AN ABSTRACT OF THE THESIS OF

Leena Leelasyatanakij for the degree of Doctoral of Philosophy in Pharmacy presented on April 22, 1996. Title: Synthetic Strategies for the Preparation of Affinity Label Dynorphin A(1-11)NH₂ Analogues.

Abstract approved: Jane V. Aldrich

Affinity labels which bind irreversibly to receptors are useful tools to study receptors. Potential affinity label derivatives of dynorphin A based on [D-Pro¹⁰]Dyn A(1-11)NH₂ were synthesized to use as pharmacological probes for studies of κ opioid receptors. A reactive functionality (e.g. isothiocyanate or bromoacetamide) was incorporated into either the "message" or "address" sequences of the parent peptide. The peptides were prepared using Fmoc-solid phase synthesis on a polyethylene glycol (PEG)-polystyrene (PS) support with a peptide amide linker (PAL). Orthogonal protection was obtained using the allyloxycarbonyl (Alloc) group which was selectively removed by palladium (0), followed by introducing the reactive functionality.

Preliminary binding assay results indicated that, except for the bromoacetamide derivative [Phe(NHCOCH₂Br)⁴,D-Pro¹⁰]Dyn A(1-11)NH₂, all peptides bound to κ opioid receptors with high affinity, with [Lys⁸,D-Pro¹⁰]Dyn A(1-11)NH₂ showing the highest affinity for κ opioid receptors. The isothiocyanate derivatives generally showed higher
affinity for \( \kappa \) opioid receptors than the bromoacetamide derivatives (the bromoacetamide was not introduced into Phe(NH\(_2\))\(^1\)). Incorporation of a reactive functional group in the "message" and "address" sequences of Dyn A yielded peptides with varied selectivity for opioid receptors; for the "message" sequence modifications \( \kappa > \mu >> \delta \), and for the "address" sequence modifications \( \kappa = \mu > \delta \).

The synthesis of [Phe(X)\(^4\),D-Pro\(^{10}\)]Dyn A(1-11)NH\(_2\) (where X = -NH\(_2\), -N=C=S and -NHCOCH\(_2\)Br) on two different resins (PAL-PEG-PS vs. PAL-PS resin) showed that Aloc deprotection rates and the purity of the crude peptide were dependent upon the type of resin used. Removal of Aloc from peptides assembled on the PAL-PEG-PS resin was much faster (3 h) than was deprotection on the PAL-PS resin (24 h), but the crude peptides from the PAL-PS resin contained fewer side products than the peptides obtained from the PAL-PEG-PS.

Radiolabeled affinity label peptides can be useful for studies of the interactions of ligands with opioid receptors. Thus, we developed strategies for the synthesis of a protected precursor for radiolabeling, using [Phe(3',5'-I,4'-NH\(_2\))\(^4\),D-Pro\(^{10}\)]Dyn A(1-11)NH\(_2\) as a model. The protected peptide was assembled on a functionalized NovaSyn TG\(^\circ\) resin with a base-labile 4-hydroxymethylbenzoic acid linkage (linker B). Assembly of the peptide on this modified resin proceeded smoothly. The protected peptide was obtained in an excellent yield (93.1% of the expected peptide substitution). The peptide-resin linkage was cleaved by ammonolysis in isopropanol, with 90% of the peptide cleaved after 1 day. The synthetic strategies described in this dissertation hopefully will be applicable for other affinity label-containing peptides.
Synthetic Strategies for the Preparation of Affinity Label Dynorphin A(1-11)NH₂ Analogues

by

Leeta Leelasvatanakij

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Leena Leelasvatanakij, Author
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CONTRIBUTION OF AUTHORS

Dr. Jane V. Aldrich provided her advice on the design and analysis of the research and writing of each manuscript. Dr. Thomas Murray and Elizabeth Olenchek were involved in the pharmacological experiments. Dr. Brian Arbogast assisted in the mass spectrometry experiments.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER 1 INTRODUCTION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Background</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Objectives and rationale</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 2 LITERATURE REVIEW: OPIOIDS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Opioid peptides</td>
<td>6</td>
</tr>
<tr>
<td>2.1.1 Pro-opiomelanocortin (POMC)</td>
<td>9</td>
</tr>
<tr>
<td>2.1.2 Proenkephalin A (PROENK)</td>
<td>9</td>
</tr>
<tr>
<td>2.1.3 Proenkephalin B or prodynorphin (PRODYN)</td>
<td>10</td>
</tr>
<tr>
<td>2.2 Dynorphin A</td>
<td>11</td>
</tr>
<tr>
<td>2.2.1 Metabolism and enzyme inhibitors</td>
<td>12</td>
</tr>
<tr>
<td>2.2.2 Conformational studies</td>
<td>14</td>
</tr>
<tr>
<td>2.2.3 Structure-activity relationships (SAR)</td>
<td>16</td>
</tr>
<tr>
<td>2.3 Opioid receptors</td>
<td>18</td>
</tr>
<tr>
<td>2.3.1 Opioid receptor types and their ligands</td>
<td>20</td>
</tr>
<tr>
<td>2.3.2 Isolation and purification of opioid receptors</td>
<td>28</td>
</tr>
<tr>
<td>2.3.3 Molecular biology and cloned opioid receptors</td>
<td>30</td>
</tr>
<tr>
<td>2.4 Affinity label-containing opioids</td>
<td>35</td>
</tr>
<tr>
<td>2.4.1 Non-peptide affinity labels</td>
<td>38</td>
</tr>
<tr>
<td>2.4.2 Peptide affinity labels</td>
<td>41</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 3 LITERATURE REVIEW: SOLID PHASE PEPTIDE SYNTHESIS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Basic synthetic strategies</td>
<td>42</td>
</tr>
<tr>
<td>3.2 Protecting groups</td>
<td>44</td>
</tr>
<tr>
<td>3.2.1 N* amino protecting groups</td>
<td>44</td>
</tr>
</tbody>
</table>
### TABLE OF CONTENTS (CONTINUED)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.2 Side chain protecting groups</td>
<td>49</td>
</tr>
<tr>
<td>3.3 Insoluble supports</td>
<td>52</td>
</tr>
<tr>
<td>3.4 Linkers</td>
<td>55</td>
</tr>
<tr>
<td>3.5 Peptide bond formation</td>
<td>60</td>
</tr>
<tr>
<td>3.6 Monitoring</td>
<td>66</td>
</tr>
</tbody>
</table>

**CHAPTER 4 SYNTHESIS OF POTENTIAL AFFINITY LABEL DERIVATIVES OF DYNORPHIN A(1-11)NH₂: MESSAGE SEQUENCE MODIFICATIONS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Abstract</td>
<td>69</td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>70</td>
</tr>
<tr>
<td>4.3 Experimental section</td>
<td>75</td>
</tr>
<tr>
<td>4.3.1 Materials</td>
<td>75</td>
</tr>
<tr>
<td>4.3.2 Methods</td>
<td>76</td>
</tr>
<tr>
<td>4.3.3 Receptor binding assays</td>
<td>82</td>
</tr>
<tr>
<td>4.4 Results and discussion</td>
<td>84</td>
</tr>
<tr>
<td>4.4.1 Amino acid syntheses</td>
<td>84</td>
</tr>
<tr>
<td>4.4.2 Peptide syntheses</td>
<td>89</td>
</tr>
<tr>
<td>4.4.3 Receptor affinity</td>
<td>92</td>
</tr>
<tr>
<td>4.5 Conclusions</td>
<td>95</td>
</tr>
</tbody>
</table>

**CHAPTER 5 SYNTHESIS OF POTENTIAL AFFINITY LABEL DERIVATIVES OF DYNORPHIN A(1-11)NH₂: ADDRESS SEQUENCE MODIFICATIONS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Abstract</td>
<td>99</td>
</tr>
<tr>
<td>5.2 Introduction</td>
<td>100</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS (CONTINUED)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3 Experimental section</td>
<td>103</td>
</tr>
<tr>
<td>5.3.1 Materials</td>
<td>103</td>
</tr>
<tr>
<td>5.3.2 Methods</td>
<td>103</td>
</tr>
<tr>
<td>5.3.3 Receptor binding assays</td>
<td>105</td>
</tr>
<tr>
<td>5.4 Results and discussion</td>
<td>105</td>
</tr>
<tr>
<td>5.4.1 Peptide syntheses</td>
<td>105</td>
</tr>
<tr>
<td>5.4.2 Receptor affinity</td>
<td>106</td>
</tr>
<tr>
<td>5.5 Conclusions</td>
<td>111</td>
</tr>
</tbody>
</table>

## CHAPTER 6 SOLID PHASE SYNTHESIS OF POTENTIAL AFFINITY LABEL DERIVATIVES OF DYNORPHIN A ASSEMBLED ON PAL-PEG-PS VS PAL-PS RESIN

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 Abstract</td>
<td>115</td>
</tr>
<tr>
<td>6.2 Introduction</td>
<td>116</td>
</tr>
<tr>
<td>6.3 Experimental section</td>
<td>120</td>
</tr>
<tr>
<td>6.3.1 Materials</td>
<td>120</td>
</tr>
<tr>
<td>6.3.2 Methods</td>
<td>121</td>
</tr>
<tr>
<td>6.4 Results and discussion</td>
<td>123</td>
</tr>
<tr>
<td>6.5 Conclusions</td>
<td>126</td>
</tr>
</tbody>
</table>

## CHAPTER 7 SYNTHESIS OF A DYNORPHIN A PRECURSOR FOR RADIOLABELING

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1 Abstract</td>
<td>133</td>
</tr>
<tr>
<td>7.2 Introduction</td>
<td>134</td>
</tr>
<tr>
<td>7.3 Experimental section</td>
<td>138</td>
</tr>
</tbody>
</table>
**TABLE OF CONTENTS (CONTINUED)**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3.1 Materials</td>
<td>138</td>
</tr>
<tr>
<td>7.3.2 Methods</td>
<td>139</td>
</tr>
<tr>
<td>7.4 Results and discussion</td>
<td>144</td>
</tr>
<tr>
<td>7.4.1 NovaSyn TG® resin functionalization</td>
<td>145</td>
</tr>
<tr>
<td>7.4.2 Peptide syntheses</td>
<td>146</td>
</tr>
<tr>
<td>7.5 Conclusions</td>
<td>152</td>
</tr>
<tr>
<td>CHAPTER 8 CONCLUSIONS</td>
<td>153</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>157</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>[D-Pro\textsuperscript{10}]Dyn A(1-11)NH\textsubscript{2} and affinity label-containing derivatives</td>
<td>4</td>
</tr>
<tr>
<td>2.1</td>
<td>Diagrammatic representation of structures of opioid peptide precursors</td>
<td>8</td>
</tr>
<tr>
<td>2.2</td>
<td>Dynorphin sequence and sites for enzymatic degradation</td>
<td>13</td>
</tr>
<tr>
<td>2.3</td>
<td>Peptidase inhibitors</td>
<td>14</td>
</tr>
<tr>
<td>2.4</td>
<td>Selected opioid receptor ligands</td>
<td>23</td>
</tr>
<tr>
<td>2.5</td>
<td>Comparison of the amino acid sequences of $\delta$, $\kappa$ and $\mu$ opioid receptors</td>
<td>31</td>
</tr>
<tr>
<td>2.6</td>
<td>Schematic diagram of protein structure for, and similarity among, the three opioid receptors</td>
<td>34</td>
</tr>
<tr>
<td>2.7</td>
<td>A schematic illustration of the principle of recognition amplification in the covalent binding of receptor type A by an affinity label containing a group-selective electrophile X</td>
<td>36</td>
</tr>
<tr>
<td>2.8</td>
<td>Chemical affinity labeling versus photoaffinity labeling</td>
<td>37</td>
</tr>
<tr>
<td>2.9</td>
<td>Affinity label agonists</td>
<td>39</td>
</tr>
<tr>
<td>2.10</td>
<td>Affinity label antagonists</td>
<td>40</td>
</tr>
<tr>
<td>3.1</td>
<td>Stepwise solid-phase synthesis of linear peptide</td>
<td>43</td>
</tr>
<tr>
<td>3.2</td>
<td>&quot;Merrifield&quot; protection scheme for solid-phase synthesis, based on graduated acidolysis</td>
<td>45</td>
</tr>
<tr>
<td>3.3</td>
<td>A mild two-dimensional orthogonal protection scheme for solid phase synthesis</td>
<td>47</td>
</tr>
<tr>
<td>3.4</td>
<td>Fmoc deprotection</td>
<td>48</td>
</tr>
<tr>
<td>3.5</td>
<td>Side chain protecting groups used in Boc SPPS</td>
<td>50</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES (CONTINUED)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6</td>
<td>Side chain protecting groups used in Fmoc SPPS</td>
</tr>
<tr>
<td>3.7</td>
<td>Insoluble supports</td>
</tr>
<tr>
<td>3.8</td>
<td>Resin linkers and handles used in Boc SPPS</td>
</tr>
<tr>
<td>3.9</td>
<td>Resin linkers and handles used in Fmoc SPPS</td>
</tr>
<tr>
<td>3.10</td>
<td>Resin linkers and handles used in both Boc and Fmoc SPPS</td>
</tr>
<tr>
<td>3.11</td>
<td>Peptide bond formation</td>
</tr>
<tr>
<td>3.12</td>
<td>Active esters of amino acids</td>
</tr>
<tr>
<td>3.13</td>
<td>Coupling reagents</td>
</tr>
<tr>
<td>3.14</td>
<td>Ninhydrin test</td>
</tr>
<tr>
<td>4.1</td>
<td>Synthesis of phenylalanine derivatives</td>
</tr>
<tr>
<td>4.2</td>
<td>Synthesis of [Phe(X)₄,D-Pro¹⁰]Dyn A(1-11)NH₂ derivatives</td>
</tr>
<tr>
<td>4.3</td>
<td>Chromatograms of FmocPhe(NH₂) (solid line) and FmocPhe(NHAloc) (dotted line.)</td>
</tr>
<tr>
<td>4.4</td>
<td>Chromatograms of BocPhe(NH₂) (solid line) and BocPhe(NHAloc) (dotted line)</td>
</tr>
<tr>
<td>4.5</td>
<td>Chromatograms of crude peptide 1b (top), 1c (middle) and 1d (bottom)</td>
</tr>
<tr>
<td>4.6</td>
<td>Chromatograms of crude peptide 2b (top) and 2c (bottom)</td>
</tr>
<tr>
<td>5.1</td>
<td>Chromatograms of crude peptides 1b (top), 1c (middle) and 1d (bottom)</td>
</tr>
<tr>
<td>5.2</td>
<td>Chromatograms of crude peptides 2b (top), 2c (middle) and 2d (bottom)</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>6.1</td>
<td>Deprotection of allyl-base protecting groups</td>
</tr>
<tr>
<td>6.2</td>
<td>Structures of PAL-PEG-PS (top) and PAL-PS (bottom) resin</td>
</tr>
<tr>
<td>6.3</td>
<td>Comparison of deprotection rates of Aloc from [Phe(NHAlloc)₄, D-Pro¹⁰]Dyn A(1-11)NH-resin synthesized on PAL-PEG-PS (top) vs and PAL-PS (bottom)</td>
</tr>
<tr>
<td>6.4</td>
<td>Chromatograms of crude peptide [Phe(NH₂)₄,D-Pro¹⁰]Dyn A(1-11)NH₂ from PAL-PEG-PS (left) and PAL-PS (right) resins</td>
</tr>
<tr>
<td>6.5</td>
<td>Chromatograms of crude peptide [Phe(N=C=S)₄,D-Pro¹⁰]Dyn A(1-11)NH₂ from PAL-PEG-PS (left) and PAL-PS (right) resins</td>
</tr>
<tr>
<td>6.6</td>
<td>Chromatograms of crude peptide [Phe(NHCOCH₂Br)₄,D-Pro¹⁰]Dyn A(1-11)NH₂ from PAL-PEG-PS (left) and PAL-PS (right) resins</td>
</tr>
<tr>
<td>7.1</td>
<td>Synthesis of tritium-labeled peptides bearing a reactive functionality</td>
</tr>
<tr>
<td>7.2</td>
<td>NovaSyn TG® resin functionalization</td>
</tr>
<tr>
<td>7.3</td>
<td>Synthesis of dynorphin A precursor for radiolabeling</td>
</tr>
<tr>
<td>7.4</td>
<td>Chromatograms of Phe(3',5'-I₂,4'-NH₂) (dotted line) and FmocPhe(3',5'-I₂,4'-NH₂) (solid line)</td>
</tr>
<tr>
<td>7.5</td>
<td>Chromatograms of crude protected peptide I after ammonolysis; 1 (top), 3 (middle) and 5 (bottom) days</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>2.1</td>
<td>Classification of opioid peptides</td>
</tr>
<tr>
<td>2.2</td>
<td>Binding profile of dynorphin A(1-13)</td>
</tr>
<tr>
<td>2.3</td>
<td>Opioid receptors and physiological effects</td>
</tr>
<tr>
<td>2.4</td>
<td>Distributions of opioid receptors in tissues used in smooth muscle assays</td>
</tr>
<tr>
<td>2.5</td>
<td>Tentative receptor classification</td>
</tr>
<tr>
<td>2.6</td>
<td>Pharmacology of the cloned opioid receptors</td>
</tr>
<tr>
<td>4.1</td>
<td>Analytical data of modified phenylalanine derivatives</td>
</tr>
<tr>
<td>4.2</td>
<td>Analytical data of potential affinity label derivatives of dynorphin A</td>
</tr>
<tr>
<td>4.3</td>
<td>Preliminary binding assay data for potential affinity label derivatives of dynorphin A</td>
</tr>
<tr>
<td>5.1</td>
<td>Analytical data of potential affinity label derivatives of dynorphin A</td>
</tr>
<tr>
<td>5.2</td>
<td>Preliminary binding assay data for potential affinity label derivatives of dynorphin A</td>
</tr>
<tr>
<td>6.1</td>
<td>Ratio of swollen/dry volumes for individual polyethylene glycol polystyrene (PEG-PS) graft beads and polystyrene (PS) support in different solvents</td>
</tr>
<tr>
<td>7.1</td>
<td>Characterization of [Phe(3',5'-I₂,4'-NH₂)₄,D-Pro¹⁰]Dyn A(1-11) NH₂ before and after ammonolysis</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aib</td>
<td>α-aminoisobutyric acid</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>BOP</td>
<td>(benzotriazolylxy) tris (dimethylamino) phosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>CTAP</td>
<td>D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂</td>
</tr>
<tr>
<td>CTOP</td>
<td>D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂</td>
</tr>
<tr>
<td>DAMGO</td>
<td>Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH</td>
</tr>
<tr>
<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIC</td>
<td>diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DIEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMA</td>
<td>N,N-dimethylacetamide</td>
</tr>
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<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DPDPE</td>
<td>cyclo[D-Pen²,D-Pen⁵]enkephalin</td>
</tr>
<tr>
<td>DSLET</td>
<td>Tyr-D-Ser-Gly-Phe-Leu-Thr-OH</td>
</tr>
<tr>
<td>Dyn A</td>
<td>dynorphin A</td>
</tr>
<tr>
<td>EDT</td>
<td>1,2-ethanedithiol</td>
</tr>
<tr>
<td>EKC</td>
<td>ethylketocyclazocine</td>
</tr>
<tr>
<td>FAB-MS</td>
<td>fast atom bombardment mass spectrometry</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>Fmoc-OSu</td>
<td>9-fluorenylmethyl succinimidyl carbonate</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Fmoc-Cl</td>
<td>9-fluorenlymethoxycarbonyl chloride</td>
</tr>
<tr>
<td>GPI</td>
<td>guinea pig ileum</td>
</tr>
<tr>
<td>HAPyU</td>
<td>$O$-(7'-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium</td>
</tr>
<tr>
<td>HATU</td>
<td>$O$-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HOAt</td>
<td>1-hydroxy-7-azabenzotriazole</td>
</tr>
<tr>
<td>HOBt</td>
<td>1-hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>MBHA</td>
<td>4-methylbenzhydrylamine</td>
</tr>
<tr>
<td>Mtr</td>
<td>4-methoxy-2,3,6-trimethylbenzenesulfonyl</td>
</tr>
<tr>
<td>MVD</td>
<td>mouse vas deferens</td>
</tr>
<tr>
<td>NaI-BzOH</td>
<td>naiotone benzoylhydrazone</td>
</tr>
<tr>
<td>nor-BNI</td>
<td>norbinaltorphamine</td>
</tr>
<tr>
<td>OPfp</td>
<td>pentafluorophenyl ester</td>
</tr>
<tr>
<td>PAL</td>
<td>peptide amide linker or 5-(4-aminomethyl-3,5-dimethoxyphenoyl)valeric acid resin</td>
</tr>
<tr>
<td>Pbf</td>
<td>2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Pen</td>
<td>penicillamine</td>
</tr>
<tr>
<td>Pmc</td>
<td>2,2,5,7,8-pentamethylchroman-6-sulfonyl</td>
</tr>
<tr>
<td>PyAOP</td>
<td>7-azabenzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>PyBOP</td>
<td>benzotriazol-1-yl-oxy-trispyrrolidino phosphonium hexafluorophosphate</td>
</tr>
</tbody>
</table>
TBTU: 2-(1H-benzotriazol-1-yl)1,1,3,3-tetramethyluronium tetrafluoroborate

t-Bu: tert-butyl

TFA: trifluoroacetic acid

THF: tetrahydrofuran

Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid

Tos: 4-toluenesulfonyl

U-50,488: (-)-(1S, 2S)-trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide

U-69,593: (5α,7α,8β)-(−)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetamide

Z: benzyloxycarbonyl
SYNTHETIC STRATEGIES FOR THE PREPARATION OF AFFINITY LABEL DYNORPHIN A(1-11)NH₂ ANALOGUES

CHAPTER 1
INTRODUCTION

1.1 Background

Opium from *Papaver somniferum* has been used for controlling pain and diarrhea since ancient times [Brownstein, 1993]. The active ingredient morphine exerts its actions, including relieving pain, reducing intestinal motility and secretion and altering mood, by binding with opioid receptors [Simon, 1991]. Morphine is clinically used as an analgesic for severe pain, but long term users can develop tolerance and physical dependence. Thus, researchers have attempted to discover novel nonaddictive analgesics. Studies of opioid receptors have made significant contributions in this search for new analgesics. Three types of opioid receptors, the µ, κ and σ receptors, were originally proposed by Martin and coworkers [Martin et al., 1976; Gilbert et al., 1976]. The σ receptor was later classified as a non-opioid receptor because its pharmacological effects are not antagonized by naloxone [Rees and Hunter, 1990; Fries, 1991]. The δ receptor was proposed as another type of opioid receptor after the discovery of the enkephalins [Lord et al., 1977]. Today the µ, κ and δ receptors are the generally accepted opioid receptor types.

Characterization of multiple opioid receptors as well as the isolation of opioid peptides has provided a better understanding of the pharmacological effects of opiates and of the physiological functions of endogenous opioid systems. For example κ opioid
receptors have been characterized by benzomorphan derivatives such as ethylketocyclazocine (EKC) and bremazocine [Romer et al., 1980], but these ligands can also interact with \( \mu \) opioid receptors [Kosterlitz et al., 1981; Wood et al., 1981; Magnan et al., 1982; Goldstein 1984]. The more recently developed ligands, including U50,488, U69,593 and their analogues, show very high \( \kappa \) receptor selectivity [Zimmerman, 1990].

Pharmacological studies of opioid receptors can be facilitated by the availability of affinity label-containing opioids including photoaffinity labels and electrophilic affinity labels (chemical affinity labels). Both types of affinity labels bind with receptors in a two-step process; first binding reversibly followed by covalent bond formation [Takemori and Portoghese, 1985]. The irreversible binding of affinity labels can be used in the isolation of receptors from cell membranes such as the isothiocyanate derivative of U50,488 which bind irreversibly to \( \kappa \) receptors [de Costa et al., 1989].

1.2 Objectives and rationale

The specific aim of this dissertation was to synthesize and characterize affinity label-containing derivatives of dynorphin A which can be used for \( \kappa \) opioid receptor characterization. These analogues are electrophilic affinity labels containing reactive functionalities including isothiocyanate (-N=C=S) and bromoacetamide (-NCOCH\(_2\)Br) groups. They will be screened for wash resistant inhibition of binding to cloned opioid receptors. Analogues that bind in a nonequilibrium (irreversible) manner to \( \kappa \) opioid receptors can be used as probes of \( \kappa \) receptor structure and receptor-ligand interaction. The synthetic strategies used to prepare affinity label-containing analogues of dynorphin A
described in this dissertation should also be applicable to other affinity label-containing peptides.

\( \kappa \)-Opioid receptor characterization is of great interest to many researchers, because these receptors are found in human brain and spinal cord in high concentrations [Pfeiffer et al., 1982; Czlonkowski et al., 1983; Gouarderes et al., 1986]. Unlike morphine, \( \kappa \) ligands produce analgesic effects without physical dependence [Millan, 1990]. Dynorphin A (Dyn A), an endogenous opioid peptide derived from prodynorphin [Simmon, 1991], binds to \( \kappa \) opioid receptors and is thought to be an endogenous ligand for these receptors [Chavkin et al., 1982]. Therefore, we developed synthetic strategies for the preparation of potential affinity label derivatives of dynorphin A (1-11)NH2. These compounds hopefully will bind selectively and irreversibly to \( \kappa \) opioid receptors and can be used as probes for \( \kappa \) receptor characterization. In addition, because Dyn A is an endogenous ligand it is important to understand its interactions with opioid receptors and its physiological functions. The truncated peptide, Dyn A(1-13)NH2, has similar activity as the heptadecapeptide in the guinea pig ileum smooth muscle assay [Goldstein et al., 1979]. The C-terminal amide derivative of Dyn A(1-13) was found to have greater metabolic stability than the C-terminal acid [Leslie and Goldstein, 1982]. Gairin [1985] found [D-Pro\(^{10}\)]Dyn A(1-11)NH2 has higher potency and selectivity (ratio of \( K_i \) values (\( \kappa \); \( \mu \); \( \delta \)) = 1: 93: 280) than Dyn A(1-13)NH2.

Therefore, affinity label-containing derivatives of [D-Pro\(^{10}\)]Dyn A(1-11)NH2 derivatives were prepared (Fig.1.1). The reactive functionalities, isothiocyanate (-N=C=S) and bromoacetamide (-NCOCH\(_2\)Br), were incorporated in both the N-terminal "message" and the C-terminal "address" sequences. In the N-terminal sequence, the reactive
|D-Pro\textsuperscript{10}|Dyn A(1-11)NH\textsubscript{2}  
H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-D-Pro-Lys-NH\textsubscript{2}

Affinity label-containing peptides with N-terminal sequence modifications

1. Phe\textsuperscript{1}-substituted analogues

H-Phe(X)-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-D-Pro-Lys-NH\textsubscript{2}

2. Phe\textsuperscript{4}-substituted analogues

H-Tyr-Gly-Gly-Phe(X)-Leu-Arg-Arg-Ile-Arg-D-Pro-Lys-NH\textsubscript{2}

Affinity label-containing peptides with C-terminal sequence modifications

1. Phe\textsuperscript{8}-substituted analogues

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Phe(X)-Arg-D-Pro-Lys-NH\textsubscript{2}

2. Lys\textsuperscript{8}-substituted analogues

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Lys(X)-Arg-D-Pro-Lys-NH\textsubscript{2}

X = -NH\textsubscript{2} (or -H for Lys\textsuperscript{8} series) (control compounds)

= -N=C=S

= -NHCOCH\textsubscript{2}Br (except for Phe\textsuperscript{3} series)

\textbf{Fig. 1.1} [D-Pro\textsuperscript{10}]Dyn A(1-11)NH\textsubscript{2} and affinity label-containing derivatives.
functionalities were introduced on the \( p \)-amino group of phenylalanine in positions 1 and 4 (see chapter 4). In the C-terminal sequence, Ile\(^8\) was replaced by \( p \)-amino phenylalanine or Lys derivatives (see chapter 5). In all cases, the amine compounds without a reactive functionality were prepared as control compounds for the pharmacological assays.

The type of resins used in solid phase synthesis can affect reaction rates and the purity of the final peptides. Therefore we investigated the synthesis of a model peptide, \([\text{Phe}(X)_4, \text{D-Pro}^{10}]\text{Dyn A}(1-11)\text{NH}_2\) (where \( X = -\text{NH}_2, -\text{N} = \text{C}=\text{S} \) and \(-\text{NHOCH}_2\text{Br}\)) on two different resins (PAL-PEG-PS vs. PAL-PS resin) (see chapter 6), and compared the rates of Aloc deprotection and purity of the crude peptides obtained from these two resins.

To facilitate the study of receptor-peptide interactions, radiolabeled peptides need to be prepared. We have developed a synthetic strategy for the preparation of radiolabeled Dyn A analogues containing an affinity label (see chapter 7). The radiolabel and reactive functionalities will be on the same residue so that they will remain associated even after proteolytic digestion of affinity labeled receptors. NovaSyn Tentagel\textsuperscript{®} resin was functionalized with a base labile (\( \text{NH}_3/i\text{-PrOH} \)) linkage and used for the preparation of a protected precursor for radiolabeling by replacing Phe by Phe\((3,5'\text{-I}_2,4'\text{-NH}_2)\). Following cleavage from the resin this protected peptide amide will be subjected to catalytic hydrogenation prior to introducing a reactive functionality. The procedures developed for this model peptide will be used for the preparation of tritiated affinity label-containing peptides. These peptides will be used as pharmacological tools for \( \kappa \) opioid receptor characterization.
CHAPTER 2
LITERATURE REVIEW: OPIOIDS

2.1 Opioid peptides

Opium alkaloids from *Papaver somniferum*, has been used as pain killers since ancient times. The active ingredient in the opium alkaloids is morphine, which binds to receptors [Evans et al., 1988] and produces potent analgesia. A search for the endogenous ligands for opioid receptors resulted in the discovery of the opioid peptides. In 1975, two pentapeptides with opiate-like activities, methionine enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) were identified from porcine brain [Hughes et al., 1975]. Since then, several endogenous opioid peptides have been isolated and characterized, including β-endorphin (β-End) and the dynorphins (Dyn).

Mammalian endogenous opioid peptides were classified into three groups according to their precursors, pro-opiomelanocortin (POMC), proenkephalin A (PROENK) and proenkephalin B or prodynorphin (PRODYN) (Table 2.1) [Negi et al., 1992]. These peptide precursors are translational products of separate but similar genes [Lutz et al., 1992]. The genes have similarity in the placement and size of their respective introns and exons. This suggests that the genes for the three precursors are derived from the same ancestoral gene by gene duplication. POMC, PROENK and PRODYN share common characteristics (Fig. 2.1) [Simon et al., 1994]; they contain almost the same number of amino acids and their sequence homology is more than 50 percent. Each precursor contains multiple opioid peptide...
### Table 2.1 Classification of opioid peptides.

<table>
<thead>
<tr>
<th>Precursors</th>
<th>Peptides</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-opiomelanocortin</td>
<td>β-endorphin</td>
<td>YGGFMTSEKSQTPLVTLFKNAIKNAYKKGE</td>
</tr>
<tr>
<td></td>
<td>δ-endorphin (β-endorphin 1-27)</td>
<td>YGGFMTSEKSQTPLVTLFKNAIKNAY</td>
</tr>
<tr>
<td></td>
<td>γ-endorphin (β-endorphin 1-17)</td>
<td>YGGFMTSEKSQTPLVTL</td>
</tr>
<tr>
<td></td>
<td>α-endorphin (β-endorphin 1-16)</td>
<td>YGGFMTSEKSQTPLVVT</td>
</tr>
<tr>
<td>Proenkephalin A</td>
<td>Met-enkephalin</td>
<td>YGGFM</td>
</tr>
<tr>
<td></td>
<td>Met-enkephalin-Arg-Phe</td>
<td>YGGFMRF</td>
</tr>
<tr>
<td></td>
<td>Met-enkephalin-Arg-Gly-Leu</td>
<td>YGGFMRFGL</td>
</tr>
<tr>
<td></td>
<td>Leu-enkephalin</td>
<td>YGGFL</td>
</tr>
<tr>
<td></td>
<td>Peptide E</td>
<td>TGGFMRRVGRPEWWMDYQKRYGFL</td>
</tr>
<tr>
<td>Proenkephalin B (Prodynorphin)</td>
<td>α-neo-endorphin</td>
<td>YGGFLRKYPK</td>
</tr>
<tr>
<td></td>
<td>β-neo-endorphin</td>
<td>YGGFLRKYP</td>
</tr>
<tr>
<td></td>
<td>Dynorphin A (1-17)</td>
<td>YGGFLRRIRPKLKWDFNE</td>
</tr>
<tr>
<td></td>
<td>Dynorphin A (1-8)</td>
<td>YGGFLRI</td>
</tr>
<tr>
<td></td>
<td>Dynorphin B</td>
<td>YGGFLRRQFKVVT</td>
</tr>
<tr>
<td></td>
<td>Dynorphin B (1-29) (Leumorphin)</td>
<td>TGGFLRRQFKVVTTRSQEDPNAYYELFDV</td>
</tr>
</tbody>
</table>
Fig. 2.1 Diagrammatic representation of structures of opioid peptide precursors. MSH and enkephalin (enk) units are shown by solid or shaded boxes. Cysteine and dibasic residue are shown in the upper portion and major peptides derived are shown in the lower portion of each diagram. LPH, lipotrophin; CLIP, corticotrophin-like intermediate lobe peptide.

sequences which are marked by pairs of basic amino acids at the C-terminus and the N-terminus contain multiple cysteine residues.

2.1.1 Pro-opiomelanocortin (POMC)

Pro-opiomelanocortin (POMC), a translated product of the POMC gene, is a 31 kDa glycoprotein consisting of 267 amino acids. The POMC gene contains 7665 base pairs (bp) including exon 1 (86 bp), exon 2 (152 bp), exon 3 (833 bp), intron A (3708 bp) and intron B (2886 bp) [Nakanishi, 1979]. The major source of POMC is in the intermediate lobe of the pituitary [Simon, 1991]. POMC has been identified as the precursor of adrenocorticotropin hormone (ACTH), β-lipotrophin (β-LPH), endorphins, γ-melanocyte-stimulating hormone (γ-MSH), α-MSH, γ-lipotrophin (γ-LPH) and the corticotropin-like intermediate lobe peptide (CLIP) [Evans et al., 1988]. β-Endorphin (Table 2.1) is the only opioid peptide derived from this precursor which possesses analgesic activity.

2.1.2 Proenkephalin A (PROENK)

Proenkephalin A (PROENK) contains 267 amino acids with multiple copies of enkephalin sequences [Evans et al., 1988]. The PROENK gene is 5200 bp long including exon 1 (70 bp), exon 2 (56 bp), exon 3 (141 bp), exon 4 (980 bp), intron A (87 bp), intron B (469 bp) and intron C (3400 bp) [Comb et al., 1982]. PROENK identified from bovine adrenal cortex contains a copy of Leu-enkephalin, four copies of Met-enkephalin, two copies of Met-enkephalin-Arg-Phe and one copy of Met-enkephalin-Arg-Phe-Leu [Undenfriend et al., 1984]. Proenkephalin A products (Table 2.1) localize in both central and peripheral
tissues [Frederickson et al., 1981; Herbert et al., 1985]. The processing of PROENK yields smaller opioid peptides by cleavage at all pairs of basic residues and at some single arginine residues. In human adrenal, the processing occurs extensively, whereas in bovine adrenal the processing is less extensive yielding higher molecular weight opioid peptides [Imura et al., 1985].

2.1.3 Proenkephalin B or prodynorphin (PRODYN)

Proenkephalin B or prodynorphin (PRODYN) contains 254 amino acids. The PRODYN gene consists of exon 1 (1400 bp), exon 2 (60 bp), exon 3 (145 bp), exon 4 (2200 bp), intron A (1200 bp), intron B (9900 bp) and intron C (1700 bp) [Horikawa, 1983]. Prodynorphin gives rise to dynorphins and neo-endorphins. The derived peptides are found in the brain and spinal cord with the highest concentrations in the anterior hypothalamic nuclei. Porcine PRODYN contains three Leu-enkephalin sequences each flanked by pairs of basic amino acids (i.e. Lys-Arg and Arg-Arg) [Kakidani et al., 1982]. When Lys-Arg pairs are the processing signals, prodynorphin will be cleaved into three opioid peptides: β-neo-endorphin [Minomino et al., 1981], dynorphin A [Goldstein et al., 1981] and leumorphin [Nakao et al., 1983]. Dyn A(1-8) is derived from the cleavage of Dyn A(1-17) between Ile<sup>8</sup> and Arg<sup>9</sup> [Minomino et al., 1980; Seizinger et al., 1981]. A similar cleavage of leumorphin between Thr<sup>13</sup> and Arg<sup>14</sup> yields dynorphin B (rimorphin) [Fischi et al., 1982; Kilpatrick et al., 1982]. Opioid peptides derived from prodynorphin (Table 2.1) share a leu-enkephalin, Tyr-Gly-Gly-Phe-Leu, sequence at the N-terminus and except for dynorphin A(1-8) and β-neo-endorphin, have selectivity for κ opioid receptors [Kosterlitz et al., 1985; Holt et al., 1983].
Processing patterns of PROENK and PRODYN are different although these precursors are co-localized [Basbaum and Fields, 1984; Evans et al., 1984]. PROENK products from cleavage at basic residue pairs are Leu-enkephalin, Met-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe. The processing of PRODYN is less extensive providing opioid peptides with paired basic residues linking C-terminal extensions to the enkephalin sequence. These C-terminal extended enkephalin sequences have higher affinities for κ opioid receptors than enkephalins themselves [James et al., 1984].

