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A Novel and Efficient Approach for the Amidation of *C*-Terminal Peptides

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This paper dedicated to Dr. Shahriyar Omidvar for his effective contribution in Iranian pharmaceutical industry

A highly efficient and practical synthesis of *C*-terminal amidated peptides has been developed. According to this approach, amidation of the *C*-terminus of peptides was carried out using NH_4Cl, alkylammonium chloride (RNH_3Cl) and semicarbazide hydrochloride in the presence of TBTU as a coupling reagent and a tertiary amine as the base at room temperature in good to high yields. Some opioid peptides such as enkephalin derivatives were synthesized according to this novel method.

Keywords: Met-enkephalin, Leu-enkephalin, Solid phase peptide synthesis, Amidation, C-Terminal amidated peptides

INTRODUCTION

The presence of a *C*-terminal of amido group on the peptide chain is essential for the biological activity of many peptide hormones. There are many commercially available GnRH peptides based drugs that contain an amide, alkyl, or semicarbazide group, such as leuprolide, buserelin, histrelin, goserelin, and nafarelin [1]. It was shown that derivatization of the terminal carboxyl function of Met-enkephalin to get alkylamides could be expected to lead to derivatives that would not only be resistant to the attack of carboxypeptidases, but would also posses higher binding affinity for the opioid receptors due to enhanced hydrophobicity at the *C*-terminus. It could also affect the lipophilicity of the peptides, and could affect the interaction with the opiate receptors and cause the activity of enkephalin derivatives. Meanwhile, lengthening and shortening of the alkyl chain was found to have an adverse effect on the antinociceptive activity of the peptide [2-5].

Amidated peptides are usually prepared by solid-phase synthesis on benzhydrylamine resins or by the aminolysis of *C*-terminal peptide esters, which can be prepared by conventional peptide synthesis. Recombinant DNA technology allows the production of longer peptides by fermentation, but these products lack the *C*-terminal amido group [5]. There are different methods for the synthesis of *C*-terminal amidated peptides such as; a) enzymatic amidation [6], b) the combination of rDNA technology with chemical modification of the *C*-terminus, c) using of amide resins in SPPS, d) using of carboxypeptidase in the presence of ammonia, e) conversion of the *C*-terminus of peptides to the methylester and addition of ammonia at low temperature.

All of the reported methods have some merit such as: a) laborious reaction conditions, b) high price of enzymes and limitation of solubility parameters, c) using of ammonia or alkylamines as gas and performing the reaction at low temperature, d) the use of HF for the cleavage of the peptide from the surface of the resin. Separation and purification of enzymes need more time and energy [7-17].

EXPERIMENTAL

General

^{1}H and ^{13}C NMR spectra were recorded on a *Bruker DRX-500 AVANCE* at 500 and 125 MHz, respectively, using TMS as internal standard and DMSO (d_6) as solvent. Mass spectra were obtained using a *MALDI-MS Bruker ApexQe FT-ICR*

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instrument.

General Procedure for the Synthesis of Protected Enkephalins (5a-f)

Peptide synthesis was carried out using 2-chlorotrityl chloride resin (1 mmol g^{-1}) following standard Fmoc strategy. Fmoc-AA-OH (AA: Leu- or Met-, 2 mmol) was attached to the 2-CTC resin with 1.4 cm^3 DIPEA (8 mmol) in 10 cm^3 anhydrous DCM: DMF (1:1) at room temperature for 2 h. After filtration, the remaining trityl chloride groups were capped by 24 cm^3 solution of DCM:MeOH:DIPEA (17:2:1) for 30 min. The resin was filtered and washed thoroughly with DCM (1 × 5 cm^3), DMF (4 × 5 cm^3) and MeOH (5 × 5 cm^3). The loading capacity was determined by weight after drying the resin under vacuum and was 0.9. The resin-bound Fmoc-amino acid was washed with DMF (3 × 5 cm^3) and treated with 12.7 cm^3 of 25% piperidine in DMF for 30 min and the resin was washed with DMF (3 × 5 cm^3) [19]. Then a solution of 0.77 g Fmoc-Phe-OH (2 mmol), 0.64 g TBTU (2 mmol), 0.8 cm^3 DIPEA (4.7 mmol) in 6.5 cm^3 DMF was added to the resin-bound free amine and shaken for 2 h at room temperature. After completion of coupling, resin was washed with DMF (4 × 5 cm^3) and DCM (1 × 5 cm^3). After Fmoc deprotection, 0.6 g Fmoc-Gly-OH (2 mmol) was coupled in the presence of TBTU, DIPEA in DMF. The coupling was repeated another time with Fmoc-Gly-OH. In all cases for the presence or absence of free primary amino groups, *Kaiser* Test was used. Fmoc determination was done using UV spectroscopy method.

