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(54) Title: AN IMPROVED PROCESS FOR THE PREPARATION OF PLECANATIDE

(57) Abstract: The present invention relates to a process for the preparation of Plecanatide, which comprises preparation of three fragments such as Fragment A (7 amino acids), Fragment B (3 amino acids), Fragment D (6 amino acids) and coupling the fragments to provide Plecanatide followed by purification using buffer system comprising Tris hydrochloride (or) Triethylammonium phosphate.



WO 2020/250102 A1

AN IMPROVED PROCESS FOR THE PREPARATION OF PLECANATIDE

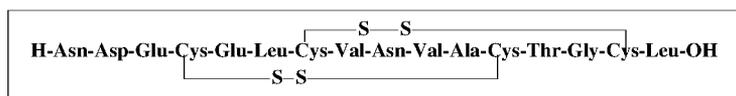
FIELD OF THE INVENTION

5 The present invention relates to a process for the preparation of Plecanatide, which comprises preparation of three Fragments such as Fragment A (7 amino acids), Fragment B (3 amino acids), Fragment D(6 amino acids) and coupling the Fragments to provide Plecanatide followed by purification using buffer system comprising Tris hydrochloride (or) Triethylammonium phosphate.

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BACKGROUND OF THE INVENTION

Plecanatide is an agonist of the guanylate cyclase type-C receptor ("GCC agonists"). Plecanatide is a 16 amino acid peptide with the following chemical name: L-Leucine, L-asparaginy-L- α -aspartyl-L- α -glutamyl-L-cysteinyl-L- α -
 15 glutamyl-L-leucyl-L-cysteinyl-L-valyl-L-asparaginy-L-valyl-L-alanyl-L-cysteinyl-L-threonylglycyl-L-cysteinyl-, cyclic (4 \rightarrow 12),(7 \rightarrow 15)-bis(disulfide). The amino acid sequence for Plecanatide is shown below:



Formula I

20 Plecanatide is approved in the United States under the trade name *TRULANCETM* for treatment of Chronic Idiopathic Constipation (CIC) in adult patients.

Plecanatide is described in US patent No. 7,041,786. The US '786 patent discloses that the peptide including plecanatide are synthesized and purified (>95% purity)
 25 using a published procedure of Klodt, et al., J. Peptide Res. 50:222-230 (1997). The article discloses general solid-phase synthesis, deprotection of Fmoc groups with 20% piperidine in NMP, deprotection of dry peptidyl resins by using a mixture of TFA/EDT/H₂O, formation of disulfide bond by air oxidation and with iodine, acidification with TFA, purification using preparative C¹⁸-HPLC column
 30 (buffer A: 0.1% TFA, buffer B: 0.1% TFA in MeCN/water, 80:20).

US Patent No. US 9,580,471 describes a process for preparation of plecanatide using combination of solid and solution phase synthesis. Further, US '471 describes purification process of peptide on RP-HPLC column followed by desalination by eluting column with aq. alcohol, concentration of obtained
 5 fractions and then precipitation with diethyl ether or MTBE to obtain Plecanatide.

The inventors of the present invention developed an improved process for the preparation of pure Plecanatide, which is simple, cost-effective, and avoids or reduces content of impurities and makes the process robust.

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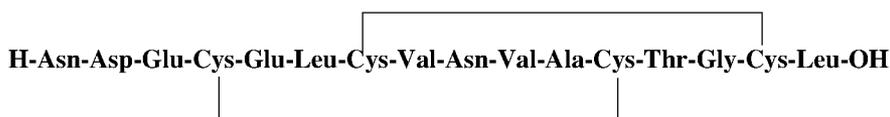
OBJECTIVE OF THE INVENTION

The objective of the present invention is to provide a process for the preparation of Plecanatide. Another objective of the present invention is to provide a process for the purification of Plecanatide.

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SUMMARY OF THE INVENTION

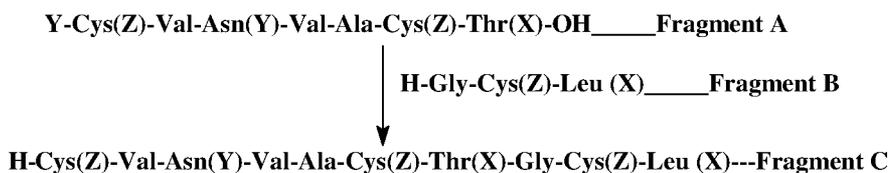
In an aspect, the present invention provides a process for the preparation of Plecanatide of Formula I:



Formula-I

20 which comprises the following steps:

a) coupling of Fragment A with Fragment B to provide Fragment C;



b) coupling of the Fragment C with Fragment D:

25



to provide protected linear peptide;

Y-Asn(Y)-Asp(X)-Glu(X)-Cys(Z)-Glu(X)-Leu-Cys(Z)-Val-Asn(Y)-Val-Ala-Cys(Z)-Thr(X)-Gly-Cys(Z)-Leu (X)

Protected linear peptide

c) deprotecting protected linear peptide to obtain linear 1-16 peptide; and

H-Asn-Asp-Glu-Cys-Glu-Leu-Cys(Z)-Val-Asn-Val-Ala-Cys-Thr-Gly-Cys(Z)-Leu-OH

Linear 1-16 peptide

d) oxidizing the linear 1-16 peptide to obtain Plecanatide.

5

In another aspect, the present invention provides a process for the purification of Plecanatide, which comprises:

- a) purification on preparative HPLC column with Tris hydrochloride (buffer A) and acetonitrile (buffer B) to obtain Plecanatide having purity >95%;
- 10 b) second purification of Plecanatide obtained from step a) on preparative HPLC column with Tris hydrochloride (buffer A) and acetonitrile (buffer B) to obtain pure Plecanatide (>99%); or
- c) purification of Plecanatide obtained from step a) on preparative HPLC column with Triethylammonium phosphate (buffer A) and acetonitrile (buffer B) to obtain
- 15 pure Plecanatide (>99%);
- d) desalting of Plecanatide obtained from step b) or step c) on preparative HPLC column with acetic acid in water and acetonitrile; and
- e) isolation of pure Plecanatide.

