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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Anti-nociceptive effect of some synthesized smaller chain tripeptides and tetrapeptides in mice

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Abstract The present study describes an approach to synthesize the smaller chain tripeptides and tetrapeptides and to test their antinociceptive potency in mice. Based on rational drug design using hydrophobic ratio and total net charge as descriptors, five leads were selected, viz., Met-Arg-Tyr (MRY), Met-Val-Tyr (MVY), Met-Ile-Cys-Tyr (MICY), Met-Trp-Lys-Tyr (MWKY) and Phe-Trp-Lys-Tyr (FWKY) from the subjected 65 templates and synthesized by dicyclohexyl carbodiimide coupling using polystyrene as solid support. All the synthesized compounds were purified by column chromatography and further confirmed by melting point, infrared, proton nuclear magnetic resonance and mass spectral datas. Acute toxicity studies were performed for dose selection in all the compounds using OECD guidelines 423 (Annexure 2b). Antinociceptive potency of peptides was tested in Swiss albino mice using acetic acid writhing and hot plate method. The LD_{50} cut-off mg/kg body weight for tripeptides (MRY, MVY) and tetrapeptides (Met-Ile-Cys-Tyr, Met-Trp-Lys-Tyr, Phe-Trp-Lys-Tyr) were found to be 500–2000 mg/kg and 200–300 mg/kg respectively. The tripeptide MVY have shown maximum antinociceptive action with an average number of writhing as 13 at 150 mg/kg body weight by writhing method, whereas the tripeptide Met-Arg-Tyr have shown maximum potency with the average reaction time of 5.75 min after 15 min at a dose of 150 mg/kg by hot plate method, which clearly indicated that the tripeptides are comparatively potent antinociceptive agents than the tetrapeptides for central neuropathic pain.

Keywords Tetrapeptides · Tripeptides · Acute toxicity · Antinociceptive activity · Drug design · Met-Arg-Tyrosine

Introduction

Central neuropathic pain is a condition arising from injury or disease of the central nervous system such as spinal cord injury, syringomyelia, multiple sclerosis, stroke (infarction or haemorrhage), traumatic brain injury, parkinson's disease, tumours and epilepsy. Large scale studies in western countries have shown that one fifth of the adult population suffer from chronic pain. Despite several available analgesics, unrelieved pain remains a major health care issue (Committee on Advancing pain research, 2011).

The use of opioid analgesics for pain management has increased dramatically over the past decade, with corresponding increase in negative sequelae, including overdose and death. There is currently no well validated objective means of accurately identifying patients likely to experience good analgesia with low side effects and abuse risk prior to initiating opioid therapy (Bruehl et al., 2013).

Oral analgesics are commonly prescribed for the treatment of acute and chronic pain, but these agents often produce adverse systemic effects, which sometimes are

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severe (Argoff, 2013). Experimental pain models have been established as cost-effective tools for assessing analgesic drug efficacy (Mogil, 2009).

Past study examined the effect of peptide analogue of thymulin in rat models of inflammatory hyperalgesia and found to have stronger anti-hyperalgesic effects as compared to the tripeptides Lys-Pro-Thr and Lys-Pro-Val in antagonizing interleukin (IL)-1β and prostaglandin E2 mechanisms (Garabedian et al., 2002). A κ-agonist tetrapeptide, FE 200665 was found to be peripherally selective with potent analgesic action (Waltraud et al., 2001). The tripeptides Lys-D-Pro-Val, Lys-Pro-Val, Lys-D-Pro-Thr and D-Lys-Pro-Thr were identified with good analgesic potency along with α-melanocyte stimulating hormone (α-melanocyte stimulating hormone) in inhibiting the hyperalgesic responses to IL-1β especially in rats (Poole et al., 1992). A bivalent opioid tetrapeptide, biphalin (Tyr-d-Ala-Gly-Phe-NH)$_2$ was found to produce significant analgesia through intravenous route in rats (Silbert et al., 1991). The synthetic pentapeptide, neo-kyotorphin (Thr-Ser-Lys-Tyr-Arg) showed the dose dependent analgesic effect in mice as similar to the potency of Leu-enkephalin (Kiso et al., 2010).

Our objective describes an approach to synthesize the smaller chain tripeptides and tetrapeptides and to test them on two models, viz., tail flick and writhing tests in mice for their potent antinociceptive action.

Materials and methods

Materials

The descriptors of test set analogues in drug design were calculated directly from the online peptide database (http://aps.unmc.edu/AP/main.php) using Windows 7, 32 bit operating system with Intel (R) Core (TM) 2 Duo CPU E 4600@2.40 GHz and 1 GB RAM in HP 7540. All chemicals and amino acids needed for synthesis were procured from CDH Laboratories, Mumbai. All glasswares for synthesis and purification were made of Borosil.

Methodology

Drug design

Boman index or protein binding potential estimates the potential for a protein to bind to other proteins (Boman, 2003). Sixty-five templates of shorter chain peptides, in which 20 have an input of 3 amino acid residues and the rest have an input of 4 amino acid residues each were selected, viz., MGY, MAY, MLY, MNY, MFY, MWY, MKY, MQY, MEY, MSY, MPY, MVY, MIY, MCY, MYY, MHY, MRY, MNY, MDY, MTY, MFGY, MGAY, MALY, MLMY, MMFY, MFWY, MWKY, MKQY, MQEY, MESY, MSPY, MPVY, MVIY, MICY, MCYY, MYHY, MHRY, MRNY, MNDY, MDTY, MTGY, FGGY, FGAY, FALY, FLMY, FMFY, FFWY, FWKY, FKQY, FQEY, FESY, FSPY, FPVY, FVIY, FICY, FCYY, FYHY, FHRY, FRNY, FNDY, FDTY, FEGY, TRRH, TKRH, THRH and subjected to descriptors such as hydrophobic ratio (%), total net charge and protein binding potential in drug design for the prediction of best antinociceptive action among all the templates theoretically.

Synthesis of tripeptides by solid phase peptide synthesis

Synthesis of tripeptide Met-Arg-Tyr (MRY) *Step (1): Protection of Tyrosine.* In a 100 mL round bottomed flask, 2.605 g of polystyrene (9.238 mmol) was taken as amino protecting agent for tyrosine (3.276 g; 18.1 mmol) and heated in an oil bath at 160–180 °C. Within 10–15 min, the mixture melted and heating was continued for another 15 min. The reaction mixture was cooled; the residue was dissolved in ethanol and filtered off. Water was added to the clear filtrate till turbid and the solution was cooled at 0 °C for 2–3 h so that crystals of polystyrene tyrosine separate out by filtration and dried. *Step (2): Coupling of Polystyrene tyrosine with Arginine.* In a 250 ml flat round bottomed flask, protected amino acid was taken and dissolved in minimum volume of dry dichloromethane. Secondly, Arginine (3.168 g; 18.1 mmol) was added to it followed by the addition of 1-hydroxybenzotriazole (7.33 g; 54.3 mmol) and 4-dimethyl amino pyridine (0.6552 g; 3.62 mmol). Then the environment of the reaction was made nitrogenous and the reaction mixture was cooled in ice-salt mixture. DCC (11.2 g; 54.3 mmol) solution was made by dissolving DCC in minimum volume of dichloromethane (60 ml). This DCC solution was injected into the reaction mixture in cold condition. Finally, few drops of dimethylformamide (DMF) were added to the solution and then, the mixture of solution was stirred overnight. Further, the reaction mixture was analyzed on thin layer chromatography (TLC) using 10 % methanol in chloroform as mobile phase. The mixture was filtered and dried in rotary vacuum evaporator and placed in desiccator for overnight drying. The dried product was dissolved in dichloromethane (DCM) and directly filtered by separating funnel to avoid wastage. The filtered product was acidified with 1N HCl, and neutralized with 1N sodium carbonate and washed with brine solution and kept it aside for some time. Then clear product was cleared out and the other one was discarded. A little quantity of sodium sulphate was added and heated on oil bath for 15 min, dried by rota evapour and placed in desiccator overnight (Tegge et al., 2010; Gao et al., 2011). *Step (3): Protection of*

Methionine. 2.7 g of methionine (18.1 mmol) was refluxed with 1.87 ml of 4-methyxybenzyl alcohol on oil bath for half an hour so that the thiol group gets protected. *Step (4): Coupling of polystyrene-tyrosine-arginine with 4-methoxybenzyl alcohol-methionine.* In a 250 ml flat round bottomed flask, protected polystyrene-tyrosine-arginine was taken and was dissolved in minimum volume of dry DCM. Then, 4-methoxybenzyl alcohol-methionine was added to it followed by the addition of 1-hydroxybenzotriazole (HOBt) and dimethylaminopyridine (DMAP). The environment of the reaction was made nitrogenous and cooled in ice-salt mixture and DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then the reaction mixture was analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotavapour and was placed in desiccator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered in separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl and then was neutralized with 1N sodium carbonate and was washed with brine solution. The product was kept aside for some time. Then separated out the clear product and discarded the other one. Then, a little quantity of sodium sulphate was added and heated it on oil bath for 15 min; then dried it in rotavapour and placed in desiccator overnight. *Step (5): Deprotection.* The protected tripeptide 4-methoxy benzyl alcohol-methionine-arginine-tyrosine-polystyrene was dissolved in 0.5 ml of anhydrous liquid hydrofluoric acid and the mixture was heated on steam bath for half an hour to deprotect the thiol group in methionine. The reaction mixture was further treated with 20 % piperidine in DMF and again heated on water bath at 50 °C for half an hour. The corresponding polystyrene was filtered off followed by crystallization from aqueous ethanol, gives the MRY as colourless crystal.