After Fmoc deprotection from the tetrapeptide-resin, a solution of 0.675 g Boc-Tyr(tBu)-OH (2 mmol), 0.64 g TBTU (2 mmol), 0.8 cm^3 DIPEA (4.7 mmol) in 6.5 cm^3 DMF was added to the resin-tetrapeptide for the formation of amide bond. After completion of coupling, resin was washed with DMF (4 × 5 cm^3), DCM (1 × 5 cm^3). The produced pentapeptides **4** were cleaved from resin by treatment of 40 cm^3 TFA (1%) in DCM and neutralization with 12 cm^3 pyridine (4%) in MeOH. The solvent was removed under reduced pressure and precipitated in water. The yields were 71.4% (AA = Leucine) and 83.2% (AA = Methionine).

General Procedure for Amidation of C-Terminus Leu- and Met-enkephalins (6a-f)

To a solution of 0.264 g TBTU (0.82mmol) and RNH_2.HCl (1.1 mmol) in 1.5 cm^3 NMP was added peptide **4** (0.55 mmol) and 0.3 cm^3 NMM (2.73 mmol). The mixture was stirred overnight. The progress of reaction was monitored using TLC. The desired peptide was precipitated in water and the C-terminal amidated enkephalins were dried. The final deprotection was done using mixture of TFA:TES:H_2O (94:5:1) (20 ml g^{-1} peptide **5**) and in this way all of protecting groups were removed. The final peptide was dried under vacuum at 40 ºC (Isolated yields 71-86%). Further purification was done using Prep-HPLC with column (ODS-C_{18}, 120 × 20 mm) and UV detector (λ = 210 nm). The elution solvent was ACN/10 mM NaH_2PO_4 buffer. The spectral data for the compounds **6a-f** are as follows:

(6a). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 0.81 (d, 3H, J = 6.5 Hz, CH_3), 0.87 (d, 3H, J = 6.5 Hz, CH_3), 1.43-1.57 (m, 3H, CH, CH_2), 2.76-2.83 (m, 2H, NCH_2), 2.92-3.03 (m, 2H, NCH_2), 3.58-3.86 (m, 5H, $2CH_2Ar$, C<u>H</u>NH_2), 4.18 (dt, 1H, J = 9.0, 5.0 Hz, NCH), 4.43 (dt, 1H, J = 9.0, 5.0 Hz, C<u>H</u>-CH_2Ar), 5.50 (brs, 2H, NH_2), 6.68 (d, 2H, J = 6.5 Hz, H-Ar), 6.97 (brs, 1H, NH), 7.02 (d, 2H, J = 8.5 Hz, H-Ar), 7.15 (m, 2H, H-Ar), 7.20-7.30 (m, 4H, NH), 8.15 (dt, 2H, J = 8.5, 2.0 Hz, H-Ar), 8.34 (t, 1H, J = 5.5 Hz, H-Ar), 8.81 (brs, 1H, OH) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ = 21.5, 22.9, 24.1, 37.2, 37.3, 40.7, 42.0, 42.2, 51.1, 54.4, 54.6, 115.2, 126.2, 128.0, 129.1, 130.2, 137.8, 156.2, 168.7, 170.7, 174.0 ppm. MS (MALDI): m/z = 555.29266 $(M+1)^+$, 577.27452 $(M+Na)^+$, 1131.55905 $(2M+Na)^+$.

