Chapter 5

Synthesis and Biological activity of Trp-Lys-Pro-Asp tetrapeptide
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5.1 Introduction

Protein Tyrosine Phosphatase 1B (PTP1B) has recently been receiving considerable attention not only in understanding the mechanism of insulin resistance in diabetes mellitus, but also as a drug target for the management of insulin resistant states such as obesity and type 2 diabetes.\(^1\) PTP1B is encoded by the PTPN1 gene which is located in 20q13; this genomic region has been linked to type 2 diabetes in multiple genetic studies. The recent studies lend considerable credence that in the mice lacking the PTP1B gene, exhibited increased insulin sensitivity and improved glucose tolerance. In addition PTP1B deficient mice were also found to be resistant to diet induced obesity.\(^2; 3\) As PTP1B has been shown to be a negative regulator of insulin signaling by dephosphorylating key tyrosine residues within the regulatory domain of the \(\beta\)-subunit of insulin receptor,\(^4\) inhibitors of PTP1B would have the potential of prolonging the phosphorylated state of the insulin receptor and hence, enhancing the downstream metabolic events in the expression of biological effects of insulin. The above evidence also strongly suggests that selective, small molecule inhibitors of PTP1B may be effective in treating insulin resistance at an early stage thereby leading to a preventive strategy for type 2 diabetes and obesity. Several PTP1B inhibitors have been developed to date using a structure-based design approach.\(^5, 6\)

The discovery of PTP1B inhibitors started with the identification of cholecystokinin derived octapeptide known as CCK-8, since then a number of peptides and peptidomimetics have been reported with interesting PTP1B inhibitory activities. The peptidomimetic based PTP1B inhibitors constitute a most important class of PTP1B inhibitors. However, a few small organic molecules are also reported with high order of PTP1B inhibitory activity. Till date, a molecule with the clinical acceptability is yet to be discovered. Several molecules are reported with high order of \textit{in vitro} PTP1B inhibition but at same time a majority of the molecules are non selective, which makes them less promising, from drug development point of view. On the other hand the \textit{in vivo} activity, bioavailability and oral efficacy are among the most prominent factors restricting a variety of compounds to reach the stage where a compound is considered for developmental work. The ideal inhibitor towards this very attractive and promising target is generally needed to address the issue of insulin resistance for which an appropriate medication is still lacking. Beside the applications of glucose homeostasis via controlling the insulin resistance, PTP1B inhibitors are also very much needed for the ailments like obesity and lipid disorders.
5.2 Basis of work

Computer assisted approach for designing of drug like molecules are being extensively used for quick discovery of mechanism based or target specific molecules. Since the protein sequence and crystal structure of PTP1B is already known and the crystal structure of PTP1B bound to certain specific inhibitors is also known, it can be of great help for *in silico* design of potent inhibitors as well as prediction of inhibitory activity of unknown molecules, if found to have a good binding affinity. The crystal structure of PTP1B bound to couple of potent inhibitors is already published which include, 3-[5-(2-Acetylamino-3-{4-[(2-carboxy-phenyl)-oxalyl-amino]-naphthalen-1-yl}-propionylamino)-pentyloxy]-naphthalene-2-carboxylic acid (XIV) and Phe-\(\psi\)(NH-CO-NH)-Tyr(O-Malonyl)-NH-C_5H_{11}(VI) (Figure 22).

![Figure 22. Chemical structures of the known diaryloxaminic acid based inhibitor (XIV) and VI](image)

Figure 23 shows the schematic representation of the hydrogen-bonding interactions between VI (red) and PTP1B (black). Bound water molecules are labeled as W. All the distances are in angstroms.
The crystal structure of PTP1B with the naphthoic acid derivative is presented in (Figures 24a and 24b). This reveals that in addition to the phosphotyrosine binding site (catalytic site) (residues Cys215-Arg221), there is a second binding site (Site 2) (Arg24 and Arg254). Inhibitors that bind to both the sites are found to be highly potent with activities in the nanomolar range. Recently, a third binding site (Site 3) (residues Tyr46-Asp48) was also found to contribute to the potency and selectivity of inhibitors. This indicated a perfect ligand based inhibitor must be able to bind at all important binding sites including the catalytic site.