2.2 Dynorphin A

Dynorphin A (Dyn), a heptadecapeptide derived from prodynorphin, was first identified and isolated from porcine pituitary and duodenum extract [Cox et al., 1975; Goldstein et al., 1981]. It has been postulated to be an endogenous ligand for κ opioid receptors [Chavkin et al., 1982]. Dyn is distributed in different organs including the pituitary, brain, hypothalamus [Goldstein et al., 1979; Kangawa et al., 1981; Khachaturicn et al., 1982] spinal cord, ganglia [Nakao et al., 1981] duodenum [Botticelli et al., 1981] and the adrenal medulla [Lamaire et al., 1983 and 1982]. Dyn A contains a Leu-enkephalin "message" sequence at the N-terminus which accounts for opioid activity, while the "address" sequence at the C-terminus directs the peptide to κ opioid receptors (Fig. 2.2) [Chavkin et al, 1982]. The last four amino acid residues (Trp-Asp-Asn-Gln), however, are not necessary for association of Dyn A with opioid receptors. The binding profile of Dyn A(1-13) to κ binding sites is comparable to that of the heptadecapeptide and also shows affinity for μ and δ opioid receptors (Table 2.2) [Corbett et al., 1982]. The potency of Dyn A(1-13) is 700 times that
of Leu-enkephalin and 200 times that of normorphine in guinea pig ileum (GPI) [Corbett et al., 1982]. The analgesic effects of Dyn are somewhat controversial, with some evidence showing that it relieves pain from chemical and pressure stimuli but not from heat [Hayes et al., 1983], and that it is effective only by intraspinal administration [Piercey et al., 1982].

**Table 2.2** Binding profile of dynorphin A(1-13).

<table>
<thead>
<tr>
<th>IC₅₀ (nM)</th>
<th>GPI(µ, κ)</th>
<th>MVD(δ)</th>
<th>Rat VD¹</th>
<th>Rabbit VD²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynorphin A(1-13)</td>
<td>0.31</td>
<td>0.33</td>
<td>10,000</td>
<td>2.43</td>
</tr>
</tbody>
</table>

¹Low sensitivity to κ-ligands.
²Sensitive to κ- but not µ- or δ-agonists.

### 2.2.1 Metabolism and enzyme inhibitors

Like other opioid peptides, dynorphin A is susceptible to enzymatic degradation. The N-terminal fragment can be inactivated by various peptidases; the Tyr-Gly bond is cleaved by an aminopeptidase (EC 3.4.11), the Gly-Phe bond by either a metalloendopeptidase EC 3.4.24.11 (enkephalinase) or angiotensin converting enzyme (Fig 2.2) [Leslie et al., 1982; Marks et al., 1980; Benuck et al., 1984]. The C-terminus of both Dyn A(1-17) and Dyn A (1-13) are cleaved by endopeptidases to provide a shorter biologically active fragment, Dyn (1-8) [Leslie and Goldstein, 1982]. Dyn (1-8), resulting from cleavage at Ile⁸-Arg⁹, is less selective toward κ binding sites and is more susceptible to enzymatic inactivation than the longer parent peptides. Several approaches, including substitution of a D-amino acid, conversion of the terminal COOH to CONH₂ [Leslie et al., 1982], conformational constraint
and use of enzyme inhibitors in assays, can prevent the metabolic degradation of dynorphin analogues.

**Fig. 2.2** Dynorphin sequence and sites for enzymatic degradation. a = aminopeptidase, b = enkephalinase and c = endopeptidase.

N-Terminal enzymatic inactivation of dynorphin can be suppressed by several enzyme inhibitors (Fig. 2.3). Bestatin is a specific aminopeptidase inhibitor [Fournie-Zaluski et al., 1985], while thiorphan is an enkephalinase inhibitor [Roques et al., 1980]. Thiorphan itself possesses antinociceptive activity by potentiating the effects of enkephalins released in response to noxious stimuli [Corbett et al., 1982]. The affinity of thiorphan for enkephalinase is independent of its stereochemistry (inhibitory potency (IC$_{50}$): S, 1.90 nM vs R, 1.60 nM).

A retro isomer of thiorphan is also an effective enkephalinase inhibitor which inhibits angiotensin converting enzyme (ACE) less than thiorphan. *Retro*-thiorphan displays a 100-fold difference in inhibitory activity by the different isomers (IC$_{50}$: S, 210 nM vs R, 2.3 nM) [Fournie-Zaluski et al., 1986]. Kelatorphan, a hydroxyamido analogue of thiorphan, is a potent enkephalinase and aminopeptidase inhibitor [Fournie-Zaluski et al., 1984]. The $RVSS$
diastereoisomeric mixture was twice as effective as thiorphan against enkephalinase [Fournie-Zaluski et al., 1984].

\[
\text{H}_2\text{NCH}(\text{CH}_2\text{Ph})\text{CH(OH)CONHCH(Ch}_2\text{CHMe}_2\text{)}\text{CO}_2\text{H}
\]

**Bestatin**

\[
\text{PhCH}_2\text{CH(Ch}_2\text{SH)CONHCHCO}_2\text{H}
\]

**Thiorphan**

\[
\text{PhCH}_2\text{CH(Ch}_2\text{SH)NHCOCHCO}_2\text{H}
\]

**retro-Thiorphan**

\[
\text{PhCH}_2\text{CH(Ch}_2\text{CONHOH)CONHCH(Me)CO}_2\text{H}
\]

**Kelatorphan**

Fig. 2.3 Peptidase inhibitors.

### 2.2.2 Conformational studies

Conformation studies of dynorphin A are problematic due to the linear nature of the peptide and the multitude of conformations it can adopt in solution [Maroun et al., 1981; Zhou et al., 1986; Renugopalakrishman et al., 1988]. Data from conformational studies using circular dichroism (CD), nuclear magnetic resonance spectroscopy (NMR including
COSY, NOESY, ROESY) and Fourier-transform infrared spectroscopy (FT-IR) techniques are important for revealing possible secondary structures of dynorphin. The results from CD studies of Dyn A(1-13) depended upon the experimental conditions (i.e. temperatures, solvents and additives) [Lancaster et al., 1991; Wu et al., 1986]. In aqueous solution, Dyn A(1-13) adopted random coil and β-sheet conformations [Vaughn and Taylor, 1989]; when sodium dodecyl sulfate was added, the α-helix content increased [Maroun and Mattice, 1981], whereas in trifluorethanol (TFE) a minimal amount of α-helix was observed [Lancaster et al., 1991]. Possible conformations of dynorphin were also studied by fluorescence energy transfer between the Tyr¹ and Trp¹⁴ in Dyn A(1-17) in aqueous solution. There was no energy transfer between these two residues indicating the distance between them was at least 20 Å; therefore the N- and C-terminus were not in close proximity [Schiller, 1983]. Fluorescence energy transfer experiments indicated that the N-terminus of [Trp¹]Dyn A(1-13) was fully extended, whereas in the corresponding [Trp¹]Leu-enkephalin analogue, it was folded [Schiller, 1983]. NMR and Raman spectroscopy studies showed dynorphin exists in a mixture of extended β-pleated sheet and random coil structure in aqueous or methanolic solution [Kallick, 1993; Lancaster et al., 1991; Rapaka et al., 1987]. In a lipid environment, Schwyzer proposed that the N-terminal sequence of dynorphin adopts an α-helix structure when the peptide binds to κ opioid receptors embedded in the hydrophobic membrane [Schwyzer, 1986].
2.2.3 Structure-activity relationships (SAR)

The heptadecapeptide dynorphin A displays limited selectivity for κ vs μ and δ receptors. The C-terminal truncated peptide, dynorphin A(1-13), shows a similar receptor selectivity profile to the Dyn A(1-17) [Chavkin et al., 1982]. A truncated sequence Dyn A(1-11) further shows 3 times higher κ selectivity towards both the μ and the δ receptors than dynorphin A [Gairin et al., 1985].

Structure-activity relationship studies have indicated which residues are important for the biological activity of the peptide. These residues are normally preserved while other residues can be modified to obtain analogues with greater selectivity and/or stability. Sequential removal of the C-terminal amino acids of Dyn A(1-13) showed that basic residues Arg\textsuperscript{7} and Lys\textsuperscript{11} are essential for high κ selectivity and/or potency [Chavkin and Goldstein, 1981]. Arg\textsuperscript{7} is important for the interaction with κ opioid receptors while Lys in position 11 is important for potency and κ selectivity. Incorporation of various residues in position 7-15 of Dyn A(1-17) suggests that Pro\textsuperscript{10} plays an important role in an amphilic β-strand structure of Dyn A [Taylor, 1990]. D-Pro substitution at position 10 of Dyn A(1-11) and Dyn A(1-13) also improves κ receptor selectivity and potency [Lemaire et al., 1986; Gairin, et al., 1984 and 1988]. D-Amino acid substitution increases Dyn A(1-13) stability against peptidases [Kawasaki et al., 1993]. Substitution of hydrophobic residues or D-amino acids for Ile\textsuperscript{8} also enhances κ receptor selectivity [Gairin et al., 1984].

An alanine scan is a standard approach to identify important residues in a peptide. Introduction of Ala into the Dyn A(1-13) sequence provided structure-activity relationship information for this peptide [Turcotte et al., 1984]. Substitution of Ala into positions 1 and
4 dramatically reduced Dyn A(1-13) activities. Thus, Tyr and Phe are crucial for potency and receptor affinity of Dyn A(1-13). [Ala]Dyn A(1-13) retained 35% of the potency in the GPI and the MVD assay and 12% of binding activity compared to Dyn A(1-13). The linear peptides [D-Ala]Dyn A(1-11)NH2 and [Ala]Dyn A(1-11)NH2 are very potent for κ opioid receptors and very selective for κ vs µ and κ vs δ receptors [Lung et al., 1995]. Replacement of Arg, Arg, Arg, and Lys by Ala decreases the potency in both GPI and binding assays. [Ala]Dyn A(1-13), however, showed improved potency in both the MVD and binding assays [Turcotte et al., 1984]. In rat, [Ala]Dyn A(1-13) was a potent analgesic, while in guinea pig it was less potent than Dyn A(1-13) itself [Jhamandas et al., 1984]. The D-Ala analogue showed only a slight improvement in κ selectivity [Lemaire et al., 1986].

The Dyn A(1-13) analogue DAKLI, [Arg,Arg,Gly]Dyn A(1-13)NH(CH2)5NH2 retains the same κ opioid receptor selectivity as the parent peptide [Goldstein et al., 1988]. DAKLI derivatives containing various labeling groups (e.g. 125I, fluorescein or biotin) also had selectivities comparable to Dyn A(1-13).

Conformationally constrained analogues of Dyn A have also been prepared and studied for opioid activity and selectivity. A disulfide bridged analogue [D-Cys,Cys]Dyn A(1-13) was the first synthesized cyclic Dyn A analogue and shows greater potency in the GPI than the parent peptide Dyn A(1-13) [Schiller et al., 1982]. This cyclic peptide, however, mainly interacts with δ opioid receptors instead of with κ opioid receptors [Sherman et al., 1985]. Although introduction of a disulfide bridge between D-Cys and D-Cys (or L-Cys) in Dyn A(1-13)NH2 gives compounds with high affinity for κ receptors, these cyclic peptides exhibit low κ over µ receptor selectivity [Kawasaki et al., 1990].
cyclic disulfide-containing analogs cyclo[L-Pen\textsuperscript{5},Cys\textsuperscript{11}]\textsuperscript{-}, cyclo[Cys\textsuperscript{4},Cys\textsuperscript{10}]\textsuperscript{-}, cyclo[Cys\textsuperscript{5}, Cys\textsuperscript{9}]\textsuperscript{-} and cyclo[Cys\textsuperscript{4},Cys\textsuperscript{9},Arg\textsuperscript{10}]\textsuperscript{Dyn A(1-11)NH\textsubscript{2}} possess high κ and μ opioid receptor affiities for the central receptor (guinea pig brain), but affect only weak potency at peripheral κ and μ opioid receptor (guinea pig ileum (GPI)) [Kawasaki et al., 1993]. A lactam analog cyclo[D-Orn\textsuperscript{2},Asp\textsuperscript{\textsuperscript{2}}]Dyn A(1-8)NH\textsubscript{2} shows greater affinity for μ opioid receptors than for κ opioid receptors [Schiller et al., 1988]. Several other lactam analogues such as, cyclo[Orn\textsuperscript{5}, Asp\textsuperscript{8}]\textsuperscript{-}, cyclo[Orn\textsuperscript{5},Asp\textsuperscript{10}]\textsuperscript{-} and cyclo[Orn\textsuperscript{5},Asp\textsuperscript{13}]\textsuperscript{Dyn A(1-13)NH\textsubscript{2}} are selective for μ opioid receptors and show weak activity in the GPI [Schiller et al., 1988].

2.3 Opioid receptors

Structure-activity relationships suggested that opiate drugs exert their effects through specific receptors. Opioid receptors were first thought to be a single class of homogeneous receptors. Martin and co-workers [Martin 1976; Gilbert 1976], however, proposed the multiple opioid receptor classification, and since then the idea has become generally accepted. In the original classification, three opioid receptor types were proposed which were named after the prototypic drugs used in the studies: mu (μ) receptors for morphine, kappa (κ) receptors for ketocyclazocine and sigma (σ) receptors for N-allylnormetazocine (SKF10047). These ligands exerted distinct pharmacological effects (Table 2.3) and were unable to replace each other in the suppression of withdrawal symptoms in chronically treated dogs. Sigma (σ) receptors are now defined as non-opioid receptors, since pharmacological effects mediated through σ receptors are not blocked by naloxone, an opioid antagonist normally used to define opioid receptors [Rees and Hunter, 1990; Fries, 1991].
Delta (δ) (from vas deferens) opioid receptors [Lord et al., 1977] were defined after the discovery of enkephalins. They are present in high concentrations in the mouse vas deferens, but functional δ receptors are absent from guinea pig ileum (Table 2.4). Mu, kappa and delta receptors are generally accepted as the major opioid receptor types. Two novel opioid receptors, epsilon (ε) and zeta (ξ) receptors, have been reported [Wuster et al., 1978; Zagon et al., 1993].

Table 2.3 Opioid receptors and physiological effects.

<table>
<thead>
<tr>
<th></th>
<th>mu (μ)</th>
<th>kappa (κ)</th>
<th>delta (δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>analgesia</td>
<td></td>
<td>analgesia</td>
<td>analgesia</td>
</tr>
<tr>
<td>constipation</td>
<td></td>
<td>sedation</td>
<td>hyperthermia</td>
</tr>
<tr>
<td>respiratory depression</td>
<td></td>
<td>diuresis</td>
<td>respiratory depression</td>
</tr>
<tr>
<td>physical dependence</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Table 2.4 Distributions of opioid receptors in tissues used in smooth muscle assays.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>μ</th>
<th>δ</th>
<th>κ</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig ileum (GPI)</td>
<td>++</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Hamster vas deferens (HVD)</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Rabbit vas deferens (LVD)</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Mouse vas deferens (MVD)</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat vas deferens (RVD)</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>
2.3.1 Opioid receptor types and their ligands

2.3.1.1 Mu (µ) receptors

Morphine and some opiates have limited selectivity for µ receptors, whereas DAMGO ([D-Ala²,MePhe⁴,Gly-ol]enkephalin, dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH₂) and DALDA (Tyr-D-Arg-Phe-Lys-NH₂, Kᵦ/Kᵦµ = 11,400) are highly selective µ agonists [Schiller et al., 1989; Erspamer, 1990]. Radiolabeled DAMGO is commonly used for radioligand binding assays for µ receptors. The δ antagonist Tyr-D-Tic-Phe-PheNH₂ (Tic = tetrahydroisoquinoline-3-carboxylic acid) also shows some activity as a µ receptor agonist [Schiller et al., 1992]. Naloxone and naltrexone are non-selective µ-antagonists. Potent µ-antagonists are somatostatin analogues including cyclo [H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol] (SMS-201995), cyclo[H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH₂] (CTP), and cyclo[H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂] (CTOP) [Maurer et al., 1982; Schiller, 1993]. Unlike SMS-201995, CTP and CTOP have high affinity for µ-receptors with reduced affinities for somatostatin receptors [Pelton et al., 1985, 1986]. The heptapeptide cyclo[H-D-Tic-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂] (TCTOP, Tic = 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid) is the most selective µ-antagonist reported (IC₅₀/IC₅₀µ = 11,400) with a very low affinity for the somatostatin receptors [Kazmierski et al., 1988].

Mu receptors have been classified into two subtypes, ū₁ and ū₂ by Pasternak [1994]. Both have some common characteristics including high affinities for morphine and similarities in regional distributions. However, they differ in binding profiles for a number of opioid ligands and opioid peptides (Table 2.5) [Pasternak, 1994]. The postulated ū₁ sites
**Table 2.5** Tentative receptor classification

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Agonists</th>
<th>Antagonists</th>
<th>Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>μ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>μ₁</td>
<td>Morphine</td>
<td>Naloxonazine</td>
<td>Supraspinal analgesia</td>
</tr>
<tr>
<td></td>
<td>Enkephalin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>μ₂</td>
<td>Morphine</td>
<td>CTOP</td>
<td>Spinal analgesia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Respiratory depression</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibition of gastrointestinal transit</td>
</tr>
<tr>
<td><strong>κ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>κ₁</td>
<td>Dynorphin A</td>
<td>nor-BNI</td>
<td>Spinal analgesia</td>
</tr>
<tr>
<td></td>
<td>U-50,488</td>
<td></td>
<td>Diuresis</td>
</tr>
<tr>
<td></td>
<td>U-69,593</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spiradoline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>κ₂</td>
<td></td>
<td></td>
<td>Pharmacology unknown</td>
</tr>
<tr>
<td>κ₃</td>
<td>NalBzOH</td>
<td></td>
<td>Supraspinal analgesia</td>
</tr>
<tr>
<td></td>
<td>Nalorphine</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>δ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ₁</td>
<td>Enkephalin; tolerant of</td>
<td>DALCE</td>
<td>Analgesia (spinal systems are more sensitive than supraspinal ones)</td>
</tr>
<tr>
<td></td>
<td>conformations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ₂</td>
<td>Met-enkephalin; not</td>
<td>nor-BNI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tolerant of conformations</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

bind to morphine and enkephalins with high affinity, whereas the \( \mu_2 \) sites have higher affinity for morphine than enkephalins. In addition, naloxonazine and naloxazone are reported to selectively inactivate \( \mu_1 \) but not \( \mu_2 \) subtypes [Pasternak, 1994]. Mu opioid receptors have been recently cloned [Chen et al., 1993; Wang, et al., 1993; Thompson, et al., 1993], and are a member of the G protein superfamily with high homology with \( \delta \) and \( \kappa \) (see section 2.3.3 below).