(6b). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 0.81 (d, 3H, J = 6.5 Hz, CH_3), 0.87 (d, 3H, J = 6.5 Hz, CH_3), 0.98 (t, 3H, J = 7.0 Hz, CH_3), 1.40-1.55 (m, 3H, CH_2, CH), 2.83 (m, 2H, NC<u>H_2</u>-CH_3), 2.94-3.05 (m, 4H, $2NCH_2$), 3.63-3.68 (dd, 2H, J = 17.0, 6.0 Hz, CH_2Ar), 3.72-3.80 (dd, 2H, J = 17.0, 6.0 Hz, CH_2Ar), 3.95 (t, 1H, J = 6.5 Hz, C<u>H</u>NH_2), 4.20 (dt, 1H, J = 9.0, 5.0 Hz, NCH), 4.48 (dt, 1H, J = 9.0, 5.0 Hz, C<u>H</u>-CH_2Ar), 6.30 (brs, 2H, NH_2), 6.68 (d, 2H, J = 8.5 Hz, H-Ar), 7.03 (d, 2H, J = 8.5 Hz, H-Ar), 7.20 (dt, 1H, J = 9.0, 4.0 Hz, H-Ar), 7.23-7.26 (m, 4H, H-Ar, NH), 7.73 (brs, 1H, NH), 8.07 (d, 1H, J = 7.5 Hz, H-Ar), 8.14 (d, 1H, J = 7.5 Hz, H-Ar), 8.28 (brs, 1H, NH), 8.83 (brs, 1H, OH) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ = 14.5, 21.6, 22.9, 24.1, 33.3, 36.3, 34.7, 40.8, 41.9, 42.1, 51.2, 54.0, 54.2, 115.2, 125.1, 126,1, 127.9, 129.1, 130.0, 137.7, 156.4, 168.5, 168.6, 169.2, 170.6, 171.4 ppm. MS (MALDI): m/z = 583.32392 $(M+1)^+$, 605.30573 $(M+Na)^+$, 1187.62206 $(2M+Na)^+$.

(6c). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 0.83 (d, 3H, J = 6.5 Hz, CH_3), 0.88 (d, 3H, J = 6.5 Hz, CH_3), 1.44-1.61 (m, 3H,

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CH_2, CH), 2.76-2.81 (m, 2H, NCH_2), 2.93-3.00 (m, 2H, NCH_2), 3.58-3.81 (m, 4H, $2CH_2Ar$), 3.87 (t, 1H, J = 7.0 Hz, C<u>H</u>NH_2), 4.20 (brs, 1H, NCH), 4.44 (dt, 1H, J = 9.0, 3.5 Hz, C<u>H</u>-CH_2Ar), 5.9 (brs, 2H, NH_2), 6.68 (d, 2H, J = 8.5 Hz, H-Ar), 7.03 (d, 2H, J = 8.5 Hz, H-Ar), 7.14-7.20 (m, 1H, H-Ar), 7.20-7.24 (brs, 4H, NH), 7.80 (brs, 1H, NH), 8.10 (d, 1H, J = 8.0 Hz, H-Ar), 8.28 (t, 1H, J = 6.0 Hz, H-Ar), 8.34 (d, 1H, J = 6.0 Hz, H-Ar), 8.80 (brs, 1H, H-Ar), 8.64 (brs, 1H, OH) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ = 21.6, 22.8, 23.9, 36.9, 37.3, 41.9, 42.1, 50.1, 54.2, 54.4, 115.2, 125.8, 126.1, 128.0, 129.1, 130.3, 137.7, 156.2, 158.8, 168.6, 168.8, 171.3, 171.5 ppm. MS (MALDI): *m/z* = 613.30920 $(M+1)^+$, 635.29117 $(M+Na)^+$, 1225.61043 $(2M+1)^+$, 1247.59246 $(2M+Na)^+$.

