PROCESS FOR THE PREPARATION OF PEPTIDES

Technical field

The present invention relates to an improved process for the preparation of N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic(1→6)-disulfide of formula (1) using solid phase Fmoc-chemistry.

Background and prior art references of the invention

US Patent No.5318899 describes N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic (1→6)-disulfide of the formula (1) as a therapeutic agent for the treatment of, and prevention of, platelet-associated ischemic disorders. It binds to the platelet receptor glycoprotein (GP) of human platelets and inhibits platelet aggregation. Platelet aggregation is mediated by GP complex on the surface of the platelet membrane. It exists on the surface of unstimulated platelets in an inactive form. When platelets are activated by adhesion and the physiological agonists, the GP also becomes activated such that it becomes a receptor for fibrinogen, von Willebrand Factor (vWF), and fibronectin. However, it is the binding of fibrinogen and/or vWF that is believed to be principally responsible for platelet aggregation and thrombus formation *in vivo*. This teaches that substances which specifically inhibit the binding of fibrinogen or vWF to GP, inhibit platelet aggregation and could be candidates for inhibiting thrombus formation *in vivo* (Eric J. Topol, Tatiana V. Byzova, Edward F. Plow and The Lancet; Vol 353; January16, 1999; pg 227-231). This article describes the compound having platelet aggregation inhibition activity but does not teach the method to synthesize the molecule.

Antagonists of platelet glycoprotein IIb/IIIa have an approved role in reducing the extent of thrombotic complications leading to myocardial damage during percutaneous coronary interventions (PCI).

Compound of formula (1) is a disulphide looped cyclic heptapeptide containing six amino acids and one mercaptopropionyl(desamino cysteinyl) residue. The disulfide bridge is formed between the cysteine amide and the mercaptopropionyl moieties. It is known to be produced by solution-phase peptide synthesis and purified by preparative

reverse phase liquid chromatography and lyophilized (www.fda.gov/cder/foi/label/1998/20718lbl.pdf).

In terms of peptide synthesis methodology, two major synthetic techniques dominate current practice. These are synthesis in solution (homogeneous phase) and synthesis on solid phase (heterogeneous phase). But solution phase route is more cumbersome as compared to the solid phase route as after each coupling the peptide formed has to be isolated, whereas in the solid phase synthesis, the excess reagents and by-products are washed off by simple filtration. In both, the desired peptide compound is prepared by the step-wise addition of amino acid moieties to a building peptide chain.

US patents 5958732 and 5318899 claim about recombinant techniques to synthesize peptides like N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic(1→6)-disulfide of the formula (1). The peptide obtained by this recombinant process is modified by solution phase synthesis for conversion of lysine residue to homoarginine residue. These patent documents also claim solid phase synthesis using Boc chemistry and the subject matter of these patents is fundamentally different from the present invention.

As compared to Boc-chemistry, Fmoc-chemistry based synthesis utilizes a mild procedure and because of the base lability of Fmoc group, acid-labile side-chain protecting groups are employed providing orthogonal protection. The rationale for use of protecting groups is that the energy of breaking a bond of a protecting group is lower than any other group.

Patents US5686566, US5686567, US5686569, US5686570 and US5756451 deal with different PAI's in their salt or other forms of the compound of formula (1) but do not teach the process for its preparation using Fmoc solid phase synthesis.

Likewise, patents US5759999, US5786333, US5770564, US5807825, US5807828, US5843897, US5968902, and US5935926 describe the method of treating platelet-associated disorders and the process for the preparation of peptide amide of formula (1) using boc chemistry.

Patents US5344783 and US5851839 deal with methods for selecting and identifying Platelet Aggregation Inhibitors (PAI) and disclose boc chemistry for the preparation of peptide amide of formula (1).

US patent 5780595 claims antibodies specific to PAI's and also discloses boc chemistry for the preparation of the peptide amide of formula (1).

The Fmoc route of synthesis of various other peptides is well-known in prior art and several documents are available for their preparation. However there is a definite need to develop a process for the preparation of compound of formula (1) which is economical, involves minimal steps and also eco-friendly.

As explained earlier, Fmoc-chemistry based synthesis utilises a mild procedure and because of the base lability of Fmoc group, acid-labile side-chain protecting groups are employed providing orthogonal protection. The protecting groups used in Fmoc chemistry are based on the tert-butyl moiety: tert-butyl ethers for Ser, Thr, tert-butyl esters for Asp, Glu and Boc for Lys, His. The trt and acm groups have been used for the protection of Cys. Theguanidine group of Arg and Har is protected by Mtr, Pmc or Pbf. Most of the Fmoc-amino acids derivatives are commercially available. However, a problem exists in the art for the preparation of some amino acid analogs like peptides containing homoarginine as well as cyclic peptide compounds based on disulfide links, because separate operations are required before purifying the end product, which increases expense and may affect final product purity and yield. Fmoc-homoarginine residue if purchased commercially for use in the assembly of the chain becomes expensive. Alternatively in the peptide assembly, the Har unit is built by guanylation of the lysine residue at the α-NH_2, which has been demonstrated to obtain vasopressin analogues for the evaluation of its biological activity (Lindeberg *et al*, Int. J. Peptide Protein Res.10, 1977, 240-244).

