesis using Fmoc strategy on Wang resin (0.25-1.2 mmol/g). Loading of first Fmoc α-amino-protected amino acids to the resin achieved and after estimating and achieving desired loading of first amino acid, sequence-dependent coupling of amino acid to one another is carried out in a step-wise manner. Each step of coupling of an amino acid is followed to removal the Fmoc protection group. Removal of fmoc protecting group is achieved by bases such as Pyrrolidine, Ethanolamine, Piperazine, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) or mixture of any of these, preferably Pyrrolidine. Pyrrolidine, also known as tetrahydropyrrole, is an organic compound with the molecular formula $(CH_2)_4NH$. It is a cyclic secondary amine, also classified as a saturated heterocycle.

According to the present aspect of the invention is an improved process for synthesis of GLP-1 analogue on solid phase peptide synthesis using Fmoc strategy on Wang resin (0.25-1.0 mmol/g) comprising following steps:

a) Loading of the first Fmoc-protected amino acid and removing Fmoc protecting group by reacting it with Pyrrolidine in Dimethylformamide (DMF)
b) Repetitive cycles of the following:
    i. Coupling of sequential next Fmoc-protected amino acid with amino acid loaded on Wang resin in presences of peptide coupling reagent Ethyl cyanohydroxyiminoacetate and N,N′-Diisopropylcarbodiimide (DIPC);
    ii. Removal of Fmoc protecting group by reacting it with Pyrrolidine in Dimethylformamide (DMF);
    iii. Coupling of sequential next Fmoc-protected amino acid in presences of peptide coupling reagent Ethyl cyanohydroxyiminoacetate and N,N′-Diisopropylcarbodiimide (DIPC);
c) Washing and drying the peptide-resin;

d) Cleavage of the protected peptide intermediate from the resin using various scavengers;
e) Precipitating the crude GLP-1 analogue from the cleavage solution;
f) Purifying the crude GLP-1 analogue by a suitable separation method including counter-ion exchange to form a GLP-1 analogue solution; and
g) Drying the GLP-1 analogue solution to obtain the GLP-1 analogue product.

DETAILED DESCRIPTION OF THE INVENTION:

As used herein, the terms below have the meanings indicated.

The singular forms "a," "an," and "the" may refer to plural articles unless specifically stated otherwise.

The term "about," as used herein, is intended to qualify the numerical values which it modifies, denoting such a value as variable within a margin of error. When no particular margin of error, such as a standard deviation to a mean value given in a chart or table of data, is recited, the term "about" should be understood to mean that range which would encompass the recited value and the range which would be included by rounding up or down to that figure as well, taking into account significant figures.

For the purpose of clarity and as an aid in the understanding of the invention, as disclosed and claimed herein, the following terms and abbreviations are defined below:

ACN acetonitrile
BOC tert-butoxycarbonyl
DIPEA N,N-Diisopropylethylamine
DMF N,N-Dimethylformamide
Fmoc 9-fluorenylmethoxycarbonyl
TBTU 2-[lH-benzotriazole- 1 -yl)- 1 , 1 ,3,3-tetramethyluronium tetrafluoroborate
HOBt N-hydroxybenzotriazole
HPLC High Performance Liquid Chromatography
SPPS solid phase peptide synthesis

TFA trifluoroacetic acid

t-Bu tert-butyl

TIS Triisopropylsilane

DIPC N,N-Diisopropylcarbodiimide

EDT Ethanedithiol

Trt trityl

OXYMA Ethyl cyanohydroxyiminoacetate

In one of the embodiments of the present invention method for preparing a glucagon-like peptide-1 (GLP-I) comprising:

a) attaching a first amino acid to a solid support resin through an ester bond to form a first-coupled-product,

b) removing the Fmoc from the first-coupled-product in presences of base and organic solvent to form a first-unprotected-coupled-product;

