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RESEARCH ARTICLE

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# Synthesis of Met-enkephalin by solution-phase peptide synthesis methodology utilizing *para*-toluene sulfonic acid as N-terminal masking of L-methionine amino acid

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The Met-enkephalin, Tyr-Gly-Gly-Phe-Met, was synthesized by the solution-phase synthesis (SPS) methodology employing -OBzl group as carboxyls' protection, while the *t*-Boc groups were employed for the N-terminal $\alpha$-amines' protection for the majority of the amino acids of the pentapeptide sequence. The L-methionine (L-Met) amino acid was used as PTSA.Met-OBzl obtained from the simultaneous protection of the $\alpha$-amino, and carboxyl group with *para*-toluene sulfonic acid (PTSA) and as-OBzl ester, respectively in a C-terminal start of the 2 + 2 + 1 fragments condensation convergent synthetic approach. The protection strategy provided a short, single-step, simultaneous, orthogonal, nearly quantitative, robust, and stable process to carry through the protected L-methionine and L-phenylalanine coupling without any structural deformities during coupling and workups. The structurally confirmed final pentapeptide product was feasibly obtained in good yields through the current approach.

**KEYWORDS**

Bulk preparation, enkephalin, Met-enkephalin, *para*-toluene sulfonic acid, peptide synthesis, PTSA. Met-OBzl, simultaneous amine and carboxyl protections, solution-phase synthesis

The discovery of enkephalin by Hughes in 1975 generated a great deal of interest in its biological activity and development of synthetic routes.[1] Both the solution[2], and solid-phase preparation methods[3,4] for the L-methionine (L-Met) and L-leucine (L-Leu) analogs of the enkephalin considering their structure–activity relationship (SAR), involved receptor subtypes,[5] peptidomimetics,[6] structural-turn mimetics,[7] and the amino acid mutant analogs[8] have been utilized over the years.[9–13] The recent spurt in synthesis[14] and molecular modeling[15] including mathematical modeling[16] of enkephalin rests on the biological activity relationship and requisite structural and topological features.[17] Moreover, toward developing a more detailed understanding of the SAR for desirable structural features *vis-a-vis* pharmacophore modeling and biological activity elicitation, structure-based bioactivity modulation, and designing of opiate-behavior molecular templates, a number of different analogs and radiolabeled enkephalin[18,19] have also been synthesized. Reports on the advances in the enkephalin receptor interactions details through various conjugates[20,21] and probe molecules toward modeling the enkephalin structures[22] are some of the recent additions. The current study is an attempt to synthesize the Met-enkephalin for biological activity studies, and for finding a feasible route for synthesis towards preparing a synthetic platform for more diversified synthetic transformations to be enable to synthesize designed peptides on preparative and scale-up levels.

## 1 | METHODS AND MATERIALS

All amino acids (AA) were of L-configuration and coupling reactions were carried out with anhydrous reactants, and in freshly prepared dry solvents, wherever required. The evaporation of solvents during workup was done *in vacuo* below 40 °C. The homogeneity of all the amino acid derivatives and peptide fragments were established by UV-active TLC

on precoated silica gel plates in specified solvents. Ninhydrin and HBr-AcOH were used as spraying reagents. The melting points were determined in a sulfuric acid bath and are uncorrected. All the single AA derivatives were compared with the known samples as otherwise stated and were used only after through comparison and identity establishment.

## 1.1 | Preparation of Boc-Gly

L-Gly (1.5 g, 20 mM) was taken in a mixture of water–dioxane (1:1, 20 mL) and cooled to 0 °C followed by the addition of 2N aq. NaOH to maintain highly basic condition (pH 11–12) with stirring for 15 min. Di-*tert*-butyl-dicarbonate (DCC) (22 mM) was added over a period of 30 min in portions with pH maintaining at the higher basic range with 2N aq. NaOH solution additions with the temperature maintained at 0 °C under continued stirring for 2 h followed by stirring at RT for another 1 h. The solvents were removed *in vacuo* below 40 °C and the resultant residue was taken in 20 mL of water, acidified with solid citric acid to pH 2. The mixture was saturated with brine, extracted with diethyl ether, and combined organic layers were dried over anhydrous $Na_2SO_4$ and evaporated to give the product as a viscous oil, which was crystallized with dry diethyl ether, yields 2.2 g (72%). TLC, co-TLC, and m.p. were compared with the known sample and product was found matching with the in-house reference standard.