WO 03/093302 discloses the synthesis of the peptide of formula (1) using Fmoc-α-nitrogen protected Cα-carboxamide cysteine. It describes the attachment of the first amino acid, cysteine in the precipitated form to the solid support 4-methoxytrityl polystyrene resin through its thiol side chain, followed by removing the α-nitrogen protecting group and assembling the peptide on the said nitrogen. However, the process uses the solid support – 4-methoxytrityl polystyrene resin which is not a

common commercial embodiment and also the Fmoc-α-nitrogen protected Cα-carboxamide cysteine is not commercially available. This enables the process having increased number of steps and also expensive with respect to the process of the present invention. The cleavage conditions utilize ethanedithiol, which makes the process highly toxic and non-environment friendly requiring the use of expensive scrubbers. The use of Fmoc-homoarginine residue in the assembly of the chain is mentioned, which if purchased commercially, also makes the process very expensive. Overall, the process claimed in this document is different from the process claimed in the present invention. In addition the process of WO 03/093302 is associated with certain limitations, which has been overcome by providing suitable modifications in the process steps of the present invention.

Thus process of the present invention is an improved and efficient process over the one described in WO03/093302-A2 patent publication as herein mentioned below.

1 Does not involve the production of -SH peptide, which is susceptible to aerial oxidation leading to the formation of impurities, which hamper the purification of the final product and yield.

2 Precise selection of protecting groups for amino acids to build the peptide chain.

3 Activation of carboxylic function of the amino acid using appropriate activating reagent to prevent the racemization of amino acids.

4 Efficient process for obtaining disulfide loop in the peptide amide of formula (1) from silver-peptide salt intermediate without isolating –SH peptide.

A considerable number of known, naturally occurring small and medium-sized cyclic peptides as well as some of their synthetic derivatives and analogs possessing desirable pharmacological properties have been synthesized. However, wider medical use is often hampered due to complexity of their synthesis and purification. Therefore, improved methods for making these compounds in simple, lesser steps and at lesser cost are desirable and it is the need of the industry and mankind.

The purity and yield of the peptide are important aspects of any route of synthesis. Yield, represented by the relative content of the pharmacologically active compound in the final product, should be as high as possible. Purity is represented by

the degree of presence of pharmacologically active impurities, which though present in trace amounts only, may disturb or even render useless the beneficial action of the peptide when used as a therapeutic agent. In a pharmacological context both aspects have to be considered. As a rule, purification becomes increasingly difficult with larger peptide molecules. In homogeneous (solution) phase synthesis (which is the current method of choice for industrial production of larger amounts of peptides) repeated purification required between individual steps provides a purer product but low yield. Thus, improvements in yield and purification techniques at the terminal stages of synthesis are needed. The present invention is an industrially feasible solid phase synthesis and is a novel process to yield a high purity product with enhanced yield.

Prior art describes the use of HOBt and DIC for activation of amino acids, which leads to the formation of the OBt ester. However, a major drawback in using this procedure is that the preparation of the OBt ester itself takes about 20 min and also the reaction has to be carried out at 0°C. The step-wise introduction of Nα-protected amino acids in SPPS normally involves in situ carboxyl group activation of the incoming amino acid or the use of pre-formed activated amino acid derivatives. In recent years, aminium and phosphonium based derivatives (HBTU, TBTU, Py Boc, and HATU) have become the preferred tools for in situ carboxyl activation. They have been shown to give superior results in terms of both coupling efficiency and suppression of enantiomerization. (Fmoc Solid Phase Peptide Synthesis by Chan W.C. and White P.D., Oxford University Press, 2000, p. 41 – 74) Use of HBTU provides high yield and high purity. It saves time in the activation step with no cooling required. Double coupling is also not required for Mpr(Acm)-OH.

Most of the Fmoc-amino acids derivatives are commercially available. However, a problem exists in the art for the preparation of some amino acid analogs like peptides containing homoarginine as well as cyclic peptide compounds based on disulfide links, because separate operations are required before purifying the end product, which increases expense and may affect final product purity and yield. Fmoc-homoarginine residue if purchased commercially for use in the assembly of the peptide chain becomes very expensive. Alternatively the peptide assembly can be built using lysine followed by guanylation of the lysine residue at the α-NH_2 (Lindeberg et al, Int.J. Peptide Protein Res.10, 1977, 240-244).

Another embodiment of the present invention utilizes an organic solvent for guanylation selected from a group consisting of DMF, ethanol and methanol.

Yet another embodiment of the present invention the guanylation of peptide is performed preferably by using the solvent DMF.

Still another embodiment of the present invention the precipitation of the peptide amide of formula (4) is performed using a solvent selected from the group consisting of acetone, acetonitrile, methanol, ethers, pentane, hexane and mixture thereof.

Still yet another embodiment of the present invention the precipitation is preferably performed using acetonitrile.

Another embodiment of the present invention) the purification of the peptide of formula (4) can be achieved by RP-HPLC.

Yet another embodiment of the present invention the peptide amide of formula (1) obtained has purity more than 99%.