c) optionally coupling a next-amino-acid to the first- unprotected -coupled-product, which comprises reacting the next-amino-acid with the first- unprotected -coupled-product in an organic solvent comprising a peptide coupling reagent to form a next- protected -coupled-product, wherein the next-amino-acid has a non-side chain amino group protected by Fmoc and if the next-amino-acid has one or more side chain functionalities then the side chain functionalities do not require protection or the side chain functionalities have protecting groups that are stable to acid and base reagents used to remove Boc or Fmoc;

d) removing the Fmoc from the next-protected-coupled-product which comprises reacting the next-protected-coupled-product with an organic base to yield a next-unprotected-coupled-product;

e) optionally repeating steps (b) and (e), each cycle forming an (X+1)-next- unprotected -coupled-product where X is the number of desired cycle repetitions;

f) coupling a next-amino-acid to the first- unprotected -coupled-product from (b), or optionally to the (X+1)-next- unprotected -coupled-product from (e), which comprises reacting the next-amino-acid with said first- unprotected -coupled-product or said (X+1)-next- unprotected -coupled-product in an organic solvent comprising a peptide coupling reagent to form a next-protected -coupled-product, wherein the next-amino-acid has a non-side chain amino group protected by Fmoc, provided that if the next-amino-acid has one or more side chain

functionalities then the side chain functionalities do not require protection or the side chain functionalities have protecting groups that are stable to base reagents used to remove Fmoc;

g) cleaving the peptide from the solid support resin of the completed-peptide-resin-product or the deprotected-completed-peptide-resin-product to yield the peptide, which comprises reacting the completed-peptide-resin-product or the deprotected-completed-peptide-resin-product with suitable cleavage solution in the presence of scavenger until the cleavage of the peptide from the resin is substantially complete.

In an embodiment the present invention provides process for preparation of GLP-1 analogue product which includes the step of:

a) Synthesis of the protected peptide was carried out by a stepwise Fmoc SPPS (solid phase peptide synthesis) procedure. The first amino acid was loaded by a regular coupling procedure. After washing of the resin with DMF, the Fmoc protecting group was removed by treatment with Pyrrolidine in DMF. After washing of residual reagents with DMF, the second amino acid was introduced to start the second coupling step. Fmoc protected amino acids were activated in situ using Ethyl cyanohydroxyiminoacetate/N,N′-Diisopropylcarbodiimide (DIPC) or TBTU/HOBt (N-hydroxybenzotriazole) or DIPC/HOBt, and subsequently coupled to the resin. After washing of the resin, the Fmoc protecting group on the -amine was removed with Pyrrolidine in DMF. These steps were repeated each time with the next amino acid according to peptide sequence. All amino acids used were Fmoc-protected. At the end of the synthesis the peptide-resin was washed with suitable solvent, followed by drying under vacuum to obtain the dry peptide-resin. The cleavage and deprotection of peptide is achieved by suitable cleavage solution in the presence of a scavanger. The crude GLP-1 analogue product obtained is subjected to purification.

The resin could be, but is not limited to, Wang or 2-chlorotrityl resin, and the permanently stable protecting group should be compatible with Fmoc strategy. In a preferred embodiment, the resin is Wang resin.

In one of the particular embodiments of the present invention deprotection is achieved by base, such as Pyrrolidine, Ethanolamine, Piperazine, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) or mixture of any of these, preferably Pyrrolidine. Pyrrolidine, also known as tetrahydropyrrole, is

an organic compound with the molecular formula (CH2)4NH. It is a cyclic secondary amine, also classified as a saturated heterocycle.

In another embodiments of the present invention, an improved process for synthesis of GLP-1 analogue on solid phase peptide synthesis using Fmoc strategy on Wang resin (0.25-1.0 mmol/g) comprising following steps:

a) Loading of the first Fmoc-protected amino acid followed by deprotection by reacting it with Pyrrolidine in Dimethylformamide (DMF)
b) Repetitive cycles of the following:
    i. Coupling of sequential next Fmoc-protected amino acid with amino acid loaded on Wang resin in presences of peptide coupling reagent Ethyl cyanohydroxyiminoacetate and N,N′-Diisopropylcarbodiimide (DIPC);
    ii. Removal of Fmoc protecting group by reacting it with Pyrrolidine in Dimethylformamide (DMF);
    iii. Coupling of sequential next Fmoc-protected amino acid in presences of peptide coupling reagent Ethyl cyanohydroxyiminoacetate and N,N′-Diisopropylcarbodiimide (DIPC)
c) Washing and drying the peptide-resin;
d) Cleaving of the protected peptide intermediate from the resin using suitable cleavage solution in presence of a scavenger;
e) Precipitating the crude GLP-1 analogue from the cleavage solution;
f) Purifying the crude GLP-1 analogue by a suitable separation method including counter-ion exchange to form a GLP-1 analogue solution; and
g) Drying the GLP-1 analogue solution to obtain the GLP-1 analogue product.

In another embodiments of the present invention, glucagon-like peptide-1 (GLP-I) analogue were prepared by standard solid phase peptide synthesis using the Fmoc strategy on Wang resin (0.25-1.2 mmol/g). Removal of Fmoc-protecting group is achieved by using 1 to 5% of pyrrolidine in DMF. Side chain was incorporated by modifying lysine as Fmoc-Lys[side chain]-OH and the N-terminal amino acid as Boc-His(Trt)-OH. After completion of the synthesis the peptide was cleaved from the resin by treatment with TFA:H_2O:TIPS:PhOH (90:2.5:5.0:2.5) for 2 to 6 hours,

followed by precipitation with ether and ether wash. The analogs were purified by RP-HPLC on a C18-column in acetonitrile/TFA, and purity and identity of the product established by HPLC and LCMS.

In another embodiments of the present invention, a method for preparing Semaglutide having (SEQ ID NO. 1)

Sequence ID NO. 1: H-His-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(SC)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH

SC = Fmoc-Lys[ODDA(OtBu)-Glu(AEEA-AEEA)-OtBu]-OH

The method for preparing Semaglutide:

Semaglutide was prepared by solid phase peptide synthesis using the Fmoc strategy on Wang resin (0.25-1.2 mmol/g). Removal of Fmoc-protecting group is achieved by using 1 to 5% of pyrrolidine in DMF. Side chain was incorporated by modifying lysine as Fmoc-Lys[ODDA(OtBu)-Glu(AEEA-AEEA)-OtBu]-OH and the N-terminal amino acid as Boc-His(Trt)-OH. After completion of the synthesis the peptide was cleaved from the resin by treatment with TFA:H_2O:TIPS:PhOH (90:2.5:5.0:2.5) for 2 to 6 hours, followed by precipitation with ether and ether wash. Crude purity by HPLC is 35-55% on area normalization method.

In one of the particular embodiments of the present invention, a method for preparing Liraglutide having (SEQ ID NO. 2)

Sequence ID NO. 2: H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(SC)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH.

SC = Palm-Glu-OH

The method for preparing Liraglutide

Liraglutide was prepared by solid phase peptide synthesis using the Fmoc strategy on Wang resin (0.25-1.2 mmol/g). Removal of Fmoc-protecting group is achieved by using 1 to 5% of pyrrolidine in DMF. Side chain was incorporated by modifying lysine as Fmoc-Lys[Palm-Glu-

OtBu]-OH and the N-terminal amino acid as Boc-His(Trt)-OH. After completion of the synthesis the peptide was cleaved from the resin by treatment with TFA : H_2O : TIPS : PhOH (90:2.5:5.0:2.5) for 2 to 6 hours, followed by precipitation with ether and ether wash. Crude purity by HPLC is 50-70% on area normalization method.

The foregoing descriptions are merely preferred embodiments of the present invention and are not intended to limit the present invention. For those skilled in the art, the present invention may have various changes and modifications. Any modification, equivalent replacement, and improvement made within the spirit and principle of the present invention shall fall within the protection scope of the present invention.