## 1.2 | Preparation of Boc-Gly-Gly-OH

Boc-Gly (1.75 g, 10 mM) was taken into dry THF (25 mL); N-methyl morpholine (NMM) (1.15 mL, 10 mM) and isobutyl chloroformate (1.35 mL, 10 mM) were added. After 2–3 min, a mixture of Boc-Gly (1.125 g, 15 mM) and 1 N aq. NaOH (16.5 mL, 11 mM) was added in one instance and the reaction mixture stirred for 3 h. The solvents were evaporated *in vacuo* below 45 °C and the resultant thick oil was crystallized with vigorous scratching in dry diethyl ether to yield the crude solid product, which was recrystallized with dry diethyl ether. Yields 1.6 g, TLC single spot $R_f$ 5 (MeOH: $CHCl_3$: AcOH: 5:93:2, upper layer), HBr-AcOH and Ninhydrin. TLC, co-TLC, and m.p. were compared with the known sample and were found matching with the in-house reference standard.

## 1.3 | Preparation of HCl. Tyr-OMe

Thionyl chloride (1.6 mL, 20 mM) was added dropwise over a period of 10 min with vigorous stirring to precooled absolute methanol (35 mL) in an RB flask fitted with calcium chloride guard tube and acetone: ethyl acetate (1:1) dry-ice mixture cold bath. To this solution, tyrosine was added (3.6 g), and the reaction mixture was further stirred for 1 h at 4 °C on crushed ice: NaCl bath, and then at RT. The RM was left in dark overnight. Next morning, TLC was monitored to confirm the completion of the reaction and the mixture was concentrated to dryness *in vacuo* and later at an elevated temperature at 45 °C. Methanol (dry) was added (30 mL), and the residue was again concentrated to dryness. The procedure was repeated three times to remove excess of HCl. Tyrosine methyl ester hydrochloride crystals were collected. The product was again recrystallized from dry methanol–dry diethyl ether to yield 4.2 g of the final product. TLC (BuOH-AcOH-$H_2O$: 4:1:5, upper layer), $R_f$ 3.5, single spot, Ninhydrin, and m.p. 188–189 °C confirmed the identity of the product on comparison with the in-house reference sample.

## 1.4 | Preparation of Boc-Tyr-OMe

HCl.Tyr-OMe (3.71 g, 15 mM) was dissolved in dry DMF, and trimethylamine (4.5 mL, 30 mM) was added to free the salt. DCC (di-tert-butyl-dicarbonate) (3.27 g, 15 mM) was added to the reaction mixture in about 30 min in small portions at 0 °C under vigorous stirring, and stirring was continued at RT for another 2 h. The solvent was evaporated *in vacuo* and residue took in EtOAc, washed with brine (3×), dried over anhydrous $Na_2SO_4$, and combined organic layers were evaporated *in vacuo* below 40 °C to give a white crude product as a solid, 3.7 g, which was recrystallized to single spot product, 3.7 g. TLC Rf 6 ($CHCl_3$: MeOH :: 95:5), Ninhydrin, single spot, dark orange in color. Co-TLC and m.p. were compared with the known sample and found matching with the in-house reference standard.