20 **BRIEF DESCRIPTION OF ABBREVIATIONS AND DEFINITIONS**

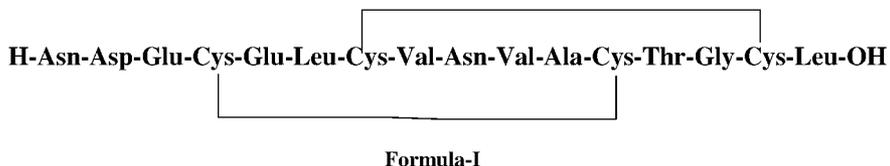
DCC:N,N'-Dicyclohexylcarbodiimide	Tris HCl: 2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride
Dcb: 2,6-Dichlorobenzyl	HBTU:O-(Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Cbz:Carboxybenzyl	BOP: Benzotriazole-1-yl-oxytris(dimethylamino) phosphonium hexafluorophosphate
CTC:Chlorotriyl chloride	HATU:O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Boc: t-Butyloxycarbonyl	Oxyma: Ethyl-2-cyano-2-(hydroxyimino)acetate
DIPEA:Diisopropylethylamine	Fmoc: Fluorenylmethyloxycarbonyl
HOBT: Hydroxy Benzotriazole	TBTU:O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
OtBu:O-t-Butyl	SPPS:Solid phase peptide synthesis

Xan: 9-Xanthenyl	PyBrOP: Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate
DIC: Diisopropylcarbodiimide	BOP-Cl:N,N-Bis-(2-oxo-3-oxazolidinyl)phosphonic dichloride
DTT: Dithiothreitol	WSCDI:1-(Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
IBCF: Iso-butylchloroformate	TNTU:2-(5-Norbornen-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate
IPCF: Isopropyl chloroformate	EEDQ: N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
TIPS/TIS:Triisopropylsilane	PPAA: Propane phosphonic acid anhydride
HOSu: N-Hydroxysuccinimide	TSTU:2-Succinimido-1,1,3,3-tetramethyluronium tetrafluoro borate
HOAt:7-Aza-1-hydroxybenzotriazole	HODhbt:1-Oxo-2-hydroxy dihydrobenzotriazine

DETAILED DESCRIPTION OF THE INVENTION

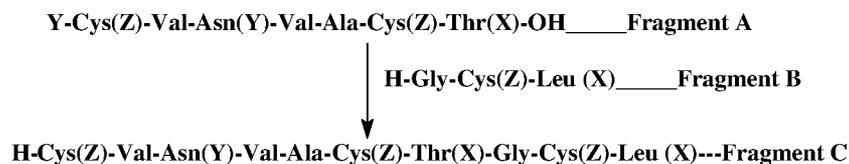
The present invention relates to a process for the preparation of Plecanatide by coupling of two or more protected fragments either by solution phase or SPPS methods.

In an aspect, the present invention provides a process for the preparation of Plecanatide of Formula I:



10 which comprises the following steps:

a) coupling of Fragment A with Fragment B to provide Fragment C;



b) coupling of the Fragment C with Fragment D:



15 to provide protected linear peptide;

Y-Asn(Y)-Asp(X)-Glu(X)-Cys(Z)-Glu(X)-Leu-Cys(Z)-Val-Asn(Y)-Val-Ala-Cys(Z)-Thr(X)-Gly-Cys(Z)-Leu (X)
Protected linear peptide

c) deprotecting the protected linear peptide to obtain linear 1-16 peptide; and

H-Asn-Asp-Glu-Cys-Glu-Leu-Cys(Z)-Val-Asn-Val-Ala-Cys-Thr-Gly-Cys(Z)-Leu-OH

Linear 1-16 peptide

5 d) oxidizing the linear 1-16 peptide to obtain Plecanatide,
 wherein Y represents amino protecting group, X represents carboxyl, phenol and
 alcoholic protecting group, Z-represents thiol protecting group.

The side chain protecting groups for a hydroxyl group in an amino acid include,
 10 but are not limited to, benzyl (Bzl), tert-butyl (tBu), acetamidomethyl (Acm), and
 trityl (Trt), tetrahydropyranyl, Cbz, and 2,5-dichlorobenzyl (Dcb). The suitable
 side chain protecting groups for a thiol group include, but are not limited to,
 acetamidomethyl (Acm), trityl (Trt), Bzl, tBu, tert-butylthio (tButhio), p-
 methoxybenzyl (pMeoBzl), and 4-methoxytrityl (Mmt). The side chain protecting
 15 groups for a carboxylic acid include, but are not limited to benzyl, 2,6-
 dichlorobenzyl, tBu, and cyclohexyl. The side chain protecting groups for amide
 group include, but are not limited to, Xan, Trt; and the side chain protecting
 groups for alpha amino protecting group include, but are not limited to Fmoc.

20 In an embodiment, the Fragment A and Fragment D are prepared in solid phase
 synthesis; Fragment-B is prepared in solution phase; and coupling of the
 fragments is performed in solution phase.

The solid phase synthesis comprises elongation of peptide sequence by coupling
 25 of protected amino acids onto a peptide resin, cleaving amino protecting group,
 coupling of second protected amino acid via peptide linkage to the carboxyl group
 of a second protected amino acid and repeating the cycle till to obtain protected
 peptide intermediates. The resin is selected from the followings: wang resin,
 TentaGel S, chlorotrityl resin (CTC), 4-methytrityl chloride resin, TentaGel TGA,
 30 Rink acid resin, NovaSyn TGT resin, HMPB-AM resin.

The process for the preparation of linear 1-16 peptide involves coupling of Fragment A (7 amino acids) with Fragment B (3 amino acid) in solution phase in the presence of a coupling reagent to produce a Fmoc-protected Decapeptide (Fragment C), which is subjected for Fmoc deprotection in the presence of base to produce a decapeptide. The obtained decapeptide is coupled with a Fragment D (6 amino acids) by solution phase in presence of a coupling agent to give a protected linear 1-16 peptide, which is then deprotected with a cocktail mixture to produce linear 1-16 peptide.

10

In an embodiment, the Fragment A is Fmoc-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(tBu)-OH, the Fragment B is H-Gly-Cys(Acm)-Leu-OtBu, the Fragment C is H-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(tBu)-Gly-Cys(Acm)-Leu-OtBu and the Fragment D is Boc-Asn(Xan)-Asp(OtBu)-Glu(OtBu)-Cys(Trt)-Glu(OtBu)-Leu-OH.