Process for preparation of Liraglutide:

Example 1:
Process of removing the Fmoc (de-protecting α-amino acids loaded on resin)

In a peptide vessel 10 g of wang resin pre-loaded with 11 amino acids sequence (Fmoc-Glu(Otbu)-Phe-Ile-Ala-Trp((Boc)-Leu-Val-Arg(pbf)-Gly-Arg(pbf)-Gly-OH) add solution of 5 mL pyrrolidine in 95 ml of DMF. The reaction mixture was stirred for 10 min, solution was separated under vacuum. Further the resin was treated with 5ml of pyrrolidine in 95 ml DMF with stirring for 15 min. the solution was separated and the resin was washed with DMF (4X100 mL), completion of reaction was confirmed by kaiser test.

Example 2:
Coupling of amino-acid to the de-protected amino acids sequence

To the de-protected amino acids loaded on resin of Example 1 add a mixture of Fmoc-Lys(Palm-Glu-OtBu)-OH (3eqv.), 1-hydroxybenzotriazole monohydrate (3 eqv) and DIPC (3 eqv) in 80 ml DMF. The reaction mixture was stirred for about 24 hrs at ambient temperature, completion of reaction was confirmed by kaiser test.

Example 3:
Coupling of amino-acid to the de-protected amino acids sequence

To the de-protected amino acids loaded on resin of Example 1 was add to a mixture of Fmoc-Lys(Palm-Glu-OtBu)-OH (3 eqv.), Oxyma (3 eqv) and DIPC (3 eqv) in 80 ml DMF. The reaction mixture was stirred for 15 hrs at ambient temperature, completion of reaction was confirmed by kaiser test.

Process for preparation of semaglutide:

Example 4:
Process of removing the Fmoc (de-protecting amino acids loaded on resin)

In a peptide vessel 10 g of wang resin pre-loaded with 11 amino acids sequence (Fmoc-Glu(Otbu)-Phe-Ile-Ala-Trp((Boc)-Leu-Val-Arg(pbf)-Gly-Arg(pbf)-Gly-OH) add solution of 5ml pyrrolidine in 95 ml of DMF. The reaction mixture was stirred for 10 min solution was separated under vacuum. Add again 5 ml of purrolidine in 95 ml DMF into resin and allow it to stir for 15 min. Remove the DMF solution and wash with DMF (4X100 ml). Completion of reaction was confirmed by kaiser test.

Example 5:
Coupling of amino-acid to the de-protected amino acids sequence

To the above de-protected resin of Example 4 was add to a mixture Fmoc-Lys[ODDA(OtBu)-Glu(AEEA-AEEA)-OtBu]-OH (3 eqv.), 1-hydroxybenzotriazole monohydrate (3 eqv) and DIPC (3 eqv) in 80 ml DMF. The reaction mixture was stirred for 24 hrs at ambient temperature Completion of reaction was confirmed by kaiser test.

Example 6:
Coupling of amino-acid to the de-protected amino acids sequence

To the above deprotected resin of Example 4 was add a mixture of Fmoc-Lys[ODDA(OtBu)-Glu(AEEA-AEEA)-OtBu]-OH (3 eqv.), Oxyma (3 eqv) and DIPC (3 eqv) in 80 ml DMF. The reaction mixture was stirred for 15 hrs at ambient temperature Completion of reaction was confirmed by kaiser test.

Comparative Study :

GLP-1 inhibitors (liraglutide and semaglutide) both having lengthy side chain at 20^{th} position. Due to lengthy side chain at 20^{th} position there is a steric effect probably hindered the coupling of the amino-acid. The present inventors have studied that using the Oxyma and DIPC as

coupling reagents result into competition of reaction, higher yield, and less impurities, due to the unreacted crude peptide the purity is increased which is more than 60% which is also reflected in the purity of the final APIs as shown in Table 1

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Table 1:

Experiment No	**Coupling reagents at 20th amino acid position**	**Duration of coupling time**	**Purity after 20_Deprotection**	**Purity of the API crude**
Example 2	HOBt and TBTU	24 Hrs	39.13%	42.54%
Example 3	Oxyma and DIPC	15 Hrs	73.19%	61.2%
Example5	HOBT and TBTU	24 Hrs	45.36%	37.6%
Example	Oxyma and DIPC	15 Hrs	66.41%	54.93%