Figure 23. Schematic representation of hydrogen bonding interactions between compound VI and PTP1B.

Figure 24a. Overall structure of the catalytic domain of PTP1B. The secondary structure elements are shown in blue (helices), yellow (β-strands), and pink (loops and turns). The three binding sites are shown in green ball-and-stick rendering.
Figure 24b. X-ray crystal structure of the complex of PTPIB with a known inhibitor [Naphthoic acid derivative]. The inhibitor is shown in red. The hydrogen bonded residues of PTPIB are shown green and the other contacting residues are shown in blue.

A wide variety of chemical structures are reported in the literature where it is very difficult to generate a correlation of SAR which poses serious limitations, for the design of new structures using computer assisted devices for the design of drug like molecules. Most of the inhibitors that have been developed so far are either non-peptidic or peptidomimetic in nature. Towards this objective, an interesting report published dealing in computer assisted design of a small peptide inhibitor, which has interactions with all the three binding sites and has potency comparable to that of the known inhibitors and is selective for PTPIB as compared to the closely related PTP's, such as TCPTP, PTP-LAR and Calcineurin, a potent serine/threonine Phosphatase. Based on the crystal structure of the complex of PTPIB with a known inhibitor, a tetrapeptide inhibitor was identified with the sequence Trp-Lys-Pro-Asp (WKPD) Figure 25.

Figure 25. Trp-Lys-Pro-Asp a tetrapeptide proposed as highly potent PTPIB inhibitor.
The final docked position of the designed inhibitor is shown in Figure 26, and the list of contacting residues (up to 4 Å) is given in Table 10. It can be seen from the Figure that the inhibitor makes hydrogen bonds with residues Ser216, Ala217 and Arg221 in the catalytic site, with Tyr46 and Asp48 in binding site 3 and has hydrophobic interactions (up to 4 Å) with Arg24 in the binding site 2. In addition, there is hydrogen bonding with Lys116. The ligand has also hydrophobic interactions with several other residues in the binding sites (colored blue in Figure 26).

![Figure 26. Final docked complex of PTP1B with designed peptide inhibitor. The color coding is the same as in Figure 24b. Hydrogen bonds are shown as black dashed lines together with distances.](image)

A comparison with the list of contacts of compound XIV (Table 10) shows a similar binding region for the designed ligand. The AutoDock 3.0 results gave a frequency of the largest cluster (which was also the top ranked cluster) of 20/50, with a free energy of binding $\Delta G = -12.26$ kcal/mole and a corresponding $K_d$ value of 1.03 nM. Thus the designed peptide has potency comparable to that of the most potent known inhibitors.
Docking results showing the comparison of docked energy and contacting residues in PTP1B for naphthoic acid and WKPD.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Docked with</th>
<th>Docked Energy (kcal mole(^{-1}))</th>
<th>Contacting residues (up to 4 Å) in final docked position (hydrogen bonded residues are highlighted in bold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naphthoic acid (XIV)</td>
<td>-93.2</td>
<td>Y20, R24, G28, D29, Y46, V49, K120, W 179, C215, S216, A217, G218, I219, G220, R221, R254, M258, G259, Q262, T263, Q266.</td>
</tr>
</tbody>
</table>

The selectivity of the identified tetrapeptide was also studied. The designed ligand was docked with T-Cell Protein Tyrosine Phosphatase (TCPTP) (PDB ID 1L8K),\(^{11, 12}\) PTP of LAR (PDB ID 1LAR)\(^3\) and Calcineurin (PDB ID 1MF8).\(^{14}\) The AutoDock 3.0 results are shown in Table 11. It can be seen that the designed peptide is 800-fold selective over TCPTP (\(K_d = 0.83 \, \mu M\)). This is much higher than the best selectivity of 10-fold achieved so far with the existing inhibitors.\(^15\) The designed peptide is also seen to have high selectivity over LAR and Calcineurin (Table 11).