## 1.5 | Preparation of Boc-Tyr-OH

Boc-Try-OMe (3.1 g, 10 mM) in methanol (75 mL) was stirred at 0 °C and 2 N aq. NaOH (20 mM) was added dropwise with continued stirring for another 1 h. The solvents were evaporated *in vacuo*, and the residue was washed with ether, acidified with (solid) citric acid to neutral pH 7 at 0 °C. EtOAc was added and the organic layers were extracted and combined, washed with brine, and dried over anhydrous $Na_2SO_4$, followed by drying *in vacuo* to give a TLC pure single spot product. Yields 2.0 g, TLC: $R_f$ 4 ($CHCl_3$: MeOH: 95:5), Ninhydrin, single spot, light orange in color. Co-TLC and m.p. were compared with the known sample and found matching with the in-house reference standard.

## 1.6 | Preparation of Boc-Phe-OH

L-Phe (2.30 g, 20 mM) was taken in water (90 mL), and 2 N aq. NaOH (10 mL) was added. After the dissolution of the starting material, dioxane (100 mL) was added, followed by DCC (4.36 g) in small portions in about 30 min with vigorous stirring at 0 °C. Stirring was continued at 0 °C for another 1.5 h and at RT for another 1 h. The solvents were concentrated up to $1/4^{th}$ of the volume, water (50 mL) was added, and the

RM was acidified to pH 2 by the addition of solid citric acid. The organic layers were extracted with EtOAc (3 × 50 mL), washed with brine (3×), and dried over anhydrous $Na_2SO_4$. The combined organic layers were dried *in vacuo* below 40 °C to give an oily product, which was recrystallized to give the solid product, yields 3.4 g. TLC, co-TLC, and m.p. were compared with the known sample and the product was found matching with the in-house reference standard.

## 1.7 | Preparation of PTSA.Met-OBzl

To a Dean-Stark assembly's fitted round-bottom flask, L-Met (2.98 g, 20 mM), *para*-toluene sulfonic acid (PTSA) (4.18 g, 22 mM), and benzyl alcohol (13 mL, 120 mM) were charged in dry benzene as solvent (30 mL). The reaction mixture was refluxed at 100 °C for 22 h and the collected water in lower portion of DS assembly's arm was removed intermittently with filling of dry benzene to facilitate the reflux and distillation off of the azeotrope formed. The RM was monitored over a period of time but at first after 8 h of the reaction. After completion of the reaction (22 h), as monitored by TLC (BuOH-AcOH-$H_2O$: 4:1:5, upper layer, $R_f$ 6, Ninhydrin), a solid product was obtained (6.1 g) after solvent removal *in vacuo* below 40 °C. The crude product was recrystallized from dry MeOH-diethyl ether, yields: 6.03 g.

## 1.8 | Preparation of Boc-Phe-Met-OBzl

Boc-Phe (2.65 g, 10 mM) was dissolved in dry DMF (30 mL), and HOBt (1.53 g, 10 mM) was added at room temperature. The mixture was kept separate under dry conditions protected by a guard tube and away from moisture. The PTSA.Met-OBzl (4.12 g) was dissolved in dry DMF (25 mL) at 0 °C, and N-methyl morpholine (NMM) (1.3 mL, 11 mM) was added to make pH 7. The Boc-Phe-HOBt and the deprotected PTSA.Met-OBzl were mixed together over 5 min period at 0 °C temperature under stirring. The reaction mixture was allowed to cool to 0 °C, and DCC (2.26 g, 11 mM) dissolved in $CH_2Cl_2$ was added. Stirring was continued for another 2 h under cool conditions and 2 h at RT. The solvents were evaporated *in vacuo* to give a residue, which was crystallized with dry MeOH to give a crude solid, 4.5 g, which was processed further as such after vacuum drying of the product over $P_2O_5$ and NaOH pellets at RT in a vacuum desiccator.