CLAIM

We Claim,

CLAIM 1. A method for preparing glucagon-like peptide-1 (GLP-I) by solid phase peptide synthesis comprising:

(a) attaching a first amino acid to a solid support resin through an ester bond to form a first-coupled-product;

(b) removing the Fmoc from the first-coupled-product in presences of a base and organic solvent to form a first-deprotected-coupled-product;

(c) optionally coupling a next-amino-acid to the first-deprotected-coupled-product, which comprises reacting the next-amino-acid with the first-deprotected-coupled-product in an organic solvent comprising a peptide coupling reagent to form a next-protected-coupled-product, wherein the next-amino-acid has an alpha (non-side chain) amino group protected by Fmoc and if the next-amino-acid has one or more side chain functionalities then the side chain functionalities do not require protection or the side chain functionalities have protecting groups that are stable to acid and base reagents used to remove Fmoc;

(d) removing the Fmoc from the next-protected-coupled-product which comprises reacting the next-protected-coupled-product with an organic base to yield a next-deprotected-coupled-product;

(e) optionally repeating steps (b) and (d), each cycle forming an (X+1)-next-deprotected-coupled-product where X is the number of desired cycle repetitions;

(f) coupling a next-amino-acid to the first-unprotected-coupled-product from (b), or optionally to the (X+1)-next-unprotected-coupled-product from (e), which comprises reacting the next-amino-acid with said first-deprotected-coupled-product or said (X+1)-next-deprotected-coupled-product in an organic solvent comprising a peptide coupling reagent to form a next-

protected-coupled-product, wherein the next-amino-acid has a non-side chain amino group protected by Fmoc, provided that if the next-amino-acid has one or more side chain functionalities then the side chain functionalities do not require protection or the side chain functionalities have protecting groups that are stable to base reagents used to remove Fmoc;

(g) cleaving the peptide from the solid support resin of the completed-peptide-resin-product or the unprotected-completed-peptide-resin-product to yield the peptide, which comprises reacting the completed-peptide-resin-product or the unprotected-completed-peptide-resin-product with scavengers until the cleavage of the peptide from the resin is substantially complete.

CLAIM 2. The method for preparing a glucagon-like peptide-1 (GLP-I) as claimed in claim 1 wherein, base is selected form Pyrrolidine, Ethanolamine, Piperazine, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) or mixture.

CLAIM 3. The method for preparing a glucagon-like peptide-1 (GLP-I) as claimed in claim 1 wherein, peptide coupling reagent is Ethyl cyanohydroxyiminoacetate and N,N′-Diisopropylcarbodiimide (DIPC).

CLAIM 4. The method for preparing a glucagon-like peptide-1 (GLP-I) as claimed in claim 1 wherein, process comprises:

(a) a solution of an amino acid or peptide of which the α-amino group is protected by Fmoc;

(b) contacting the support with the solution in the presence of reagent for forming a peptide bond between a carboxyl group of the amino acid or peptide and solid support;

(c) removal of Fmoc protecting group in presences of a base and organic solvent;

wherein base is selected form Pyrrolidine, Ethanolamine, Piperazine, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) or mixture of any of these, preferably Pyrrolidine.

CLAIM 5. The method for preparing a glucagon-like peptide-1 (GLP-I) as claimed in claim 1 wherein the glucagon-like peptide-1 (GLP-I) that is synthesized is Liraglutide comprises:

(a) Loading Fmoc-protected amino acid Fmoc-Glu(Otbu)-Phe-Ile-Ala-Trp((Boc)-Leu-Val-Arg(pbf)-Gly-Arg(pbf)-Gly-OH on Wang resin (0.25-1.0 mmol/g);

(b) De-protecting Fmoc protecting group by reacting with Pyrrolidine in Dimethylformamide (DMF);

(c) contacting Fmoc-Lys(Palm-Glu-OtBu)-OH with Glu(Otbu)-Phe-Ile-Ala-Trp((Boc)-Leu-Val-Arg(pbf)-Gly-Arg(pbf)-Gly-OH on Wang resin in presences of peptide coupling reagent Ethyl cyanohydroxyiminoacetate and N,N′-Diisopropylcarbodiimide (DIPC).