Still yet another embodiment of the present invention the preparation of the peptide of formula (1) by solid phase synthesis is carried out using Fmoc chemistry.

Further embodiment of the present invention uses heavy metal salt for removal of acm selected from thallium trifluoromethane sulphonate, mercuric acetate or silver trifluoromethane sulphonate, preferably silver trifluoromethane sulphonate.

Another embodiment of the present invention the heavy metal peptide salt is obtained by preferably treating peptide of formula (4) with silver trifluoromethane sulphonate in TFA.

Yet another embodiment of the present invention the precipitation of the heavy metal-peptide salt of Formula (5) is preferably carried out using an etheral solvent and more preferably disopropyl ether.

Still another embodiment of the present invention the heavy metal-peptide salt may be treated with HCl and DMSO to simultaneously remove the heavy metal and to oxidize the resulting peptide to yield crude peptide amide of formula (1).

Still yet another embodiment of the present invention the crude peptide amide of formula (1) may be purified by RP-HPLC.

Another embodiment of the present invention the purification of crude peptide amide of formula (1) is preferentially performed by RP-HPLC using C-4, C-8 or C-18 silica or polymer reverse phase columns using methanol and/or acetonitrile in combination with aqueous TFA(0-0.5%) as mobile phase

Still another embodiment of the present invention uses methanol (AR grade) for purification of crude peptide enabling the process inexpensive.

Yet another embodiment of the present invention provides process for preparation of an intermediate peptide of formula (2) as given under:

(Acm)Mpr-Lys(Boc)-Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-Resin

Formula (2)

Still another embodiment of the present invention provides process for preparation of an intermediate peptide of amide formula (3) as given under:

(Acm)Mpr-Lys-Gly-Asp-Trp-Pro-Cys(Acm)-$CONH_2$

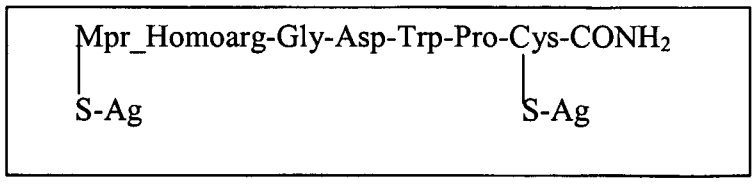
Formula (3)

Still yet another embodiment of the present invention provides process for preparation of a peptide amide of formula (4) as given below:

(Acm)Mpr-Homoarg-Gly-Asp-Trp-Pro-Cys(Acm)-$CONH_2$

Formula (4)

Yet another embodiment of the present invention provides process for preparation of an intermediate peptide amide silver salt of formula (5) as given under:



Formula (5)

The following examples are illustrative of the present invention and not to be construed to limit the scope of the invention.

EXAMPLES

EXAMPLE (1) Chemical synthesis of linear peptide

(Acm)Mpr-Lys(Boc)-Gly-Asp(OBut)-Trp-Pro-Cys(Acm)-Resin

Formula (2)

General Procedure:

The assembly of the peptide chain is carried out in the following manner. The resin is transferred to the RV of the peptide synthesizer [CS936, CS BIO, Calif. Peptide Synthesizer] and the linear peptide is assembled on it using 1.5 – 4.0 times mole excess amino acid derivatives, on the peptide synthesizer. The first amino acid, Fmoc-Cys (C), is coupled to the resin by deprotecting the Fmoc-group on the resin, followed by activating the Fmoc-Cys(C) by HBTU in the presence of NMM. For coupling of the next amino acid, Proline, the α-nitrogen of the first amino acid i.e. Fmoc-Cys(C), is deprotected followed by activating the Fmoc-Pro by HBTU in the presence of NMM. This process is repeated with all the amino acids till the entire linear peptide chain is assembled on the solid support. The Mpr is assembled at the end. Each coupling is carried out for a time range of 45-90 min. The coupling steps are followed by capping with acetic anhydride for 30-60 min. After the coupling are complete, the resin is washed with organic solvent/s which may be selected from the range of DMF, N-methyl pyrrolidone or DCM, preferably DMF followed by DCM, and then dried under vacuum. The linear peptide of formula (2) is obtained.

The peptide was synthesized as peptide amide by solid phase peptide synthesis technology on rink amide resin using Fmoc chemistry.

Instrument	CS936, CS BIO, Calif. Peptide synthesizer
Resin	Rink amide resin (0.65mm/g)
Activator	HBTU/0.4M NMM
Solvent	Dimethyl Formamide
Deprotection	20%Piperidine

The resin (15.38g–rink amide, 10 mmole) was transferred to the RV of the CS936 and swollen in DMF.

(i) Synthesis of Fmoc Cys(Acm)-resin by coupling of Fmoc-Cys(Acm)/HBTU to the resin. The pre-swollen resin (10mmole) was washed twice with DMF followed by removal of Fmoc by treatment with 20% piperidine twice. The resin was washed 6 times with DMF. Fmoc Cys(Acm)(20mmoles) and HBTU (equimole to amino acid) were dissolved in 0.4M NMM and added to the resin. Coupling was carried out for 60min under optimized stirring. The resin was washed once again with DMF thrice. After the coupling, the free amino groups were capped by acetic anhydride (2.5M) for 45 min followed by washing with DMF three times. This HBTU process is a one-step process wherein ester is not isolated.