15

The coupling reagent is used in presence or absence of additive. The coupling reagents includes but are not limited to DIC, DCC, HATU, HBTU, TBTU, BOP, BOP-Cl, PyBOP, PyBrOP, IBCF, WSCDI, EEDQ, IPCF, TNTU, PPAA, TSTU, PyClOP, Oxyma pure, TCTU, COMU, HOSu, The additive includes but are not limited to HOBt, HODhbt, HOAt, 6-CF₃-HOBt 6-NO₂-HOBt, Oxyma or mixture thereof. In one embodiment, the coupling agent and additive is of HATU/HOAt.

20

The base used for coupling is organic or inorganic base. The inorganic base comprises potassium carbonate, lithium carbonate, sodium carbonate, sodium bicarbonate, potassium bicarbonate, sodium hydroxide, potassium hydroxide, ammonium hydroxide, and mixtures thereof; the organic base comprises t-butylamine, 4-Dimethylaminopyridine (DMAP), diisopropylamine, DIPEA, triethylamine, dimethylamine, trimethyl amine, isopropyl ethylamine, pyridine, N-methyl morpholine, piperidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and/or mixtures thereof. The solvent may be used for coupling reaction that comprises

25
30

dimethylformamide (DMF), dimethylsulfoxide (DMSO), N-Methyl pyrrolidine (NMP), Dimethylacetamide (DMAC), dichloromethane (DCM), methanol, isopropanol, dichloroethane, 1,4-dioxane, tetrahydrofuran (THF), 2-methyl tetrahydrofuran, ethyl acetate, acetonitrile, acetone or mixtures thereof.

5

The base used for Fmoc deprotection includes but are not limited to t-butylamine, piperidine, diethyl amine, DBU, piperazine, pyrrolidine, derivatives of piperadine, piperazine and pyrrolidine in presence of solvent comprises alcohol, amide or ether. In one embodiment, the base and solvent used for Fmoc deprotection are t-

10 butylamine and DMF.

In an embodiment, the cleavage and deblocking is performed in presence of cocktail mixture i.e. TFA: TIPS: DTT: solvent (or) TFA: TIPS: DTT: water: solvent (or) TFA: TIS: solvent. The solvent comprises water, dimethyl sulfide

15 (DMS), alcohol solvent selected form methanol, ethanol, 1-propanol, isopropanol, n-butanol; chlorinated solvent is selected form dichloromethane, dichloroethane, chlorobenzene; ether solvent selected form diethyl ester, THF, diisopropylether and/or combination thereof. The additional cocktail reagents is selected from 1,2-ethanedithiol (EDT), DMS, thioanisole, phenol, anisole etc. In

20 an embodiment, the present invention provides deblocking of protected linear peptide using a mixture of TFA: TIPS: DTT: water: DMS.

In one embodiment, the cleavage and deblocking is performed in presence of cocktail mixture is Cocktail of 84% TFA (Trifluoroacetic acid): 5% TIPS

25 (Triisopropylsilane): 5% H₂O: 5% DTT (Dithiothreitol): 5% DMS.

After completion of cleavage and deprotection, the linear peptide or its TFA salt is precipitated by using suitable solvent, which is selected from ether solvent like diethyl ether, diisopropyl ether, methyl tertiary butyl ether, ethyl acetate and

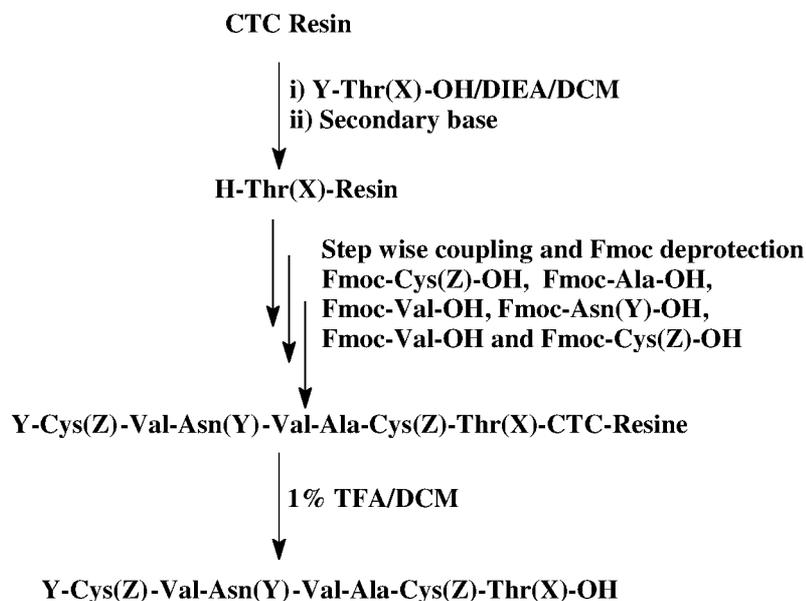
30 tetrahydrofuran.

After completion of cleavage and deprotection, the linear peptide is oxidized with molecular oxygen and/or oxidizing agent comprises hydrogen peroxide, dimethyl sulfoxide in presence or absence of solvent, which is selected from water, nitrile, alcoholic solvent or combination thereof. The obtained peptide optionally treated
 5 with Iodine in a solvent, which is selected from nitrile to provide dicyclised peptide. The oxidation may be performed at a pH range from 3 to 4.

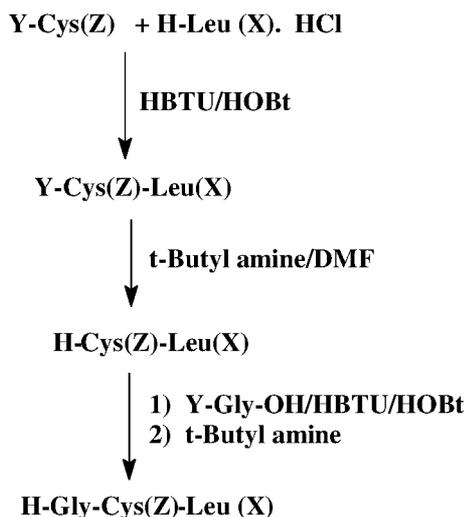
The oxidation of open-chain peptide containing two free and/or two protected sulfhydryl groups with hydrogen peroxide. The protected/non-protected linear
 10 peptide may be subjected for pH adjustment of 3 to 4 or 6 to 7 by using acid/base followed by air oxidation using hydrogen peroxide and compressed air to afford mono-cyclized 1-16 peptide. The mono-cyclized peptide may be further treated with iodine in a solvent comprising nitrile solvent to provide disulfide 1-16 peptide i.e. Plecanatide.

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In another embodiment, the present invention provides a process for the preparation of Fragment A is shown in the below Scheme-1.