CLAIM 6. The method for preparing a glucagon-like peptide-1 (GLP-I) as claimed in claim 1 wherein the glucagon-like peptide-1 (GLP-I) that is synthesized is semaglutide comprises:

(a) Loading Fmoc-protected amino acid Fmoc-Glu(Otbu)-Phe-Ile-Ala-Trp((Boc)-Leu-Val-Arg(pbf)-Gly-Arg(pbf)-Gly-OH on Wang resin (0.25-1.0 mmol/g);

(b) De-protecting Fmoc protecting group by reacting with Pyrrolidine in Dimethylformamide (DMF);

(c) contacting Fmoc-Lys[ODDA(OtBu)-Glu(AEEA-AEEA)-OtBu]-OH with Glu(Otbu)-Phe-Ile-Ala-Trp((Boc)-Leu-Val-Arg(pbf)-Gly-Arg(pbf)-Gly-OH on Wang resin in presences of peptide coupling reagent Ethyl cyanohydroxyiminoacetate and N,N′-Diisopropylcarbodiimide (DIPC).

CLAIM 7. The method for preparing a glucagon-like peptide-1 (GLP-I) as claimed in claim 1 wherein, process for synthesis of GLP-1 analogue on solid phase peptide synthesis using Fmoc strategy on Wang resin (0.25-1.0 mmol/g) comprising following steps:

a) Loading of the first Fmoc-protected amino acid on Wang resin and removing Fmoc protecting group by reacting it with Pyrrolidine in Dimethylformamide (DMF)

b) Repetitive cycles of the following:

   i. Coupling of sequential next Fmoc-protected amino acid with amino acid loaded on Wang resin in presences of peptide coupling reagent Ethyl cyanohydroxyiminoacetate and N,N′-Diisopropylcarbodiimide (DIPC);

   ii. Removal of Fmoc protecting group by reacting it with Pyrrolidine in Dimethylformamide (DMF);

   iii. Coupling of sequential next Fmoc-protected amino acid in presences of peptide coupling reagent Ethyl cyanohydroxyiminoacetate and N,N′-Diisopropylcarbodiimide (DIPC);

c) Washing and drying the peptide-resin;

d) Cleavage of the protected peptide intermediate from the resin using various scavengers;

e) Precipitating the crude GLP-1 analogue from the cleavage solution;

f) Purifying the crude GLP-1 analogue by a suitable separation method including counter-ion exchange to form a GLP-1 analogue solution; and

g) Drying the GLP-1 analogue solution to obtain the GLP-1 analogue product.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2020/050869

A. CLASSIFICATION OF SUBJECT MATTER
C07K14/605 Version=2021.01

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)

C07K

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

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

TotalPatent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO2016046753A1, NOVETIDE, LTD. [IL], 31 MARCH 2013 (31/03/2013) claims 1-104	1-7
A	WO2006127948A2, BRISTOL-MYERS SQUIBB COMPANY [US], 30 NOVEMBER 2006 (30/11/2006) claims 1-48	1-7
A	WO2011080102A2, NOVO NORDISK A/S [DK], 7 JULY 2011 (07/07/2011) claims 1-13	1-7
A	WO2008101017A2, INDIANA UNIVERSITY RESEARCH AND TECHNOLOGY CORPORATION [US], 21 AUGUST 2008 (21/08/2008) claims 1-65	1-7

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"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)
"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
"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
"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
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12-02-2021	12-02-2021
Name and mailing address of the ISA/ Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Authorized officer Chanchal Kumar Malav Telephone No. +91-1125300200

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IN2020/050869

Citation	Pub.Date	Family	Pub.Date
WO 2016046753 A1	31-03-2013	US 20190177392 A1	13-06-2019
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