Synthesis of tripeptide Met-Val-Tyr (MVY)iop *Step (1): Protection of tyrosine.* In this protection, 2.605 g of polystyrene (9.238 mmol) was taken as amino protecting agent for tyrosine (3.276 g; 18.1 mmol) and heated in an oil bath at 160–180 °C. Within 10–15 min, the mixture melted and heating was continued for another 15 min. The reaction mixture was cooled; the residue was dissolved in ethanol and filtered off. Water was added to the clear filtrate till turbid and the solution was cooled at 0 °C for 2–3 h so that crystals of polystyrene tyrosine separate out by filtration and dried. *Step (2): Coupling of polystyrene tyrosine with valine.* In a 250 ml flat round bottomed flask, protected amino acid was taken and dissolved it in minimum volume of dry DCM. Secondly, valine (2.118 g; 18.1 mmol) was added to it followed by the addition of 1-hydroxybenzotriazole (7.33 g; 54.3 mmol) and 4-dimethyl amino pyridine (0.6552 g; 3.62 mmol). Then the environment of the reaction was made nitrogenous and cooled in ice-salt mixture. Then DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then, the reaction mixture was analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotary vacuum evaporator and placed in desiccator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered by separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl, and neutralized it with 1N sodium carbonate and washed it with brine solution, then kept it aside for some time. Then separated out the clear product and discarded the other one. Then added a little quantity of sodium sulphate and heated it on oil bath for 15 min, dried it by rotavapour and placed in desiccator overnight. *Step (3): Protection of Methionine.* 2.7 g of methionine (18.1 mmol) was refluxed with 1.87 ml of 4-methyxybenzyl alcohol on oil bath for half an hour so that the thiol group gets protected. *Step (4): Coupling of polystyrene-tyrosine-valine with 4-methoxy benzyl alcohol-methionine.* In a 250 ml flat round bottomed flask, protected polystyrene-tyrosine-valine was taken and dissolved it in minimum volume of dry DCM. Then, secondly 4-methoxybenzyl alcohol-methionine was added to it followed by the addition of 1-HOBt and DMAP. The environment of the reaction was made nitrogenous and cooled in ice-salt mixture and DCC solution was made by dissolving DCC in minimum volume of DCM. The DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then the reaction mixture was analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotavapour and placed in desiccator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered in separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl and then neutralized it with 1N sodium carbonate and washed it with brine solution, kept it aside for some time. Then separated out the clear product and discarded the other one. Then, added little quantity of sodium sulphate and heated it on oil bath for 15 min, then dried it in rotavapour and placed in desiccator overnight. *Step (5): Deprotection.* The protected tripeptide 4-methoxy benzyl alcohol-methionine-valine-tyrosine-polystyrene was dissolved in 0.5 ml of anhydrous liquid hydrofluoric acid and the mixture was heated on steam bath for half an hour to deprotect the thiol group in methionine. The reaction mixture was further treated with 20 % piperidine in DMF and

again heated on water bath at 50 °C for half an hour. The corresponding polystyrene was filtered off followed by crystallization from aqueous ethanol, gives the MVY as colourless crystal. The scheme for the synthesis of tripeptide MVY are outlined in Fig. 1.

Synthesis of tetrapeptide Met-Ile-Cys-Tyr (MICY) *Step (1): Protection of tyrosine.* In this protection, 2.605 g of polystyrene (9.238 mmol) was taken as amino protecting agent for tyrosine (3.276 g; 18.1 mmol) and heated in an oil bath at 160–180 °C. Within 10–15 min, the mixture melted and heating was continued for another 15 min. The reaction mixture was cooled; the residue was dissolved in ethanol and filtered off. Water was added to the clear filtrate till turbid and the solution was cooled at 0 °C for 2–3 h so that crystals of polystyrene tyrosine separate out by filtration and dried. *Step (2): Coupling of polystyrene tyrosine with cysteine.* In a 250 ml flat round bottomed flask, protected amino acid was taken and dissolved it in minimum volume of dry DCM. Secondly, cysteine (2.190 g; 18.1 mmol) was added to it followed by the addition of 1-hydroxybenzotriazole (7.33 g; 54.3 mmol) and 4-dimethyl amino pyridine (0.6552 g; 3.62 mmol). Then the environment of the reaction was made nitrogenous and cooled in ice-salt mixture and dicyclohexyl carbodimide (DCC, 11.2 g; 54.3 mmol) solution was made by dissolving DCC in minimum volume of DCM (60 ml). This DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then, the reaction mixture analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotary vacuum evaporator and placed in desiccator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered by separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl, and neutralized it with 1N sodium carbonate and washed it with brine solution, then kept it aside for some time. Then separated out the clear product and discarded the other one. Then added little quantity of sodium sulphate and heated it on oil bath for 15 min, dried it by rotavapour and placed in desiccator overnight. *Step (3): Coupling of polystyrene tyrosine-cysteine with isoleucine.* In a 250 ml flat round bottomed flask, protected amino acid as a product from previous step was taken and dissolved it in minimum volume of dry DCM. Secondly, isoleucine (2.371 g; 18.1 mmol) was added to it followed by the addition of 1-hydroxybenzotriazole (7.33 g; 54.3 mmol) and 4-dimethyl amino pyridine (0.6552 g; 3.62 mmol). Then the environment of the reaction was made nitrogenous and cooled in ice-salt mixture and DCC (11.2 gm; 54.3 mmol) solution was made by dissolving DCC in minimum volume of DCM (60 ml). This DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then, the reaction mixture was analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotary vacuum evaporator and placed in desiccator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered by separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl, and neutralized it with 1N sodium carbonate and washed it with brine solution, then kept it aside for some time. Then separated out the clear product and discarded the other one. Then added little quantity of sodium sulphate and heated it on oil bath for 15 min, dried it by rotavapour and placed in desiccator overnight. *Step (4): Protection of Methionine.* 2.7 g of methionine (18.1 mmol) was refluxed with 1.87 ml of 4-methyxybenzyl alcohol on oil bath for half an hour so that the thiol group gets protected. *Step (5): Coupling of polystyrene-tyrosine-cysteine-isoleucine with 4-methoxy benzyl benzyl alcohol-methionine.* In a 250 ml flat round bottomed flask, protected polystyrene-tyrosine-cysteine-isoleucine was taken and dissolved it in minimum volume of dry DCM. Then, secondly 4-methoxybenzyl alcohol-methionine was added to it followed by the addition of 1-HOBt and DMAP. The environment of the reaction was made nitrogenous and cooled in ice-salt mixture and DCC solution was made by dissolving DCC in minimum volume of DCM. The DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then the reaction mixture was analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotavapour and placed in dessicator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered in separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl and then neutralized it with 1N sodium carbonate and washed it with brine solution, kept it aside for some time. Then separated out the clear product and discarded the other one. Then, added little quantity of sodium sulphate and heated it on oil bath for 15 min, then dried it on rotavapour and placed in desiccator overnight. *Step (6): Deprotection.* The protected tetrapeptide 4-methoxy benzyl alcohol-methionine-isoleucine-cysteine-tyrosine-polystyrene was dissolved in 0.5 ml of anhydrous liquid hydrofluoric acid and the mixture was heated on steam bath for half an hour to deprotect the thiol group in methionine. The reaction mixture was further treated with 20 % piperidine in DMF and again heated on water bath at 50 °C for half an hour. The corresponding polystyrene was filtered off followed by crystallization from aqueous ethanol, gives the MICY as

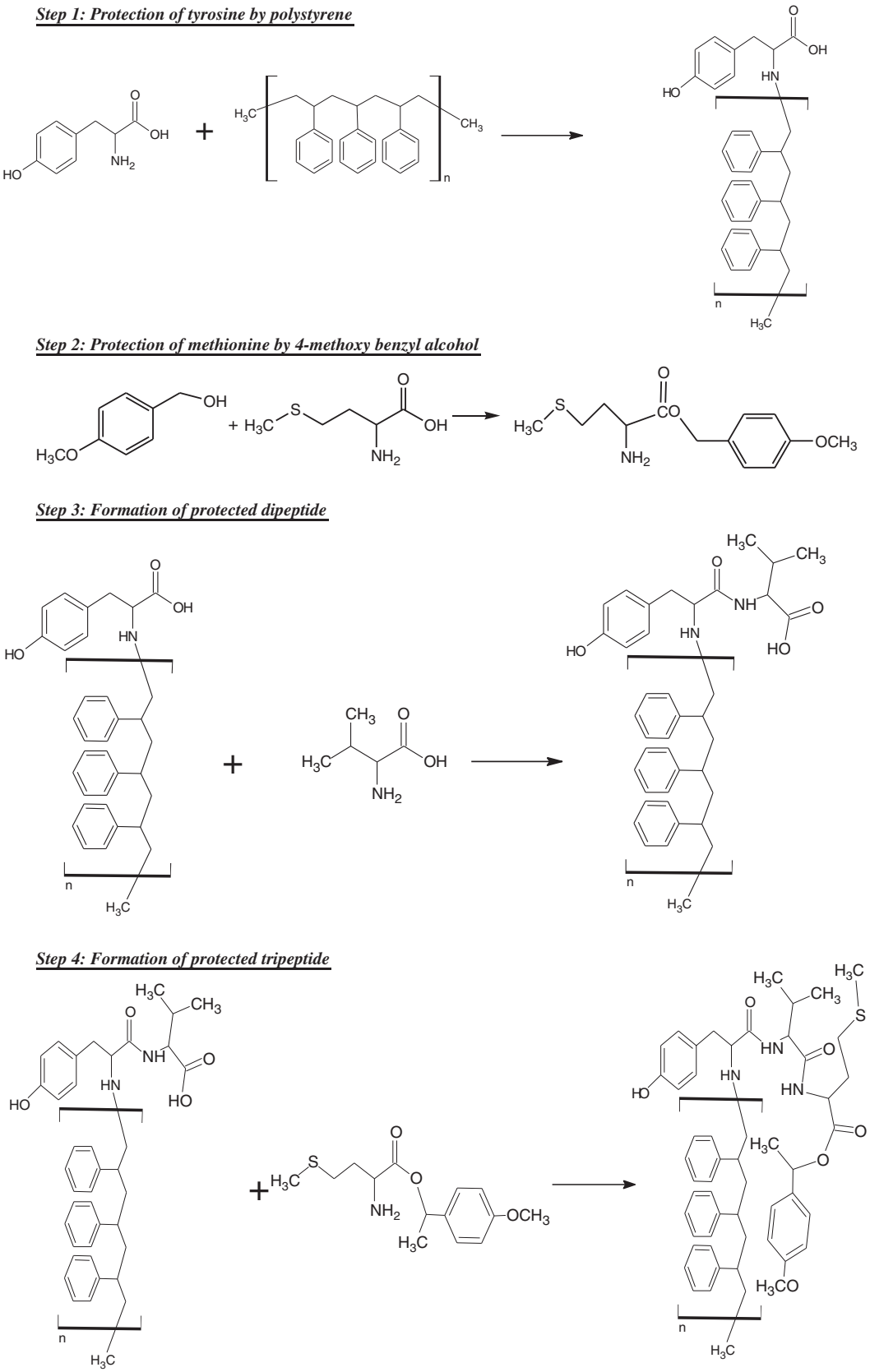


Fig. 1 Scheme for the synthesis of tripeptide Met-Val-Tyr (MVY)

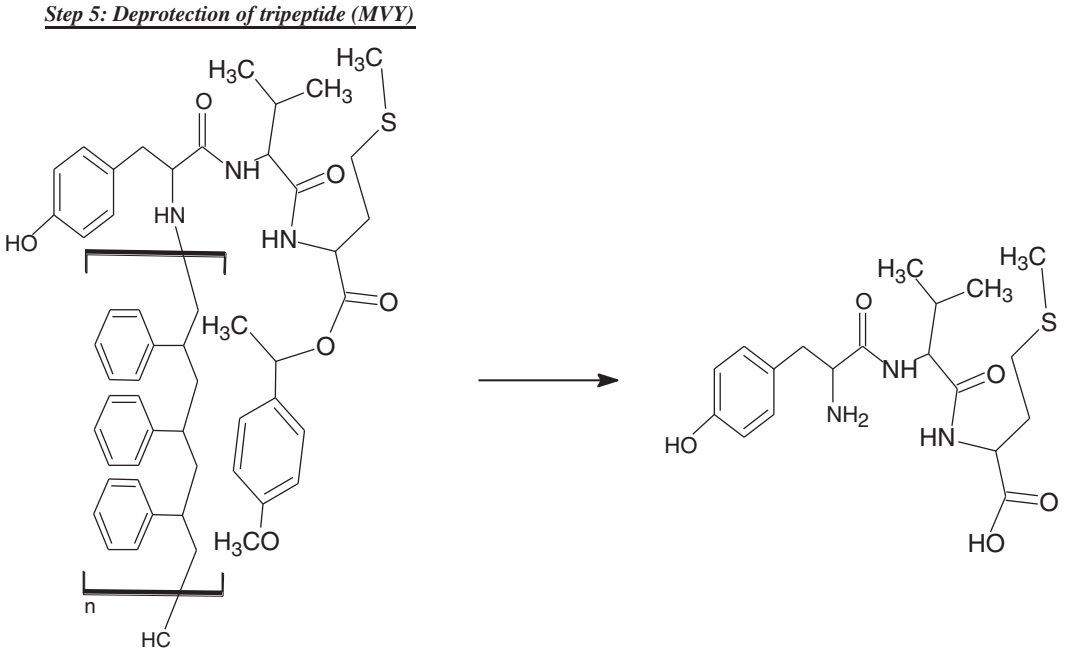


Fig. 1 continued

colourless crystal. The scheme for the synthesis of tetrapeptide MICY is outlined in Fig. 2.