(6d). ^1H NMR (500 MHz, DMSO-d_6): δ = 1.80-1.92 (m, 2H, CH_2), 2.02 (s, 3H, SCH_3), 2.30-2.45 (m, 2H, CH_2), 2.75-3.10 (m, 4H, $2NCH_2$), 3.59-3.82 (m, 4H, CH_2Ar), 3.90 (t, 1H, J = 7.0 Hz, C<u>H</u>NH_2), 4.23 (dt, 1H, J = 8.5, 4.5 Hz, NCH), 4.43 (dt, 1H, J = 8.5, 4.5 Hz, C<u>H</u>-CH_2Ar), 6.68 (d, 2H, J = 8.5 Hz, H-Ar), 7.02 (d, 2H, J = 8.5 Hz, H-Ar), 7.05 (brs, 2H, NH), 7.12 (brs, 1H, NH), 7.17 (brs, 1H, NH), 7.22 (brs, 1H, NH), 7.24 (d, 2H, J = 3.0 Hz, H-Ar), 7.25 (brs, 2H, NH_2), 8.15 (d, 1H, J = 8.0 Hz, H-Ar), 8.25 (d, 1H, J = 8.0 Hz, H-Ar), 8.35 (t, 1H, J = 5.6 Hz, H-Ar), 8.85 (brs, 1H, OH) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ = 14.5, 29.6, 31.4, 36.9, 37.2, 42.0, 42.2, 51.9, 54.4, 54.5, 115.2, 125.8, 126.2, 128.0, 129.1, 130.3, 137.8, 156.3, 168.8, 170.0, 171.0, 173.1 ppm. MS (MALDI): *m/z* = 573.24890 $(M+1)^+$, 595.23086 $(M+Na)^+$, 1167.47197 $(2M+Na)^+$.

(6e). ^1H NMR (500 MHz, DMSO-d_6): δ = 0.98 (t, 3H, J = 7.2 Hz, CH_3), 1.80-1.95 (m, 2H, CH_2), 2.01 (s, 3H, SCH_3), 2.34-2.41 (m, 2H, CH_2), 2.68-2.87 (dd, 2H, J = 14.0, 8.0 Hz, NCH_2), 2.90-3.03 (dd, 2H, J = 14.0, 8.0 Hz, NCH_2), 3.05 (m, 2H, J = 7.2 Hz, NC<u>H_2</u>CH_3), 3.58-3.80 (m, 5H, $2CH_2$, C<u>H</u>NH_2), 4.23 (dt, 1H, J = 8.5, 4.5 Hz, NCH), 4.42 (dt, 1H, J = 8.5, 4.5 Hz, C<u>H</u>-CH_2Ar), 5.20 (brs, 2H, NH_2), 6.67 (d, 1H, J = 8.5 Hz, H-Ar), 7.01 (d, 1H, J = 8.5 Hz, H-Ar), 7.18 (m , 1H, H-Ar), 7.24 (brs, 4H, NH), 7.66 (t, 1H, J = 5.0 Hz, H-Ar), 8.15 (d, 1H, J = 8.5 Hz, H-Ar), 8.35 (t, 1H, J = 5.0 Hz, H-Ar), 8.73 (brs, 1H, OH) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ = 14.5, 14.6, 29.6, 31.5, 33.4, 37.2, 37.9, 42.1, 42.2, 52.0, 54.5, 55.0, 115.1, 126.2, 126.3, 128.0, 129.1, 130.2, 137.7, 156.0, 168.8, 168.9, 170.5, 171.0 ppm. MS (MALDI): *m/z* = 601.28038 $(M+1)^+$, 623.26216 $(M+Na)^+$, 1223.53452 $(2M+Na)^+$.