The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	10min	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	ACT	30sec	X1	DISSOLVES Fmoc-Cys (Acm)/HBTU
5	AA	45min	X1	Fmoc-Cys (Acm) COUPLING
6	SOLV	30sec	X3	WASHES RESIN

(ii) Synthesis of Fmoc-Pro-Cys(Acm)-resin by coupling Fmoc-Pro/HBTU to Fmoc-Cys(Acm)-resin. The reaction was carried out as in step 1. The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	ACT	30sec	X1	DISSOLVES Fmoc-Pro/HBTU
5	AA	45min	X1	COUPLING Fmoc-Pro
6	SOLV	30sec	X3	WASHES RESIN

(iii)Synthesis of Fmoc-Trp-Pro-Cys(Acm)-resin by coupling Fmoc-Trp/HBTU to Fmoc-Pro- Cys(Acm)-resin. The reaction was carried out as in step 1. The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN

2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	ACT	30sec	X1	DISSOLVES Fmoc-Trp/HBTU
5	AA	45min	X1	COUPLING Fmoc-Trp
6	SOLV	30sec	X3	WASHES RESIN

(iv) Synthesis of Fmoc-Asp(Obut)-Trp-Pro-Cys(Acm)-resin by coupling Fmoc-Asp (Obut)/HBTU to Fmoc-Trp-Pro- Cys(Acm)-resin. The reaction was carried out as in step 1. The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	ACT	30sec	X1	DISSOLVES Fmoc-Asp(Obut)/HBTU
5	AA	45min	X1	COUPLING Fmoc-Asp(Obut)
6	SOLV	30sec	X3	WASHES RESIN

(v) Synthesis of Fmoc-Gly-Asp (Obut)-Trp-Pro-Cys(Acm)-resin by coupling Fmoc-Gly/HBTU to Fmoc-Asp(Obut)-Trp-Pro-Cys(Acm)-resin . The reaction was carried out as in step 1. The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	ACT	30sec	X1	DISSOLVES Fmoc-Gly/HBTU
5	AA	45min	X1	COUPLING Fmoc-Gly
6	SOLV	30sec	X3	WASHES RESIN

(vi) Synthesis of Fmoc-Lys(Boc)-Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-resin by coupling Fmoc-Lys(Boc)/HBTU to Fmoc -Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-resin . The reaction was carried out as in step 1.The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN

2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	ACT	30sec	X1	DISSOLVES Fmoc-Lys(Boc)/HBTU
5	AA	45min	X1	COUPLING Fmoc-Lys(Boc)
6	SOLV	30sec	X3	WASHES RESIN

(vii) Synthesis of Mpr(Acm)-Lys(Boc)-Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-resin by coupling Mpr(Acm)/HBTU to Fmoc-Lys(Boc)-Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-resin. The reaction was carried out as in step 1. The synthesis cycle was programmed as follows:

Step	**Reagent**	**Time**	**Repeat**	**Activity**
1	SOLV	30sec	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	ACT	30sec	X1	DISSOLVES Mpr(Acm)/HBTU
5	AA	45min	X1	COUPLING Mpr(Acm)
6	SOLV	30sec	X3	WASHES RESIN

EXAMPLE (2) Chemical synthesis of linear peptide

(Acm)Mpr-Lys(Boc)-Gly-Asp(OBut)-Trp-Pro-Cys(Acm)-Resin

Formula (2)

General Procedure:

The assembly of the peptide chain is carried out in the following manner. The resin is transferred to the RV of the peptide synthesizer [PS3, Protein Technologies, Peptide Synthesizer] and the linear peptide is assembled on it using 1.5 – 4.0 times mole excess amino acid derivatives, on the peptide synthesizer. The first amino acid, Fmoc-Cys (C), is coupled to the resin by deprotecting the Fmoc-group on the resin, followed by activation of Fmoc-Cys(C). Fmoc-Cys(C) (1.3 mmole) and HOBt (2.6 mmole) were dissolved in DMF (5.0ml) and cooled to less than 10^0C in an ice bath. DIC (1.74 mmole) was added to the reaction mixture as a single aliquot. The mixture was then agitated for 6 minutes before being charged to the damp resin in the reaction vessel. The coupling reaction takes place for 60mins.

For coupling of the next amino acid, Proline, the α-nitrogen of the first amino acid i.e. Fmoc-Cys(C), is deprotected. This is followed by activation of Fmoc-Pro by DIC/HOBt in cold conditions as described above and then transfer of this mixture to the reaction vessel. This process is repeated with all the amino acids till the entire linear peptide chain is assembled on the solid support. The Mpr is assembled at the end. Each coupling is carried out for a time range of 45-90 min. Coupling of Mpr is repeated. The coupling steps are followed by capping with acetic anhydride for 30-60 min. After the coupling are complete, the resin is washed with organic solvent/s which may be selected from the range of DMF, N-methyl pyrrolidone or DCM, preferably DMF followed by DCM, and then dried under vacuum. The linear peptide of formula (2) is obtained.