Synthesis of tetrapeptide Phe-Trp-Lys-Tyr (FWKY) *Step (1): Protection of tyrosine.* In this protection, 2.605 g of polystyrene (9.238 mmol) was taken as amino protecting agent for tyrosine (3.276 g; 18.1 mmol) and heated in an oil bath at 160–180 °C. Within 10–15 min, the mixture melted and heating was continued for another 15 min. The reaction mixture was cooled; the residue was dissolved in ethanol and filtered off. Water was added to the clear filtrate till turbid and the solution was cooled at 0 °C for 2–3 h so that crystals of polystyrene tyrosine separate out by filtration and dried. *Step (2): Coupling of polystyrene tyrosine with lysine.* In a 250 ml flat round bottomed flask, protected amino acid was taken and dissolved it in minimum volume of dry DCM. Secondly, lysine (2.661 g; 18.1 mmol) was added to it followed by the addition of 1-hydroxybenzotriazole (7.33 g; 54.3 mmol) and 4-dimethyl amino pyridine (0.6552 g; 3.62 mmol). Then the environment of the reaction was made nitrogenous and cooled in ice-salt mixture and DCC (11.2 g; 54.3 mmol) solution was made by dissolving DCC in minimum volume of DCM (60 ml).
This DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then, the reaction mixture analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotary vacuum evaporator and placed in desiccator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered by separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl, and neutralized it with 1N sodium carbonate and washed it with brine solution, then kept it aside for some time. Then separated out the clear product and discarded the other one. Then added little quantity of sodium sulphate and heated it on oil bath for 15 min, dried it by rotavapour and placed in desiccator overnight. *Step (3): Coupling of polystyrene tyrosine-lysine with tryptophan.* In a 250 ml flat round bottomed flask, protected amino acid as a product from previous step was taken and dissolved it in minimum volume of dry DCM. Secondly, tryptophan (3.692 g; 18.1 mmol) was added to it followed by the addition of 1-hydroxybenzotriazole (7.33 g; 54.3 mmol) and 4-dimethyl amino pyridine (0.6552 g; 3.62 mmol). Then the environment of the reaction was made nitrogenous and cooled in ice-salt mixture and DCC (11.2 gm; 54.3 m.mol) solution was made by dissolving DCC in minimum volume of DCM (60 ml). This DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then, the reaction mixture analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotary vacuum evaporator and placed in desiccator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered by separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl, and neutralized it with 1N sodium

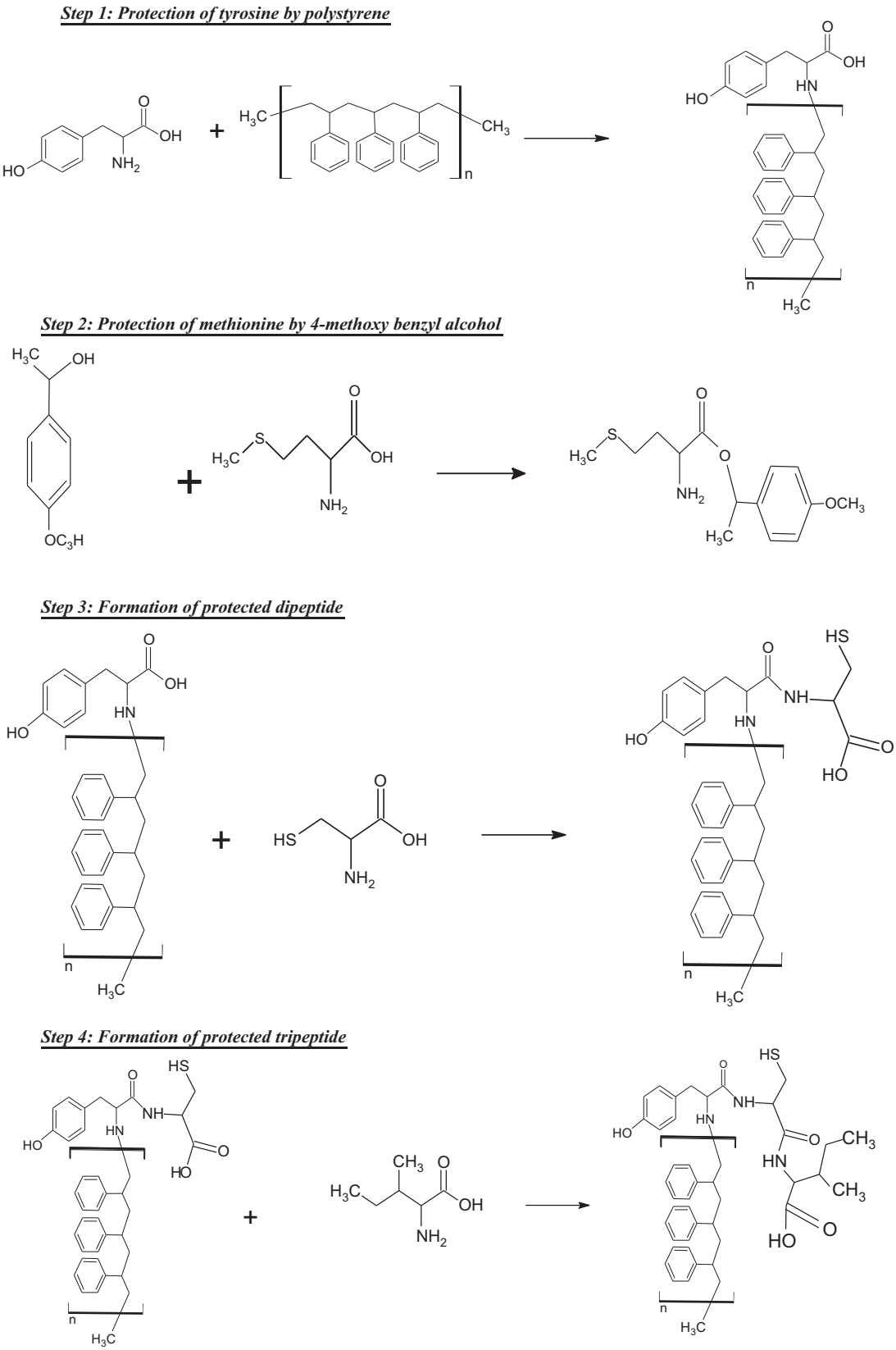


Fig. 2 Scheme for the synthesis of tetrapeptide Met-Ile-Cys-Tyr (MICY)