(6f). ^1H NMR (500 MHz, DMSO-d_6): δ = 1.80-1.90 (m, 2H, CH_2), 2.05 (s, 3H, SCH_3), 2.40-2.48 (m, 2H, CH_2), 2.60-3.03 (m, 4H, $2NCH_2$), 3.55-3.80 (m, 5H, $2CH_2Ar$, C<u>H</u>NH_2), 4.26 (brs, 1H, NC<u>H</u>), 4.44 (brs, 1H, C<u>H</u>CH_2Ar), 5.88 (brs, 2H, NH_2), 6.66 (d, 2H, J = 8.0 Hz, H-Ar), 7.00 (d, 2H, J = 8.0 Hz, H-Ar), 7.15 (m, 1H, H-Ar), 7.20-7.25 (brs, 4H, NH), 7.79 (brs, 1H, NH), 8.15 (d, 1H, J = 7.5 Hz, H-Ar), 8.25 (brs, 1H, H-Ar), 8.40 (brs, 1H, H-Ar), 9.50 (brs, 1H, OH) ppm. ^{13}C NMR (125 MHz, *DMSO-d_6*): δ = 14.5, 29.3, 31.5, 37.2, 37.2, 41.9, 42.1, 51.0, 54.3, 55.2, 115.1, 126.2, 128.0, 129.1, 130.2, 137.8, 156.0, 168.8, 168.9, 170.7, 171.0 ppm. MS (MALDI): *m/z* = 631.26572 $(M+1)^+$, 653.24756 $(M+Na)^+$, 1283.50508 $(2M+Na)^+$.

RESULTS AND DISCUSSION

Following discovering enkephalins in 1975 (Hughes), it is now well established that enkephalins play several important roles in CNS in addition to their role in the process of analgesia. Composition based findings showed that endorphine, which is a 31 amino acid peptide having the Met-enkephalin sequence at its C-terminus produces a powerful analgesia 100 times more potent than morphine [18].

Herein, we wish to report an efficient route for the amidation, alkylamidation and semicarbazidation of the *C*-terminus Met- and Leu-enkephalins and some GnRH pharmaceutical peptides using ammonium chloride, alkylammonium chloride, semicarbazide hydrochloride in solution phase at room temperature. The details for the synthesis of Met- and Leu-enkephalins as model were summarized in Scheme 1.

Tetrapeptides (**2**) were synthesized manually using the standard solid phase peptide synthesis *via* Fmoc strategy. First of all, Fmoc-Leu-OH or Fmoc-Met-OH was loaded on the surface of 2-chlorotrityl chloride resin (**1**) and Fmoc-protected amino acids were coupled according to known methods. The tetrapeptide (**2**) after reaction with Boc-Tyr(tBu)-OH afforded the resin-boundpentapeptide (**3**). Cleavage of the desired peptides from the surface of the resin provided the *N*-Boc protected peptides (**4**) that could be amidated with ammonium chloride, ethylammonium chloride and semicarbazide hydrochloride and led to the amidated form of *C*-terminus

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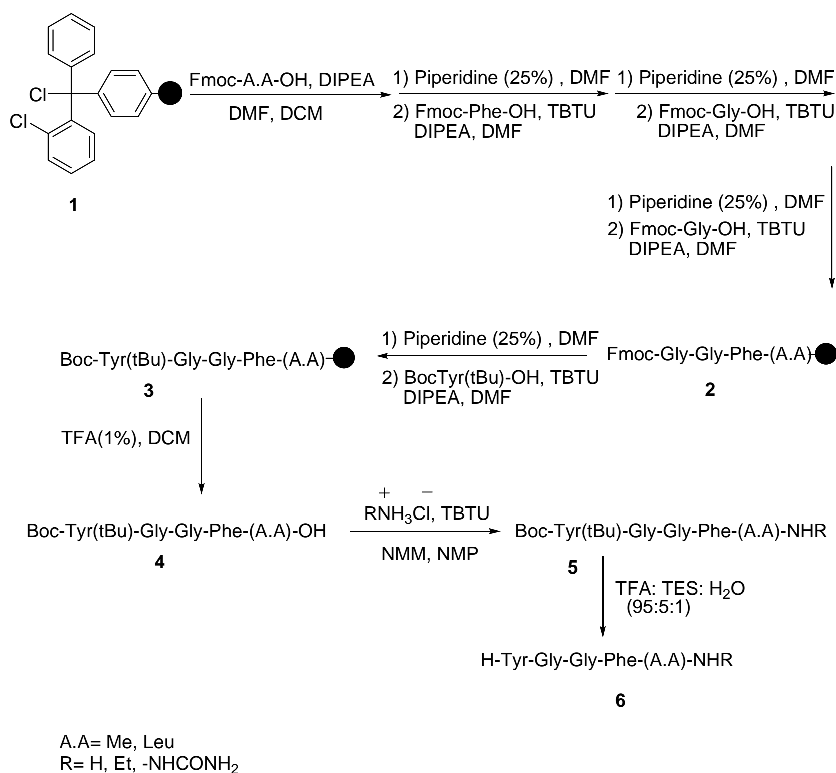