2.3.1.2 Kappa (\( \kappa \)) receptors

Early studies of \( \kappa \) opioid receptors were complicated by the lack of \( \kappa \)-selective ligands. Benzomorphans (e.g. bremazocine and ethylketocyclazocine) were often used to study \( \kappa \) receptors, but, they have affinity for other opioid receptors as well and are not very selective for \( \kappa \) receptors. The development of the highly selective \( \kappa \)-ligand, U-50,488\{trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolindinyl)cyclohexyl]-benzeneacetamide\} [Von Voighlander et al., 1983] (Fig. 2.4) and the discovery of high levels of \( \kappa \) binding sites in guinea pig ileum [Kosteliz et al., 1981] have greatly facilitated the study of \( \kappa \)-opioid receptors. The endogenous opioid peptide dynorphin A preferentially binds to \( \kappa \) opioid receptors, but it also shows affinity for both \( \mu \) and \( \delta \) opioid receptors. \([\text{Ala}^8]\text{Dyn A}(1-13)\) is four times more \( \kappa \)-selective over both the \( \mu \)- and \( \delta \)-receptors than Dyn A(1-13), while slight improvements in \( \kappa \)-selectivity were found for \([\text{Trp}^8]\text{Dyn A}(1-13)\) and \([\text{D-Pro}^{10}]\text{Dyn A}(1-13)\) [Lemaire et al., 1986]. The shorter Dyn analogue \([\text{D-Pro}^{10}]\text{Dyn A}(1-11)\) shows an increase in \( \kappa \)-selectivity [Gairin et al., 1984], and 200- to >1,000-fold selectivity for \( \kappa \) over \( \mu \) receptors was found in the N-monoalkylated \([\text{D-Pro}^{10}]\text{Dyn A}(1-11)\) derivatives [Choi et al., 1992].
Fig. 2.4 Selected opioid receptor ligands.

Fig. 2.4 (cont.) Selected opioid receptor ligands.
The bivalent ligand TENA is the first selective κ opioid receptor antagonist [Portoghese and Takemori, 1985]. TENA consists of two naltrexone-derived pharmacophores connected by a triethylene glycol spacer (Fig. 2.4). Binaltorphimine and nor-binaltorphimine (norBNI) contain a very short and rigid spacer (i.e. a pyrrole ring) are highly potent and selective κ opioid receptor antagonists [Portoghese et al., 1987].

At least three κ-opioid receptor subtypes, κ₁, κ₂ and κ₃, have been proposed, κ₁-Receptors are defined by the highly κ selective agonists, U-50,488 and U-69,593 (Fig. 2.4) [Zukin et al., 1988]. κ₁-Binding sites bind with high affinity to arylacetamides U-50,488 and U-69,593, while the κ₂-sites have lower affinities (Table 2.5) [Zukin et al., 1981]. Further classification of κ₁-receptors into κ₁ₐ and κ₁ₐ [Clark et al., 1989] has been proposed, but, their pharmacology remains unclear. κ₂-Receptors, which are U-50,488-insensitive, were described in rat brain using ³H-ethylketocyclazocine plus unlabeled μ, δ and κ₁ ligands to block these receptors [Zukin et al., 1988]. Another U-50,488-insensitive binding site, the κ₃-receptor, was proposed based upon the activity of naloexone benzylhydrazone [Gistrak et al., 1989; Paul et al., 1990]. κ₃-Binding sites appear to predominate in calf striatum, where their density is twice that of either μ or δ receptors [Clark et al., 1989]. κ₃-Receptors have also been proposed as the iso-receptor of μ receptors [Wollemann et al., 1992; Barden and Simon, 1993]. The cloned κ opioid receptors are proposed to be the κ₁ subtype [Raynor et al., 1994].
2.3.1.3 Delta (δ) receptors

Delta receptors were identified after the discovery of enkephalins [Lord et al., 1977]. Selective enkephalin derivatives for δ receptors include the linear analogues DSLET and DTLET ([D-Ser²,Leu⁴,Thr⁶]- and [D-Thr²,Leu⁴,Thr⁶]enkephalin) and the cyclic analogues DPDPE ([D-Pen²,D-Pen⁵]enkephalin) and its derivatives [Schiller, 1993]. Derivatives of frog skin deltorphins, D-Met-deltorphin (Tyr-D-Met-Phe-His-Leu-Met-Asp-NH₂), [D-Ala²] deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂) and [D-Ala²] deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂), also show high affinity and selectivity for δ binding sites [Erspamer, 1992]. The N,N-dialkylated enkephalin derivative ICI 174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu) (Aib = aminoisobutyric acid) [Cotton et al., 1984] shows antagonism for δ receptors, whereas TIPP (Tyr-Tic-Phe-Phe-OH) possesses higher potency than ICI 174,864 [Schiller et al., 1992]. Recently developed non-peptide δ-selective agonists are BW373U86 [Chang et al., 1993] and SIOM (7-spiroindino-oxymorphone) [Portoghese et al., 1993], while nonpeptidic δ antagonists are naltrindole and the naltrindole derivatives naltriben [Portoghese et al., 1991] and BNTX (7-benzidenenaltrexone) [Portoghese et al., 1992].

Two subtypes of δ opioid receptors, δ₁ and δ₂, have been proposed using agonists (DPDPE for δ₁, and [D-Ala²]deltorphin II for δ₂) [Mattia et al., 1991 and 1992; Jian et al., 1991], antagonists (BNTX for δ₁ [Portoghese et al., 1992], and naltriben for δ₂ [Portoghese et al., 1992]) and affinity labels (DALCE (Tyr-D-Ala-Gly-Phe-Leu-CysOH) for δ₁ [Bowen et al., 1987], and naltrindole isothiocyanate for δ₂ [Portoghese et al., 1990]). The cloned δ receptor has similar a pharmacological profile to δ₂ sites [Raynor et al., 1994].
2.3.1.4 Epsilon (ε) receptors

Epsilon (ε) receptors in rat vas deferens (RVD) have been proposed by Schulz [1981] and Gillan [1981]. Met-enkephalin and Leu-enkephalin have low affinities for these binding sites (IC$_{50}$ > 100 nM), whereas β-endorphin show higher affinity (IC$_{50}$ = 74-90 nM) [Wuster et al., 1978]. It was suggested that the β-endorphin (1-31) sequence was required for binding to ε-receptors. In addition, when RVD was made highly tolerant to etorphine, it was only moderately tolerant to β-endorphin [Wuster et al., 1978]. The high degree of tolerance to etorphine was thought to be due to a small population of μ receptors in the RVD. The presence of ε-receptors in other tissues is inconclusive. The benzomorphan binding sites in rat brain may also be the ε-receptors [Chang et al., 1984]. Nock and co-workers (1990), however, have reported that the binding sites, previously characterized by them as κ$_2$-receptors, may be ε-receptors.

2.3.1.5 Zeta (ζ) receptors

Zeta (ζ) receptors have recently been postulated based on studies in S20Y murine neuroblastoma cells. Some opioids showed growth inhibitory effects in neuroblastomas and in cell cultures derived from neural tumors which have been suggested to be mediated via zeta bindings sites [Zagon et al., 1989]. Zeta (ζ) receptors have been proposed as opioid growth factor receptors [Zagon et al., 1991 and 1993]. Most peptides derived from proenkephalin A and some from prodynorphin have high affinities for zeta receptors, while other alkaloids and selective ligands for μ, κ, δ, σ and ε receptors have low affinity for zeta binding sites [Zagon et al., 1989].
2.3.2 Isolation and purification of opioid receptors

Studies of opioid receptor structure have involved isolation and purification of the receptors. One approach involves a two-step procedure to obtain a specific opioid binding site, first labeling receptors with affinity labels or crosslinking, followed by isolation and purification of the receptor molecules [Simon and Hiller, 1994]. \[^{3}H\]Etorphine was used to label opioid binding sites from rat brain [Simon et al., 1975], and the labeled complex subsequently solubilized by detergents such as digitonin, lysophosphatidylcholine and \(3-[(3\text{-chloamidopropyl})\text{ dimethylamino}]\)-1-propanesulfonate\) (CHAPS). CHAPS is a zwitter-ionic derivative of deoxycholate which has an advantage over digitonin in that agonist binding is readily restored by a simple precipitation of the solubilized receptor protein with polyethylene glycol (PEG) [Simon and Gioannini, 1993]. The \[^{3}H\]etorphine-labeled receptor was unstable under conditions for further purification although it was useful for receptor characterization. The development of FIT (fentanylisothiocyanate) and superFIT (methyl-fentanylisothio-cyanate) have enhanced opioid receptor purification [Simonds et al., 1985]. \[^{3}H\]FIT was successfully utilized to label opioid receptors on the neuroblastoma glioma hybrid NG108-15 cell line which contains only \(\delta\) opioid receptors. The labeled receptors were solubilized and purified to homogeneity using chromatography on wheat germ agglutinin (WGA)-agarose followed by chromatography on a column of immobilized antibodies to FIT. The isolated receptors were \(\delta\) binding sites with a molecular weight of 58 kDa on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

An affinity crosslinking technique has been utilized to separate \(\mu\)- and \(\delta\)-binding sites using human \[^{125}\]I\(\beta\)-endorphin (\(\beta\text{-End}_{\text{h}}\)) [Toogood et al., 1986]. Tyr\(^{27}\) was iodinated instead
of Tyr', since the iodination of Tyr' significantly decreases endorphin's binding affinity. 

$[^{125}\text{I}-\text{Tyr}^{27}]\beta$-End$_h$ bound to $\mu$- and $\delta$-receptors with negligible affinity for $\kappa$-receptors, and thus both $\mu$- and $\delta$-receptors could be studied in the same tissue. The membrane-bound opioid receptors were labeled with $[^{125}\text{I}]\beta$-End$_h$ and washed to remove free or loosely bound ligands. Then they were crosslinked with bis[2-(succinimidoxycarbonyloxy)ethyl] sulfonate (BSCOES), which forms linkages between amino groups on the ligand and receptor. The crosslinked membranes were solubilized in SDS, separated by SDS-PAGE and subjected to autoradiography [Howard et al., 1985]. A band of $M_r = 65$ kDa was present in all tissues containing $\mu$ receptors, while a band of $M_r = 53$ kDa existed only in $\delta$-receptor containing tissues. To confirm the identity of these bands, crosslinking experiments were performed in the presence of ligands highly selective for $\mu$ or $\delta$ receptors [Howard et al., 1986]. The radioactive band at 65 kDa disappeared when the binding of $^{125}\text{I}$-Tyr$^{27}$-$\beta$-End$_h$ was carried out in the presence of DAMGO (a highly $\mu$-selective ligand), while the band at 53 kDa was eliminated when the binding was carried out in the presence of DPDPE (a highly $\delta$-selective ligand).

Kappa-opioid receptors were similarly isolated from guinea pig cerebellum membrane by affinity crosslinking technique using labeled [D-Pro$^{10}$]Dyn A(1-11) [Yao et al., 1989]. Bands at $M_r = 55$ kDa and $M_r = 35$ kDa were obtained. The 55 kDa band was completely eliminated in the presence of U-50,488 (a highly $\kappa$-selective ligand), while the 35 kDa band was reduced in intensity but did not completely disappear, even when using higher concentrations of $\kappa$ ligands. The band at 55 kDa was proposed to be high-affinity $\kappa$-binding sites, whereas the band at 35 kDa was thought to be low-affinity $\kappa$-binding sites.
or a combination of κ-binding sites and non-opioid binding sites [Yao et al., 1989]. Itzhak and co-workers [1984] separated κ-binding sites from other receptors using a one-step procedure. Guinea pig brain membranes were solubilized with digitonin and layered on top of a sucrose density gradient devoid of sodium and containing only a low concentration of digitonin (0.02%). The mixture was centrifuged, fractions were collected and assayed for binding activities with [3H]bremazocine. Two peaks were observed, the first one corresponded to κ-binding sites and the second one was a combination of μ- and δ- binding sites.

Active kappa receptors had been purified by different techniques. Simon and co-workers [1987] used affinity chromatography on a matrix of DADLE coupled to epoxy-activated Sepharose 4B, followed by gel permeation chromatography on Sepharose 6B to purify κ-binding sites from frog brain (Rana esculenta), whereas Mollereau and co-workers [1988] purified κ-receptors from frog brain (Rana ridibunda) using affinity chromatography on a dynorphin-agarose column.

2.3.3 Molecular biology and cloned opioid receptors

The first opioid receptors successfully cloned were δ receptors from NG 108-15 cells reported in 1992 [Kieffer et al., 1992; Evans et al., 1992]. Since then μ, κ and δ-receptors have been cloned from rat, guinea pig, mouse, and human brain [Yasuda et al., 1993; Chen et al., 1993; Li et al., 1993; Fukuda et al., 1993; Nishi et al 1993; Abood et al., 1994; Zastawny et al., 1994]. Amino acid sequences of all three cloned receptors are highly homologous (Fig. 2.5), but their pharmacological effects profiles are distinct (Table 2.6)
Fig. 2.5 Comparison of the amino acid sequences of δ, κ, and µ opioid receptors. The sequence of mouse δ (mOPRD1), mouse κ (mOPRK1) and rat µ (rOPRM1) opioid receptors are shown using the single-letter abbreviations for the amino acids. Residues that are identical in two or more of the proteins are boxed. Gaps introduced to generate this alignment are represented by dashes. The seven predicted transmembrane domains are noted (TM1-TM7) and are shown by shading. The potential sites for N-linked glycosylation in the extracellular amino domains of these proteins are: mouse δ receptor, residues 18 and 33; mouse κ receptor, residues 25 and 39; rat µ receptor, residues 9, 31, 38, 46 and 53.

Table 2.6 Pharmacology of the cloned opioid receptors.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$\delta$ (nM)</th>
<th>$\kappa$ (nM)</th>
<th>$\mu$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>$&gt;1000$</td>
<td>163</td>
<td>1.4</td>
</tr>
<tr>
<td>Naloxone</td>
<td>95</td>
<td>16</td>
<td>3.9</td>
</tr>
<tr>
<td>DAMGO</td>
<td>$&gt;1000$</td>
<td>$&gt;1000$</td>
<td>0.87</td>
</tr>
<tr>
<td>DADLE</td>
<td>2.8</td>
<td>514</td>
<td>6.4</td>
</tr>
<tr>
<td>DPDPE</td>
<td>7.6</td>
<td>$&gt;1000$</td>
<td>$&gt;1000$</td>
</tr>
<tr>
<td>Dynorphin A(1-17)</td>
<td>45</td>
<td>5.48</td>
<td>120</td>
</tr>
<tr>
<td>U-50,488</td>
<td>$&gt;1000$</td>
<td>1.4</td>
<td>$&gt;1000$</td>
</tr>
<tr>
<td>U-69,593</td>
<td>$&gt;1000$</td>
<td>2.3</td>
<td>$&gt;1000$</td>
</tr>
</tbody>
</table>

[Reisine and Bell, 1993]. The cloned mouse \( \delta \) receptor contains 372 amino acid proteins with 97% identity with rat \( \delta \) receptors. Rat \( \mu \) opioid receptors contain 398 amino acids with 58% and 57% identity and 66% and 68% similarity to the sequences of mouse \( \delta \) and \( \kappa \) receptors, respectively. Rat \( \kappa \) receptors, which consist of 380 amino acids, have 61% amino acid identity and 70% similarity to the sequence of the mouse \( \delta \) receptors. Human \( \mu \), \( \delta \), and \( \kappa \)-receptors have been recently cloned [Wang et al., 1994; Knapp et al., 1994; Simonin et al., 1994; Yasuda et al., 1994].

Opioid receptors are guanine nucleotide-binding regulatory proteins (G-proteins) coupled receptors [Wollemann, 1990]. The receptor protein consists of extracellular regions (the N-terminus and extracellular loops), seven putative transmembrane (TM) regions, and intracellular regions (the C-terminus and intracellular loops). The highest sequence homology among rat \( \mu \), rat \( \kappa \) and mouse \( \delta \) receptors exist in TM2, TM3 and TM7, and highly preserved sequences are also found in the 2nd and the 3rd intracellular loops (Fig. 2.6) [Chen et al., 1993]. The sequences of TM2 and TM3 each contain a conserved Asp residue which may interact with protonated amine groups of opioid ligands. Such an acidic residue has been proposed to mediate sodium inhibition of agonist binding in the \( \alpha_2 \)-adrenergic and somatostatin receptors [Horstman et al., 1990]. The 3rd intracellular loop is required for the interactions with G-proteins. The 2nd and 3rd extracellular loops and TM1 and TM4-6 are less conserved, while the extracellular N-terminus and the intracellular C-terminus have the greatest diversities. The 1st and 2nd extracellular loops contain Cys residues which may be involved in a disulfide linkage. The N-terminal extracellular domain contains several potential sites for N-linked glycosylation, and the intracellular loops and the C-terminus
Fig. 2.6 Schematic diagram of protein structure for, and similarity among, the three opioid receptors.

contain several sites for phosphorylation by cAMP-dependent protein kinases and protein kinase C.

2.4 Affinity label-containing opioids

Affinity labels are ligands that have a very high affinity (i.e. low degree of dissociation) for receptors and bind to target sites in a nonequilibrium manner [Takemori and Portoghese, 1985]. The interactions may or may not involve covalent bonds. Affinity labels that bind covalently to receptors are useful in opioid receptor labeling experiments for differentiating various opioid receptor types and subtypes. Many selective non-peptide and peptide affinity labels, both chemical affinity and photoaffinity labels, have been developed. Affinity label compounds bind to receptors in a two step recognition process (Fig. 2.7). Reversible binding in the first step determines receptor affinity, while covalent bond formation in the second step depends on proper orientations of the electrophile group and the receptor-based nucleophile. Chemical affinity labels contain an electrophile which interacts with a nucleophile on the receptors. Selectivities of chemical affinity labels are dependent upon the affinity of the ligand for the receptors, location of the electrophilic group on the ligand, and the chemical selectivity of the electrophile. Photoaffinity labels require a photolysis step (e.g. irradiation at 254 nm) yielding a photoactivated intermediate which could subsequently bind to receptors (Fig. 2.8). Photoaffinity labels have advantages over chemical labels in that they are stable in aqueous solution until activation and they do not require nucleophile group for reaction with the binding sites [Alex et al., 1992]. However, photoaffinity labels cannot be used in vivo and photoactivation by short-wavelength
Fig. 2.7 A schematic illustration of the principle of recognition amplification in the covalent binding of receptor type A by an affinity label containing a group-selective electrophile $X$. Note that receptor types A-C have similar topographic features that lead to reversible binding ($1^\circ$ degree recognition). However, the receptor types differ with respect to the reactivity ($G^1$ versus $G^2$ in A and B) and location ($G^1$ in A and C) of nucleophiles. Only in A is the nucleophile $G^1$ reactive with respect to $X$ and within covalent binding distance ($2^\circ$ degree recognition).

Fig. 2.8 Chemical affinity labeling versus photoaffinity labeling.

ultraviolet light causes losses of opioid receptor-binding capacity [Glasel and Venn, 1981; Smolarsky and Koshland, 1980].