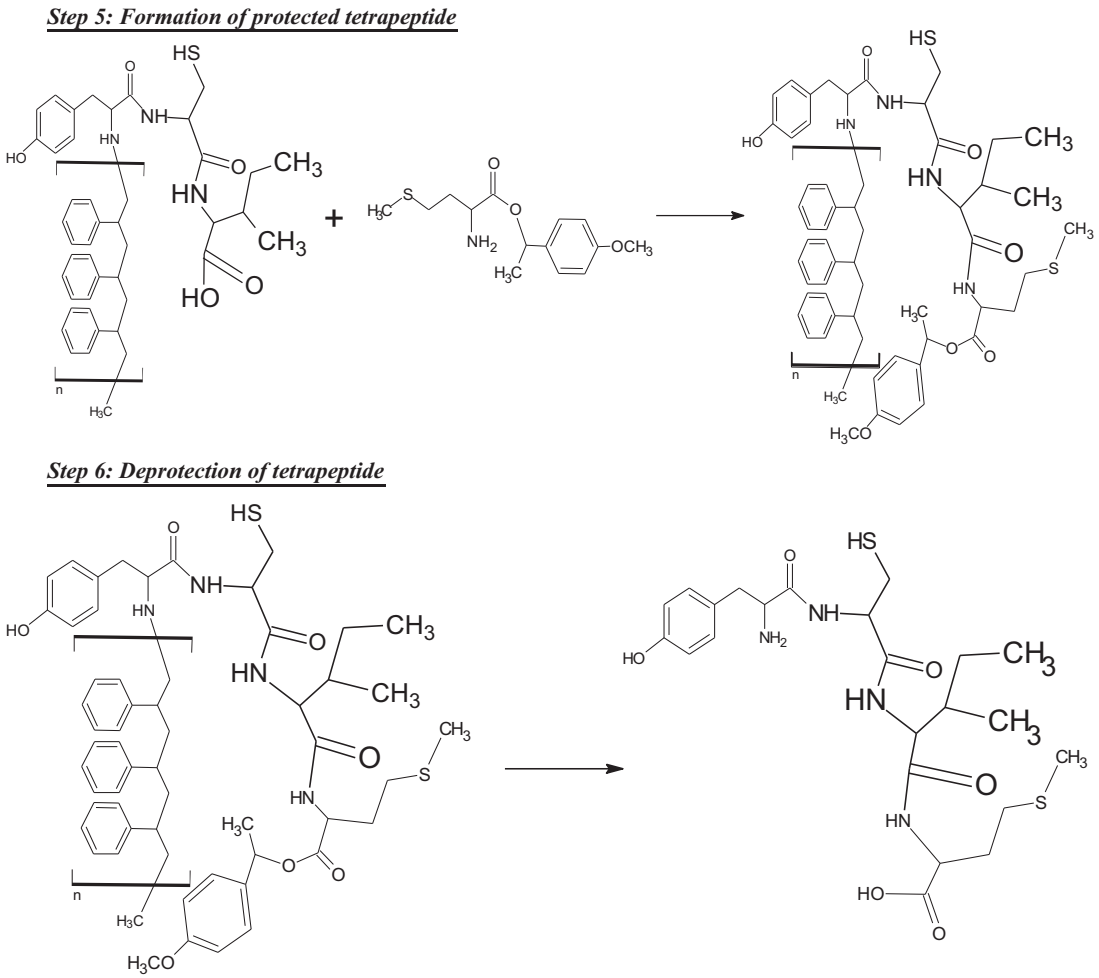


Fig. 2 continued

carbonate and washed it with brine solution, then kept it aside for some time. Then separated out the clear product and discarded the other one. Then added little quantity of sodium sulphate and heated it on oil bath for 15 min, dried it by rotavapour and placed in desiccator overnight. *Step (4): Protection of Phenylalanine.* 2.987 g of phenylalanine (18.1 mmol) was refluxed with 2.1 ml of 4-methyxybenzyl alcohol on oil bath for half an hour so that the carboxy group gets protected. *Step (5): Coupling of polystyrene-tyrosine-lysine-tryptophan with 4-methoxy benzyl alcohol-phenylalanine.* In a 250 ml flat round bottomed flask, protected polystyrene-tyrosine-lysine-tryptophan was taken and dissolved it in minimum volume of dry DCM. Then, secondly 4-methoxybenzyl alcohol-phenylalanine was added to it followed by the addition of 1-HOBt and DMAP. The environment of the reaction was made nitrogenous and cooled in ice-salt mixture and DCC solution was made by dissolving DCC in minimum volume of DCM. The DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then the reaction mixture was analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in Rotavapour and placed in dessicator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered in separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl and then neutralized it with 1N sodium carbonate and washed it with brine solution, kept it aside for some time. Then separated out the clear product and discarded the other one. Then, added little quantity of sodium sulphate and heated it on oil bath for 15 min, then dried it in rotavapour and placed in desiccator overnight. *Step (6): Deprotection.* The protected tetrapeptide 4-methoxy benzyl alcohol-phenylalanine-tryptophan-lysine-tyrosine-polystyrene was dissolved in 0.5 ml of anhydrous liquid hydrofluoric acid and the mixture was heated on steam bath for half an hour to deprotect the carboxy group in phenylalanine. The reaction mixture was further treated with 20 % piperidine in DMF

and again heated on water bath at 50 °C for half an hour. The corresponding polystyrene was filtered off followed by crystallization from aqueous ethanol, gives the FWKY as colourless crystal.

Synthesis of tetrapeptide Met-Trp-Lys-Tyr (MWKY) *Step (1): Protection of tyrosine.* In this protection, 2.605 g of polystyrene (9.238 mmol) was taken as amino protecting agent for tyrosine (3.276 g; 18.1 mmol) and heated in an oil bath at 160–180 °C. Within 10–15 min, the mixture melted and heating was continued for another 15 min. The reaction mixture was cooled; the residue was dissolved in ethanol and filtered off. Water was added to the clear filtrate till turbid and the solution was cooled at 0 °C for 2–3 h so that crystals of polystyrene tyrosine separate out by filtration and dried. *Step (2): Coupling of polystyrene tyrosine with lysine.* In a 250 ml flat round bottomed flask, protected amino acid was taken and dissolved it in minimum volume of dry DCM. Secondly, lysine (2.661 g; 18.1 mmol) was added to it followed by the addition of 1-hydroxybenzotriazole (7.33 g; 54.3 mmol) and 4-dimethyl amino pyridine (0.6552 g; 3.62 mmol). Then the environment of the reaction was made nitrogenous and cooled in ice-salt mixture and DCC (11.2 g; 54.3 mmol) solution was made by dissolving DCC in minimum volume of DCM (60 ml).
This DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then, the reaction mixture was analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotary vacuum evaporator and placed in desiccator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered by separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl, and neutralized it with 1N sodium carbonate and washed it with brine solution, then kept it aside for some time. Then separated out the clear product and discarded the other one. Then added little quantity of sodium sulphate and heated it on oil bath for 15 min, dried it by rotavapour and placed in desiccator overnight. *Step (3): Coupling of polystyrene tyrosine-lysine with tryptophan.* In a 250 ml flat round bottomed flask, protected amino acid as a product from previous step was taken and dissolved it in minimum volume of dry DCM. Secondly, tryptophan (3.692 g; 18.1 mmol) was added to it followed by the addition of 1-hydroxybenzotriazole (7.33 g; 54.3 mmol) and 4-dimethyl amino pyridine (0.6552 g; 3.62 mmol). Then the environment of the reaction was made nitrogenous and cooled in ice-salt mixture and DCC (11.2 g; 54.3 mmol) solution was made by dissolving DCC in minimum volume of DCM (60 ml). This DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then, the reaction mixture analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotary vacuum evaporator and placed in desiccator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered by separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl, and neutralized it with 1N sodium carbonate and washed it with brine solution, then kept it aside for some time. Then separated out the clear product and discarded the other one. Then added little quantity of sodium sulphate and heated it on oil bath for 15 min, dried it by rotavapour and placed in desiccator overnight. *Step (4): Protection of Methionine.* 2.7 g of methionine (18.1 mmol) was refluxed with 1.87 ml of 4-methyxybenzyl alcohol on oil bath for half an hour so that the thiol group gets protected. *Step (5): Coupling of polystyrene-tyrosine-lysine-tryptophan with 4-methoxy benzyl alcohol-methionine.* In a 250 ml flat round bottomed flask, protected polystyrene-tyrosine-lysine-tryptophan was taken and dissolved it in minimum volume of dry DCM. Then, secondly 4-methoxybenzyl alcohol-methionine was added to it followed by the addition of 1-HOBt and DMAP. The environment of the reaction was made nitrogenous and cooled in ice-salt mixture and DCC solution was made by dissolving DCC in minimum volume of DCM. The DCC solution was injected to the reaction mixture in cold condition. Finally, few drops of DMF were added to the solution and then the mixture of the solution was stirred overnight. Then the reaction mixture was analyzed on TLC using 10 % methanol in chloroform as mobile phase. After this, the mixture was filtered and dried in rotavapour and placed in desiccator for drying fully overnight. Next day, the dried product was dissolved in DCM and then directly filtered in separating funnel to avoid wastage. Then after, the filtered product was acidified with 1N HCl and then neutralized it with 1N sodium carbonate and washed it with brine solution, kept it aside for some time. Then separated out the clear product and discarded the other one. Then, added little quantity of sodium sulphate and heated it on oil bath for 15 min, then dried it in rotavapour and placed in desiccator overnight. *Step (6): Deprotection.* The protected tetrapeptide 4-methoxy benzyl alcohol-methionine-tryptophan-lysine-tyrosine-polystyrene was dissolved in 0.5 ml of anhydrous liquid hydrofluoric acid and the mixture was heated on steam bath for half an hour to deprotect the thiol group in methionine. The reaction mixture was further treated with 20 % piperidine in DMF and again heated on water bath at 50 °C for half an hour. The corresponding polystyrene was filtered off followed by crystallization from aqueous ethanol, gives the MWKY as colourless crystal.

Purification and preliminary identification of synthesized peptides

The purification of the synthesized compounds was performed by column chromatography. In column chromatography, the mobile phase is again a combination of solvents by successive solvents of their nonpolar–polar end, and the stationary phase is a finely divided solid, such as silica gel or neutral alumina. The compounds MRY, MVY uses silica gel as stationary phase whereas rest of the compounds MICY, MWKY and FWKY uses neutral alumina as stationary phase. There is an element of trial and error involved in selecting a suitable solvent and column for the purification of synthesized moiety.

A small amount of tripeptide/tetrapeptide sample (1 g) was taken in a china dish or petridish separately and either silica gel (30–70 mesh size) or neutral alumina was then added (double the quantity of compound) slowly with continuous mixing by adding sufficient quantity of DCM, till the desired consistency of mixtures were obtained. It was air dried and larger lumps were broken to get a smooth free flowing mixture.

A column of 5 feet length and 16 mm of internal diameter was taken separately for all the five test compounds and dried. The lower end of the column was plugged with absorbent cotton wool. The columns were clamped and fitted in vertical position on a stand. The column was then half-filled with *n*-hexane, silica gel or neutral alumina (21 g) was then poured and allowed to settle gently with the immiscible solvent *n*-hexane, until the necessary length of the column was obtained. The dried silica gel or alumina slurry containing the test compounds were poured in to the columns separately and then eluted successively with different solvents, in the order of petroleum ether (60–80 °C), petroleum ether: benzene (5:5), benzene, chloroform, chloroform: methanol (9:1), chloroform: methanol (4.5:5.5), methanol and methanol: acetic acid (9:1). Eight fractions were collected in conical flask and marked. The marked fractions were subjected to TLC to check homogeneity of various fractions (Stahl, 1969). Chromatographically identical various fractions (having same R_f values) of single spot were combined together and concentrated. The pure compounds were checked for melting point determinations using Digimelt MPA 161, Stanford Research System (SRS), USA.

Physico-chemical characterization of the tripeptides & tetrapeptides

All the purified column eluted tripeptides (MRY, MVY) and tetrapeptides (MICY, MWKY, FWKY) were further identified by infrared (IR), proton nuclear magnetic resonance (H NMR) and mass spectral analysis.

IR spectral analysis The IR spectra were recorded on Bruker's FT-IR-4100 type A spectrometer at Indian Institute of Technology (IIT), New Delhi. All the test compounds were subjected to IR by nujol mull technique in the range (4000–500 cm^{-1}) with the study of absorption of IR radiation functional groups clearly (Silverstein and Webster, 2005) and it is a powerful tool for structure elucidation. Fig. 3 illustrates the IR spectra of the tripeptide MVY.

^{1}H NMR analysis The ^{1}H NMR spectra of all the test compounds were recorded on Bruker's 400 MHz instrument using DMSO-d6 as the solvent at Indian Institute of Technology (IIT), New Delhi. The test compounds nucleus can be revealed and the number of protons predicted the structures of corresponding tripeptides and tetrapeptides with the help of H NMR analysis. Fig. 4 illustrates the tripeptide MVY.

Mass spectral analysis The electro spray ionization mass spectra for all the test compounds were recorded on Bruker's Micro TOFQ II 10262 mass spectrometer at IIT, New Delhi. The mass spectra give information on various types of peaks and determining the molecular formula for the test compound after successful interpretation (Gross, 2010). Fig. 5 illustrates the mass spectra of tripeptide MVY.

Toxicity study

Animals used Female Wistar rats weighing 150–200 g were obtained from the animal house of KIET School of Pharmacy, Ghaziabad. The rats were maintained in a well-ventilated room with 12:12 h light/dark cycle in polypropylene cages. The present study was approved by Institutional Animal Ethical Committee (IAEC) of KIET School of Pharmacy, Ghaziabad constituted under CPCSEA (IAEC ref. no. KSOP-02/IAEC/2012).