Scheme 1. Total synthesis of amidated enkephalins

Table 1. Synthesis of Amidated C-Terminus Enkephalins

Product	A.A	R	Yield (%)[a]	$(M+1)^+$
6a	Leu	-H	86	555.29
6b	Leu	-Et	83	583.32
6c	Leu	$-NHCONH_2$	81	613.31
6d	Met	-H	76	573.25
6e	Met	-Et	85	601.28
6f	Met	$-NHCONH_2$	71	631.27

[a]Yields of isolated amides.

enkephalins (**5**). This amidation reaction was carried out using TBTU as a coupling reagent and DIPEA as a base at room temperature. The reaction yields are good to high and details were shown in Table 1.

For full deprotection of the pentapeptides, a mixture of TFA:TES:H_2O (94:5:1) was used. The synthetic details were clarified in the experimental section. The crude products were in general, of good purity as shown by analytical HPLC analysis. We used this methodology for the synthesis of some pharmaceutical GnRH analogues such as, leuprolide, buserelin and fertirelin acetate *via* amidation of the *C*-terminus.

This novel strategy is the combination of solid and solution phase peptide synthesis, and it has some advantages compared to the previously reported methods, such as mild reaction conditions, cost effectiveness, and reducing of the reaction steps. All products were characterized using ^{1}H and ^{13}C NMR spectroscopy and MALDI mass spectrometry. In Figs. 1 and 2 were shown MALDI mass spectra of Leu-Enk-NH_2 and Met-

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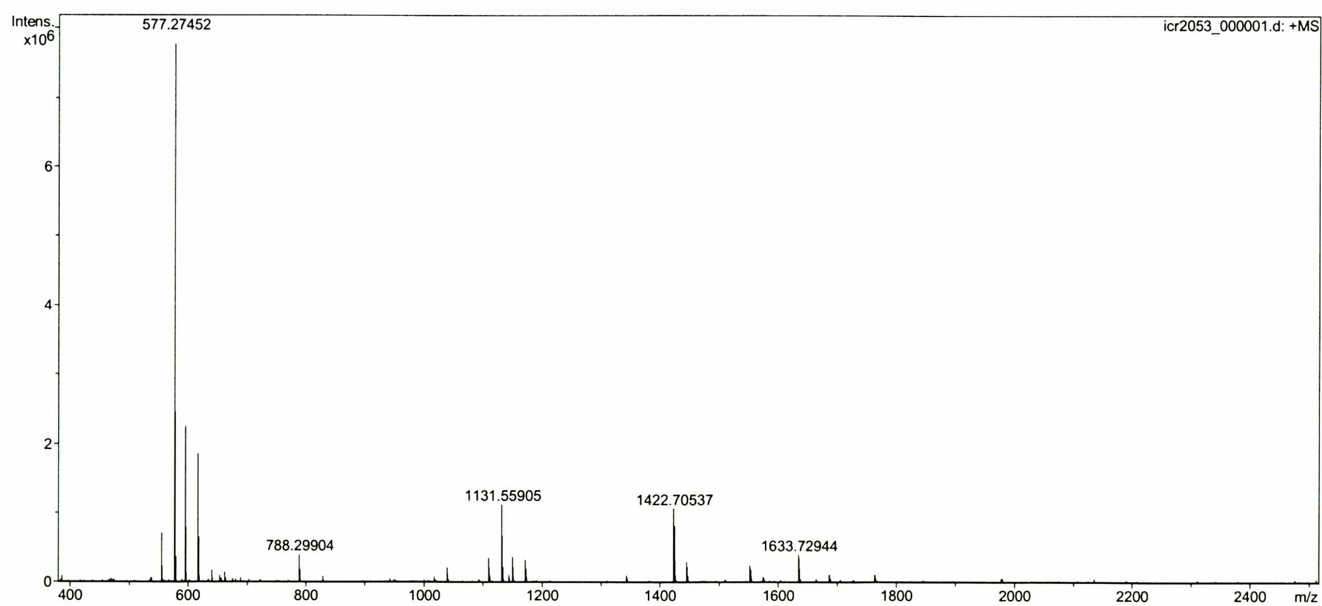

Fig. 1. MALDI spectra of compound.