The peptide was synthesized as peptide amide by solid phase peptide synthesis technology on rink amide resin using Fmoc chemistry.

Instrument	**PS3, Protein Technologies, Peptide synthesizer**
Resin	Rink amide resin (0.65mm/g)
Activator	DIC/HOBT
Solvent	Dimethyl Formamide
Deprotection	20%Piperidine

The resin (1g–rink amide, 0.65 mmole) was transferred to the RV of the PS3 and swollen in DMF.

Synthesis of Fmoc Cys(Acm)-resin by coupling of activated Fmoc-Cys(Acm) to the resin. The pre-swollen resin (0.65mmole) was washed twice with DMF followed by removal of Fmoc by treatment with 20% piperidine twice. The resin was washed 6 times with DMF. Fmoc Cys(Acm)(1.3mmoles) and HOBt (2.6 mmole) were dissolved in DMF (5.0ml) and cooled to less than 10^0C in an ice bath. DIC (1.74 mmole) was added to the reaction mixture as a single aliquot. The mixture was then agitated for 6 minutes before being charged to the damp resin. Coupling was carried out for 60min under optimized stirring. The resin was washed once again with DMF thrice. After the coupling, the free amino groups were capped by acetic anhydride (2.5M) for 45 min followed by washing with DMF three times. This DIC/HOBt process is a manual and multistep process.

The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	10min	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	Manual addition of activated Fmoc amino acid.			
5	AA	45min	X1	Fmoc-Cys (Acm) COUPLING
6	SOLV	30sec	X3	WASHES RESIN

(ii) Synthesis of Fmoc-Pro-Cys(Acm)-resin by coupling activated Fmoc-Pro to Fmoc- Cys(Acm)-resin. The reaction was carried out as in step 1. The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	Manual addition of activated Fmoc amino acid.			
5	AA	45min	X1	COUPLING Fmoc-Pro
6	SOLV	30sec	X3	WASHES RESIN

(iii)Synthesis of Fmoc-Trp-Pro-Cys(Acm)-resin by coupling activated Fmoc-Trp to Fmoc-Pro- Cys(Acm)-resin. The reaction was carried out as in step 1. The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	Manual addition of activated Fmoc amino acid.			
5	AA	45min	X1	COUPLING Fmoc-Trp
6	SOLV	30sec	X3	WASHES RESIN

(iv)Synthesis of Fmoc-Asp(Obut)-Trp-Pro-Cys(Acm)-resin by coupling Fmoc-Asp to Fmoc-Trp-Pro- Cys(Acm)-resin. The reaction was carried out as in step 1. The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	Manual addition of activated Fmoc amino acid.			
5	AA	45min	X1	COUPLING Fmoc-Asp(Obut)
6	SOLV	30sec	X3	WASHES RESIN

(v) Synthesis of Fmoc-Gly-Asp (Obut)-Trp-Pro-Cys(Acm)-resin by coupling Fmoc-Gly to Fmoc-Asp(Obut)-Trp-Pro-Cys(Acm)-resin . The reaction was carried out as in step 1. The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	Manual addition of activated Fmoc amino acid.			
5	AA	45min	X1	COUPLING Fmoc-Gly
6	SOLV	30sec	X3	WASHES RESIN

(vi)Synthesis of Fmoc-Lys(Boc)-Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-resin by coupling Fmoc-Lys(Boc) to Fmoc -Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-resin. The reaction was carried out as in step 1.The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	Manual addition of activated Fmoc amino acid.			
5	AA	45min	X1	COUPLING Fmoc-Lys(Boc)
6	SOLV	30sec	X3	WASHES RESIN

(vii) Synthesis of Mpr(Acm)-Lys(Boc)-Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-resin by coupling Mpr(Acm) to Fmoc-Lys(Boc)-Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-

resin. The reaction was carried out as in step 1. The synthesis cycle was programmed as follows:

Step	Reagent	Time	Repeat	Activity
1	SOLV	30sec	X3	WASHES RESIN
2	DEP	5min	X2	DEP N-TERMINUS
3	SOLV	30sec	X6	WASHES RESIN
4	Manual addition of activated Fmoc amino acid.			
5	AA	45min	X1	COUPLING Mpr(Acm)
6	SOLV	30sec	X3	WASHES RESIN

In the synthesis coupling of Mpr(Acm) had to be carried out twice to complete the coupling reaction.

EXAMPLE (3) CLEAVAGE OF THE PEPTIDE FROM THE RESIN TO YIELD PEPTIDE AMIDE (Acm)Mpr-Lys-Gly-Asp-Trp-Pro-Cys(Acm)-$CONH_2$ (Formula (3))

The assembled peptide resin (from Example 1 or 2) is treated with 500 ml of cleavage cocktail consisting of TFA (95%): TIS(2.5%) : H_2O(2.5%) : EDT(0%) : Phenol (0%) for 2 hrs at R.T in CS936. The reaction mixture is filtered through RV, and TFA was evaporated on Rotavap. Precipitation of the peptide was carried out at -20°C by addition of 300 ml of cold di isopropyl ether with constant stirring. The crude peptide precipitate in the solvent is let to stand at –20°C for 10 hrs. The peptide was isolated by filtering through Whatman paper no. 5, followed by cold solvent wash (100ml x 3) to remove the scavengers used in the cleavage cocktail. The crude peptide precipitate is dried under vacuum over P_2O_5, and characterized by RP-HPLC **(Fig.1 and 2).**