2.4.1 Non-peptide affinity labels

The first non-peptide potential affinity label agonists were N-2-bromoalkyl substituted benzomorphans [May et al., 1968]. Their analgesic effects, however, have not been determined. More recently, a number of non-peptide affinity labels for various opioid receptors have been developed [Takemori and Portoghese, 1985; Zimmerman and Leander, 1990]. Selective affinity label agonists include the isothiocyanate derivatives of fentanyl (FIT and superFIT), U50,488 (UPHIT) and etonitazene (BIT) and the fumaramido derivative of endothenotetra-hydrooripavine (FAO) (Fig. 2.9) [Rice et al., 1983; Burke et al., 1986]. FIT, superFIT and FAO are highly selective for δ opioid receptors, whereas BIT is selective for μ opioid receptors. A nitrogen mustard derivative of the opioid antagonist naltrexone, β-chloralnaltrexamine (β-CNA) (Fig.2.10), is a non-selective irreversible opioid antagonist which can block all three opioid receptors [Portoghese et al., 1978 and 1979], whereas β-funaltrexamine (β-FNA) is selective for μ opioid receptors [Portoghese et al., 1980] and naltrindole isothiocyanate (Fig. 2.10) is an irreversible δ-antagonist [Portoghese et al., 1990], while the isothiocyanate derivative of U50,488 is an irreversible κ-receptor blocker [de Costa et al., 1989 and 1990].
Fig. 2.9 Affinity label agonists.
Fig. 2.10 Affinity label antagonists.
2.4.2 Peptide affinity labels

Reported peptide-based electrophilic affinity labels (chemical affinity labels) are derivatives of enkephalins and include chloromethyl ketone derivatives DALECK (H-Tyr-D-Ala-Gly-Phe-Leu-CH₂Cl) and DAMK (H-Tyr-D-Ala-Gly-MePhe-CH₂Cl) [Bowen, et al., 1987]. DALECK binds preferentially to μ-receptors (IC₅₀[^μ]/IC₅₀[^δ] = 14.3) [Venn and Barnard, 1981; Szőcs et al., 1987; Newman and Barnard, 1984], and DAMK shows higher selectivity for μ-receptors (IC₅₀[^δ]/IC₅₀[^μ] = 40) [Benhye et al., 1987]. DALCE covalently binds to δ-receptors by forming a disulfide bridge between Cys⁶ and a sulphydryl group on the binding site and shows moderate δ-selectivity (IC₅₀[^δ]/IC₅₀[^μ] = 0.0745) [Bowen et al., 1987].

Photoaffinity labels are ligands containing a photoreactive moiety such as an azido or a nitrophenylazido group [Alex et al., 1992]. These groups can selectively and covalently label receptors providing high ratios of specific to non-specific bindings. The azide derivatives of DAMGO (H-Tyr-D-Ala-Gly-MePhe(pN₃)-Gly-ol), TAPP (H-Tyr-D-Ala-Phe-Phe(pN₃)-NH₂) and CTP (H-D-Phe-Cys-Phe(pN₃)-D-Trp-Lys-Thr-Lys-Thr-Pen-Thr-NH₂) show high μ-receptor selectivities (IC₅₀[^μ]/IC₅₀[^δ] = 136, 107 and 408, respectively) [Garbay-Jaureguiberry et al., 1984; Schiller et al. 1989; Landis et al., 1989], whereas the incorporation of p-benzoylphenylalanine (Bpa) in a morphinceptin analog gave a compound (H-Tyr-Pro-BpaNH₂) which was less μ selective (IC₅₀[^μ]/IC₅₀[^δ] = 13.7) [Herblin et al., 1987].

Photoaffinity labels for δ-receptors have been prepared by similar strategies providing the azide derivatives of DTLET (H-Tyr-D-Thr-Gly-Phe(pN₃)-Leu-Thr-OH, IC₅₀[^δ]/IC₅₀[^μ] = 0.0194) [Garbay-Jaureguiberry et al., 1984] and DPDPE (H-Tyr-D-Pen-Gly-Phe(pN₃)-D-Pen-OH, IC₅₀[^δ]/IC₅₀[^μ] = 0.00913) [Landis et al., 1989].
CHAPTER 3

LITERATURE REVIEW: SOLID PHASE PEPTIDE SYNTHESIS

3.1 Basic synthetic strategies

Chemical peptide syntheses can be achieved by two strategies, solution (conventional) and solid phase syntheses. Solution synthesis requires optimization of reaction conditions and purification of intermediates following each step. These can be time-consuming and result in physical losses during synthesis and purification. In addition, the unpredictable solubility and crystallization characteristics of intermediates can be problematic.

Solid phase peptide synthesis (SPPS) was first developed by Merrifield [1963]. SPPS differs from solution synthesis in that a peptide chain is attached to an insoluble polymeric support and the anchored peptide is subsequently extended from the C-terminus toward the N-terminus by cycles of deprotections and couplings (Fig. 3.1) [Grant, 1992]. The C-terminal amino acid, containing a "temporary" protecting group (T) at the N*-amine and when needed a "permanent" protecting group on the side chain, is attached to the insoluble polymeric support (R) bearing reactive groups (X) by either an ester linkage for a peptide acid or an amide bond for a peptide amide. In each repetitive cycle, the "temporary" protecting group is removed to free the N*-amine and the next incoming protected amino acid is then coupled to the amine via the activated carboxyl group. Excess soluble reagents are required to drive the reactions to completion and to obtain a homogenous product. At the end of the synthesis surplus amino acids, soluble reagents and side products are removed.
Fig. 3.1 Stepwise solid-phase synthesis of linear peptide. R, insoluble polymeric support; AA^1, AA^2,..., AA^n, amino acid residues numbered starting from C-terminus; T, "temporary" protection; O, "permanent" protection; □, free carboxyl; □, free amino group.

simply by filtration and washing without mechanical losses of the attached peptides. After the peptide chain assembly has been completed, the crude peptide is cleaved from the support, concurrent with removal of the permanent protecting groups, and purified to give the pure desired peptide. The advantages of SPPS over solution synthesis are simplicity, speed and automation.

3.2 Protecting groups

3.2.1 N[^a]-amino protecting groups

In classical solid phase peptide synthesis, N[^a]-amine groups are protected by the acid labile N[^a]-tert-butyloxy carbonyl (Boc) group (Fig. 3.2) [Merrifield, 1963]. The Boc group is introduced into amino acids using di-tert-butyl dicarbonate or 2-tert-butyloxy carbonyl-oximino-2-phenylacetonitrile (Boc-ON) in aqueous 1,4-dioxane containing NaOH or triethyl-amine (TEA) [Bodanszky and Bodanszky, 1984]. It is stable to alkali and nucleophiles but readily removed by inorganic and organic acids (e.g. 20-50% trifluoroacetic acid (TFA) in dichloromethane (DCM) or 4 N HCl in 1,4-dioxane) [Barany and Merrifield, 1979]. A neutralization step using TEA or N,N-diisopropylethylamine (DIEA) is usually required following Boc deprotection. Coupling amino acids without prior neutralization is feasible in the presence of DIEA or N-methylmorpholine (NMM) (in situ neutralization) [Suzuki, 1975; Schnolzer et al., 1992]. Permanent side chain protecting groups in Boc chemistry are benzyl-type blocking groups. Strong acids either inorganic or organic acids (e.g. liquid anhydrous hydrogen fluoride (HF) at 0°C or trifluoromethane-
Fig. 3.2 "Merrifield" protection scheme for solid-phase synthesis, based on graduated acidolysis. Temporary N*-amino protection is provided by the Boc group, removed at each step by the moderately strong acid TFA. Permanent Bzl-based and cHex side-chain protecting groups, and the PAM linkage, are then cleaved simultaneously by HF or other strong acids, with a free peptide acid being formed in high yield.

sulfonic acid (TFMSA) at 25 °C) in the presence of suitable scavengers are utilized for final deprotection.

An alternative N*-amine protection strategy involves the use of the N*-9-fluorenlymethoxycarbonyl (Fmoc) group (Fig. 3.3). The Fmoc protecting group was developed by Carpino and Han [1972] to avoid the repetitive treatment of peptides with acids which can cause loss of peptides from the support and side reactions during the final cleavage. Several reagents including fluorenylmethyl succinimidyl carbonate (Fmoc-OSu) or Fmoc-Cl in a partially aqueous/organic mixture in the presence of base, and [4-(9-fluorenylmethoxy-carbonyloxy)phenyl]dimethylsulfoniu methyl sulfate (Fmoc-ODSP) in H₂O with Na₂CO₃ or TEA [Azuse et al., 1989] can be used to introduce an Fmoc group onto the N*-amine. The Fmoc-Cl reagent, however, can cause formation of the undesired Fmoc dipeptide (2-20%) [Milton et al., 1987]. The Fmoc group is stable to acids but susceptible to secondary amines such as piperidine [Fields and Fields, 1991] and diethylamine. Piperidine in dimethylformamide (20-55%) is widely used because the reactive dibenzofulvene intermediate generated from proton abstraction at position 9 of the fluorene ring system and β-elimination is readily trapped by excess secondary amine to form a stable harmless tertiary amine adduct (Fig. 3.4) [Carpino and Han, 1972]. A non-nucleophilic base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) can be used for removal of Fmoc in continuous flow synthesis. DBU does not form adducts with the dibenzofulvene intermediate, and therefore it must be washed from the peptide resin immediately [Wade et al., 1991]. Permanent side chain protecting groups in Fmoc chemistry are tert-butyl-type blocking groups which can be removed by TFA in the final deprotection.
Fig. 3.3 A mild two-dimensional orthogonal protection scheme for solid phase synthesis. Temporary $\text{N}^\alpha$-amino protection is provided by the Fmoc group, removed by the indicated base-catalyzed $\beta$-elimination mechanism. Permanent $t\text{Bu}$-base side chain protecting groups and the HMP/PAB ester linkage are both cleaved by treatment with TFA to yield the free peptide acid. A third dimension of orthogonality may be added with an acid-stable, photolabile anchoring linkage.

Fig. 3.4 Fmoc deprotection.

3.2.2 Side chain protecting groups

A key to the success of solid phase peptide synthesis is dependent on the availability of appropriate side chain protecting groups for different amino acids. Certain side chains of amino acids such as the ε-amino group of Lys, the side chain carboxyls of Asp and Glu and the mercapto group of Cys require side chain protection. The hydroxyl groups of Ser, Tyr, and Thr, and the basic groups of Arg and His are usually protected. Choices of side chain protecting groups should be compatible with the Nα-amine protecting groups (Boc or Fmoc) and should minimize side reactions which may occur during cycles of deprotection/coupling and during final cleavage of the peptide from the resin.

Benzyl-type and tert-butyl-type derivatives are the two commonly used side chain protecting groups for Boc and Fmoc SPPS, respectively. (Fig. 3.5 and 3.6). The ε-amino group of Lys is protected by the 2-chlorobenzyloxy carbonyl (2-ClZ) [Erickson and Merrifield, 1973] or Fmoc group in Boc-based syntheses and, reciprocally by the Boc group for Fmoc chemistry. An allyloxycarbonyl (Aloc) protecting group is compatible with both Boc-and Fmoc-base syntheses. Aloc is insensitive to acids and bases and can be removed by a palladium (0) catalyst [Lyttle and Hudson, 1992]. The highly basic guanidino side chain of Arg is usually protected by the 4-toluenesulfonyl (Tos) [Tam and Merrifield, 1987] or mesitylene-2-sulfonyl (Mts) [Yajima et al., 1988] in Boc SPPS, and either 4-methoxy-2,3,6-trimethylbenzene-sulfonyl (Mtr) [Fujino, 1981], 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) [Ramage and Green, 1987] or 2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonyl (Pbf) [Carpino, et al., 1993; Fields and Fields, 1993] in Fmoc SPPS. Pbf is more readily removed than the corresponding Pmc by factors of 1.2-1.4 for TFA/H₂O (95/5; 80/20)
Fig. 3.5 Side chain protecting groups used in Boc SPPS.
Fig. 3.6 Side chain protecting groups used in Fmoc SPPS.
at temperatures between ambient and 37 °C [Carpino et al., 1993]. The side chain carboxyls of Boc-Asp and Boc-Glu are usually protected by the benzyl ester (OBzl), while tert-butyl esters are used for Fmoc-Asp and Fmoc-Glu. The aspartamide rearrangement may occur with Boc-Asp(OBzl) in a susceptible sequence [Bodanszky et al., 1978; Nicolas et al., 1989]. The 2-adamantyl (O-2-Ada) [Okada and Iguchi, 1988], cyclohexyl (OcHex) [Tam et al., 1988] 9-fluorenlymethyl (OFm) [Albericio et al., 1990] ester are recommended to minimize this side reaction.

The mercapto group of Boc-Cys can be protected by 4-methyl-benzyl (Meb) [Erickson and Merrifield, 1973], and 3-nitro-2-pyridinesulfenyl (Npys) [Rosen et al., 1990]. The acetamidomethyl (Acm) [McDurdy, 1989] and trimethylacetamido-methyl (Tacm) [Kiso et al., 1990] groups can be used for both Boc-Cys and Fmoc-Cys. The side chain hydroxyls of Ser, Tyr and Tyr are often protected as Bzl and t-Bu in Boc and Fmoc chemistry, respectively. The 2,6-dichlorobenzyl (2,6-Cl₂Bzl) [Erickson and Merrifield, 1973b] and 2-bromobenzzyloxy-carbonyl (2-BrZ) are preferred to Bzl for Boc-Tyr since the Bzl protecting group can yield in a side product due to the migration of Bzl to the 3-position of the phenol ring.

3.3 Insoluble supports

Polymeric supports of choice must have sufficient mechanical stability and appropriate physicochemical properties which are compatible with solid phase peptide synthesis. Different insoluble supports are compared in Fig. 3.7. The most widely used solid support is a polystyrene polymer cross-linked with 1% 1,3-divinylbenzene (substitution
Fig. 3.7 Insoluble supports.

value 0.2-1.0 mmol/g) [Merrifield, 1963]. The resin swells 2.5-6.2 fold in volume in DCM and DMF allowing rapid penetration of solvents and reagents into the resin beads and thus facilitating chemical reactions [Sarin et al., 1980].

Copolymerized dimethylacrylamide, N,N'-bisacryloethylenediamine, and acryloyl-sarcosine methyl ester resins (commercially known as Pepsyn, substitution value 0.3 mmol/g) [Atherton and Sheppard, 1989] were developed in order to increase the compatibility of the peptide polarity and the insoluble support. Unlike polystyrene resins, polyamide resins also swell sufficiently in polar aprotic solvents. Pepsyn K is an encapsulated polydimethyl-acrylamide resin with a Kieselguhr matrix which provides rigidity to the support (substitution value 0.1-0.2 mmol/g) [Atherton et al., 1981]. Polyhipe is a derivatized polyamide support with high porosity, permeability and physical stability (substitution value 0.3-1.8 mmol/g) [Small and Sherrington, 1989]. Both Pepsyn K and Polyhipe withstand high back pressures, and thus they are suitable for continuous flow synthesis. The Sparrow resin is a cross-linked bead of dimethylacrylamide (substitution value 0.65-0.7 mmol/g) which has the advantage of being swollen in aqueous buffers as well as the polar organic solvents used in peptide synthesis [Sparrow et al., 1990; Kanda et al., 1991]. A polyethylene glycol polystyrene (PEG-PS) graft support (substitution value 0.1-0.4 mmol/g) is a newly developed resin with increased swelling properties [Zalipsky et al., 1985; Barary et al., 1992]. In addition to appreciable swelling in a wide range of solvents the PEG-PS support has improved physical and mechanical properties for both batchwise and continuous-flow SPPS.
3.4 Linkers

A key principle in solid phase peptide synthesis is the stepwise chain elongation starting from the C-terminus and proceeding to the N-terminus to avoid racemization. The C-terminal amino acid is attached to the functionalized resin either directly or by an appropriate linker (handle) [Mitchell et al., 1978]. Most handles contain two functional groups; one has a free or activated carboxyl group which can be coupled to a functionalized amino group on the insoluble support, while the other is a labile protecting group which can be removed to attach the peptide chain. Suitable linkers should provide desired peptide acids or amides upon final cleavage.

Benzyl ester linkers (Fig. 3.8) (e.g. hydroxymethyl resin and 4-(hydroxymethyl) phenylacetic acid (PAM) linker) are commonly utilized for synthesis of peptide acids using Boc chemistry [Mitchell, 1978; Stewart and Young, 1984]. These linkers are cleaved simultaneously with the removal of benzyl-type side chain protecting groups by strong acids (e.g. anhydrous HF and HBr in TFA). The 4-(2-hydroxyethyl)-3-nitrobenzoic acid (NPE) [Eritja et al., 1991] and 9-(hydroxymethyl)-2-fluoreneacetic acid (HMFA) [Lui et al., 1990] linkers are used to prepare protected Boc-peptide fragments since they are stable to acids. Both NPE and HMFA are cleaved by bases, but HMFA can also be cleaved by free N*-amino groups on the peptide resin. Addition of 1-hydroxybenzotriazole (HOBt) during SPPS can prevent premature cleavage of peptides from the HMFA linker [Lui et al., 1990]. Linkers for peptide amides during Boc SPPS are benzhydrylamine derivatives (Fig. 3.8) such as benzhydrylamine (BHA) [Pietta and Marshall, 1970] and 4-methylbenzhydrylamine (MBHA) [Matsueda and Stewart, 1981].
Fig. 3.8 Resin linkers and handles used in Boc SPPS. (P = polymeric support)
Classical resins for Fmoc chemistry peptide acids are the 4-alkoxybenzyl alcohol resin (Wang resin) and the 4-hydroxymethylphenoxyacetic acid (HMPA) linker (Fig. 3.9) [Wang, 1973; Sheppard and Williams, 1982; Bernatowicz et al., 1990]. Peptides are cleaved from these resins by 50-100% TFA at 25°C within 1-2 h. Novel linkers (Fig. 3.9) with increased acid liability are 3-methoxy-4-hydroxymethylphenoxyacetic acid [Sheppard and Williams, 1982], 4-(2',4'-dimethoxyphenylhydroxymethyl)phenoxy (Rink acid) resin [Rink, 1987], 2-methoxy-4-alkoxybenzyl alcohol (SASRIN™; Super Acid Sensitive ResIN) [Mergler, et al., 1988], 2-chlorotrityl resin [Barlos et al., 1989; Barols et al., 1991] and 5-(4-hydroxymethyl-3,5-dimethoxyphenoxy) valeric acid (HAL; Hypersensitive Acid Labile) [Albericio and Barany, 1991]. Peptides can be cleaved from these resins under mild acid conditions (1-2% TFA), and thus they are suitable for preparation of protected peptide fragments. Fmoc SPPS peptide amide linkers are based on the benzylhydrylamide structure with the introduction of an electron-donating methoxy groups into the MBHA structure to enhance the linkers' acid liability. These linkers including 5-(4-aminomethyl-3,5-dimethoxyphenoxy) valeric acid (PAL; Peptide Amide Linker) [Albericio and Barany, 1987], 5-(9-aminoxanthene-2-oxy) valeric acid (XAL) [Sieber, 1987; Bontems et al., 1992], 4-(2',4-dimethoxyphenylamino-methyl)phenoxy resin (Rink amide) [Rink, 1987], 4-(4'-methoxybenzhydryl)phenoxy acetic acid (Dod) [Stuber, et al., 1989], 3-(amino-4-methoxybenzyl)-4,6-dimethoxyphenyl-propionic acid (Breipohl amide linker) [Breipohl, et al., 1989] and 4-succinylamino-2,2',4'-trimethoxy-benzhydrylamine (SAMBHA) [Penke et al., 1988] are sensitive to 50% TFA.
Fig. 3.9 Resin linkers and handles used in Fmoc SPPS. (P = polymeric support)


Fig. 3.9 (cont.) Resin linkers and handles used in Fmoc SPPS. (P = polymeric support)
Linkers which are compatible with both Boc and Fmoc SPPS peptide acids are hydroxycrotonyl (HYCRAM™) [Kunz and Combo, 1988] and 3-nitro-4-hydroxymethyl benzoic acid (Onb) [Rich and Gurwara, 1975; Kneib-Cordonier et al., 1990] (Fig. 3.10). The HYCRAM™ linker is cleaved by Pd (0) in presence of weak nucleophiles such as N-methylmorpholine or dimedone, whereas the ONb linker is cleaved photolytically at 350 nm. For peptide amides, the photolizable 3-nitro-4-aminomethylbenzoic (Nonb) linker (Fig. 3.10) is compatible with both Boc- and Fmoc-based syntheses.