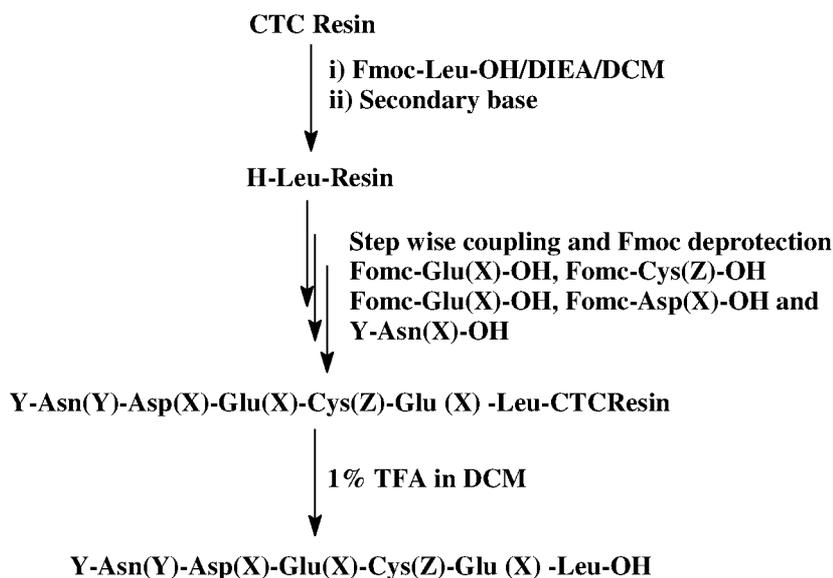
**Scheme-1**

In yet another embodiment, the present invention provides a process for the
 20 preparation of Fragment B is shown in the below Scheme-2.



Scheme-2

In yet another embodiment, the present invention provides a process for the process for the preparation of Fragment D is shown in the below Scheme-3.



Scheme-3

- 5 The crude Plecanatide obtained by the process of present invention can be purified using preparative column chromatography or reverse phase column chromatography (RPHPLC).

In another aspect of the present invention, there is provided a process for the
 10 purification of Plecanatide of Formula I, which comprises:

- 5 a) purification on preparative HPLC column with Tris hydrochloride (buffer A) and acetonitrile (buffer B) to obtain Plecanatide having purity >95%;
- b) second purification of Plecanatide obtained from step a) on preparative HPLC column with Tris hydrochloride (buffer A) and acetonitrile (buffer B) to obtain pure Plecanatide (>99%); or
- 10 c) purification of Plecanatide obtained from step a) on preparative HPLC column with Triethylammonium phosphate (buffer A) and acetonitrile (buffer B) to obtain pure Plecanatide (>99%);
- d) desalting of Plecanatide obtained from step b) or step c) on preparative HPLC column with acetic acid in water and acetonitrile; and
- e) isolation of pure Plecanatide.

The present application relates to a purification process of crude Plecanatide or a reaction mixture containing Plecanatide comprising preparative reverse phase column chromatography. The column in preparative HPLC may be packed with reverse phase C18 hybrid silica. Suitable silica gel types, which can be selected from, but are not limited to the following silica gel sorbents: Kromasil™C18 100 - 16, Kromasil™C18 100 - 10, Kromasil™C8 100 - 16, Kromasil™C4 100 - 16, Kromasil™ Phenyl 100 - 10, Kromasil™ CI 8 Eternity 100 - 5, Kromasil™ C4 Eternity 100 - 5, Chromatorex™ CI 8 SMB 100-15 HE, Chromatorex™ C8 SMB 100-15 HE, Chromatorex™ C4 SMB 100-15 HE, Daisopak™ SP 120-15 ODS-AP, Daisopak™ SP 120-10-C4-Bio, Daisopak™ SP 200-10-C4-Bio, Zeosphere™ C18 100-15, Zeosphere™ C8 100-15, Zeosphere™ C4 100-15, SepTech ST 150-10 C18, Luna C18 100-10, Gemini C18 110-10, YMC Triart C18 120-5 and YMC Triart C8 200-10.

The column is packed with silica using Tris HCl having pH of 6 to 8 or pH of 7 and nitrile solvent such as acetonitrile, as buffers to obtain fractions having purity of about 94% by HPLC. The obtained fractions may be re-purified using column by packing with reverse phase C18 hybrid silica using Tris HCl.

The Plecanatide obtained according to the present invention has purity greater than 95% and preferably greater than 99% (by HPLC). The yields of the Plecanatide obtained according to present invention are consistent.

- 5 The Plecanatide, as produced by any one of the reaction conditions and purification process described above, undergoes desalting process by ion-exchange/preparative column chromatography. The resultant pure Plecanatide is subjected for precipitation, lyophilization or spray drying techniques to provide amorphous or crystalline Plecanatide.

10

Having described the invention with reference to certain aspects and embodiments, which will become apparent to one skilled in the art from consideration of the specification. The invention is further defined by reference to the following examples. It will be apparent to those skilled in the art that many
15 modifications, both to materials and methods, may be practiced without departing from the scope of the invention.

EXAMPLES

EXAMPLE 1: Synthesis of H-Gly-Cys(Acm)-Leu-Otbu (Fragment B)

Step –I: Synthesis of H-Cys(Acm)-Lue-Otbu.

- 20 H-Leu-(Otbu).HCl (356.27gm,1.1eq), HOBT(222.97gm,1.0eq) were added to a solution of Fmoc-Cys(Acm)-OH (600.0 g, 1.0eq) in DMF (3.0L) and then cooled to 5-10°C. HBTU (603.9gm, 1.1 eq) and DIPEA(883.4 ml, 3.5eq) were added to the reaction mass. After completion of the reaction, the product was extracted with ethyl acetate (6.0L) and then washed with HCl, 5% aq sodium bicarbonate
25 soln, 10%NaCl solution and water. The organic layer was collected, filtered and the filtrate was concentrated to obtain Fmoc-protected dipeptide. The obtained product was proceeded for next step without any further purification. Yield: 945 g Fmoc-Cys (Acm)-Leu-Otbu (945 g, 1eq) was taken in flask containing DMF (1.89 L) and cooled the solution to 5-10°C. Tertiary butyl amine (255 ml 1.5eq) was
30 added slowly to the solution and stirred for 15min. After completion of reaction, the reaction mass was cooled to 5-10°C. Water and 1N HCl were added to the

solution and the obtained aqueous solution was washed with hexane. The pH of product was adjusted to 8 to 8.5 with saturated sodium bicarbonate solution and the product was extracted with ethyl acetate. The ethyl acetate layer was washed with 10% NaCl solution and water and then filtered. The obtained filtrate was
5 evaporated completely to obtained light yellow thick residue H-Cys(Acm)-Leu-Otbu. Yield: 450 g.