Selectivity of the designed ligand WKPD against PTPases.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Target</th>
<th>Estimated free energy of binding ((\Delta G)) (kcal mole(^{-1}))</th>
<th>Corresponding (K_d) value at 298 K ((\mu M))</th>
<th>Selectivity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PTP1B</td>
<td>-12.26</td>
<td>0.001</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>TCPTP</td>
<td>-8.29</td>
<td>0.83</td>
<td>809</td>
</tr>
<tr>
<td>3</td>
<td>LAR</td>
<td>-8.38</td>
<td>0.72</td>
<td>696</td>
</tr>
<tr>
<td>4</td>
<td>Calcineurin</td>
<td>-9.26</td>
<td>0.16</td>
<td>159</td>
</tr>
</tbody>
</table>

The Docking calculations indicate that this peptide is highly potent as compared to existing inhibitors. Moreover, the peptide is also found to be selective for PTP1B with a greatly reduced potency against other biologically important protein tyrosine phosphatases such as PTP-LAR, Calcineurin and the highly homologous T-Cell Protein Tyrosine Phosphatase (TCPTP). Therefore it can be concluded, that using an in silico structure-based approach, a small peptide Trp-Lys-Pro-Asp is identified as potent and selective inhibitor of PTP1B. Docking studies show that the designed peptide has potency comparable to that of the best known non-peptidic and peptidomimetic inhibitors. Dynamics simulations on a fully
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hydrated model show that the potency and strong binding are retained even in the presence of explicit solvent. Docking calculations with closely related TCPTP show a very high selectivity of 800 for PTP1B and also high selectivity over PTP-LAR and Calcineurin. The above results suggest that the designed tetrapeptide is a potent and selective inhibitor of PTP1B and is a suitable lead compound for the development of new drugs against PTP1B. Although peptides are generally known to have undesirable pharmacokinetic properties, yet they have provided novel lead compounds and in several cases, modified peptide analogs have been developed as drugs. With recent drug delivery techniques, the opportunities for peptide drug development have been significantly enhanced. Thus, the designed tetrapeptide is a suitable lead compound for the development of new drugs against type 2 diabetes and obesity.

Attempts towards further optimization of the lead thus generated need for the validation of in silico results with the experimental data. Since the peptide based inhibitor is unique in respect to its chemical structure, it could be exceptionally rewarding target for the development of highly selective PTP1B inhibitors. The most striking feature of chemical structure of the tetrapeptide is the absence of any phosphate mimic pharmacophore. However, the C-terminal Asp residue is providing anionic cluster for hydrogen binding. Yet another strange structural feature is the presence of cationic clusters at N-terminal part viz. alpha-amino group of Trp. and ω-amino of Lys-residue. The activity in the cationic molecules or molecules with free amino group is usually unprecedented. In view of these critical and contrasting structural features, it was considered essential to validate the predicted inhibition with the experimental data. To generate experimental data, synthesis of the tetrapeptide is essential. Therefore, the tetrapeptide was synthesized and characterized. The synthesized tetrapeptide Trp-Lys-Pro-Asp was also evaluated for its inhibition potential using the kit based assay. The details of the peptide synthesis and PTP1B inhibition activity is presented in following description.

5.3 Chemistry

The synthesis of tetrapeptide Trp-Lys-Pro-Asp has been carried out in solution phase employing the methods reported for peptide synthesis. Boc group was used as α-amino protecting group while benzyl ester or benzylxycarbonyl groups are used for the α and ω carboxy and ω-amino of Lysine respectively. The NH group of the indole moiety
remained unprotected for the synthesis. The peptide was synthesised in a step wise chain elongation method starting from the coupling of Boc-Pro with dibenzylaspartate to get the dipeptide Boc-Pro-Asp(OBzl)-(OBzl) (41). The Boc group was deblocked with HCl/Dioxane at room temperature and the resulting amine was coupled with the Boc-Lys(Z)-OH\textsuperscript{22} which was prepared by the known procedure reported in the literature to get the tripeptide (42) in good yield and purity after column chromatography. Boc was removed from the tripeptide (42) and the resulting amine was condensed with Z-Trp to get the protected tetrapeptide (43) which on catalytic hydrogenation\textsuperscript{17} over Pd-C resulted in the formation of desired tetrapeptide (44). DCC/HOBt\textsuperscript{18} has been used as coupling reagent during all the coupling reactions. The synthetic steps involved and the reagent used for the synthesis of tetrapeptide are summarized in scheme 9.