3.5 Peptide bond formation

Coupling of amino acids to one another requires converting the carboxyl components into acylating agents. This can be achieved by replacement of the OH with an electron-withdrawing group. The electrophilicity of the carbonyl of the amino acid is increased, and therefore, the carbonyl is prone to nucleophilic attack by the amino group of the amino acid to be acylated (Fig. 3.11) [Bodanszky, 1988]. Amino acid chlorides are powerful acylating agents. Conversion of the carboxyl group of the amino acid to the chloride is carried out with phosphorus pentachloride or thionyl chloride. Both reagents give HCl as a byproduct, and therefore the use of amino acid chlorides has been limited in SPPS, particularly for Boc chemistry [Grant, 1992]. Acid fluorides prepared by cyanuric fluoride are compatible with both Boc and Fmoc SPPS [Carpino et al., 1990]. Fmoc amino acid chlorides and fluorides are excellent acylating agents for SPPS in the presence of HOBT/DIEA and DIEA, respectively. Both are useful for either solution or solid phase peptide synthesis providing low levels of racemization [Carpino et al., 1990].
Fig. 3.10 Resin linkers and handles used in both Boc and Fmoc SPPS.

Acylation of amino acids with carboxylic acid anhydrides is the simplest and one of the most efficient methods for peptide bond formation [Merrifield, et al., 1982; Yamashiro, 1987]. The highly reactive preformed symmetrical anhydrides (PSA) are generated *in situ* from the corresponding N*-protected amino acid (4-12 equiv) plus *N*, *N*-diisopropylcarbodiimide (DIC, 1-2 equiv in DCM). PSA’s are not widely used for FmocSPPS due to the wastefulness of the process. Only one amino acid molecule in the anhydride is incorporated into the product, whereas the second amino acid is regenerated but normally not recovered. Mixed or unsymmetrical anhydrides have been developed in several laboratories [Chen and Benoiton, 1987], but the inherent ambiguity of synthesis via mixed anhydrides limits their use in SPPS [Barany et al., 1987]. Activation of amino acids to acyl azides has been used in peptide synthesis. Amino acids can either undergo hydrazinolysis of alkyl esters and conversion of the hydrazides to acyl azides with nitrous acid, or direct conversion to acid azides with diphenylphosphoryl azide [Bodanszky, 1988]. An advantage of the acid azide method is that only negligible racemization occurs.

Preactivated amino acids can alternatively be prepared by the formation of active esters. Aryl esters with electron-withdrawing substituents such as 2-(Ono) and 4-nitrophenyl (Onp) esters (Fig. 3.12) were used by Bodanszky and co-workers [1973]. Neither Ono nor Onp are popular for SPPS due to their low reactivities even in the presence of the additive hydroxybenzotriazole (HOBT). Recently developed active esters (Fig. 3.12) including pentafluorophenyl (OPfp) and 3-hydroxy-2,3-dihydro-4-oxobenzotriazine (ODhbt) esters have been successfully used in SPPS. Addition of 1-hydroxybenzotriazole (HOBT) (1-2 equiv) to an OPfp ester increases coupling rates both in Boc and Fmoc SPPS [Atherton et
Fig. 3.12 Active esters of amino acids. (W = protecting groups)

al., 1988; Hudson, 1990]. Boc- and Fmoc-amino acid OBt [Harrison et al., 1989; Fields et al., 1989] and ODhbt [Konig and Geiger, 1970] esters are both highly reactive. N\textsuperscript{a}-Protected amino acid OBt and ODhbt esters reduce racemization levels during couplings. Fmoc amino acid ODhbt esters are more stable than OBt esters, whereas the 1-hydroxy-7-azabenzotriazole (OAt) esters are more powerful acylating agents than OBt esters [Carpino, 1993].

Peptide bonds can be formed \textit{in situ} by using coupling reagents (Fig. 3.13). \textit{N},\textit{N}'-Dicyclohexylcarbodiimide (DCC) was the first coupling reagent introduced to SPPS [Rich and Singh, 1979; Merrifield et al., 1988]. Unlike DCC, DIC gives a soluble urea byproduct which can be easily washed away during synthesis [Sarantakis et al., 1976]. Addition of HOBt [Konig and Geiger, 1970; Mojosov et al., 1980] or HOAt [Carpino, 1993] can enhance coupling reactions, suppress racemization and inhibit dehydration of the carboxamide side chains of Asn and Gln to the corresponding nitriles.

New phosphonium and uronium coupling reagents (Fig. 3.13) are highly efficient reagents for SPPS [Fields et al., 1992]. Phosphonium reagents include benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP). BOP generates the carcinogen hexmethylphosphoramide as a byproduct, while PyBOP gives faster reaction rates and less harmful side products [Coste et al., 1990]. Uronium coupling reagents are 2-(H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) [Dourtoglou et al., 1978], (O-(7-azabenzotriazol-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) [Carpino, 1993], 2-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
Fig. 3.13 Coupling reagents.
(TBTU) [Knorr et al., 1989], and 2-[2-oxo-1(2H)-pyridyl]-1,1,3,3-bispentamethylene-uronium tetrafluoroborate (TOPPipU) [Knorr et al., 1990]. BOP, HBTU, TBTU and TOPPipU require a tertiary amine (e.g. NMM or DIEA) for their optimal efficiency [Gausepohl et al., 1988]. Excess amounts of HBTU or TBTU can trap free amino groups giving undesired Schiff base byproducts [Gausepohl et al., 1992].

3.6 Monitoring

SPPS consists of repetitive cycles of deprotection and coupling. Each step has to be driven to completion to obtain a homogenous final product. The ninhydrin test is commonly used to monitor for the presence of unreacted amines after an acylation step [Kaiser et al., 1970]. This classical color reagent can detect nanomolar quantities of free primary N\textsuperscript{α}-amine which yields the Ruhemann's purple product (Fig. 3.14). The secondary amine of proline gives a yellow color compound which can be monitored at 440 nm.

![Chemical structure](image)

**Fig. 3.14** Ninhydrin test.

CHAPTER 4

SYNTHESIS OF POTENTIAL AFFINITY LABEL DERIVATIVES OF DY NOPHIN A(1-11)NH₂: MESSAGE SEQUENCE MODIFICATIONS

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Abbreviations:

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature in *Biochem. J.* 1984, 219, 345-373. Amino acids are in the L-configuration except when indicated. Additional abbreviations used are as follows: Aloc, allyloxycarbonyl; Boc, *t*-butyloxycarbonyl; *t*-Bu, *tert*-butyl; DCM, dichloromethane, DIC, *N*,*N*′-diisopropylcarbodiimide; DIEA, *N*,*N*′-diisopropylethylamine; DMA, *N*,*N*-dimethylacetamide; Dyn A, dynorphin A; DMF, *N*,*N*-dimethylformamide; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (9-fluorenylmethoxy)carbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; NMM, *N*-methylmorpholine; PAL, peptide amide linker, PEG, polyethylene glycol; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PS, polystyrene; TFA, trifluoroacetic acid, THF, tetrahydrofuran; Z, benzoxycarboxyl.
4.1 Abstract

Potential affinity label derivatives of dynorphin (Dyn) A(1-11)NH₂ with modifications in the N-terminal "message" sequence involved incorporation of a reactive functionality (i.e. isothiocyanate and/or bromoacetamide) into the p-amino group of Phe(NH₂) in position 1 or 4 of [D-Pro¹⁰]Dyn A(1-11)NH₂. Syntheses of these derivatives were performed on a PAL-PEG-PS resin using Fmoc chemistry. An allyloxy carbonyl (Aløc) protecting group was introduced at the p-amino group of Phe(NH₂) using allyl chloroformate, followed by protecting the Nα-amine using Fmoc-Cl. The Aløc group was selectively removed by Pd(0), followed by incorporation of the reactive functionality. Except for the isothiocyanate and bromoacetamide derivatives of [Phe(NH₂)⁴,D-Pro¹⁰]Dyn A(1-11)NH₂, the peptides were obtained in excellent purity.

Preliminary binding assay results indicated that the control amine compounds (without a reactive functional group) in both the 1 and 4 positions and the isothiocyanate derivative of [Phe(NH₂)⁴,D-Pro¹⁰]Dyn A(1-11)NH₂ had high affinity for κ opioid receptors. The bromoacetamide derivative of [Phe(NH₂)⁴,D-Pro¹⁰]Dyn A(1-11)NH₂ and isothiocyanate of [Phe(NH₂)¹,D-Pro¹⁰]Dyn A(1-11)NH₂, however, showed lower affinity for κ receptors. The amine compounds as well as the potential affinity label derivatives of [D-Pro¹⁰]Dyn A(1-11)NH₂ had significantly lower affinity for both µ and δ opioid receptors. These peptides are being examined for wash-resistant inhibition of binding to opioid receptors.
4.2 Introduction

Endogenous mammalian opioid peptides (e.g. enkephalins, dynorphins and endorphins) have been discovered in both the central and peripheral nervous systems [Konig, 1993]. These ligands bind to specific opioid receptors and produce various physiological and pharmacological effects [Simon and Hiller, 1994]. The existence of multiple opioid receptors, \( \mu \), \( \kappa \) and \( \delta \) receptors, is well established [Paterson et al., 1984; Simmon, 1991]. Characterization of opioid receptors is important for understanding opiates’ functions at a molecular level as well as the physiological and pharmacological roles of opioid receptors.

Highly selective ligands which bind irreversibly to receptors have been valuable tools to study opioid receptor structure and function. These ligands can block certain receptor populations in tissues which contain multiple receptor types, so that the remaining receptor types can be characterized.

Affinity label-containing compounds which covalently bind to receptors differ from reversible ligands [Takemori and Portoghese, 1985]. They are classified into two classes, chemical affinity and photoaffinity labels. The irreversible binding of chemical affinity labels is due to the acylation or alkylation by the electrophilic group on the affinity label of a nucleophilic site on the receptors [Takemori and Portoghese, 1985; Newman, 1991]. Photoaffinity labels, however, require a photolysis step (UV irradiation) which prevents their use for in vivo study of opioid receptors [Glasel and Venn, 1981]. Many affinity labels including nonpeptide and peptide-based ligands have been prepared and used for opioid receptor characterizations [Zimmerman and Leander, 1990; Newman, 1991]. Selective nonpeptide affinity labels for \( \mu \) opioid receptors are \( \beta \)-funaltrexamine (\( \beta \)-FNA) [Portoghese
et al., 1980] and the etonitazene derivative BIT [Rice et al., 1983]. The fumaramido
derivative of endoethenotetrahydrooripavine (FAO) and the isothiocyanate derivatives of
fentanyl FIT and superFIT selectively alkylate δ receptors [Rice et al., 1983; Burke et al.,
1986]. Alkylating agents for κ receptors are isothiocyanate derivatives of U-50,488
[de Costa et al., 1989; 1990]. Peptide-based affinity labels such as DALECK (H-Tyr-D-Ala-
Gly-Phe-Leu-CH₂Cl) [Venn and Bernard, 1981], DAMK (H-Tyr-D-Ala-Gly-MePhe-CH₂Cl)
[Benyhe et al., 1987] and DALCE (H-Tyr-D-Ala-Gly-Phe-Leu-CysOH) [Bowen et al., 1987]
are enkephalin derivatives.

Dynorphin A (Dyn A), a 17-amino acid peptide derived from prodynorphin, is an
endogenous ligand for κ opioid receptors [Cox et al., 1975]. The last four amino acids at the
C-terminus (Trp-Asp-Asn-Gln) are not important for the association of Dyn A to opioid
receptors, and Dyn A(1-13) accounts for essentially all of the activity of the parent peptide
[Goldstein et al., 1981]. The N-terminal region (Tyr-Gly-Gly-Phe) of Dyn A was proposed
as a “message” sequence, while the sequence containing amino acid residues 5-13 was
considered an “address” sequence [Chavkin and Goldstein, 1981]. The message sequence,
which is also common in other mammalian opioid peptides, is important for opioid activity,
whereas the “address” sequence accounts for binding of dynorphin to κ receptors. Dyn
A(1-13)NH₂, however, binds with only modest selectivity to κ receptors (Kᵢ ratios μ: δ: κ =
30: 80: 1.0) [Goldstein, 1984]. [D-Pro¹⁰]Dyn A(1-11)NH₂ shows an increase in κ selectivity
(Kᵢ ratios μ: δ: κ = 93: 280: 1.0) [Gairin et al., 1985, 1988]. Thus, [D-Pro¹⁰]Dyn A(1-
11)NH₂ has been used as a prototype for the synthesis of potential affinity label Dyn A
analalogues in our laboratory. The peptide structure of Dyn A provides flexibility in the
placement of the affinity labeling groups. We, therefore, incorporated reactive functionalities including isothiocyanate (-N=C=S) and bromoacetamide (-NHCOCH₂Br) in both the N- and C-terminus of [D-Pro¹⁰]Dyn A(1-11)NH₂ to obtain affinity label Dyn A analogues. The peptides' affinity (IC₅₀ values) for κ, μ and δ opioid receptors were evaluated by radioligand binding assays using cloned opioid receptors stably expressed in Chinese hamster ovary (CHO) cells. The peptides' ability to inhibit radioligand binding in a wash-resistant manner will be determined in the same assays, using concentrations which gave maximum inhibition of binding under standard conditions.

The synthesis of Dyn A analogues containing affinity labels are described in this chapter ("message" sequence modifications) and chapter 5 ("address" sequence modifications). Modifications in the "message" sequence involved Tyr in position 1 and Phe in position 4. Since opioid peptides share the same "message" sequence, affinity labels with modifications in this region could provide insight into whether or not this part of the binding site is similar for opioid receptors. Structure-activity relationship studies indicate that the phenol ring of Tyr¹ and the aromatic ring of Phe⁴ in the "message" sequence are critical for the interaction of opioid peptides with opioid receptors [Chavkin and Goldstein, 1981; Turcotte et al., 1984]. Studies of [Phe(NO)₂]⁴Dyn A(1-13) showed that minor modifications at position 4 of dynorphin A are well tolerated [Schiller et al., 1982]. Thus, we incorporated both isothiocyanate (-N=C=S) and bromoacetamide (-NHCOCH₂Br) in the para position of Phe⁴. The aromatic ring of non-peptide ligands (e.g. ethylketocyclazocine) mimics the Tyr¹ of Dyn A. Therefore, we replaced Tyr¹ with an isothiocyanate phenylalanine derivative (Phe(N=C=S)¹). The bromoacetamide derivative was not incorporated into position 1 because
of its steric hindrance and its lower affinity towards kappa receptors in position 4 of Phe\(^{4}\) comparing to the isothiocyanate derivative. The \(p\)-amino (-NH\(_{2}\)) compounds were included as reversible controls for pharmacological assays. Peptide amides were preferred to peptide acids because of their improved metabolic stability [Leslie and Goldstein, 1982].

The original approach for preparing the potential affinity label dynorphin A derivatives by solid phase peptide synthesis was to cleave the protected peptide from the resin followed by introducing the reactive functionalities in solution [Story and Aldrich, 1992]. Synthesis of the model peptide Boc-Tyr(t-Bu)-Gly-Gly-Phe-Leu-Arg(Pmc)NH\(_{2}\) on two different commercially available resins, the Rink amide resin (4-(2',4'-dimethoxyphenyl-Fmoc-amino-methyl phenoxy) and the Pepsyn resin (a polyamide resin with the 4-hydroxy-methyl benzoic acid linker) gave unsatisfactory results. Therefore, a modified methylbenz-hydryamine (MBHA) resin with a base-labile linker was developed, and a high yield (>80%) of the model peptide was obtained after ammonolysis in isopropanol. When the modified MBHA resin was utilized to synthesize the longer protected peptide amides, [Phe(NO\(_{2}\))\(^{4}\),D-Pro\(^{10}\)]DynA(1-11)NH\(_{2}\) and [Phe(NHZ\(^{4}\))]Dyn A(1-13)NH\(_{2}\), however, low recoveries were obtained (25% and 38% yields, respectively). These results may be due to the steric hindrance of the D-amino acid at the C-terminal (i.e. D-Pro\(^{10}\) in the Dyn A(1-11)NH\(_{2}\) derivative) and/or the hydrophobic character of the polystyrene supports.