## 1.9 | Preparation of Boc-Gly-Gly-Phe-Met-OBzl

Boc-Phe-Met-OBzl was deprotected using 5N HCl in dioxane in the presence of thioanisole and mercaptoethanol under stirring at RT for 15 min. The solvents were evaporated below 40 °C *in vacuo* to give the product, HCl.Phe-Met-OBzl, which was dissolved in dry DMF and chilled to 0 °C. N-methyl morpholine (NMM) was added to neutralize the mixture (pH 7) and Boc-Gly-Gly-OH in dry DMF followed by HOBt (225 mg) were added under stirring at 0 °C. The reaction mixture was kept stirring and chilled at 0 °C, and DCC (350 mg) in dry $CH_2Cl_2$ was added. After 2 h of stirring at 0 °C, which was followed by another 2 h of stirring at RT, solvents were evaporated *in vacuo* below 40 °C to give the solid product, which was recrystallized from dry MeOH-dry diethyl ether, 2.6 g. TLC: $R_f$ 3.5 (MeOH:$CHCl_3$ :: 5:95), single spot, HBr-AcOH, Ninhydrin.

## 1.10 | Deblocking of Boc-Gly-Gly-Phe-Met-OBzl

5N HCl.dioxane was added to Boc-Gly-Gly-Phe-Met-OBzl (2 g) followed by thioanisole and mercaptoethanol (0.2 mL each), and the reaction mixture was kept at room temperature for 1 h. The solvents were evaporated *in vacuo*, and the residue was triturated with dry ether to give the HCl.Gly-Gly-Phe-Met-OBzl as an amorphous solid, which was processed further as such after drying *in vacuo* over $P_2O_5$ and NaOH pellets at RT in a vacuum desiccator.

## 1.11 | Preparation of Boc-Tyr-Gly-Gly-Phe-Met-OBzl

HCl.Gly-Gly-Phe-Met-OBzl (600 mg) was dissolved in dry DMF and cooled to 0 °C followed by addition of NMM to free the hydrochloride salt. Boc-Tyr-OH (0.25 g) was dissolved in dry DMF and HOBt was added (0.08 g), followed by mixing of free amine $H_2N$-Gly-Gly-Phe-Met-OBzl and Boc-Tyr-OH in one instance. DCC was added as a $CH_2Cl_2$ solution, and stirring was continued at 0 °C for 2 h followed by further stirring at room temperature for 2 h. The solvents were removed *in vacuo* and residue was taken in water, extracted with EtOAc, and the combined organic layers were dried over anhydrous $Na_2SO_4$. The dried organic layer was removed *in vacuo* below 40 °C to give the amorphous solid (610 mg), which was recrystallized from dry MeOH to give white crystalline material, 560 mg, $R_f$ 6.5 (MeOH:$CHCl_3$ :: 10:1), single spot, Ninhydrin.

## 1.12 | Preparation of Tyr-Gly-Gly-Phe-Met-OH

Boc-Tyr-Gly-Gly-Phe-Met-OBzl (250 mg) was dissolved in 5% formic acid in methanol (50 mL), and 10% Pd-C was added under stirring at RT for 20 min. After completion of the reaction as monitored by TLC, the catalyst was filtered off and solvents removed *in vacuo* below 40 °C. The residue was treated with 5N HCl-dioxane at 0 °C under stirring for 30 min in the presence of thioanisole and mercaptoethanol (0.2 mL each). The solvents were again evaporated *in vacuo* below

40 °C, and the residue was treated with NMM in MeOH to get solution's pH slightly above neutral. The reaction mixture was again evaporated *in vacuo* at 40 °C. The solid residue, so obtained, was washed (3 × 50 mL) with dry ether, and the crude product was recrystallized with dry MeOH–dry diethyl ether, yields 187 mg (66%). m.p., 197–98 °C, TLC, and co-TLC, were compared with the known in-house standard sample. TLC: $R_f$ 6 (n-BuOH: AcOH: $H_2O$: 4:1:4), ($[\alpha]_D$ −22° (c = 1.1, DMF), elemental analysis: found $C_{56.45}$ $H_{6.22}$ $N_{12.3}$%, required. $C_{56.55}$ $H_{6.15}$ $N_{12.2}$%. PMR (DMSO-$d_6$): $\delta$ 1.51–2.02 (m, 7H, Met-$CH_3$, 2x$CH_2$), 3.01–3.40 (m, 4H, Tyr, Phe 2x$CH_2$), 3.71(brs, 4H, $Gly^{2,3}$-CH), 3.78–4.56 (m, 3H, Tyr, Phe, Met-CH), 6.84 (dd, 4H, $Tyr^{aromatic}$H), 7.30 (brs, 5H, $Phe^{aromatic}$H), 8.1-8.7(NH), unequivocally confirmed the identity of the product.