\[
\text{Boc-Pro + Asp(OBzl)OBzl (37) } \xrightarrow{a} \text{ Boc-Pro-Asp(OBzl)-OBzl (41) } \xrightarrow{b} \text{ Boc-Lys(Z)-Pro-Asp(OBzl)-OBzl (42) } \xrightarrow{c} \text{ Z-Trp-Lys(Z)-Pro-Asp(OBzl)-OBzl (43) } \xrightarrow{d} \text{ Trp-Lys-Pro-Asp (44) }
\]

**Reagents and conditions:**
(a) DCC, HOBt, DCM, DMF (75 \%); (b) HCl/dioxane, Et\textsubscript{3}N, Boc-Lys(Z)-OH, DCC, HOBt, DCM, DMF (90 \%); (c) HCl/dioxane, Et\textsubscript{3}N, Z-Trp, DCC, HOBt, DCM, DMF (63 \%); (d) Pd-C, methanol (90 \%).

Scheme 9 Stepwise synthesis of tetrapeptide Trp-Lys-Pro-Asp (44).
5.4 Results and Discussions

The synthetic tetrapeptide Trp-Lys-Pro-Asp is identified by the computer modeling and docking studies as highly potent and selective PTP1B inhibitor with $K_i$ value in the range of 1 nM, as predicted by the *in silico* assessment of PTP1B inhibition potential. We have synthesized this peptide and evaluated for their PTP1B inhibitory activity *in vitro*. The assay was performed using a kit supplied by BIOMOL, USA. The activity was performed at 100 μM and 10 μM concentration of the tetrapeptide. We have used Boc-Phe-Tyr(OMal)-pentylamide (V) as the positive control. The % inhibition was calculated from the triplicate of each experiment.

Table 12  Inhibition of PTP1B activity by the tetrapeptide Trp-Lys-Pro-Asp (44)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compound</th>
<th>Concentration (in μM)</th>
<th>% inhibition PTP1B</th>
<th>% inhibition LAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44</td>
<td>100</td>
<td>67.00</td>
<td>48.00</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
<td>10</td>
<td>47.00</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>V</td>
<td>100</td>
<td>83.00</td>
<td>57.81</td>
</tr>
<tr>
<td>4</td>
<td>V</td>
<td>10</td>
<td>51.00</td>
<td>-</td>
</tr>
</tbody>
</table>

PTP1B enzyme is human, recombinant (residues 1-322; MW=37.4 kDa). LAR enzyme is LAR-D1 Fragment. Substrate for PTP1B is the p-nitro-phenyl phosphate (p-NPP). V was taken as test control.

The data of PTP1B inhibition of the synthetic tetrapeptide 44 is presented in table 12. The tetrapeptide has been found to exhibit significant activity at both the concentrations. The tetrapeptide has shown 67% inhibition at 100 μM and 47% at 10 μM respectively whereas, compound V has shown 83% inhibition at 100 μM and 51% inhibition at 10 μM concentrations respectively. The inhibitory activity against LAR was also evaluated at 100 micromolar concentration. The tetrapeptide 44 exhibited 48% inhibition of LAR while compound V showed 57% inhibition. This data suggest that the tetrapeptide 44 has been found to be moderately active and equally selective as compared to compound V in the *in vitro* test system.

The PTP1B inhibitory activity of the tetrapeptide 44 in the experimental model could not be correlated with the predicted activity, both in terms of inhibition potential as well as
selectivity but the data is highly significant for the future design of novel PTP1B inhibitors. The tetrapeptide derived inhibitors of this class of compounds have special structural features as they are lacking any phosphate isostere in the structure. The PTP1B inhibitors designed by optimizing the structural features of the tetrapeptide 44 could be suitable for clinical development as this class of molecules are able to bind at all the three putative sites of PTP1B to completely and selectively block the protein activity. Therefore, it can be concluded that the PTP1B activity of the tetrapeptide 44 in the experimental model is significant achievement and would be highly useful for future design of novel PTP1B inhibitors.