In this research, we developed an alternative strategy for the synthesis of potential affinity label Dyn A analogues. First, all synthetic steps including incorporation of the reactive functionality were performed on the solid support instead of in solution. Second, an allyl-based protecting group (i.e. allyloxy carbonyl or Aloc) was used on the \(p\)-amino
group of $p$-amino-phenylalanine to obtain orthogonal protection during solid phase synthesis [Greene and Wut, 1991; Loffet et al., 1992; Lyttle et al., 1992; Kunz et al., 1988; Barany and Albericio, 1985]. Aloc is stable under acidic and basic conditions, but can be selectively deprotected by palladium (0) [Genet et al., 1993, 1994; Loffet and Zhang, 1993; Kate et al., 1993; Williams et al., 1991; Dangles et al., 1987; Guibe et al., 1986]. Therefore, it was suitable for the synthesis of dynorphin A analogues containing affinity labeling groups. The peptides were assembled on the resin and after the Aloc deprotection, the affinity labeling group could then be introduced while other amino acid side chains remained protected. Lastly, the newly developed polyethylene glycol/polystyrene support with a peptide amide linker (5-(aminomethyl-3,5-dimethoxy-phenoxy)valeric acid) (PAL-PEG-PS resin), was utilized to enhance solvent compatibilities during peptide synthesis. PAL-PEG-PS resin has proved to offer excellent coupling and deblocking efficiency [Kates et al., 1993; Barany et al., 1993; Auzanneau et al., 1995]. Moreover, PAL-PEG-PS resin has high levels of solvation due to the derivatized polyethylene glycol spacer between the functional group on the polystyrene gel bead and the handle or attachment point of the synthetic peptides. It also swells appreciably in a wide range of both organic and inorganic solvents [Auzanneau et al., 1995] which provides advantages for the synthesis of potential affinity label analogues of Dyn A.
4.3 Experimental section

4.3.1 Materials

The PAL-PEG-PS resin and \( N,N \)-diisopropylethylamine (DIEA) were purchased from Perseptive (Bedford, MA). All amino acids except BocPhe(NHAloc) and FmocPhe (NHAloc) were obtained from Bachem Inc. (Torrance, CA). BocPhe(NHAloc) and FmocPhe (NHAloc) were synthesized in this laboratory as described below. Other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI), and all solvents used (HPLC grade) were obtained from Burdick & Jackson (Muskegon, MI).

The phenylalanine derivatives and crude peptides were analyzed on a Beckman model 431 A gradient high performance liquid chromatography (HPLC) system. The HPLC column was a Vydac analytical column \( (C_{18}, 300 \text{ Å}, 5 \mu, 4.6 \times 250 \text{ mm}) \) equipped with a Vydac guard cartridge. The samples were eluted using a linear gradient of 0%-75% solvent B over 50 min with a flow rate of 1.5 mL/min and detected at 214 nm; solvent A was aqueous 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA. Preparative reversed phase HPLC was performed on a Rainin gradient HPLC system using a Vydac semipreparative column \( (C_{18}, 300 \text{ Å}, 10 \mu, 21 \times 250 \text{ mm}) \) equipped with a Vydac guard cartridge. Amino acid analyses were done by the Central Service Laboratory, Center for Gene Research and Biotechnology, Oregon State University, using standard hydrolysis conditions (6 N HCl plus 1.0% phenol at 110°C for 20 h). Each amino acid was resolved using a step gradient on a Beckman Spherogel 52C ion exchange column \( (4.6 \times 150 \text{ mm}) \) equipped with post-column ninhydrin detection on a Beckman 126A System Gold HPLC.
4.3.2 Methods

4.3.2.1 Synthesis of FmocPhe(NHAloc) (Fig. 4.1a and Table 4.1)

Allyl chloroformate (1.06 mL, 11 mmol) was added to a solution of \( p \)-amino phenylalanine (1.08 g, 11 mmol) in citrate buffer, pH 4.6 (10 mL), and the mixture stirred under pH control (pH = 4.6) [Fahrenholz and Thierauch, 1980] for 2.5 h. Phe(NHAloc) was collected by filtration and washed with H\(_2\)O, Et\(_2\)O, EtOH and EtOAc. The white precipitate was dried \textit{in vacuo} (1.55 g, 86% yield). Fmoc-Cl (1.53 g, 5.9 mmol) in dioxane (7 mL) was added to a solution of Phe (NHAloc) (1.55 g, 5.9 mmol) in 10% Na\(_2\)CO\(_3\)/dioxane (1/1, 10 mL) and the mixture was stirred at 0 °C for 1 h and at room temperature for 12 h. The mixture was then poured into ice water (20 mL) and extracted with ether (2 x 20 mL). The aqueous layer was chilled in an ice bath and acidified with HCl to pH 2, the white precipitate collected by filtration, washed with 0.1 N HCl H\(_2\)O and dried \textit{in vacuo}. The residue was dissolved in EtOAc and the solution, washed with 0.1 N HCl and H\(_2\)O, and dried with MgSO\(_4\). Fmoc Phe(NHAloc) was recrystallized from EtOAc/hexane (1/10) (1.50 g, 97% yield).
Fig. 4.1 Synthesis of phenylalanine derivatives.
4.3.2.2 Synthesis of BocPhe(NHAloc) (Fig. 4.1b and Table 4.1)

Allyl chloroformate (1.15 mL, 10.93 mmol) was added to a solution of BocPhe(NH$_2$) (3.06 g, 10.93 mmol) in methanol/water (1/1, 10 mL). DIEA was added to maintain the pH of the solution at pH 4-5 and the mixture was stirred overnight [Fahrenholz and Thierauch, 1980]. BocPhe (NHAloc) was collected by filtration, washed with H$_2$O and recrystallized from tetrahydrofuran (THF).

4.3.2.3 Protected [Phe(NHAloc)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 1a-resin) and [Phe(NHAloc)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 2a-resin) (Fig. 4.2)

The peptides were synthesized on a Biosearch 9500 automated peptide synthesizer (Novato, CA) using Fmoc-amino acids, generally according to the standard procedure [Snyder et al., 1992] except that $N,N$-dimethylacetamide (DMA) was used in place of dimethylformamide (DMF) as a solvent. Boc-Tyr(t-Bu) and FmocPhe(NHAIoc) were incorporated into the first and fourth positions, respectively, of protected 1a-resin and BocPhe(NHAloc) was incorporated into the first position of protected 2a-resin. The protected peptides were assembled on the PAL-PEG-PS resin (1% cross linked polyvinyl-styrene, 0.17 mmol/g). The resin was swollen in dichloromethane (DCM) for 20 min and washed with DCM (5x) and DMA/DCM (1/1, 5x). The repetitive cycle for synthesis was performed by the following steps: (1) removal of the Fmoc protecting group using a piperidine/toluene/DMA (30%/35%/35%) mixture; (2) addition a 4-fold excess of Fmoc-amino acid in DMA (0.4 M) which had been activated in-line for 2 min with diisopropyl-carbodiimide (DIC) in DCM; (3) mixing for 2 h (completeness of coupling was monitored
Fig. 4.2. Synthesis of \([\text{Phe}(X)^4, \text{D-Pro}^{10}]\text{Dyn A}(1-11)\text{NH}_2\) Derivatives.
by the ninhydrin test); and (4) washing with DMA/DCM (1/1, 10x). After attachment of the N-terminal amino acid, the protected peptide-resins were washed with DCM (5x) and dried in vacuo.

4.3.2.4 Protected [Phe(NH$_2$)$_4$, D-Pro$_{10}$]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 1b-resin), [Phe(NH$_2$)$_4$, D-Pro$_{10}$]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 2b-resin)

The Biosearch 9500 automated peptide synthesizer was used for Aloc deprotection from the peptide-resins. Protected 1a-resin (0.21 g, 24.4. µmol) and protected 2a-resin (0.21 g, 25.2 µmol) were separately swollen in DCM for 15 min. A solution of Pd(PPh$_3$)$_4$ (0.14 M, 3 equiv) in 92.5% DCM/5% HOAc/2.5% N-methylmorpholine (NMM) was manually added to the peptide-resins. The reaction was mixing under N$_2$, and a sufficient amount of DCM was added regularly due to the evaporation during mixing. Quantitative Kaiser test (see chapter 6) was performed after 1 h, 2 h, and 3 h; the reaction was complete after 3 h. The protected peptide-resins were washed with the following solvents: THF (3 x 2 min), DMF (3 x 2 min), DCM (3 x 2 min), 0.5% DIEA in DCM (3 x 2 min), 0.02 M sodium diethyldithiocarbamate in DMF (3 x 15 min), DMF (5 x 2 min) and DCM (3 x 2 min) and then dried in vacuo.

4.3.2.5 Protected [Phe(N=C=S)$_4$, D-Pro$_{10}$]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 1c-resin) and [Phe(N=C=S)$_4$, D-Pro$_{10}$]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 2c-resin)

Protected 1b-resin (0.106 g, 21.0 µmol) and protected 2b-resin (0.208 g, 40.6 µmol) were swollen in DCM (3.0 mL) for 15 min, DIEA (2.5 equiv) was added, followed by adding
thiophosgene (Cl₂-C=S, 1.5 equiv). The ninhydrin test was monitored until a negative result was obtained (2 h). The resins were filtered, washed with DCM (5 x 2) and dried in vacuo.

4.3.2.6 Protected [Phe(NHCOCH₂Br)₄,D-Pro¹⁰]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 1d-resin)

Protected 1b-resin (0.06 g, 7.57 µmol) was swollen in DCM (3.0 mL) for 15 min and chilled in an ice bath. DIEA (2 equiv) was added to the peptide-resin, followed by adding bromoacetyl bromide (BrCH₂COBr, 1.5 equiv). The mixture was stirred at 0°C for 1 h, and at room temperature until the ninhydrin test gave a negative result (24 h). The peptide-resin was filtered, washed with DCM (5 x 2) and dried in vacuo.

4.3.2.7 Cleavage reactions

Protected peptide-resins 1a-1d and 2a-2c were treated with 10 mL of modified reagent K (87.5% TFA/2.5% thioanisole/5% phenol/5% H₂O) [King et al., 1990] under N₂ and protected from light for 4 h at room temperature. The resins were filtered and washed with 2 mL TFA and the filtrates diluted with 10 mL of 10% acetic acid and extracted with ether (3 x 10 mL). The crude peptides were obtained by lyophilization of the aqueous extracts.

4.3.2.8 Purification

All crude peptides were purified on a Rainin preparative gradient HPLC system. The column used was a Vydac semipreparative column with a Vydac guard cartridge. The
eluents were monitored at 280 nm using an ISCO UA-5 UV-visible detector. The peptides were eluted using a linear gradient of 0%-50% solvent B over 50 min with a flow rate of 20 mL/min; solvent A was 0.1% TFA in H₂O and solvent B was 0.1% TFA in AcCN. The homogeneity of the peptides was determined by analytical HPLC on a Vydac analytical column equipped with a Vydac guard cartridge using a linear gradient of 0%-75% solvent B over 50 min with a flow rate of 1.5 mL/min and detection at 214 nm. The pure fractions were collected and lyophilized to obtain the desired peptides. All peptide were > 98% pure as determined by analytical HPLC.

4.3.3 Receptor binding assays

Radioligand binding assays of the affinity label derivatives were performed with Chinese hamster ovary (CHO) cells which express rat cloned κ and μ [Bunzow et al., 1995] and mouse δ opioid receptors. Cells were harvested 72 h following transfection in 50 mM Tris buffer, pH 7.4, at 4 °C and homogenized using a Dounce homogenizer. The homogenate was then centrifuged at 45,000 x g for 10 min at 4 °C. The pellet was washed twice by resuspension and recentrifugation as in the previous step. The pellet was resuspended in 50 mM Tris buffer, pH 7.4, at 4 °C to yield a protein concentration of 30-60 μg/mL. Incubations were performed for 90 min at 22 °C with [³H]diprenorphine, [³H]DPDPE (cyclo[D-Pen²,D-Pen⁵]enkephalin) and [³H]DAMGO ([D-Ala²,MePhe⁴,Gly-ol] enkephalin) for κ, δ and μ receptors, respectively. Kᵦ values for [³H]diprenorphine, [³H]DPDPE and [³H]DAMGO were 0.45, 1.76 and 0.49 nM, respectively. Binding assays were carried out in mixtures containing 100 μg membrane protein, 3 mM Mg²⁺, and
peptidase inhibitors (10 µM bestatin, 30 µM captopril and 50 µM L-leucyl-L-leucine) in a final volume of 2 mL 50 mM Tris buffer, pH 7.4, at 22 °C. Nonspecific binding was determined in the presence of 10 µM unlabeled Dyn A(1-13)NH₂, DPDPE and DAMGO, for κ, δ and μ receptors respectively. The reactions were terminated by rapid filtration over Whatman GF/B glass fiber filters using a Brandel M24-R Cell Harvester. The filters had been presoaked for at least 2 h in 0.5% polyethylenimine to decrease nonspecific binding. The filter disks were then placed in minivials with 4 mL Cycocint (ICN radiochemicals) and allowed to elute for at least 6 h before counting in a Beckman LS 6800 scintillation counter. IC₅₀ values were then derived from nonlinear regression analysis of competition curves.

Potential affinity label derivatives of dynorphin A are being examined for wash-resistant inhibition of binding to opioid receptors. Chinese hamster ovary (CHO) cell membranes expressing κ receptors are incubated in the presence or absence of the dynorphin analogues for 90 min at room temperature. The homogenates are then centrifuged at 40,000 x g for 15 min at 4 °C and the pellet resuspended in 50 mM Tris buffer, pH 7.4, at 4 °C. The centrifugation and resuspension procedure is repeated four times. After the fifth resuspension (wash) step, the homogenate is recentrifuged and the final pellet resuspended in 50 mM Tris buffer, pH 7.4, at 4 °C as previously described. These final CHO cell membrane homogenates are then subjected to radioligand binding experiments as described above, and the radioligand binding to membranes treated with the dynorphin analogues compared to binding to untreated control membranes.
4.4 Results and discussion

4.4.1 Amino acid syntheses

Solid phase synthesis of peptide-based affinity labels requires selective protection of the side chain to which the affinity label group (reactive functionality) is to be attached. We designed derivatives in which the reactive functionalities were attached to the aniline group of Phe(NH₂)₄ or Phe(NH₂)¹ in derivatives of [D-Pro¹⁰]Dyn A(1-11)NH₂. This aniline group can only be protected by groups which can be introduced using alkyl chloroformates or anhydrides (i.e. allyloxycarbonyl chloroformate (AlocCl) or (Boc)₂O) [J. Aldrich unpublished results]. The use of Boc or Fmoc for N°-amine protection and Aloc for the aniline group of Phe(NH₂) provided orthogonal protection [Dangles et al., 1987; Guibe et al., 1986; Merzouk et al., 1992; Kates et al., 1994] which was suitable for the synthesis of potential affinity label Dyn A derivatives. The synthesis of FmocPhe(NHAloc) was performed in a two-step reaction. First, the p-amino group of Phe(NH₂) was selectively protected by the Aloc group, followed by protection of the N°-amine using Fmoc-Cl. The p-amino group can be preferentially acylated by allyl chloroformate in citrate buffer under pH control (pH = 4.6) while the N°-amine remains unreacted (Fig. 4.1a) [Fahrenholz et al., 1980]. This selectivity is due to the higher basicity of the N°-amine (pKₐ = 9.1) compared to the p-amino group (pKₐ = 4.3). The final product FmocPhe(NHAloc) was obtained in a high yield (97%, Fig. 4.3 and Table 4.1). The Aloc group was also attached to the aniline amine of BocPhe(NH₂) by a one-step acylation reaction (Fig. 4.1b). The reaction was straightforward and the final product BocPhe (NHAloc) was also obtained in a high yield (74.7%, Fig. 4.4 and Table 4.1).
Fig. 4.3 Chromatograms of FmocPhe(NH₂) (solid line) and FmocPhe(NHAlloc) (dotted line). Column: Vydac (C₁₈). Solvent: A = aqueous 0.1%TFA, B = 0.1%TFA/ACCN. Flow rate: 1.5 mL/min. Gradient: 0-75%B over 50 min.
Fig. 4.4 Chromatograms of BocPhe(NH$_2$) (solid line) and BocPhe(NHAlolc) (dotted line). For chromatographic conditions see Fig. 4.3.
<table>
<thead>
<tr>
<th></th>
<th>Phe(NHAloc)</th>
<th>FmocPhe(NHAloc)</th>
<th>BocPhe(NHAloc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_R ) (min)</td>
<td>16.2</td>
<td>36.3</td>
<td>28.0</td>
</tr>
<tr>
<td>FAB-MS (M+H)^+</td>
<td>265.2 (265)</td>
<td>487.4 (487)</td>
<td>365.4 (365)</td>
</tr>
<tr>
<td>([\alpha]_D^{25})</td>
<td>+3.71</td>
<td>+12.86</td>
<td>+17.92</td>
</tr>
<tr>
<td>MP (°C)</td>
<td>225-227</td>
<td>178-181</td>
<td>138-139</td>
</tr>
<tr>
<td>Elemental analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formula</td>
<td>( \text{C}<em>{13}\text{H}</em>{16}\text{N}_2\text{O}_4 )</td>
<td>( \text{C}<em>{28}\text{H}</em>{26}\text{N}_2\text{O}_6 )</td>
<td>( \text{C}<em>{18}\text{H}</em>{24}\text{N}_2\text{O}_6 )</td>
</tr>
<tr>
<td>% C</td>
<td>58.9 (59.1)</td>
<td>69.4 (69.1)</td>
<td>59.4 (59.3)</td>
</tr>
<tr>
<td>% H</td>
<td>6.0 (6.1)</td>
<td>5.3 (5.4)</td>
<td>6.4 (6.6)</td>
</tr>
<tr>
<td>% N</td>
<td>10.3 (10.6)</td>
<td>5.9 (5.8)</td>
<td>7.7 (7.7)</td>
</tr>
<tr>
<td>TLC</td>
<td>0.47</td>
<td>0.90</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.87</td>
<td>0.56</td>
<td>0.90</td>
</tr>
</tbody>
</table>
Theoretical values are shown in parentheses.

For chromatographic conditions see Fig. 4.3.

1c = 0.5, 0.2 and 0.6 in MeOH for Phe(NHAIoc), FmocPhe(NHAIoc) and BocPhe(NHAIoc), respectively.

Solvent = butanol: acetic acid: water (3:1:1)

Solvent = chloroform: methanol: acetic acid (77.5:17.5:7.5)

Solvent = methanol: water: acetic acid (4:1:1)

Solvent = chloroform: methanol: acetic acid (17:2:1)

Solvent = butanol: acetic acid: water (4:2:2)
4.4.2 Peptide syntheses

The protected peptides 1a and 2a were assembled on a PAL-PEG-PS resin using standard Fmoc chemistry. After the peptide chain assembly was complete, the Aloc protecting group was removed by a solution of Pd(PPh$_3$)$_4$ (see experimental section). The Aloc deprotection reaction monitored by quantitative ninhydrin test was complete within 3 h yielding protected 1b- and 2b-resins. The aniline amines of protected 1b- and 2b-resins were converted to the isothiocyanate derivatives protected 1c- and 2c-resins by reacting with thiophosgene under basic conditions; the reactions gave a negative ninhydrin test after 2 h. The bromoacetamide group was incorporated into the aniline amine of protected 1b-resin to give the protected 1d-resin using bromoacetyl bromide under basic conditions; the reaction was complete after 24 h. Final cleavage of the peptides from the resins as well as removal of the remaining side chain protecting groups and the N-terminal Boc protecting group were achieved using modified reagent K [King et al., 1990].