Acute toxicity study Toxicity is the degree to which a substance is able to damage an exposed organism. In screening drugs, determination of LD_{50} (the dose which has proved to be lethal causing death to 50 % of the tested group of animals) is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance. It is an initial assessment of toxic manifestations (provides information on health hazards likely to arise from short-term exposure to drugs) and is one of the initial screening experiments performed with all tripeptides and tetrapeptides. Fifteen rats were used for the tripeptides and nine rats were used for tetrapeptides and hence a total of twenty-four rats were used for the acute toxicity study. According to methodology of OECD guidelines 423 (Annexure 2b) adopted, the animals were fasted overnight, provided only water after which tripeptides and tetrapeptides were

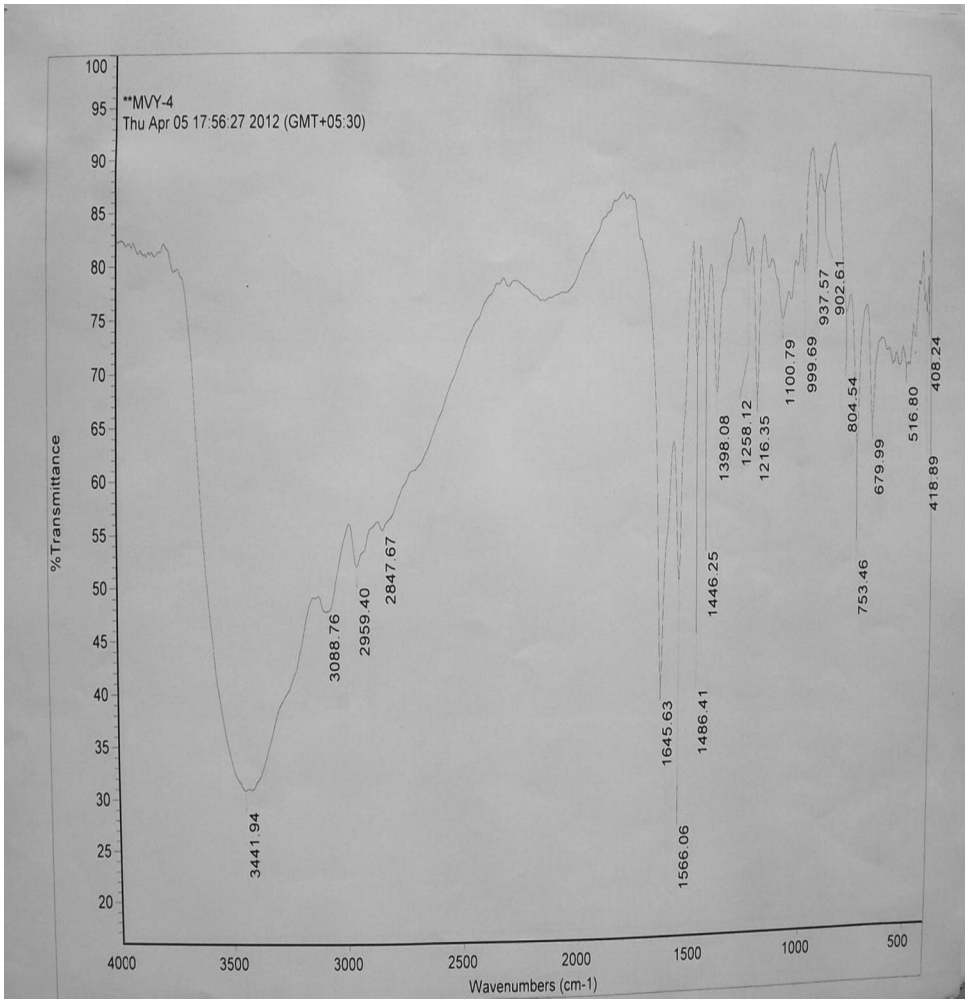


Fig. 3 Infrared spectrum of tripeptide Met-Val-Tyr (MVY)

administered separately to the group of 3 animals initially by gavage using a stomach tube at the dose level of 50 mg/kg body weight p.o.and the groups were observed for 14 days. The animals were observed for toxic symptoms such as behavioural changes, locomotion, convulsions and mortality were noted subsequently. (Koeter, 1990; Wilcox, 1994).

Antinociceptive activity of tripeptides and tetrapeptides

Animals used Albino Swiss mice weighing 40–50 g of either sex were used. The animals were grouped and housed in polyacrylic cages (38 × 23 × 10 cm) with not more than five animals per cage and maintained under standard laboratory conditions (temperature 25 + 2 °C) , relative humidity (50 % ± 5 %) and 12 h light/ dark cycle. They were allowed free access to standard pellet rat chow diet (Amrut feeds, Pranav Agro Industries Ltd., New Delhi, India) and water ad libitum. The mice were acclimatized to laboratory condition for 10 days before commencement of the experiment.

Drugs and chemicals The drug Aspirin (100 mg/kg) and the chemicals, Sodium Carboxy Methyl Cellulose (0.2 mg/100 ml), acetic acid (1 % v/v) were procured from CDH, Mumbai. Tripeptides were administered in the doses of 50 mg/kg, 100 mg/kg, 150 mg/kg, whereas the tetrapeptides were administered as 20 mg/kg, 25 mg/kg and 30 mg/kg body weight of mice. All the test drugs were administered through intraperitoneal route.

Acetic acid induced writhing method The mice were weighed, divided into seven groups, each consisting of four animals. I group served as control group (acetic acid 1 % v/v; 1 ml/100 g of body weight), II group received standard aspirin (100 mg/kg) + acetic acid (1 ml/100 g); III group received tripeptide (MRY) + acetic acid; IV group received tripeptide (MVY) + acetic acid; V group was treated with tetrapeptide (MICY) + acetic acid; VI group was treated with tetrapeptide (FWKY) + acetic acid and VII group was

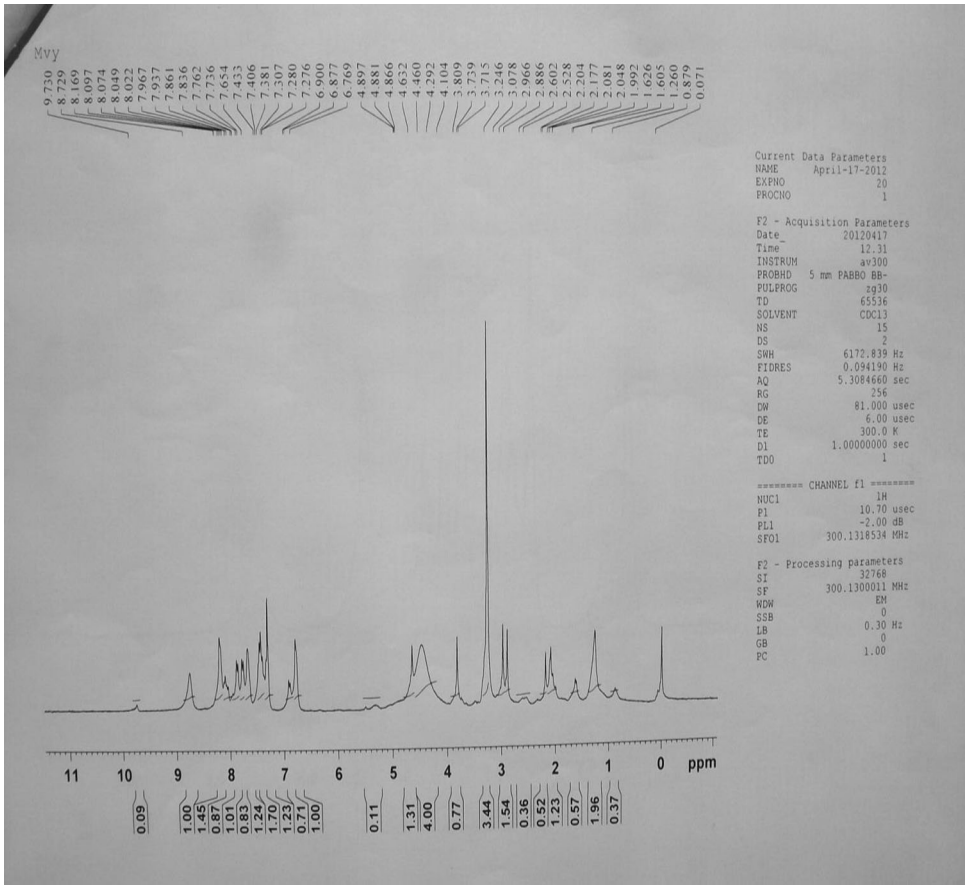


Fig. 4 ^{1}H-NMR spectrum of tripeptide Met-Val-Tyr (MVY)

treated with tetrapeptide (MWKY) + acetic acid. The appropriate volume of acetic acid solution was administered to the first group, which serves as control. The mice were placed individually under glass jar for observation and the onset on writhes were noted. The number of abdominal contractions, trunk twist response and extension of hind limbs, as well as the number of animals that showed such response during a period of 10 min were also recorded. To the second group of animals, test drug was injected and thereafter 15 min later, acetic acid solution was administered to these animals in appropriate volume. The onset and severity of writhing response was noted as done in the previous step (10 min). The mean of scores in control and test drug treated group were calculated. The inhibition of pain response by test drug was noted. Aspirin was used as standard drug for comparison of the results obtained. All the datas obtained were analysed statistically by one way ANOVA followed by Dunnett's multiple comparision test (Bhargave, 2013).

Tail flick method using hot plate The animals were weighed and numbered. The basal reaction time to radiant heat from the hot plate was taken by placing the tip (last 1–2 cm) of the animal tail on the radiant heat source. The tail withdrawal from the heat (flicking response) is taken as the end point. Normally, a mouse withdraws its tail within 3–5 s. A cut-off period of 10–12 s was observed to prevent damage to the tail. Any animal failing to withdraw its tail in 3–5 s is rejected from the study undertaken. At least 3–5 basal reaction time should be taken for each mouse at a gap of 5 min to confirm the normal behaviour of the animal. Six groups were selected containing four animals in each group. I group received standard drug—aspirin (100 mg/kg); II, III group received tripeptides MRY, MVY; and IV, V and VI group received tetrapeptides MICY, FWKY and MWKY, respectively (Pawar et al., 2011). The test drug having respective dose as similar to previous method was injected and the reaction time at 15, 30 and 45 min after the administration of test drugs was noted. When the reaction time reaches 10 s, it is considered maximum antinociceptive action and the tail is removed from the source of heat to avoid tissue damage. The percentage increase in reaction time (index of antinociception) at each time interval was calculated by the formula:

$$\%\,\text{Change} = \frac{\text{Activity (average of all animals)} - \text{Control}}{\text{Control}} \times 100$$

All the datas obtained were analysed statistically by one way ANOVA followed by Dunnett's multiple comparision test.