5.5 Experimental

All the amino acids used for this study are of L configuration. The coupling reactions were carried out in anhydrous solvents. Evaporation of solvents was carried out in vacuo at temperatures not exceeding 50 °C. Completion of the reactions was monitored and purity of the products were established by TLC on readymade silica gel plates (Merck, UV active) using following solvent systems.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>n-Butanol-Water-Acetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>CHCl₃-MeOH</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>CHCl₃-MeOH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4:1:1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9.5:0.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9:1)</td>
</tr>
</tbody>
</table>

The plates were developed either under iodine vapours or seen directly under UV-light (254 nm) and if necessary, the TLC plates were exposed to 2N HBr/AcOH followed by 0.2% Ninhydrin solution in acetone and heating the plates at approx. 80-90 °C in hot air oven for 15 minutes. Compounds with free amino group were detected by spraying the plates with Ninhydrin solution straight away and heating. Column chromatography was performed over silica gel (230–400 mesh).

Melting points (mp) were taken in open capillaries on Complab melting point apparatus and are uncorrected. Characterization of all the derivatives as well as final compounds were done with the help IR, NMR and Mass spectroscopy. The ¹H spectra were obtained with Bruker DRX-300 MHz FT-NMR spectrometers. The chemical shifts were reported as parts per million (δ ppm) taking tetramethylsilane (TMS) as an internal standard.
Infrared (IR) spectra were recorded on an FT-IR Perkin-Elmer spectrometer and reported in wave number (cm\(^{-1}\)). Mass spectra were obtained on Micromass Quattro II, Spectrometer using Electron spray ionization mass spectroscopy (ESI MS positive).

**Asp(OBzl)-OBzl (37)**

L-Aspartic acid (665 mg, 5 mmoles) and p-toluenesulphonic acid (1.91 g, 10 mmoles) were dissolved in 5 ml benzyl alcohol and 10 ml toluene. The mixture was heated to reflux by the use of Dean and Stark trap (Temperature should not rise above 90 °C as above this temperature dibenzyl ester undergoes hydrolysis to monobenzyl ester). When water ceased to distill over, the reaction mixture was cooled to room temperature and anhydrous ether was added. After standing for one hour at 4 °C, the crystalline solid dibenzyl ester p-toluenesulphonate was collected and recrystallised from ethanol-ether. To a suspension of amino acid dibenzyl ester paratoluenesulphonate in 20 ml of chloroform at 5 °C, triethylamine (5 mmoles, 0.5 ml) was added for a period of 10 minutes. To it was added 100 ml ether and the mixture was allowed to stand for 10 minutes. Precipitated triethylammonium paratoluenesulphonate was filtered off and the ethereal solution was concentrated in vacuo to obtain L-Asp(OBzl)-OBzl. This compound was obtained as a white solid. (Yield = 1.4 g, 90 %), mp 140 °C (138-140 °C); IR (KBr) 3442 cm\(^{-1}\), 1616 cm\(^{-1}\); \(^1\)H NMR; 300 MHz, CDCl\(_3\) (δ ppm): 3.2 (m, 2H, Asp \(\sim\)CH\(_2\)), 4.4 (br s, 1H, Asp COH), 5.0 (s, 2H, CH\(_2\)OBr), 7.30-7.35 (m, 10H, Ar CH); ESI-MS m/z 314 [M+H\(^+\)].

**Lys(Z)-OMe.HCl (38)**

A solution of Di-(Z)-Lys-OH\(^{21}\) (2.48 g, 6 mmoles) in 40 ml of dry ether was treated gradually with thionyl chloride (1.28 ml, 17.4 mmole). After being stirred at -10 °C for 30 minutes and at 0 °C for 30 minutes, the mixture was refluxed for 5 hours to form the corresponding N-carboxyanhydride, which was reacted, in turn, with methanol (2 ml) at 10 °C. The reaction mixture was kept at room temperature for 16 hours to afford the corresponding methyl ester. After that, the reaction mixture was filtered off and the crystalline product was washed with dry ether. This compound was obtained as a white solid. (Yield = 1.4 g, 71 %); R\(_f\) 0.2 (B). \(^1\)H NMR; 300 MHz, CD\(_2\)OD (δ ppm): 1.35 -1.50 (m, 4H, Lys CH\(_2\)), 1.52 -1.61 (m, 2H, Lys CH\(_2\)), 3.15 (m, 2H, Lys CH\(_2\)N), 3.85 (s, 3H, 3H,
OCH₃), 4.02 (m, 1H, Lys C₅H), 4.92 (br s, 1H, NH), 5.08 (s, 2H, PhCH₂), 7.31 - 7.37 (m, 5H, Ar CH); ESI-MS m/z 295 [M+H⁺].