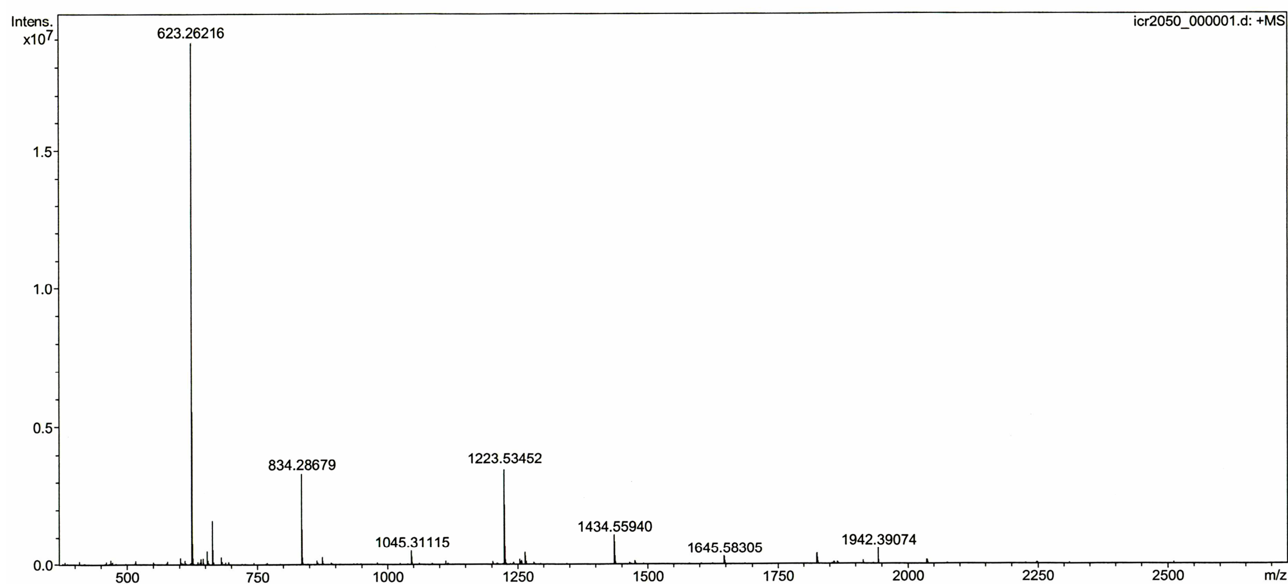

Fig. 2. MALDI spectra of compound **6e**.

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Enk-NHEt. The molecular weight of all target compounds were confirmed. The molecular ion peak $(M+1)^+$ was identified at m/z = 555.29266, for **6a**. Other distinguished peaks were shown at m/z = 577.27452 and 1131.55905 that are related to $(M+Na)^+$ and $(2M+Na)^+$ respectively. All NMR measurements were performed in commercially available DMSO-d_6. The ^{1}H NMR spectrum of amidated enkephalins exhibited distinguished peaks for Ph CH_2 (δ = 3.60-3.90 ppm, dd) and glycine CH_2 groups and also three different -CH groups. Carbonyl amide bonds in ^{13}C NMR resonate at δ = 167-174 ppm.

CONCLUSIONS

We have demonstrated an efficient strategy *via* combination of solid and solution phase peptide synthesis using Fmoc and Boc strategy and finally amidation reaction using ammonium chloride, alkylammonium chloride and semicarbazide hydrochloride that leads to the formation of amidated *C*-terminus Met- and Leu-enkephalin derivatives. This novel amidation strategy was used for the synthesis of some pharmaceutical peptides. It has some advantages in comparison to known amidation methods, such as generality, more efficiency, simplicity and high yields. The research for finding biologically activity of these novel peptides is still in progress in our laboratory.

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