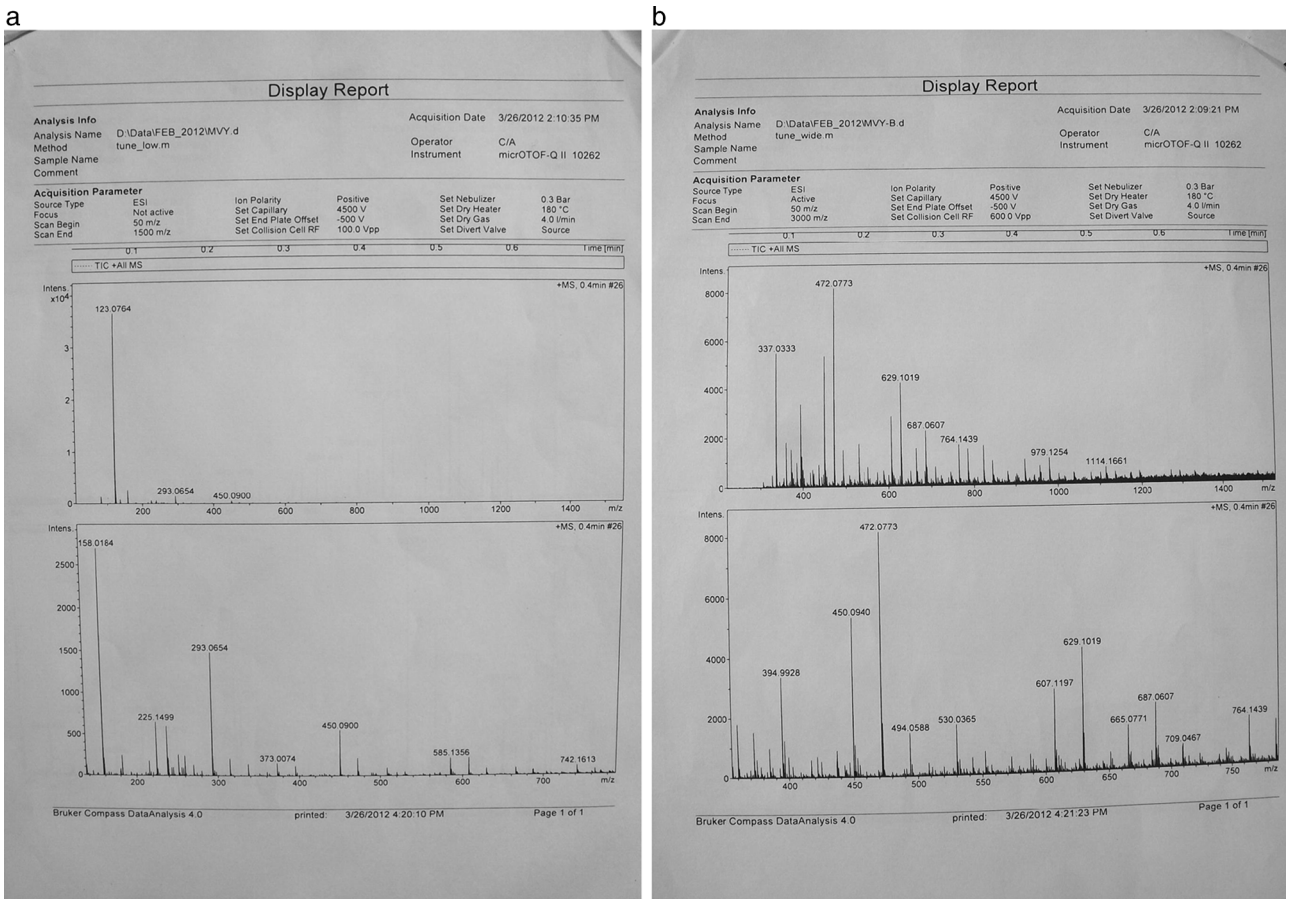


Fig. 5 **a** Mass spectrum of tripeptide Met-Val-Tyr (MVY) in low tune mode. **b** Mass spectrum of tripeptide Met-Val-Tyr (MVY) in wide tune mode

Results

Drug design

Among the 65 templates subjected to Boman index, only 18 template analogues are selected with the rational drug design approach as far as the antinociceptive potency is concerned and from these analogues, further five analogues, namely, MRY, MVY, MICY, MWKY and FWKY are selected along with their corresponding hydrophobic ratios with an optimal total net charge, are depicted as final outcome from Table 1.

The results from Table 1 clearly indicated that, for central antinociceptive action, drugs hydrophobicity plays a crucial role in addition with the Boman index value and these test compounds are further synthesized to predict the confirmation of antinociceptive potency practically in animal models.

Yield value and preliminary identification of tripeptides and tetrapeptides

All the tripeptides (MRY, MVY) and tetrapeptides (MICY, MWKY, FWKY) were synthesized and their yield values along with melting point and TLC R_f values for preliminary identification are reported in Table 2.

Table 1 Rational drug design outcome for antinociceptive peptide leads with their hydrophobicity ratios

S. no.	Selected lead set	Hydrophobicity ratio (%)	Total net charge	Boman index (kcal/mol)
1	MRY	33	+1	4.23
2	MVY	66	0	–2.08
3	MICY	75	0	–2.1
4	MWKY	50	+1	0.25
5	FWKY	50	+1	0.09

Physico-chemical characterization of the tripeptides and tetrapeptides

The test compounds were synthesized in good yield with sufficient purity and their spectral data by IR, ^{1}H NMR and mass spectroscopy are presented in Table 3, 4, 5, 6 and 7 for the tripeptides MRY, MVY and tetrapeptides MICY,

Table 2 Yield value and preliminary identification of synthesized peptides by TLC and melting point

S. no.	Test Compound	Practical yield (g)		% Yield	Melting point	R_f values MP=C:M/9:1
		Before purification	After purification	After purification	(°C)	
1	MRY	12.5	0.34	4.01	94	0.21
2	MVY	14	0.56	7.6	102	0.32
3	MICY	16.4	0.24	2.5	96	0.41
4	FWKY	15.78	0.43	3.7	112	0.48
5	MWKY	11.14	0.39	3.4	106	0.51

M.P. mobile phase; *C:M* chloroform:methanol

FWKY, MWKY, respectively. The detailed description of IR data of test compounds is given below.

Met-Arg-Tyr (MRY)

The IR spectral data of the tripeptide MRY has shown characteristic absorption bands, viz. O–H stretch: 3353.75 cm^{-1}, O↓H bend: 1298.98 cm^{-1}, Ar C–H stretch: 2980.09 cm^{-1}, Ar C–O stretch: 1205.45 $cm^{-1,}$ Alkanes C–H stretch 2933.48 cm^{-1}, Alkanes C↓H bend: 1397.82 cm^{-1}, Imines C=N stretch: 1545 cm^{-1}, C=O stretch (amide I band): 1629.47 cm^{-1}, N↓H bend and C–N stretch (amide II band): 1568.67 cm^{-1} and thiols C–S stretch: 701.18 cm^{-1}, respectively, which cleanly indicated. The formation tripeptide MRY in the mid IR region.

MVY

The IR spectral data of the tripeptide MVY has shown characteristic absorption bands, viz., N↓H stretch: 3441.94 cm^{-1}, Ar C–H stretch:3088.76 cm^{-1}, Alknaes C–H stretch: 2959.40 cm-1; Alkanes C↓H bend: 1398.08 cm^{-1}, Ar C–O stretch: 1258.12 cm^{-1}, C=O stretch (amide I band): 1645.63 cm^{-1}, N↓H δ bend & C–N stretch (amide II band): 1566.06 cm^{-1}. Thiols C–S stretch: 679.99 cm^{-1}, respectively which clearly indicated the formation of tripeptide MVY in the mod IR region.

MICY

Tetrapeptide Ar C–H stretch: 3068.61 cm^{-1}, Ar C↓H bend: 805.39 cm^{-1}, N–H stretch for amines: 3426.23 cm-1, alkanes C–H stretch: 2951.78 cm^{-1}, alkanes C↓H bend: 1399.72 cm^{-1}, C=O stretch (amide I band): 1644.99 cm^{-1}, N↓H δ bend (amide I band): 1565.52 cm^{-1}, Thiols S–H stretch 2951.78 cm^{-1}, respectively.

FWKY

Tertra peptide Ar C–H stretch: 3069.72 cm^{-1}; C=O stretch (amide I band):1644.42 cm^{-1}; N↓H bend and C–N stretch (amide II band): 1565.51 cm^{-1}; alkanes C–H stretch: 2955.76 cm^{-1}; alkanes C↓H bend: 1398.70 cm^{-1}; hetro aromatic pyrrole N–H stretch: 3422.65 cm^{-1} and hetroaromatic pyrrole CH δoop band: 742.78 cm^{-1}, respectively.

MWKY

Aromatic C–H stretch: 3080 cm^{-1}; alkanes C↓H Stretch 2957.59 cm^{-1}; alkanes C–H bend: 1298.53 cm^{-1}; C=O stretch (amide I band): 1644.43 cm^{-1}; N↓H δ bend and C–N stretch (amide II band): 1566.05 cm^{-1}; thiols C–S stretch: 607.51 cm^{-1}; hetroaromatic pyrrole N–H stretch: 3470.10 cm^{-1} and hetroaromatic pyrrole CH δoop band: 754.50 cm^{-1}, respectively.

Acute toxicity testing

The LD_{50} cut-off mg/kg body weight for the tripeptides MRY and MVY was found to be 500–2000 mg/kg (Category 4), whereas the LD_{50} cut-off mg/kg body weight for the tetrapeptides MICY, FWKY and MWKY were found to be 200–300 mg/kg (Category 3). 1/10th of dose (maximum dose) tested for LD_{50} of individual peptides was considered for the testing of antinociceptive activity. From the above study, it is concluded that tripeptides are safer than the tetrapeptides in higher doses of drug administration to animals.