Step –II: Synthesis of H-Gly-Cys (Acm)-Lue-Otbu.

H-Cys (Acm)-Leu-Otbu (450.0 g, 1.1eq) was taken in flask containing DMF (1.68 L). Fmoc-Gly-OH (336.45g, 1.0eq), HOBt (174.2g, 1.0eq) were added to the
10 solution and then cooled to 5-10°C. HBTU (472g, 1.1eq) and DIPEA (414.15ml, 2.1eq) were added and stirred. After completion of the reaction, ethyl acetate was added to the above reaction mass and washed with pre-cooled 0.5N HCl, 5% aqueous NaHCO₃ solution (pre-cooled), 10%NaCl solution and water. The resultant organic layer was evaporated completely to give Fmoc-Gly-Cys (Acm)-
15 Leu-Otbu. Yield: 825 g.

Fmoc-Gly-Cys(Acm)-Leu-Otbu (825g, 1eq) was taken in flask containing THF (1650ml) and cooled to 5-10° C. Tertiary butyl amine (338 ml 2.5eq) was added slowly to the solution. After completion of reaction, the solution was cooled to 5-
20 10°C and then water (2.48L) was added to the reaction mass. The aqueous reaction mass was washed with hexane, 50% ethyl acetate in hexane, then the product was extracted with dichloromethane. The dichloromethane layer was washed with 10%NaCl solution and water. The (collected) organic layer was dried with anhydrous sodium sulphate and then filtered. The obtained filtrate was
25 evaporated completely to obtain H-Gly-Cys (Acm)-Leu-Otbu as light yellow semi solid. Yield: 392 g; HPLC purity: ~92%.

EXAMPLE 2: Fmoc-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(Otbu)-2CTC. (Fragment-A)

Step A: CTC resin (750gm) was taken in a SPPS reactor, 7.5L of dry
30 dichloromethane (DCM) was added and allowed it to swell for 20min and drained.

Step B: A solution of Fmoc-Thr(otbu)-OH (954gm, 2eq) and DIEA (627.6ml, 3eq) in dry dichloromethane (3.75L) were added to the resin at step A and stirred for at room temperature and drained.

The resin was then capped with DIEA (1%) solution in DCM: methanol (1:1) and
5 then drained. Thereafter, washed the resin with one bed volume of DMF (2 times), DCM (2 times) and MTBE (2 times), isolated and dried. **Yield: 1150gm**

The above resin was deblocked with 20% piperidine in DMF and washed with DMF (2times), IPA (2 times) and DMF (2 times).

Step C: To a solution of Fmoc-Cys (Trt)-OH (808g, 1.5eq.) and HOBT (212.5g, 1.5eq) in DMF, DIC (323ml, 2.25eq) was added. The obtained reaction mixture
10 was added to the resin in Step B and stirred. After completion of the reaction the resin was drained and washed with DMF (2 times) and then resin was deblocked with 20% piperidine in DMF and then washed with DMF, IPA and DMF.

Step-D: Followed by sequential coupling of Fmoc-Ala-OH, Fmoc-Val-OH,
15 Fmoc-Asn (Trt)-OH, Fmoc-Val-OH similar to the procedure described in step-C.

Step E: To a solution of Fmoc-Cys (acm)-OH (572g, 1.5eq.) and HOBT (212.5g, 1.5eq) in DMF, DIC (323ml, 2.25eq) was added. The obtained reaction mixture
20 was added to the resin in Step D and stirred. After completion of the reaction, the resin was drained and washed with DMF (2 times) DCM (2 times) and MTBE (2 times). It was isolated and dried to give Fmoc-Cys (Acm)-Val-Asn (Trt)-Val-Ala-Cys (Trt)-Thr(otbu)-2CTC Resin. Yield: 2.17 kg.

Step F: Selective cleavage of 2-chloro trityl resin from the Fmoc-Cys(acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(otbu)-2CTC Resin was performed with a mixture
25 1%TFA in dichloromethane and then above peptidyl resin was taken in SPPS reactor and treated with a solution of 1% TFA in DCM and drained. The filtrate was immediately neutralized with precooled saturated NaHCO₃ Solution to precipitate the product. The same process was repeated 3 more times and dried to obtain off-white solid. The obtained solid was further purified by treating with
30 MTBE (2ml/g of product) to give Fmoc-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(tbu)-OH. Yield: 1133gm; Purity: 94.29%.

EXAMPLE-3: Synthesis of Boc-Asn(Xan)-Asp(Otbu)-Glu(Otbu)-Cys(Trt)-Glu(Otbu)-Leu-2CTC (Fragment D)

Step A: CTC resin (445gm) was taken in a SPPS reactor, 4.45L of dry dichloromethane was added and allowed it to swell for 20min and drained.

- 5 **Step B:** A solution of Fmoc-Leu-OH (525gm, 2eq) and DIEA (388.6ml, 3eq) in dry dichloromethane (2.22L) was added to the resin at step A and stirred at room temperature and drained.

The resin was then capped with DIEA (1%) solution in DCM: methanol (1:1). Thereafter, washed the resin with one bed volume of DMF (2 times), DCM (2
10 times) and MTBE (2 times), isolated and dried. Yield: 700gm.

The above resin was deblocked with 20% piperidine in DMF for 10 and 15 minutes and washed with of DMF (2times), IPA (2 times) and DMF (2 times).

- 15 **Step C:** To a solution of Fmoc-Glu(Otbu)-OH (553gm, 2.0eq.) and HOBT (200.5gm, 2.0eq) in DMF, DIC (305ml, 3.0 eq) was added. It was added to the resin in Step B. After completion of the reaction, the resin was drained and washed with DMF.

The above resin was deblocked with 20% piperidine in DMF for 10 and 15 minutes and washed with of DMF (2times), IPA (2 times) and DMF (2 times).

- 20 **Step D:** Sequential coupling of Fmoc-Cys(Trt)-OH, Fmoc-Glu(Otbu)-OH, Fmoc-Asp(Otbu)-OH, Fmoc-Asp(Otbu)-OH similar to the procedure of step-C.