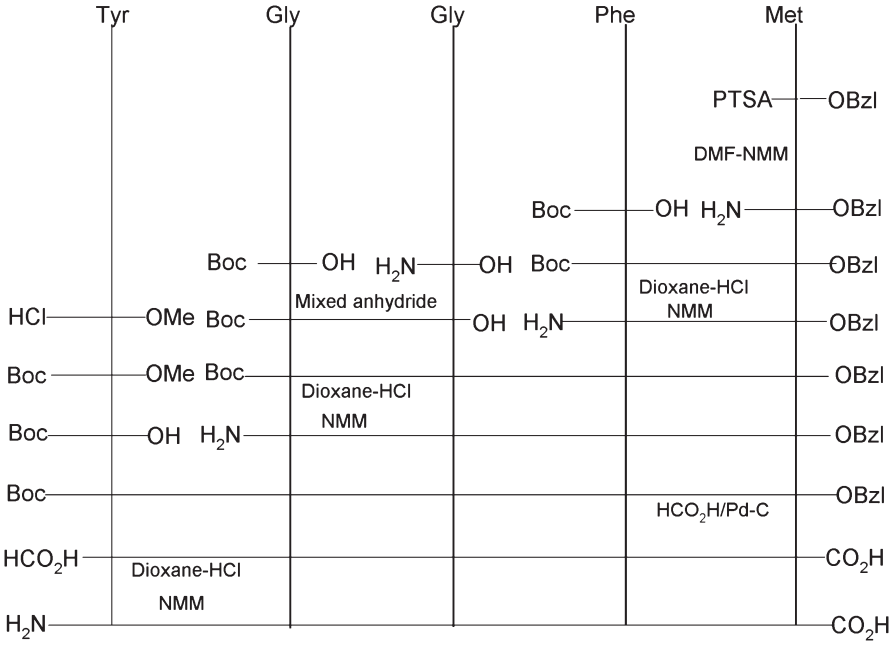
## 2 | RESULTS

The pentapeptide, Tyr-Gly-Gly-Phe-Met, was synthesized in good yields (66%) following the solution-phase peptide synthesis methodology using the PTSA-Met-OBzl as the coupling AA component in the sequence assembly involving *t*-Boc and -OBzl groups as the amino and carboxyl protections for the other four amino acids (AA) in the sequence. The final product's identity was confirmed by spectro-analytical techniques and a through comparison with an in-house reference sample.

## 3 | DISCUSSION

The quest for the development of a new method for synthesis of Met-enkephalin was driven by the need to establish a comparatively simple, synthetically effective, convenient in handling, robust, and cost-reducing method utilizing milder synthetic conditions with convergent procedure requiring shorter time span with improved optical purity and high yields for preparative scale synthesis of the pentapeptide product. Toward this effect, the synthesis was started with suitably protected amino acids. The *t*-butyl-oxy-carbonyl (*t*-Boc) group was employed (Scheme 1) for the protection of the $\alpha$-amino groups of the majority of the amino acids during the peptide synthesis owing to its established and facilitated protection–deprotection state.[23–25] The carboxylic ends were selectively masked with a neutral -OBzl group,[26] which again proved to be a versatile and efficient carboxylic protection strategy with its easy removal in catalytic transfer hydrogenation employing low concentrations of formic acid as the hydrogen donor.[27]