**Boc-Lys(Z)-OMe (39)**

H-Lys(Z)-OMe (1.22 g, 4 mmoles) was dissolved in dioxane and stirred in an ice-cold bath. To this mixture, triethylamine (1 ml, 7.5 mmoles) was added. Ditertiarybutylpyrocarbonate (981 mg, 4.5 mmoles) was slowly added to the reaction mixture and the stirring was continued for 2 hrs under ice cold conditions and for 2 hrs at room temperature. The pH of the reaction mixture was kept >9 and was monitored during the reaction. This compound was obtained as a white solid. (Yield = 1.28 g, 81 %); mp 118-120 °C; Rf 0.7 (B); IR (KBr) 3402 cm⁻¹, 1697 cm⁻¹;¹H NMR; 300 MHz, CDCl₃ (δ ppm): 1.43 (s, 9H, (CH₃)₃C), 1.35 -1.59 (m, 6H, Lys CH₂), 3.2 (m, 2H, Lys CH₂N), 3.7 (s, 3H, OCH₃), 4.13 (m, 1H, Lys CaH), 4.35 (br s, 1H, NH), 4.92 (br s, 1H, NH), 5.1 (s, 2H, PhCH₂), 7.2 -7.36 (m, 5H, Ar CH); ESI-MS m/z 395 [M+H⁺], 417 [M+ Na⁺].

**Boc-Lys(Z) (40)**

To the solution of Boc-Lys(Z)-OMe (800 mg, 2.1 mmoles) in MeOH (20 ml), was added a solution of 2N NaOH (3.13 ml, 6.1 mmoles) in water. The mixture was stirred at room temperature for 2 hrs. Solvent was evaporated and the mixture was neutralized with citric acid and extracted with EtOAc. The organic phase was washed with brine and dried over Na₂SO₄. The solvent was removed in vacuo to give Boc-Lys(Z)-OH. This compound was obtained as a white solid. (Yield = 745 mg, 90 %); Rf = 0.2 (B); IR (KBr) 3446 cm⁻¹, 1648 cm⁻¹;¹H NMR; 300 MHz, CD₃OD(δ ppm): 1.26 - 1.34 (m, 4H, Lys CH₂), 1.48 (s,9H, (CH₃)₃C), 1.52 (m, 2H, Lys CH₂), 3.17 (m, 2H, Lys CH₂N), 4.11-4.19 (m, 3H; 1H, Lys C₅H, 2H, NH), 5.1 (2H, s, PhCH₂), 7.33 -7.39 (m, 5H, Ar CH); ESI-MS m/z 381 [M+H⁺], 403 [M+ Na⁺].

**Boc-Pro-Asp(OBzl)-OBzl (41)**

Boc-Pro²⁴ (860 mg, 4 mmoles) was dissolved in DCM, followed by the addition of solution of HOBt (540 mg, 4 mmoles) solution in DMF, the reaction mixture was stirred for 2
minutes at 0 °C. To the mixture, was added DCC (924 mg, 4 mmol) dissolved in DCM at 0 °C. After 2 minutes when DCU started precipitating, Asp(OBzl)OBzl (1.25 g, 4 mmole) was added. The reaction mixture was allowed to reach at room temperature and was stirred for 4 hrs. The Dicyclohexylurea (DCU) was filtered off and the filtrate was evaporated under reduced pressure. The oily residue was dissolved in EtOAc, washed with 5% aqueous sodium bicarbonate, brine, 5% citric acid solution and finally with brine. The organic layer was dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The crude was purified by flash silica gel column chromatography and purified product was characterized with the help of mass and NMR spectroscopy. This compound was obtained as a white solid. (Yield = 1.53 g, 75 %); Rf 0.8 (B) ; IR (KBr) 3419 cm⁻¹, 1684 cm⁻¹; H NMR; 300 MHz, CDCl₃ (δ ppm): 1.45 (s, 9H, (CH₃)₃C), 1.92 (m, 2H, CH₂-Pro), 2.07 (m, 2H, CH₂-Pro), 2.93 (1H, dd, j=0.81, Asp- CδH), 3.06 (1H, dd, j=0.71 CβH), 3.38 (m, 2H, Pro CH₂), 4.24 (m, 1H, Pro CαH), 4.84-4.9 (br s, 1H, Asp CαH), 5.1 (m, 2H, CH₂ OBzl), 5.13 (s, 2H, CH₂ OBzl), 7.38-7.48 (m, 10H, Ar CH); ESI-MS m/z 511 [M+H⁺], 533 [M+ Na⁺].