Example 1	**Example 2**
Yield: 58.73	**Yield: 48.73**
% purity of peptide: 90%	**%purity of peptide: 79.68%**

EXAMPLE (4) GUANYLATION OF CRUDE PEPTIDE TO YIELD (Acm)Mpr-Homoarg-Gly-Asp-Trp-Pro-Cys(Acm)-$CONH_2$ (formula (4))

The peptide (1g, 1.157mmole) is dissolved in 15 ml of DMF, the pH adjusted to 9.0 with TEA. The reagent 3,5-dimethylpyrazole-1-carboxamidine nitrate (931.5mg) in

DMF (15ml) is added to the peptide. The reaction mixture is stirred at 30°C for 4 days with multiple additions of one time excess of reagent 3,5-dimethylpyrazole-1-carboxamidine nitrate.

The peptide is precipitated from the reaction mixture by the addition of 280ml of acetonitrile (pH adjusted to 8.0 with TEA). The mix is further kept at –20°C for 10 hrs. It is filtered through Whatman no. 5 filter paper and washed with acetonitrile (pH 8.0) 3 times, followed by plain acetonitrile to neutralize the pH. The precipitate is dried under high vacuum overnight. Yield: 85%. The peptide was characterized by RP-HPLC **(Fig.2).**

EXAMPLE (5) DE-ACM OF THE GUANYLATED PEPTIDE FOLLOWED BY OXIDATION TO YIELD

(S)Mpr-Homoarg-Gly-Asp-Trp-Pro-Cys(S)-$CONH_2$

Formula (1)

TFA (134.9ml) and anisole (2.7ml) are mixed, cooled in ice, added to 658 mg of pre-cooled peptide from example 3 and saturated with nitrogen. This is followed by addition of AgOTf (3.47g) and stirred for 2hrs in an ice bath. TFA is removed under high vacuum and silver salt of the peptide was precipitated by addition of diisopropyl ether (~400ml). The reaction mixture is filtered through G-4 sintered funnel and precipitate (silver-peptide) is re-suspended in diisopropyl ether (60ml x3), washed as above and dried over P_2O_5 under vacuum.

The oxidation silver peptideis carried out by dissolving 10 mg of the silver-peptide salt in 15.6ml of 50% DMSO / IM HCl in ice-cold condition. The reaction mixture is stirred for 3 hrs at 25 °C. The precipitate is filtered through a G-4 sintered funnel or Hyflo bed to remove silver chloride. The filtrate is checked for completion of oxidation **(Fig.4)**.On completion of the reaction crude peptide of formula (1) is obtained.Percentage purity: 85%

EXAMPLE (6) PURIFICATION OF S-S PEPTIDE

The crude disulfide looped peptide of formula (1) is loaded on to prep C-18 column (50 x 250mm, 100Å). The peptide is purified by using aqueous TFA (0.1%) and methanol in a gradient program **(Fig.5)**. This is followed by an isocratic run using the above said solvent systems on a Shimadzu preparative HPLC System consisting of

a controller, 2 LC8A pumps, UV-Vis detector. The purified peptide amide of formula (1) is analysed by analytical RP-HPLC **(Fig.6)**. The mass is determined by Mass Spectrophotometer **(Fig. 7)**.

EXAMPLE (7): PURIFICATION OF S-S PEPTIDE

The purification was carried out in the same manner as Example 5, except that Acetonitrile was used instead of methanol to obtain peptide amide of formula (1).

EXAMPLE (8): DE-ACM OF THE GUANYLATED PEPTIDE USING MERCURIC (II) ACETATE

Same as in Example (4), except that the Acm group protection of cysteineis removed from the guanylated peptide by treatment with mercury (II) acetate.

The peptide (13.4mg) estimated by Lowry's method, of Cys-Acm) is dissolved in 400µl of acetic acid (10%). Ten times excess of mercury (II) acetate (82.96mg) is added to it, the reaction mass vortexed and kept at R.T. for 5 hrs. 100 times excess of β-mercaptoethanol (181.37µl) is added, the solution vortexed and let to stand overnight at room temperature. The reaction mixture is centrifuged for 4min, and supernatant collected. The precipitate is extracted with 400µl x 3 of 10% acetic acid by centrifugation. The filtrates are pooled and percentage purity determined by RP-HPLC is 55% **(Fig 3)**.

EXAMPLE (9) : DE-ACM OF THE GUANYLATED PEPTIDE USING IODINE

Same as in Example (4), except that the Acm group protection of cysteine is removed from the guanylated peptide by treatment with iodine.

The peptide (9.18mg, estimated by Lowry's method, of Cys-Acm)is dissolved in 17.8ml of acetic acid (80%) and purged with N_2 for 15mins. 1mM solution of I_2 (in 80%acetic acid, ~4ml)is added to the peptide solution, over a period of 1hr, till there is a persistent yellow color. The mixture is stirred for an additional 30-mins followed by neutralisation with 1N $Na_2S_2O_3$, till the yellow color disappeared, and lyophilized. Estimation of 'SH' is done by Ellman Test, which is negative indicating that removal of ACM has not been achieved.