Antinociceptive activity of peptides

Acetic acid induced writhing test

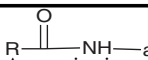
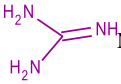
The results of test peptides by acetic acid induced writhing are expressed in Table 8. From the above data of Table 8, it is found that the tripeptide MVY have shown

Table 3 Physico-chemical characterization for Met-Arg-Tyr (MRY)

3.1 Physico-chemical characterization of the tripeptide: Met-Arg-Tyr by infrared spectroscopy

S. no.	Wavenumber (cm^{-1})	Characteristic functional group feasible	Compound type
1	3353.75	O–H st.	Alcohols
		N–H st.	Corboxylic amides
			Amino acids and amines
		C–H st.	Cyclic alkanes
2	2980.09–2933.48	C–H st.	Alkanes and cycloakanes
		N^+–H st.	Amines
3	1629.47	ar C–C st.	Aromatic hydrocarbon
4	1568.67	N–H δ bend	Amines
		N^+–H δ bend	Amines
5	1629.47–1568.67	N–H δ and N–C=O st. sy.	Amide II of carboxylic amides
6	1454.45	CH_3 δ asy. bend	Alkanes
		CH_2 δ bend	Alkanes
7	1205.42–1045.40	C–O(H) st.	Alcohols
8	798.49–701.18	ar C–H δoop bend	Aromatic hydrocarbons
9	751.42	CH_2 γ bend	Alkanes
10	701.18	C–S st.	Sulphates

3.2 Physico-chemical characterization of the tripeptide: Met-Arg-Tyr by NMR spectroscopy

S. no.	δ value(ppm)	Nature of peak	Significance and resemblance
1	8.101	Small singlet broad	 amide proton
2	7.232	Doublet	Aromatic ring proton
3	6.877	Doublet	Aromatic "OH" proton
4	6.600	Doublet	 NH proton of
5	2.944	Sharp singlet	NH_2 proton
6	2.506	Quartet	S–CH_3 proton
7	1.8	Doublet	C–H proton
8	1.248	Doublet	CH_2 proton

3.3 Physico-chemical characterization of the tripeptide: Met-Arg-Tyr by mass spectrometry

S. no.	Name of the ion	Mass	Product ion and composition of the neutral particle lost	Substructure or compound type	Peak *m/z* ratio (Mol. wt: 440)
1	C_2^+	24	$[M-24]^{+\cdot}$	—	432
2	$CONH^+$	43	$[M-43]^{+\cdot}$ (C_3 H_7)	Non-specific; Abundant: Cyclo alkyl amines	413
3	$C_5H_6^+$	66	$[M-66]^{+\cdot}$ (C_5H_6)	Cyclopentenes	390
4	$C_4H_{10}N^+$	72	$[M-72]^{+\cdot}$	Aliphatic amines "N" indicator	384
5	$C_4H_8O_2^+$	88	$[M-88]^{+\cdot}$	Ethyl esters of carboxylic acids.	368

maximum antinociceptive action (average number of writhing = 13) at its higher dose of 150 mg/kg body weight. Among the tetrapeptides tested, it is found that FWKY exerts maximum antinociceptive action at 30 mg/kg body weight with an average number writhing of 16 in mice. In comparison with all the test compounds, tripeptides are found to have proficient antinociceptive action than the tetrapeptides as similar to the safety profile of tripeptides under acute toxicity testing.

Tail flick by hot plate method

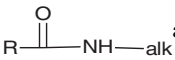
The results of tail flicking by hot plate method for the tested peptides are expressed in Table 9. The maximum

Table 4 Physico-chemical characterization for Met-Val-Tyr (MVY)

4.1 Physico-chemical characterization of the tripeptide: Met-Val-Tyr by infrared spectroscopy

S. no.	Wavenumber (cm^{-1})	Characteristic functional group feasible	Compound type
1	3441.94	O–H st.	Alcohols
		N–H st.	Amides, amino acids, amines
		C–H st.	Cyclic alkanes
		COO–H st.	Carboxylic acids
2	3088.76	H–C(=C) st.	Aromatgic hydrocarbon
		ar C–H st.	
3	2959.40	CH st.	Alkanes and cycloalkanes
4	2847.67	H-C(–N) st. Combination band	Amines aldehydes
5	1645.63	C=C st. C=O st.	Alkenes, cycloalkenes aldehydes
6	1645.63–1566.06	N–H δ bend. N–H δ and N–C=O st. sy. (Amide II)	Amines amides and lactam
7	1486.41	—	Monosubstituted aromatic
8	1446.25	ar C–C st.	Aromatic hydrocarbon
9	1258.12–999.69	C–O(H) st.	Alcohols
10	999.69–679.99	H–C(=C)δoop bend	Alkene, cycloalkane
11	902.61–679.99	ar C–H δoop bend	Aromatic hydrocarbon
12	753.46	CH_2 γ bend	Alkanes

4.2 Physico-chemical characterization of the tripeptide: Met-Val-Tyr by NMR spectroscopy

S. no.	δ value(ppm)	Nature of peak	Significance and resemblance
1	9.730	Singlet narrow	Aldehydic CHO proton
2	8.101	Small singlet broad	 amide proton
3	7.307	Doublet	Aromatic ring proton
4	6.900	Doublet	Aromatic "OH" proton
5	3.809	Sharp singlet	Amide methine proton
6	2.602	Singlet broad	S–CH_3 proton
7	1.605	Doublet	CH proton
8	1.260	Singlet broad	CH_2 proton
9	0.879	Doublet broad	CH_3 proton
10	0.071	Singlet sharp	Proton of internal reference standard TMS

4.3 Physico-chemical characterization of the tripeptide: Met-Val-Tyr by mass spectrometry

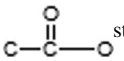
S. no.	Name of the ion	Mass	Product ion and composition of the neutral particle lost	Substructure or compound type	Peak *m/z* ratio (Mol. wt: 469)
1	$C_2H_3^+$	27	$[M-27]^+$. (C_2H_3)	Terminal vinyl, some ethyl esters	373
2	$C_5H_3^+$	63	$[M-63]^{+\cdot}$	—	337

antinociceptive potency was observed with tripeptide-MRY at a dose of 150 mg/kg with the average reaction time of 5.75 min after 15 min, which is almost comparable with the standard drug aspirin (6.75 min after 15 min). The same drug MRY also showed the reaction time of 8.25 min after 30 min interval, which clearly indicated the tripeptide MRY has better onset of action and longer duration of antinociceptive action.

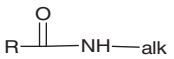
Similarly the tetrapeptides MWKY (5.5 min) and MICY (5.25 min) also showed efficient antinociceptive action next only to the tripeptide MRY after 15 min time interval. The tetrapeptide MICY showed antinociceptive action with longer duration of action, as evidenced with twice the analgesia index (%) after 15 min (75 %) and 30 min (150 %) interval (Table 9).

Table 5 Physico-chemical characterization for Met-Ile-Cys-Tyr (MICY)

5.1 Physico-chemical characterization of the tetrapeptide: Met-Ile-Cys-Tyr by infrared spectroscopy

S. no.	Wavenumber (cm^{-1})	Characteristic functional group feasible	Compound type
1	3426.23	O–H st.	Alcohol, phenol
—	—	N–H st.	Amides, amines
—	—	COO–H st.	Carboxylic acids
2	3068.61	H–C(=C) st.	Alkenes, cyclo alkenes
—		ar C–H st.	Aromatic hydrocarbon
3	2951.78	N^+–H st.	Amines
4	2850	Two weak band combinations	Aldehyde
5	2600	S–H st.	Thiols and sulfides
6	2096.03	Overtone bond assigned to a combination of NH_3^+ bend asy. And torsional oscillation of NH_3^+ gp.	Amines
7	1644.99	C=C st.	Alkenes, cycloalkenes
		C=O st.	Aldehydes
8	1565.52	N–H δ and N-C=O st. sy. (amide II)	Amides
9	1445.07	CH_2–(C=C)δ bend.	Alkenes, cycloalkenes
		ar C–C st.	Aromatic hydrocarbon
10	1216.41–999.64	C–(H) st.	Alcohols, phenols
11	1216.41	 st.	Amino acids
12	999.64–681.69	H–C(=C) δ oop. Bend.	Alkenes, cycloalkenes
13	805.39–681.69	ar C–H δoop bend	Aromatic hydrocarbon
14	752.64	CH_2 γ bend	Alkanes, cycloalkanes
15	681.69–607.65	C–S st.	Sulfides

5.2 Physico-chemical characterization of the tetrapeptide: Met-Ile-Cys-Tyr by NMR spectroscopy

S. no.	δ value (ppm)	Nature of peak	Significance and resemblance
1	12.461	Singlet broad	Intra molecular H-bonded phenols
2	8.412	Singlet broad	 amide proton
3	7.425	Pentet	Aromatic ring proton
4	6.793	Doublet	Aromatic OH proton
5	3.244	Sharp singlet	HS–CH_2 proton
6	2.820	Doublet	S–CH_3 proton
7	1.209	Singlet	CH_2 proton
8	0.000	Small narrow singlet	Proton of internal reference standard TMS

5.3 Physico-chemical characterization of the tetrapeptide: Met-Ile-Cys-Tyr by mass spectrometry

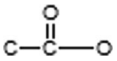
S. no.	Name of the ion	Mass	Product ion and composition of the neutral particle lost	Substructure or compound type	Peak *m/z* ratio (Mol. wt: 423)
1	$CH_2O^{+\cdot}$, $CH_2NH_2^+$ "N" indicator	30	$[M-30]^+.(CH_2O)$	Cyclic ethers, primary alcohols	487

Discussion

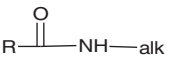
There is a good correlation achieved between the selected templates from drug design and the practically observed moieties for antinociceptive testing. MRY, MWKY and MICY showed significant hydrophobicity ratio, total net charge along with Boman index value and upon antinociceptive testing by tail flick method in mice, the same tripeptides and tetrapeptides were found to have good potency among all other peptides. This

Table 6 Physico-chemical characterization for Phe-Trp-Lys-Tyr (FWKY)

6.1 Physico-chemical characterization of the tetrapeptide: Phe-Trp-Lys-Tyr by infrared spectroscopy

S. no.	Wavenumber (cm^{-1})	Characteristic functional group feasible	Compound type
1	3422.65	O–H st.	Alcohols, phenols
		N–H st.	Amines, amides
		ar N–H st.	Hetero aromatic compounds
2	3069.72	H–C(=C) st.	Alkenes, cyclo alkenes
		ar C–H st.	Aromatic hydrocarbon and heteroaromatic compounds
3	2955.76	N^+–H st.	Amines
4	2097.44	Overtone region of NH_3^+ asy. bend and torsional oscillation of NH_3^+ gp.	Amines
5	1644.42	C=O st.	Aldehydes
6	5565.51	N–H δ and N-C=O st. sy. (amide II)	Amides
7	1446.64	CH_2–(C=C) δ bend	Alkene, cycloalkane
		ar C–C st.	Aromatic hydrocarbon
8	1216.54–1001.53	C–O(H) st.	Alcohols, phenols
9	1216.54	 st.	Amino acids
10	1001.53	H–C(=C) δ opp. Bend.	Alkenes, cycloalkenes
11	1057.52–681.91	ar C–H δ oop bend.	Heteroaromatic compound
12	74.78–681.91	ar C–H δ oop bend.	Aromatic hydrocarbon
13	606.36	C–S st. (weak)	Sulfides