Boc-Lys(Z)-Pro-Asp(OBzl)-OBzl (42)

The dipeptide Boc-Pro-Asp(OBzl)-OBzl (1.53 g, 3 mmole) was treated with 15% (6 ml) HCl/Dioxane for 2 hours at room temperature. The solvent was evaporated under reduced pressure and the residual oil precipitated from ether, filtered and dried .The hydrochloride thus obtained was dissolved in dry DMF and neutralized with triethylamine (0.6 ml, 4.5 mmole) at 0 °C. In a separate flask, Boc-Lys(Z)-OH 25 (1.19 g, 3 mmole) and HOBt (405 mg, 3 mmole) dissolved in DCM/DMF mixture was stirred at 0 °C. To the mixture was added DCC (693 mg, 3 mmole) dissolved in DCM at 0 °C. After 2 minutes when DCU started precipitating, then was added neutralized dipeptide. The reaction mixture was allowed to reach at room temperature and was stirred for 4 hrs. The Dicyclohexylurea (DCU) was filtered off and the filtrate was evaporated under reduced pressure. The oily residue was dissolved in EtOAc, washed with 5% aqueous sodium bicarbonate, brine, 5% citric acid solution and finally with brine. The organic layer was dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The crude was purified by flash silica gel column chromatography and purified product was characterized with the help of mass and NMR spectroscopy. This compound was obtained as a white solid. (Yield = 2.08 g, 90 %); Rf 0.4 (B) ; IR (KBr) 3368 cm⁻¹, 1641 cm⁻¹; H NMR; 300 MHz, CDCl₃ (δ ppm): 1.43 (s, 9H, (CH₃)₃C), 1.25 -1.64 (m, 6H, Lys CH₂), 1.92 (m, 2H, Pro CH₂), 2.83 -2.96 (m, 4H; 2H-
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CH₂-Pro, 2H- Asp βCH₂), 3.17 (m, 2H, Lys CH₂N), 3.72 (IH, m, C₆H₄-Lys), 4.24 (m, 1H, Pro C₆H), 4.47(m, 2H, Pro CH₂), 4.84-4.9 (m, 1H, Asp C₆H), 5.04-5.08 (m, 6H, CH₂OBzl), 5.2(m, 1H, CH), 5.3 (m, 1H, CH), 7.22-7.7 (m, 15H, Ar CH); ESI-MS m/z 772 [M+H⁺].

Z-Trp-Lys(Z)-Pro-Asp(OBzl)-OBzl (43)

The protected tripeptide (771 mg, 1 mmole) was treated with 15% HCl/Dioxane (2 ml) for 2 hours at room temperature. The solvent was evaporated under reduced pressure and the residual oil precipitated from ether, filtered and dried. The hydrochloride thus obtained was dissolved in dry DMF and neutralized with triethylamine (0.2 ml, 1.5 mmole) at 0 °C. In a separate flask Z-Trp (337 mg, 1 mmole) and HOBT (135 mg, 1 mmole) dissolved in DMF and was stirred at 0 °C. To the mixture was added DCC (220 mg, 1 mmole) dissolved in DCM at 0 °C. After 2 minutes when DCU started precipitating, then was added neutralized tripeptide. The reaction mixture was allowed to reach at room temperature and was stirred for 4 hrs. The Dicyclohexyurea (DCU) was filtered off and the filtrate was evaporated at reduced pressure. The oily residue was dissolved in EtOAc, washed with 5% aqueous sodium bicarbonate, brine, 5% citric acid solution and finally with brine. The organic layer was dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The crude tetrapeptide was purified by flash silica gel column chromatography and was characterized with the help of mass and NMR spectroscopy. This compound was obtained as a white solid. (Yield = 625 mg, 63 %); IR (KBr) 3413 cm⁻¹, 1713 cm⁻¹; ¹H NMR; 300 MHz, CDCl₃ (δ ppm): 1.26 (m, 6H, Lys CH₂), 1.92 (m, 2H, CH₂-Pro), 2.8-3.2 (m, 6H; 2H-CH₂ Pro, 2H-βCH₂ Asp, 2H- βCH₂Trp), 3.44 (3H, m; IH-Lys C₆H, 2H-CH₂N), 4.24 (m, 1H, Pro C₆H ), 4.47 (m, 2H, Pro CH₂), 4.84-4.9 (m, 1H, Asp C₆H), 5.0-5.13 (m, 8H, CH₂ OBzl), 5.3 (m, 1H, CH), 5.4 (m, 1H, Lys C₆H), 6.7 (br s, 1H, NH), 7.05-7.7 (m, 20H, Ar CH); ESI-MS m/z 993 [M+H⁺].