- Step-E:** To a solution of Boc-Asn(xan)-OH (537gm, 2.0eq.) and HOBT (200.5gm, 2.0eq) in DMF, DIC (305ml, 3.0eq) were added and stirred. It was added to the resin in Step F and stirred. After completion of the reaction, the resin was drained and washed with DMF (2 times), DCM (2 times) and MTBE (2
25 times). It was isolated and dried to give Boc-Asn(Xan)-Asp(Otbu)-Glu(Otbu)-Cys(Trt)-Glu(Otbu)-Leu-2CTC resin. Yield: 1.37kg.

Step H: Selective cleavage of 2-chloro trityl resin from the Boc-Asn(Xan)-Asp(Otbu)-Glu(Otbu)-Cys(Trt)-Glu(Otbu)-Leu-2CTC resin was performed with a mixture 1%TFA in dichloromethane.

- 30 The above peptidyl resin was taken in SPPS reactor and treated with a solution of 1% TFA in DCM. The filtrate was neutralized with precooled saturated NaHCO₃

solution. The same process was repeated 3 more times, the collected organic solution was washed with water, dried with sodium sulphate and evaporated to obtain off-white solid. The obtained solid was further purified by treating with MTBE (2ml/g of product) to give Boc-Asn(Xan)-Asp(Otbu)-Glu(Otbu)-Cys(Trt)-
5 Glu(Otbu)-Leu-OH. Yield: 720g; Purity: 90.74%.

EXAMPLE 4: Synthesis of H-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(tbu)-Gly-Cys(Acm)-Leu-Otbu.

Fmoc-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(tbu)-OH (Fragment-A)
10 (500.0 g, 1.0eq) was taken in a round bottom flask containing DMF (2.5L). H-Gly-Cys (Acm)-Leu-Otbu (Fragment B) (209g, 1.5eq) was added to the reaction mass and cooled to -10 to -15°C. HOAT (50g, 1.1eq) and HATU (151.75g , 1.2 eq) were added to reaction mass and then DIPEA (116ml, 2eq) was added drop wise while stirring at -10 to-15°C. After completion of the reaction, methanol
15 (15.0L) was added to the above reaction mass (solid formation was observed), then pH of the reaction mixture was adjusted with 1N HCl up to pH 3, stirred for one hour, filtered and dried to give decapeptide. Yield: 700g.

The above obtained decapeptide (700g, 1.0eq) was taken in a round bottom flask containing DMF (4.2 L) and cooled the solution to 5-10°C. Tertiary butyl amine
20 (77.30ml 2.0eq) was added and stirred the reaction mass for 15min at 5-10°C and then stirred at R.T. After completion of the reaction, methanol (25.20L) was added to precipitate the product. The obtained solid was filtered and dried to give NH₂-Cys (Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(otbu)- Gly-Cys (Acm)-Lue-Otbu. Yield: 437g; HPLC purity: ~97%

25

EXAMPLE 5: Synthesis of Boc-Asn(Xan)-Asp(Otbu)-Glu(Otbu)-Cys(Trt)-Glu(Otbu)-Leu-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(otbu)-Gly-Cys(Acm)-Leu-Otbu.

NH₂-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(otbu)-Gly-Cys-(Acm)-Lue-
30 Otbu, (435.0g,1.0eq) and Boc-Asn(Xan)-Asp(Otbu)-Glu(Otbu)-Cys(Trt)-Glu(Otbu)-Leu-OH (Fragment D),(365.0 g, 0.9eq) were taken in a round bottom

flask containing DMF (2.61 L). The solution obtained was cooled to -10 to -15°C and then HOAT (42.0g, 1.2eq) and HATU (118g, 1.2eq) were added. DIPEA (90 ml, 2.0eq) was added to the reaction mass and stirred at -10 to -15°C. Methanol (13.0L) was added to the above reaction mass to precipitate the product. The
5 obtained solid was filtered and dried to give Boc-Asn(Xan)-Asp(Otbu)-Glu(Otbu)-Cys(Trt)-Glu(Otbu)-Leu-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(otbu)-Gly-Cys(Acm)-Leu-Otbu as off-white solid. Yield: 750g.

EXAMPLE 6: Synthesis of H-Asn-Asp-Glu-Cys-Glu-Leu-Cys(Acm)-Val-Asn-Val-Ala-Cys-Thr-Gly-Cys(Acm)-Leu-OH.

Protected peptide (500.0 g) obtained in example 5 was treated with a pre-cooled solution of 84% TFA (4200 ml), 5% TIPS (250 ml), 5% H₂O (250 ml), 5% DTT (250 gm), 1%DMS (50ml) for 2 hrs at R.T. The product was precipitated by the addition of reaction mass to the pre-cooled MTBE, filtered the product under
15 nitrogen and washed with MTBE and dried. Yield: 310.0g; Purity: 73 %.

EXAMPLE 7: PREPARATION OF CRUDE PLECANATIDE

Linear 1-16 peptide obtained from example 6 was dissolved in degassed 0.015 M ammonium hydroxide solution at a concentration of 1g /0.75 L, pH was adjusted
20 between 8.5 to 9.0 by using ammonia solution. After dissolution of compound, H₂O₂ (200 µl / g) was added. After completion of oxidation, the pH was adjusted between 3 to 4 by 1N HCl to obtain mono cyclized 1-16 peptide solution and then solution was treated with 5% iodine in acetonitrile, till the yellow color persist. The reaction mixture with excess of iodine was quenched with 0.1M aqueous
25 ascorbic acid solution and the pH was adjusted between 6.5 and 7 by using ammonia solution. The solution was filtered through 2.4 micron. The obtained filtrate was used as such for next stage purification.

EXAMPLE 8: PURIFICATION OF PLECANATIDE

30 Stage 1: Crude Plecanatide solution obtained from example 7 was purified on preparative HPLC, column was packed with reverse phase C18 hybrid silica using

Tris HCl pH 7(as buffer A) and 100% acetonitrile (as buffer B).The fractions were collected and purity of fractions were monitored by analytical HPLC. Fractions containing > 94% pure Plecanatide were pooled as main pool; and fractions not meeting the pooling criteria were re-processed in a similar manner.