6.2 Physico-chemical characterization of the tetrapeptide: Phe-Trp-Lys-Tyr by NMR spectroscopy

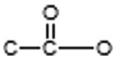
S. no.	δ value(ppm)	Nature of peak	Significance & resemblance
1	12.3	Singlet broads	Aldehydic CHO proton
2	9.9	Tiny singlet	NH ring proton of Indole
3	8.149	Doublet	 amide proton
4	7.940	Triplet	Proton "c" of indole ring
5	7.529	Triplet	Proton "f" of indole ring
6	7.309	Doublet	Proton "a" of indole ring
7	7.204	Singlet	Proton "e" of indole ring
8	6.864	Doublet	Proton "d" of indole ring
9	6.759	Doublet	Aromatic "OH" proton
10	6.496	Singlet broad	Proton "b" of indole ring
11	4.438	Singlet small	Amide methine proton
12	3.235	Sharp singlet	NH_2 proton
13	2.873	Doublet	Benzylic CH_2 proton
14	1.253	Triplet	CH_2 proton
15	0.000	Singlet sharp	Proton of internal reference standard TMS

6.3 Physico-chemical characterization of the tetrapeptide: Phe-Trp-Lys-Tyr by mass spectrometry

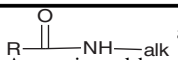
S. no.	Name of the ion	Mass	Product ion and composition of the neutral particle lost	Substructure or compound type	Peak *m/z* ratio (Mol. wt: 425)
1	$C_{12}H_{15}O_4^+$	223	$[M-223]^+$	Phalates	413

Table 7 Physico-chemical characterization for Met-Trp-Lys-Tyr (MWKY)

7.1 Physico-chemical characterization of the tetrapeptide: Met-Trp-Lys-Tyr by infrared spectroscopy

S. no.	Wavenumber (cm^{-1})	Characteristic functional group feasible	Compound type
1	3470.10	O–H st.	Alcohols, phenols
		N–H st.	Amines, amides
		ar N–H st.	Hetero aromatic compounds
2	3050	H–C(=C) st.	Alkenes, cyclo alkenes
		ar C–H st.	Aromatic hydrocarbon and heteroaromatic compounds
3	2957.59	N^+–H st.	Amines
4	2157.31	Overtone region of asy. NH_3^+ bend and torsional oscillation of NH_3^+	Amines
5	1644.43	C=O st.	Aldehydes
6	1566.05	N–H δ and N-C=O st. sy. (amide II)	Amides
7	1445.15	CH_2–(C=C)δ bend. ar C-C st.	Alkene, cycloalkane aromatic hydrocarbon
8	1216.74–1001.61	C–O (H) st.	Alcohols, phenols
9	1216.74	 st.	Amino acids
10	1001.61–680.82	ar C–H δoop bend	Hetero aromatic compound
11	1001.61	H–C(=C) δoop bend	Alkenes, cycloalkenes
12	754.50	ar C–H δoop bend	Aromatic hydrocarbons

7.2 Physico-chemical characterization of the tetrapeptide: Met-Trp-Lys-Tyr by NMR spectroscopy

S. no.	δ value(ppm)	Nature of peak	Significance and resemblance
1	8.101	Sharp singlet	 amide proton
2	7.209–6.854	Doublet, triplet	Aromatic and hetero aromatic ring proton
3	6.616	Doublet	Aromatic "OH" proton
4	4.143	Tiny broad singlet	Amide methine proton
5	2.962	Sharp singlet	NH_2 proton
6	2.737	Tiny broad singlet	S–CH_3 proton
7	1.247	doublet	CH_2 proton

7.3 Physico-chemical characterization of the tetrapeptide: Met-Trp-Lys-Tyr by mass spectrometry

S. no.	Name of the ion	Mass	Product ion and composition of the neutral particle lost	Substructure or compound type	Peak *m/z* ratio (Mol. wt: 390)
1	$C_2H_6S^{+\cdot}$	62	$[M-62]^+$. (C_2H_6S)	Ethyl sufides	553

clearly indicates that there is a rationale behind our drug design study in choosing the right parameter for antinociception.

The thermal methods generally require higher antinociceptive dose rates to prevent a pain response than other methods, and the duration of antinociception is shorter than for mechanical methods. Therefore, it is suggested that drug dosages based on thermal assessment may be more clinically relevant than dosages based on other techniques.

Hence, based on the thermal method, hot plate method, we found that the tripeptide MRY exerts maximum antinociceptive potency among all the tri and tetrapeptides tested. Biologically, tested potent compounds' amino acid sequence (MRY) made from the present investigation contains Arginine and Tyrosine, which is also present as amino acid sequence in the pentapeptide neo-kyotorphin (Thr-Ser-Lys-Tyr-Arg) isolated from bovine brain (Kiso et al., 2010). Similar results of amino acid sequence are observed with dermorphin tetrapeptide N^{α} amidino-Tyr-D-Arg-Phe-MeβAla-OH(ADAMB), which showed longer acting antinociception using tail pressure test in mice (Ogawa et al., 2002).

In addition, drugs containing dynorphin-like analgestic peptide, CH_3-[^{125}I]Tyr-Gly-Gly-Phe-Leu-Arg-CH'_3-Arg-D-

Table 8 Antinociceptive effects of the tripeptides and tetrapeptides by chemical induced writhing method

Groups	Treatment	Dose	Average body weight (g)	Average number of writhing (10 min)
I	Control: acetic acid	1 ml/100 g body weight	45	45 ± 2.58
II	Standard: aspirin + acetic acid	100 mg/kg + 1 ml/100 g	45	$9 \pm 1.63^{a,b}$
III	Treipeptide MRY + acetic acid	50 mg/kg	45	$24 \pm 1.29^{a,b}$
	—	100 mg/kg	45	$18 \pm 0.81^{a,b}$
	—	150 mg/kg	45	$14 \pm 2.94^{a,b}$
IV	Tripeptide MVY + acetic acid	50 mg/kg	45	$22 \pm 1.41^{a,b}$
		100 mg/kg	45	$16 \pm 1.41^{a,b}$
		150 mg/kg	45	$13 \pm 1.63^{c,b}$
V	Tetrapeptide MICY + acetic acid	20 mg/kg	45	$30 \pm 2.44^{a,b}$
		25 mg/kg	45	$22 \pm 1.29^{a,b}$
		30 mg/kg	45	$18 \pm 0.81^{a,b}$
VI	Tetrapeptide FWKY + acetic acid	20 mg/kg	45	$29 \pm 1.63^{a,b}$
		25 mg/kg	45	$20 \pm 1.50^{a,b}$
		30 mg/kg	45	$16 \pm 0.81^{a,b}$
VII	Tetrapeptide MWKY + acetic acid	20 mg/kg	45	$30 \pm 1.41^{a,b}$
		25 mg/kg	45	$23 \pm 0.95^{a,b}$
		30 mg/kg	45	$20 \pm 1.41^{a,b}$

Statistical significance analysed by one way ANOVA followed by Dunnett's multiple comparision test. All datas are expressed as mean ± SD

[a] Highly significant ($p < 0.001$) as compared to control-acetic acid

[b] Significant ($p < 0.01$) as compared to standard-aspirin

[c] Significant ($p < 0.01$) as compared to control-acetic acid

Table 9 Antinociceptive effects of tripeptides and tetrapeptides by tail flick method in mice

Groups	Treatment	Dose	Average body weight (g)	Basal reacti on time (s)	Reaction time (average)			Analgesia index (%)		
					15 min	30 min	45 min	15 min	30 min	45 (mts)
I	Standard aspirin	100 mg/kg	45	3 ± 0.81	6.75 ± 0.95^{a}	10 ± 0.00^{a}	—	108.33	233.33	—
II	Tripeptide MRY	50 mg/kg	45	—	4 ± 0.81^{ns}	$6.5 \pm 0.57^{a,b}$	10	33.33	116.67	233.33
		150 mg/kg	45	—	$5.75 \pm 0.50^{a,b}$	8.25 ± 0.50^{a}	10	91.67	175	233.33
III	Tripeptide MVY	50 mg/kg	45	—	4 ± 0.81^{ns}	$6 \pm 0.81^{a,b}$	10	33.33	100	233.33
		150 mg/kg	45	—	5 ± 0.81^{a}	$7.5 \pm 0.57^{a,b}$	10	66.67	150	233.33
IV	Tetrapeptide MICY	20 mg/kg	45	—	4 ± 0.81^{ns}	$6.25 \pm 0.50^{a,b}$	10	33.33	108.33	233.33
		30 mg/kg	45	—	5.25 ± 0.50^{a}	$7.5 \pm 0.57^{a,b}$	10	75	150	233.33
V	Tetrapeptide FWKY	20 mg/kg	45	—	4 ± 0.81^{ns}	$5.5 \pm 0.57^{a,b}$	10	33.33	83.33	233.33
		30 mg/kg	45	—	5 ± 0.81^{a}	$7.25 \pm 0.5^{a,b}$	10	66.67	141.67	233.33
VI	Tetrapeptide MWKY	20 mg/kg	45	—	4.25 ± 0.95^{ns}	$6 \pm 0.81^{a,b}$	10	41.66	100	233.33
		30 mg/kg	45	—	$5.5 \pm 0.57^{a,b}$	$7.25 \pm 0.5^{a,b}$	10	83.33	141	233.33

Statistical significance analysed by one way ANOVA followed by Dunnett's multiple comparision test. All datas are expressed as mean ± SD

[a] Significant ($p < 0.01$) as compared to control-basal reaction time

[b] Non significant ($p > 0.05$) as compared to standard-aspirin

[ns] Non significant ($p > 0.05$) as compared to control

Leu-NH C_2H_5([^{125}I]E-2078) also possesses arginine and tyrosine, residue, which is also present as amino acid sequence in our studies having most potent tripeptide MRY, which clearly shows that our identified and tested drug shall have the tendency to cross the blood brain barrier in a similar fashion as that of E-2078 (Terasaki et al., 1991).

Vanilloid receptors (VRs) play a fundamental role in the transduction of peripheral tissue injury and inflammation responses and the molecules that antagomize VR channel activity may act as selective and potent antinociceptive agent. Dynorphins are the natural arginine-rich peptides, which blocked VR1 activity with micromolar affinity (Cases et al., 2000). From the above observations, it can be inferred that the most potent antinociceptive tripeptide (MRY) tested also contains arginine residue and thus indicating that the most potent test drug MRY may probably act by blocking the VR-1 channels with antinocecptive activity.

The hot plate and tail flick methods are the most common tests of nociception that is based on a phasic stimulus of high intensity. The nociceptive experience is short lasting and it is well accepted that agonist of μ-opioid receptors produce analgesia in acute pain model. Acetic acid causes inflammatory pain by inducing capillary permeability and liberating endogenous substances that excite pain nerve endings. NSAIDs can inhibit COX in peripheral tissues and therefore interfere with the mechanism of transduction of primary afferent nociceptors (Barua et al., 2010). Our synthesized peptides are expected to act in the similar manner. Further investigations may be carried out to know the exact mechanism.

Since peptides are more biofriendly drugs as compared with all other heterocyclics, they are increasingly receiving more attention and a large number of new compounds or derivatives from peptides are under investigation. With this and a better understanding of the mechanism of nociception, more targets will be validated, and hopefully a pattern will emerge that will help us reach the goals of more potent lead compounds that allow these peptide drug targets for antinociceptive effect for humans in near future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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