Trp-Lys-Pro-Asp (44)

The protected tetrapeptide (198 mg, 0.2 mmole) was dissolved in Methanol and subjected to catalytic hydrogenation using Pd/C as catalyst. After completion of reaction as monitored by TLC, catalyst was filtered off and the filtrate was concentrated in vacuo and was dried over anhydrous P₂O₅ in vacuum desiccator. This compound was obtained as a
white solid. (Yield = 98 mg, 90 %), mp >225 °C; Rf 0.25 (A); IR (KBr) 3413 cm⁻¹, 1713 cm⁻¹; ¹H NMR; 300 MHz, DMSO-d₆ (δ ppm): 1.34-2.04 (m, 10H; 4H-CH₂-Pro, 6H-CH₂-Lys), 2.41-2.91 (m, 6H; CH₂-Trp, 2H, Pro CH₂, 2H- Lys CH₂), 3.57-3.63 (m, 3H; 2H- Pro CH₂, 1H- Trp CH₂), 4.12 (m, 1H, Lys CH₁), 4.38 (m, 1H, Pro CH₁), 4.6 (m, 1H, Asp CH₁), 6.84-7.69 (m, 6H, ArCH); ESI-MS m/z 545 [M+H⁺].

**Biological activity**

The ability of the tetrapeptide Trp-Lys-Pro-Asp for PTP1B inhibition was studied *in vitro* using a kit based assay as per the procedure described in chapter 1.

All the assays were conducted at room temperature in a total volume of 0.1 mL that contained assay buffer [Hepes buffer (50 mM, pH 7.2), EDTA (1 mM), DTT (1 mM), 0.01% NP-40, bovine serum albumin (BSA) (0.1 mg/ml)], PTP1B (2.5 ng/mL), pNPP (2.5 mM) and test compounds or inhibitors (not added in control) in different concentrations as mentioned below.

Inhibitors were dissolved in DMSO to get 10 mM stock solution and the final inhibitor concentrations were achieved through the serial dilutions with the above buffer system. For enzyme dilution, 0.2 mg/ml BSA was used. Appropriate volumes of assay buffer were added to each well (Table 3, chapter 2). In case of control, 5μl of PTP1B enzyme was added to each well and for inhibitor assay, 10 μL of test samples/inhibitors were added to appropriate wells followed by the addition of 5 μl of PTP1B enzyme to each well and in case of blank, neither enzyme nor inhibitor was added (Table 3). The plate was warmed for 5 minutes. 50 μL of warm pNPP substrate was added and the air bubbles were removed. OD’s of sample wells were measured kinetically at 1 minute interval for a total of 30 minutes. The amount of product *p*-nitrophenol was determined from the absorbance at 405 nm. Percentage inhibitions were calculated as follows.

\[
\text{% inhibition} = 100 - \left( \frac{\text{Change in OD of inhibitor}}{\text{Change in OD of control}} \right) \times 100
\]

where, Change in OD of sample = Final OD of sample - Initial OD of sample

From the same kit, we have performed the LAR assay, the only difference was the enzyme was LAR and buffer was at pH 6.8.
Chapter 5 Synthesis and Biological activity of Trp-Lys-Pro-Asp tetrapeptide

References