5 Stage 2: The main pool obtained from stage 1 purification were diluted with equal amount of purified water and re-purified on preparative HPLC, column was packed with reverse phase C18 hybrid silica using Tris HCl pH 7(as buffer A) and 100% acetonitrile (as buffer B).The fractions were collected and purity of fractions were monitored by analytical HPLC. Fractions containing > 98.5% pure
10 Plecanatide were pooled as main pool for desalting. **(OR)**

The main pool obtained from stage-1 purification were diluted with equal amount of purified water and re-purified on preparative HPLC, column was packed with reverse phase C18 hybrid silica using TEAP (as buffer A) and acetonitrile (as buffer B).The fractions were collected and purity of fractions were monitored by
15 analytical HPLC. Fractions containing > 98.5% pure Plecanatide were pooled as main pool for desalting.

EXAMPLE 9: DE-SALTING AND LYOPHILIZATION

The main pool obtained from the purification were diluted with equal amount of
20 purified water and loaded on preparative HPLC, column was packed with reverse phase C18 hybrid silica.

De-salting was done by passing 5 void volume of 0.1% acetic acid in purified water followed by elution of product from the column by using 30% acetonitrile (HPLC grade) in purified water containing 0.1% acetic acid. The fractions were
25 collected and purity of fractions were monitored by analytical HPLC.

The fractions containing pure Plecanatide (>98.5%) were pooled; the organic modifier was removed under reduced pressure and filtered through 0.2 micron filter. The resulting peptide solution was freeze-dried to isolate Plecanatide.

After completion of lyophilization cycle, the compound was unloaded and
30 dissolved in purified water at a concentration of 70 g /L, filtered through 0.2

micron filter. The resulting peptide solution was freeze-dried to obtain white solid lyophilized powder as Plecanatide. **Purity: 99.1%**

EXAMPLE 10: PREPARATION OF MONO CYCLIZED PLECANATIDE

5 Linear 1-16 peptide obtained from example 6 was dissolved in degassed 0.015 M ammonium hydroxide solution at a concentration of 1g /0.75 L, the pH was adjusted between 8.5 to 9.0 by using ammonia solution. After dissolution of compound, H₂O₂ (200µl / g) was added and stirred for 30 minutes. The progress of oxidation was monitored by analytical reverse phase HPLC & Ellman's test.
10 After completion of oxidation, the pH was adjusted between 6.5 and 7 by using 1N HCl to obtain mono cyclized 1-16 peptide solution.

EXAMPLE 11: PURIFICATION OF MONO CYCLIZED PLECANATIDE

Mono cyclized solution obtained from example 10 was purified on preparative
15 HPLC, column packed with reverse phase C18 hybrid silica using Tris HCl pH 7 (as buffer A) and 100% acetonitrile (as buffer B). The fractions were collected and purity were monitored by analytical HPLC. Fractions containing > 95% pure Plecanatide were pooled as main pool; and fractions not meeting the pooling criteria were re-processed in a similar manner.

20

EXAMPLE 12: PREPARATION OF CRUDE PLECANATIDE

The resultant purified solution was diluted with equal amount of water, adjusted pH 3.5 with 1N HCl and treated with 5% iodine in acetonitrile, till the yellow color persist and the reaction mass was stirred for two hours.
25 The completion of oxidation was monitored by analytical reverse phase HPLC; quenched the excess iodine with 0.1 M aqueous ascorbic acid solution and then pH was adjusted between 6.5 and 7 by using ammonia solution. The solution was filtered through 2.4 micron filter and used as such for next stage purification.

30 **EXAMPLE 13: PURIFICATION OF PLECANATIDE**

The main pool obtained from stage 1 purification were purified on preparative HPLC, column was packed with reverse phase C18 hybrid silica using Tris HCl pH 7 (buffer A) and 100% acetonitrile (buffer B). The fractions were collected and purity of fractions were monitored by analytical HPLC. Fractions containing
5 > 98.5% pure Plecanatide were pooled as main pool for desalting. **(OR)**

The main pool obtained from stage 1 purification further purified on preparative HPLC, the column was packed with reverse phase C18 hybrid silica using TEAP (as buffer A) and acetonitrile (as buffer B). Fractions containing > 98.5% pure Plecanatide were pooled as main pool for desalting.

10

EXAMPLE 14: DE-SALTING AND LYOPHILIZATION

The main pool obtained from the purification were diluted with equal amount of purified water and loaded on preparative HPLC, column packed with reverse phase C18 hybrid silica.

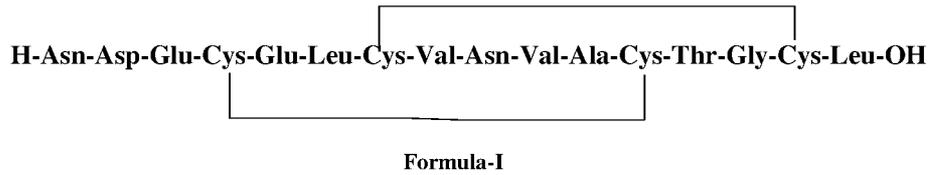
15 De-salting was done by passing 5 void volume of 0.1% acetic acid in purified water followed by elution of product from the column by using 30% acetonitrile (HPLC grade) in purified water containing 0.1% acetic acid. The fractions were collected and purity of fractions were monitored by analytical HPLC. The fractions containing pure Plecanatide (>98.5%) were pooled; the organic modifier
20 was removed under reduced pressure and filtered through 0.2 micron filter. The resulting peptide solution was freeze-dried to isolate Plecanatide.

After completion of lyophilization cycle, unload the compound and dissolve in purified water at a concentration of 70 g /L, filtered through 0.2 micron filter. The resulting peptide solution was freeze-dried to obtain white solid lyophilized
25 powder as Plecanatide.

Purity: 99% by HPLC

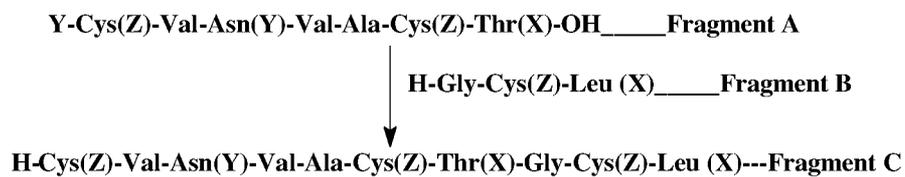
WE CLAIM:

1. A process for the preparation of Plecanatide of Formula I:



which comprises the following steps:

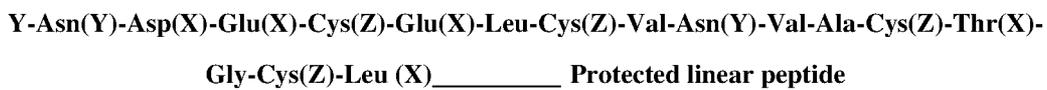
5 a) coupling of Fragment A with Fragment B to provide Fragment C;



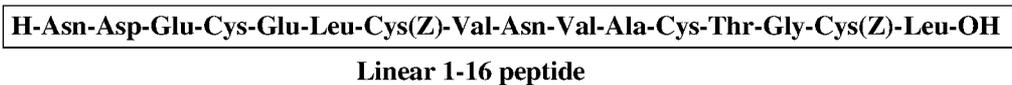
b) coupling of the Fragment C with Fragment D:



10 to provide protected linear peptide:



c) deprotecting protected linear peptide to obtain linear 1-16 peptide; and



15 d) oxidizing the linear 1-16 peptide to obtain Plecanatide.