EXAMPLE (10): PURIFICATION OF THE DE-ACM PEPTIDES

The mercury (II) acetate treated and I_2 treated peptide samples were desalted by RP-HPLC, using the hyperprep (250 x 10mm, 12µ ,C-18 column).

EXAMPLE (11): PLATELET AGGREGATION INHIBITION ASSAY TO CHECK THE BIOACTIVITY OF FORMULA (1)

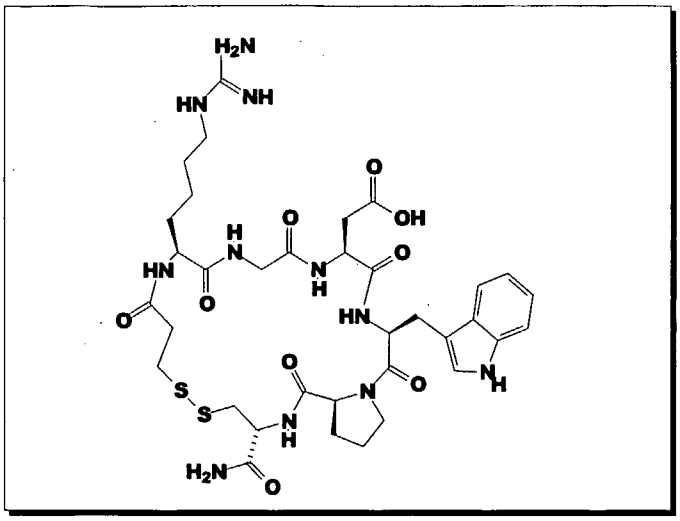
The bioactivity of peptide of formula (1) is checked using platelet aggregation inhibition assay using 4X Laser Aggregometer (EMA). Freshly venous blood from consented human donors are drawn and collected in citrated buffer. The platelet rich plasma (PRP) and platelet poor plasma are separated by centrifugation. Platelet count in PRP is adjusted to 2-3 $x10^8$ platelets / ml. After adjusting the baseline aggregation with PPP, the PRP was treated with 10-20 mM ADP and checked the percent total aggregation. The PRP is then first incubated with varying concentrations of reference standard and synthesized peptide of formula (1). ADP is then added to check the inhibition of aggregation. The reproducibility of bioactivity of synthesized peptide of formula 1is checked several times and compared with reference standards. **Table 1** represents one of many experiments (from 12 experiments). The IC_{50} dose for synthesized peptide (SP) was less than 140nM as compared to commercial reference standard. There is more than 50% inhibition of ADP induced platelet aggregation with SP seen in most of the samples and results are comparable with commercial reference standard.

Table 1: ***Inhibition of ADP induced aggregation by synthesized peptide (SP) of formula 1***

Donor Number	Percent Aggregation by ADP	Concentration (nM)	% Inhibition by	
			SP of formula 1	Reference Standard
1.	89.4%	70	ND	ND
		140	44.6	31.20
		280	61.40	52.50
2.	92.13	70	46.66	30.31
		140	**51.16**	45.18
		280	67.74	60.92
3.	54.14	70	57.42	27.41
		140	**60.28**	49.20
		280	ND	ND
4.	68.11	70	ND	ND
		140	20.52	23.06
		280	42.73	40.53
5.	66.50	70	52.63	45.68
		140	**87.21**	57.44
		280	ND	ND
6.	66.17	70	ND	ND
		140	**82.92**	70.56
		280	ND	ND

We Claim:

1. A process for the preparation of a peptide N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic(1→6)-disulfide of formula (1) on a solid phase,



the said process comprising:

a. assembling a peptide chain comprising of six amino acids and a mercaptoalkyl thioalkyl carboxylic acid in a required sequence on a solid support resin, by coupling to directly join one another by peptide bonds to obtain a peptide bound resin of formula (2) as given below:

(Acm)Mpr-Lys(Boc)-Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-Resin

Formula (2)

b. capping the free amino groups after each coupling with acetic anhydride;

c. cleaving and deprotecting, all groups except acm group, the peptide of step (a) from the resin to obtain peptide amide of formula (3) as given below:

(AAcm)Mpr-Lys-Gly-Asp-Trp-Pro-Cys(BAcm)-$CONH_2$

Formula (3)

d. guanylating the peptide at ϵ-lysine-NH_2 in an organic solvents followed by precipitating with another solvent to obtain peptide of formula (4) as given below, by precipitation with another solvent;

(AAcm)CysMpr-Homoarg-Gly-Asp-Trp-Pro-Cys(BAcm)-$CONH_2$

Formula (4)

e. treating the peptide of Formula (4) with a heavy metal salt in an appropriate solvent, followed by precipitation of the heavy metal-peptide salt using an organic solvent to obtain the heavy metal-peptide salt of formula (5);

Mpr-Homoarg-Gly-Asp-Trp-Pro-Cys
| |
S-Ag S-Ag

Formula (5)

f. treating the heavy metal-peptide salt of step (e) with a thiol followed by desalting and oxidation to obtain a peptide of formula (1); and

g. purifying the crude peptide of step (f) or (g) by RPHPLC in isolation or in combination with HIC and IEC chromatographic techniques.