The approach to 2 + 2 + 1 fragments' coupling plan followed with the preparation of Boc-Tyr-OMe, Boc-Gly-Gly-OH, and Boc-Phe-Met-OBzl as the protected, independent fragments. The Boc-Phe-Met-OBzl was prepared from Boc-Phe-OH and its coupling with the $H_2N$-Met-OBzl. An *in situ* coupling of the Boc-Phe-OH and PTSA.Met-OBzl was also tried but with purity and yields issues present, was not pursued further. However, the free amine, $H_2N$-Met-OBzl, was obtained from the PTSA.Met-OBzl deprotection (Scheme 1), while the PTSA.Met-OBzl itself was prepared (three batches) from L-Met treatment with *para*-toluene sulfonic acid (PTSA) and its simultaneous condensation in heating conditions (100 °C) with the benzyl alcohol to mask the carboxyl group of the L-Met amino acid. The coupling was mediated by dicyclohexyl carbodiimide (DCC) in dry $CH_2Cl_2$ at 0 °C for 30 min with monitoring by TLC. The Boc-Gly-Gly-OH dipeptide fragment was approached through mixed anhydride method[28,29]




**SCHEME 1** Synthetic strategy for the preparation of Met-enkephalin, Tyr-Gly-Gly-Phe-Met-OH

of peptide synthesis protocol through the coupling of the Boc-Gly-OH and $H_2N$-Gly-OH. A 2 + 2 DCC-HOBt (hydroxy benzotriazole)-mediated coupling[30,31] of the $H_2N$-Phe-Met-OBzl and Boc-Gly-Gly-OH yielded the tetrapeptide, Boc-Gly-Gly-Phe-Met-OBzl, which was deprotected to free amine using 5N HCl-dioxane followed by resulting hydrochloride salt neutralization with N-methyl morpholine (NMM) and condensing the obtained free amine to Boc-Tyr-OH, again mediated by DCC-HOBt, at cooled conditions. The Boc-Tyr-OH was prepared from Boc-Tyr-OMe, which itself was obtained from HCl.$H_2N$-Tyr-OMe starting from the L-Tyr amino acid. The protected pentapeptide sequence, Boc-Tyr-Gly-Gly-Phe-Met-OBzl, was hydrogenated for 20 min over 10% Pd-C with formic acid in a transfer hydrogenation step.[32–34] The obtained formate salt, a highly hygroscopic product, was converted to HCl.Tyr-Gly-Gly-Phe-Met-OH by 5N HCl-dioxane treatment in the presence of thioanisole and mercaptoethanol. The hydrochloride salt was neutralized, worked up, and recrystallized to give the desired pentapeptide sequence, $H_2N$-Tyr-Gly-Gly-Phe-Met-OH, in 66% yields with its identity confirmed through comparison with an in-house reference standard and spectro-analytical techniques. Thus, the desired pentapeptide was synthesized in a stepwise and linear elongation of the peptide chain starting from C-terminus (Scheme 1) in a 2 + 2 + 1 fragments convergent approach.

## 4 | CONCLUSION

The current approach in protection and deprotection strategy for L-Met in the synthetic methodology of Met-enkephalin preparation is easy to handle, robust, and stable during coupling and workups. The methodology is orthogonal in nature and provides a simultaneous single-step protocol for amino and carboxyl groups' protections for Met AA. The method has been proved to be a smooth synthetic transformation without the presence of harsh preparation or deprotection conditions including many workups. The transformation also provided near quantitative yields and is capable of easy scale-up with cost-viable protection strategy for the sulfur-containing L-Met amino group *in lieu* of the *t*-Boc protection strategy without any structural deformity during the sequence assembly. The strategy may also have the potential for polymer-based different SPPS strategies involving L-Met and other sulfur-containing AA in a variety of sequences and synthetic conditions.

## CONFLICT OF INTEREST

There is no conflict of interest.

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