2. The process as claimed in claim 1, wherein the Fragment A and Fragment D are prepared in solid phase synthesis, and Fragment-B is prepared in solution phase.

20 3. The process as claimed in claim 1, wherein the X represents carboxyl, phenol and alcoholic protecting group comprises Bzl, tBu, Acn, Trt, tetrahydropyranyl, Cbz, and Dcb, 2,6-dichlorobenzyl, cyclohexyl, and Fmoc; the Y represents amino protecting group comprises Xan, Trt; and the Z-represents thiol protecting comprises Acn, Trt, Bzl, tBu, tButhio, pMeoBzl, and Mmt.

4. The process as claimed in claim 1, wherein the coupling is conducted in presence of coupling reagent comprises DIC, DCC, HATU, HBTU, TBTU, BOP, BOP-Cl, PyBOP, PyBrOP, IBCF, IPCF, TNTU, PPAA, TSTU and PyClOP.
- 5 5. The process as claimed in claim 4, wherein the coupling reagent is HATU.
6. The process as claimed in claim 1, wherein the coupling of step a) produces is further comprises Fmoc deprotection with a tertiary-butylamine in presence of DMF.
- 10 7. The process as claimed in claim 1, wherein the deprotection of step c) is conducted in presence of cocktail mixture comprising TFA: TIPS: DTT: water: DMS or TFA: TIS: DMS.
- 15 8. The process as claimed in claim 7, wherein the cocktail is 84% TFA: 5% TIPS: 5% H₂O: 5% DTT: 5% DMS.
9. The process as claimed in claim 1, wherein the oxidation is conducted with oxidizing agent comprises H₂O₂.
- 20 10. The process as claimed in claim 1, wherein the Fragment A is Fmoc-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(tBu)-OH, the Fragment B is H-Gly-Cys(Acm)-Leu-OtBu, the Fragment C is H-Cys(Acm)-Val-Asn(Trt)-Val-Ala-Cys(Trt)-Thr(tBu)-Gly-Cys(Acm)-Leu-OtBu and the Fragment D is Boc-
- 25 Asn(Xan)-Asp(OtBu)-Glu(OtBu)-Cys(Trt)-Glu(OtBu)-Leu-OH.
11. A process for the purification of Plecanatide of Formula I, which comprises:
- a) purification on preparative HPLC column with Tris hydrochloride (buffer A) and acetonitrile (buffer B) to obtain Plecanatide having purity >95%;

- b) second purification of Plecanatide obtained from step a) on preparative HPLC column with Tris hydrochloride (buffer A) and acetonitrile (buffer B) to obtain pure Plecanatide (>99%); or
- 5 c) purification of Plecanatide obtained from step a) on preparative HPLC column with Triethylammonium phosphate (buffer A) and acetonitrile (buffer B) to obtain pure Plecanatide (>99%);
- d) desalting of Plecanatide obtained from step b) or step c) on preparative HPLC column with acetic acid in water and acetonitrile; and
- e) isolation of pure Plecanatide.

10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2020/055350

A. CLASSIFICATION OF SUBJECT MATTER
C07K7/08, C07K1/02, C07K1/04, C07K1/06 Version=2020.01

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.
Y	WO 2012/118972 A2 (SYNERGY PHARMACEUTICALS INC [US]) 07 SEPTEMBER 2012 (07.09.2012) *Paras [51-54]; schemes 1-4; pages 13-18*	1-11
Y	CN 108003222 A (SINOPEP ALLSINO BIO PHARMACEUTICAL CO LTD [CN]) 08 MAY 2018 (08.05.2018) *paras [0031-0047, 0216]* [FAMILY: NONE]	1-11
Y	WO 2018/205401 A1 (HYBIO PHARMACEUTICAL CO LTD [CN]) 15 NOVEMBER 2018 (15.11.2018) *abstract; paras [0012-0065]; claims*	1-11
Y	WO 2014/197720 A2 (SYNERGY PHARMACEUTICALS INC [US]) 11 DECEMBER 2014 (11.12.2014) *Paras [112-115]; pages 13-17, 21-24; examples 1-5 and 8*	1-11

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

23-10-2020

Date of mailing of the international search report

23-10-2020

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2020/055350

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
The present application comprises the following inventions, which are not so linked as to form a single general inventive concept, as required by Rule 13.1 of PCT:

Group I: Claims 1-10

These claims relate to a process for the preparation of plecanatide of formula-I.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

Continuation of Observations where unity of invention is lacking (Box III)

Group II: Claim 11

The claim 11 relates to a process for the purification of plecanatide of Formula-I.

These groups of inventions are not so linked as to form a single general inventive concept as required under Rule 13.1 of PCT for the following reasons.

The special technical feature is an essential feature common to all embodiments of the claimed invention (and responsible for the inventive effect) and which defines a contribution with each of the claimed inventions over prior art (Rule 13.2 of PCT). Upon prior art search, it was found that the plecanatide, is already known from the prior arts. The only linking feature in the group of inventions is the peptide plecanatide, which is already known. Hence, here it is considered that the common technical link in the above-mentioned groups is not novel. Therefore, the above-mentioned both groups lack a common feature which could be regarded as the special technical feature providing unity to the claims. Consequently, the application may be objected for lacking unity 'a posteriori'.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/IB2020/055350

Citation	Pub.Date	Family	Pub.Date
WO 2012118972 A2	07-09-2012	US 9580471 B2	28-02-2017
WO 2018/205401 A1	15-11-2018	CN 108864254 A	23-11-2018
WO 2014197720 A2	11-12-2014	US 10011637 B2	03-07-2018
		EP 3004138 A2	13-04-2016