2. A process as claimed in claim 1, wherein the reaction of amino and carboxylic equivalent of compounds forms the said peptide bond.

3. A process of claim 1, wherein the C-terminal of the protected first amino acid is bound to a solid phase through a linker to obtain a solid phase bound amino acid.

4. A process of claim 1, wherein the solid support used is any amide resin, preferably Rink Amide Resin.

5. A process of claim 1, wherein the first protected amino acid is a thiol protected Fmoc Cysteine.

6. A process of claim 1, wherein in the cleavage of the resin with the linker leads to the release of assembled peptide amide.

7. A process of claim 1, wherein the peptide amide compound is a compound joined to each of the terminal functionalities by a peptide bond and wherein each terminal functionalities is an amino or carboxylic acid group or derivatives thereof.

8. A process of claim 1, wherein the amino acids used are selected from the group consisting of Cys, Pro, Trp, Asp, Lys, Gly, Arg, Har, Leu and Glu.

9. A process of claim 1, wherein the mercaptoalkyl thioalkyl carboxylic acid used is 2-mercaptopropionic thiopropionic acid.

10. A process of claim 1 wherein, the protecting group for -NH_2 functional group of an amino acid is Fmoc or Boc.

11. A process of claim 1, wherein the protecting group for -COOH functional group is unprotected or protected O-tBu esteror free.

12. A process of claim 1, wherein the protecting group for SH-function is Acm group.

13. A process of claim 1, wherein in step (c), the peptide is cleaved from solid support resin using the reagents TFA, TIS, EDT, DCM, Phenol and water in a defined ratio, preferably TFA(85-98%) : TIS(0-5%) : H_2O(0-5%) : EDT(0-5%): Phenol(0-5%), more preferably TFA(94.5-95.5%) : TIS(0-2.5%) : H_2O(0-3%) : EDT(0-2.5%).

14. A process of claim 1, wherein in step (d), the organic solvent used for guanylation is selected from the group consisting of DMF, ethanol and methanol.

15. A process of claim 1, wherein in the step (d), the precipitation of the peptide of formula 4 is carried out by performed using a solvent selected from the group consisting of acetone, acetonitrile, methanol, ethers, pentane, hexane and mixture thereof.

16. A process as claimed in claim 15, wherein the precipitation is carried out in preferably performed using acetonitrile.

17. A process of claim 1, wherein in the step (g), the peptide of formula (1) obtained has purity more than 99%.

18. A process as claimed in claim 1, wherein the preparation of the peptide of formula (1) by solid phase synthesis is carried out using Fmoc chemistry.

19. A process as claimed in claim 1, wherein the assembly of the amino acids gives a peptide bound resin of formula 2,

(Acm)Mpr-Lys(Boc)-Gly-Asp(Obut)-Trp-Pro-Cys(Acm)-Resin

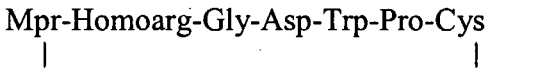
Formula 2

20. A process as claimed in claim 1, wherein in the step (d), the guanylation of peptide of formula 3 is carried out performed preferably by using the solvent in DMF.

21. A process as claimed in claim 1, wherein the purification of the peptide of Formula (4) may be carried out achieved by RP-HPLC.

22. A process as claimed in claim 1, wherein the heavy metal salt used for the treatment of peptide of Formula (4) is silver trifluoromethane sulphonate in TFA.

23. A process as claimed in claim 1, wherein the precipitation of the heavy metal-peptide salt of Formula (5) is preferably carried out using an ethereal solvent and more preferably diisopropyl ether.



Formula (5)

24. A process as claimed in claim 1, wherein in step (f), the heavy metal-peptide salt may be treated with HCl and DMSO to simultaneously remove the heavy metal and to oxidize the resulting peptide to yield a crude peptide amide of Formula (1).

25. A process as claimed in claim 1, wherein the crude peptide amide of Formula (1) is may be purified by RP-HPLC.

26. A process as claimed in claim 1, wherein the purification of crude peptide amide of formula (1) is preferentially performed by RP-HPLC using C-4, C-8 or C-18 silica or polymer reverse phase columns using methanol and/or acetonitrile in isolation or combination with aqueous TFA(0-0.5%) as the mobile phase.

27. An intermediate peptide of formula (2)

(Acm)Mpr-Lys(Boc)-Gly-Asp(Obut)-Trp-Pro-Cys("Acm)-Resin

Formula 2

28. An intermediate peptide of formula (3)

(Acm)Mpr-Lys-Gly-Asp-Trp-Pro-Cys(Acm)-CONH2

Formula (3)

29. An intermediate peptide of formula (4)

(Acm)Mpr-Homoarg-Gly-Asp-Trp-Pro-Cys(Acm)-CONH2

Formula (4)

30. An intermediate peptide salt of the formula (5)

Mpr-Homoarg-Gly-Asp-Trp-Pro-Cys
| |
S-Ag S-Ag

Formula (5)