All crude peptides were analyzed by analytical HPLC prior to purification. The control compounds 1b and 2b were obtained in excellent purity ($t_R = 17.4$ min (Fig. 4.5) and 16.8 min (Fig. 4.6), respectively). The isothiocyanate derivative 2c was also obtained in excellent purity following cleavage from the resin ($t_R = 22.9$ min, Fig. 4.6). Treatment of 1b with thiophosgene and bromoacetyl bromide to yield crude peptides 1c and 1d, respectively, however, resulted in side products (Fig. 4.5). Analyses by reverse phase HPLC showed 70% of the desired peptide 1c ($t_R = 23.9$ min) plus the control compound 1b ($t_R = 17.2$ min) and other impurities (Fig. 4.5). The bromoacetamide derivative 1d and its hydroxyl derivative were eluted with similar retention times (2:1 ratio, $t_R = 20.7$ and 19.8 min,
Fig. 4.5 Chromatograms of crude peptide $Ib$ (top), $Ic$ (middle) and $Id$ (bottom). For chromatographic conditions see Fig. 4.3.
Fig. 4.6 Chromatograms of crude peptide 2b (top) and 2c (bottom). For chromatographic conditions see Fig. 4.3.
respectively, Fig. 4.5). All crude peptides along with the reversible controls were purified by preparative reverse phase HPLC. Purified fractions were analyzed by analytical reverse phase HPLC and FAB-MS, and the data (Table 4.2) are consistent with that expected for the desired peptides.

4.4.3 Receptor affinity

Potential affinity label Dyn A analogues 1c, 1d and 2c and the reversible control peptides 1b and 2b were evaluated for their binding affinity using CHO cells expressing cloned κ, μ and δ opioid receptors. Preliminary binding assay data are shown in Table 4.3. Incorporation of affinity labeling groups in the “message” sequence of dynorphin A(1-11)NH₂ resulted in different binding affinities for the three opioid receptors. The control peptides 1b and 2b and the isothiocyanate 1c derivative exhibited high affinities (IC₅₀ = 2.34-5.75 nM), whereas 2c showed a somewhat lower affinity (IC₅₀ = 14.1 nM) for κ receptors. The isothiocyanate analogue 1c of [Phe(X)⁴,D-Pro¹⁰]Dyn A(1-11)NH₂ showed greater affinity for κ receptors than the bromoacetamide analogue 1d (IC₅₀ = 5.57 vs 27.4 nM). The affinity of these analogues for κ receptors is 1/20 to 1/110 that of the parent peptide [D-Pro¹⁰]Dyn A(1-11). In the [Phe(X)⁴,D-Pro¹⁰]Dyn A(1-11)NH₂ series, the control peptide 1b and the isothiocyanate 1c surprisingly tended to have comparable affinities for each opioid receptor type, while distinct affinities were found in the [Phe(X)¹,D-Pro¹⁰]Dyn A(1-11)NH₂ series. The control peptide 2b had 7-fold higher selectivity for κ receptors than the isothiocyanate 2c. Incorporating either isothiocyanate or bromoacetamide into the p-amino group of Phe in position 1 or 4 caused dramatic decreases in affinity for both μ and δ opioid
### Table 4.2 Analytical data of potential affinity label derivatives of dynorphin A.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>FAB-MS&lt;sup&gt;2&lt;/sup&gt;(M+H)&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Amino acid analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tyr</td>
</tr>
<tr>
<td>[Phe(X)⁴,D-Pro¹⁰]Dyn A(1-11)NH₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b, X= NH₂</td>
<td>17.4</td>
<td>1376.9</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1377)</td>
<td></td>
</tr>
<tr>
<td>1c, X= N=C=S</td>
<td>23.9</td>
<td>1418.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1419)</td>
<td></td>
</tr>
<tr>
<td>1d, X= NHCOCH₂Br</td>
<td>20.7</td>
<td>1498.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1499)</td>
<td></td>
</tr>
<tr>
<td>[Phe(X)¹,D-Pro¹⁰]Dyn A(1-11)NH₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b, X= NH₂</td>
<td>16.8</td>
<td>1361.0</td>
<td>-³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1361)</td>
<td></td>
</tr>
<tr>
<td>2c, X= N=C=S</td>
<td>22.9</td>
<td>1403.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1303)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>For chromatographic conditions see Fig. 4.3

<sup>2</sup>Theoretical values are shown in parentheses.

<sup>3</sup>[Phe(X)¹,D-Pro¹⁰]Dyn A(1-11)NH₂ series contain no Tyr.
Table 4.3 Preliminary binding assay data for potential affinity label derivatives of dynorphin A

<table>
<thead>
<tr>
<th>Compound name</th>
<th>IC₅₀ (nM) ±SE</th>
<th>IC₅₀ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>κ</td>
<td>μ</td>
</tr>
<tr>
<td>[p-Phe(X)⁴, D-Pro¹⁰]Dyn A(1-11)NH₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b, X = NH₂</td>
<td>5.75 ± 2.95</td>
<td>146 ± 16</td>
</tr>
<tr>
<td>1c, X = N=C=S</td>
<td>5.57 ± 0.97</td>
<td>328 ± 58</td>
</tr>
<tr>
<td>1d, X = NHCOCH₂Br</td>
<td>27.4 ± 5.9</td>
<td>173 ± 5.5</td>
</tr>
<tr>
<td>[p-Phe(X)¹, D-Pro¹⁰]Dyn A(1-11)NH₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b, X = NH₂</td>
<td>2.34 ± 0.31</td>
<td>78.9</td>
</tr>
<tr>
<td>2c, X = N=C=S</td>
<td>14.1 ± 1.8</td>
<td>92.5 ± 10.5</td>
</tr>
<tr>
<td>[D-Pro¹⁰]Dyn A(1-11)</td>
<td>0.25 ± 0.03</td>
<td>1.04 ± 0.15</td>
</tr>
</tbody>
</table>

¹Values without SE are from one experiment.
receptors, resulting in IC$_{50}$ values greater than 75 nM. For the [Phe(X)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$ series, the isothiocyanate $Ic$ showed better $\kappa$ selectivity (\$\kappa/\mu/\delta$ ratio = 1/48/57) than the bromoacetamide $Id$ (\$\kappa/\mu/\delta$ ratio = 1/6/60).

For the [Phe(X)$_1$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$ series, the control compound $2b$ unexpectedly had greater $\kappa$ selectivity (\$\kappa/\mu/\delta$ ratio = 1/34/244) than the isothiocyanate $2c$ (\$\kappa/\mu/\delta$ ratio = 1/6/37).

4.5 Conclusions

Potential affinity label derivatives of Dyn A(1-11)NH$_2$ assembled on PAL-PEG-PS resin have been successfully prepared using standard Fmoc chemistry and the Aloc protecting group. Removal of the Aloc group from the protected peptide-resins was complete in 3 h. Conversion of the protected [Phe(NH$_2$)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH-resin and the protected [Phe(NH$_2$)$_1$,D-Pro$^{10}$]Dyn A(1-11)NH-resin to their isothiocyanate derivatives was complete in 3 h, whereas conversion to their bromoacetamide derivatives took 24 h to go to completion. The control compounds [Phe(NH$_2$)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$ and [Phe(NH$_2$)$_1$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$ and the isothiocyanate [Phe(N=C=S)$_1$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$ were obtained in excellent purity. However, the isothiocyanate [Phe(N=C=S)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$ and the bromoacetamide [Phe(NHCOCH$_2$Br)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$, contained side products, namely the control compound [Phe(NH$_2$)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$ and the hydroxyl side product [Phe(NHCOCH$_2$OH)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$ respectively.

Preliminary binding assay data showed that the control amine compounds and the isothiocyanate [Phe(N=C=S)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$ had high affinity for $\kappa$ opioid
receptors, whereas the bromoacetamide [Phe(NHCOCH₂Br)⁴, D-Pro¹⁰]Dyn A(1-11)NH₂ and isothiocyanate [Phe(N=C=S)¹,D-Pro¹⁰]Dyn A(1-11)NH₂ exhibited lower affinity for κ opioid receptors. All peptides had markedly decreased affinity for both μ and δ opioid receptors. Thus, they showed higher selectivity for κ opioid receptor than the parent peptide [D-Pro¹⁰]Dyn A(1-11)NH₂. Additional pharmacological experiments with these compounds are in progress.
CHAPTER 5

SYNTHESIS OF POTENTIAL AFFINITY LABEL DERIVATIVES OF DYNORPHIN A(1-11)NH₂: ADDRESS SEQUENCE MODIFICATIONS

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¹College of Pharmacy, Oregon State University
²School of Pharmacy, University of Maryland at Baltimore
Abbreviations:

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature in *Biochem. J*. 1984, 219, 345-373. Amino acids are in the L-configuration except when indicated. Additional abbreviations used are as follows: Aloc, allyloxycarbonyl; Boc, t-butyloxycarbonyl; t-Bu, tert-butyl; DCM, dichloromethane, DIC, N,N'-diisopropylcarbodiimide; DIEA, N,N'-diisopropylethylamine; DMA, N,N-dimethylacetamide; Dyn A, dynorphin A; DMF, N,N-dimethylformamide; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (9-fluorenylmethoxy)carbonyl; HOBt, 1-hydroxy-benzotriazole; HPLC, high performance liquid chromatography; NMM, N-methylmorpholine; PAL, peptide amide linker, PEG, polyethylene glycol; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PS, polystyrene; TFA, trifluoroacetic acid, THF, tetrahydrofuran; Z, benzyloxy-carbonyl.
5.1 Abstract

The "address" sequence of dynorphin (Dyn) A is important for directing the peptide to kappa opioid receptors. We replaced Ile\(^8\) of [D-Pro\(^{10}\)]Dyn A(1-11)NH\(_2\) with either isothiocyanate or bromoacetamide derivatives of para-substituted phenylalanine or lysine. Syntheses of affinity label derivatives of [D-Pro\(^{10}\)]Dyn A(1-11)NH\(_2\) were achieved by the strategy described in chapter 4. Crude peptides in the [Lys(X)\(^8\),D-Pro\(^{10}\)]Dyn A(1-11)NH\(_2\) series were obtained in high purity, whereas those from [Phe(X)\(^8\),D-Pro\(^{10}\)]Dyn A(1-11)NH\(_2\) series contained minor side products (where X = NH\(_2\) (for amine compounds) or a reactive functional group (e.g.-N=C=S or -NHCOCH\(_2\)Br)).

Preliminary binding assay data revealed that the amine compounds (without a reactive functional group) as well as the affinity label derivatives of [D-Pro\(^{10}\)]Dyn A(1-11)NH\(_2\) had high affinity for both \(\kappa\) and \(\mu\) opioid receptors (IC\(_{50}\) = 0.16-1.54 and 0.77-2.58 nM, respectively). Among these peptides, the amine compound [Lys\(^8\),D-Pro\(^{10}\)]Dyn A(1-11)NH\(_2\) exhibited the highest affinity for \(\kappa\) opioid receptors (IC\(_{50}\) = 0.16 nM) with modest selectivity (IC\(_{50}\) ratio (\(\kappa/\mu/\delta\) = 1/4/31). Affinity of all compounds for \(\delta\) opioid receptors (IC\(_{50}\) = 4.92-13.9 nM) were lower than affinity for \(\kappa\) and \(\mu\) opioid receptors.
5.2 Introduction

The concept of multiple opioid receptors which was first proposed in 1965 [Portoghese, 1965], is supported by many *in vitro* and *in vivo* studies [Lord et al., 1977; Martin, 1976]. Opioid receptors were initially designated as μ, κ and σ receptors by the prototype ligands used in the study (morphine, ketazocine and N-allylnormetazocine (SKF 10,047), respectively) [Gilbert and Martin, 1976]. The σ receptors are no longer considered as opioid receptors since their effects are not reversed by naloxone [Fries, 1991]. An additional type of opioid receptor, the δ receptors, was identified after isolation of the enkephalins [Hughes et al., 1975; Lord et al., 1977]. *In vitro* studies showed that the guinea-pig ileum (GPI) is rich in μ and κ receptors, whereas δ receptors are predominant in the mouse vas deferens (MVD) [Takemori, 1976; Ward et al., 1985].

Irreversible ligands specific for a certain receptor type are useful tools to elucidate the involvement of different receptor types in physiological and pharmacological responses. Affinity label derivatives including chemical affinity labels (electrophilic ligands) and photoaffinity labels require two recognition steps for covalent bonding; 1) primary recognition which is a reflection of the reversible affinity of the ligand for the receptor, and 2) secondary recognition which involves the reaction of the electrophilic center with a proximal nucleophile [Portoghese and Takemori, 1985]. Photoaffinity labels are not useful for *in vivo* studies since UV irradiation is required to convert these ligands to highly reactive intermediates. Electrophilic affinity labels), however, can be used for both *in vitro* and *in vivo* studies. Alkylating groups have been introduced into opiates with the epoxymorphinan structure by converting an amino derivative to the corresponding isothiocyanate or
bromoacetamido derivatives [Rice et al., 1983]. Examples of compounds containing an
isothiocyanate group are 6α- and 6β-isothiocyanato-4,5-epoxymorphinans [Sayre et al.,
1983], fentanyl isothiocyanates derivatives FIT, SUPERFIT, naltrindole and BIT [Klee et
al., 1982; Rothman et al., 1989], N-alkyl-6,14-endo-ethanotetrahydrooripavine-derived
1990]. Studies of κ receptors were initially difficult due to the lack of selective ligands.
Benzomorphan derivatives such as ethylketocyclazocine (EKC) and bremazocine were often
used for κ receptor characterization, but these analogs also show affinity for μ receptors
[Paterson et al., 1984]. The benzacetamide U-50,488 was the first non-peptide κ-selective
ligand [Szumuszkovicz and Voigtlander, 1982], and many derivatives of this ligand have
been prepared [Zimmerman and Leander, 1990]. U-50,488 and U-69,593 are utilized to
distinguish two populations of κ binding sites in rat brain, a U-69,593- (or U-50,488-)
sensitive κ₁ subtype and a U-69,593- (or U-50,488-) insensitive κ₂ subtype [Zukin et al.,
1988]. Isothiocyanate derivatives of U-50,488 selectively and irreversibly block binding
sites which recognize [³H]U-69,593 but not those which bind [³H]bremazocine [de Costa,
1989; 1990].

Research in our laboratory focuses on opioid peptide analogues as tools to
c characterize multiple types of opioid receptors. In the present study, we are interested in
preparing affinity labels for κ opioid receptors based on dynorphin A (Dyn A). These
analogues can be used as probes of κ receptor structure and function. Dyn A is a 17-amino
acid endogenous opioid peptide for κ receptors [Chavkin et al., 1982], and thus it is
important to understand its interaction with opioid receptors and physiological functions.
Dyn A, however, displays limited selectivity for $\kappa$ versus $\mu$ and $\delta$ receptors [Gairin et al., 1984; Corbett et al., 1982]. The C-terminally truncated peptide Dyn A(1-13) shows similar receptor selectivity profile to Dyn A(1-17) [Lemaire et al., 1986], whereas [D-Pro$^{10}$]Dyn A(1-11) exhibits a high binding affinity ($K_i = 0.032$ nM) against the $\kappa$ ligand [$^{3}$H] bremazocine. Therefore, we prepared affinity label derivatives of Dyn A using [D-Pro$^{10}$]Dyn A(1-11) as the parent compound. We incorporated a reactive functionality (e.g. isothiocyanate (-N=C=S) or bromoacetamide (-NHCOCH$_2$Br)) into either the N-terminal "message" (see chapter 4) or the C-terminal "address" (Leu-Arg-Arg-Ile-Arg-D-Pro-Lys) sequence.

Modifications in the C-terminus of [D-Pro$^{10}$]Dyn A(1-11) are described in this chapter. The "address" sequence of Dyn A plays an important role in directing the peptide to the kappa opioid receptors. Structure-activity relationship studies indicated that the basic residues Arg$^7$ and Lys$^{11}$ are the most important for potency and kappa receptor selectivity [Chavkin and Goldstein, 1981; Turcotte et al., 1984; Snyder et al., 1992]. The positive charges of these residues may form two electrostatic interactions with negative charges on the kappa binding site, which would increase binding energy and stabilize the bioactive conformation [Hruby and Gehrig, 1989]. Replacements of the basic amino acids in position 7 and 11 cause a larger decrease in opioid activity in the GPI than do substitution in positions 8 and 10 [Turcotte, 1984]. Substitution of Ile$^8$ by Ala or D-Ala enhances kappa binding affinity [Lemaire et al., 1986]. Since structural changes in the position 8 are well tolerated, we selected the nonbasic residue Ile$^8$ as a site for introducing a reactive functionality in our studies. Ile$^8$ was replaced with either isothiocyanate or bromoacetamide derivatives of para-substituted phenylalanine or lysine. The amine containing peptides, [Phe(NH$_2$)$_8$,D-Pro$^{10}$]
Dyn A(1-11)NH₂ and [Lys⁸,D-Pro¹⁰] Dyn A(1-11)NH₂ were included as reversible controls in the pharmacological assays. The synthetic strategy which includes the use of the allyloxycarbonyl (Aloc) protecting group and the novel polyethylene glycol (PAL-PEG-PS) resin were described in detail in chapter 4.

5.3 Experimental section

5.3.1 Materials

The reagents and instrumentation used were those described previously (see chapter 4) except for FmocLys(Aloc), which was purchased from Perseptives (Bedford, MA).

5.3.2 Methods

5.3.2.1 Protected [Phe(NH₂)⁸,D-Pro¹⁰]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 1a-resin) and [Lys(NH₂)⁸,D-Pro¹⁰]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 2a-resin), protected [Phe(NH₂)⁸,D-Pro¹⁰]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 1b-resin) and [Lys(NH₂)⁸,D-Pro¹⁰]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 2b-resin)

The protected peptides 1a and 2a were assembled on PAL-PEG-PS resin (1% cross linked polyvinyl-styrene, 0.17 mmol/g, 1.0 g) using standard Fmoc chemistry procedures as described in chapter 4. Either FmocPhe(NH₂) or FmocLys(Aloc) were incorporated into position 8 where the reactive functionality was to be introduced into the peptide chain. Other side chain functionalities and the N-terminal amine were protected with TFA-labile protecting groups as described in chapter 4. Protected peptide 1a (0.14 g, 17 µmol) and 2a
(0.75 g, 89.8 μmol) were then treated with Pd(PPh₃)₄ for 3 h and 2 h, respectively, as described in chapter 4, providing protected peptide 1b- and 2b-resins.

5.3.2.2 Protected [Phe(N=C=S)⁸,D-Pro¹⁰] Dyn A(1-11)NH-PAL-PEG-PS resin (protected 1c-resin) and [Lys(N=C=S)⁸,D-Pro¹⁰] Dyn A(1-11)NH-PAL-PEG-PS resin (protected 2c-resin), protected [Phe(NHCOCH₂Br)⁸,D-Pro¹⁰] Dyn A(1-11)NH-PAL-PEG-PS resin (protected 1d-resin) and [Lys(NHCOCH₂Br)⁸,D-Pro¹⁰] Dyn A(1-11)NH-PAL-PEG-PS resin (protected 2d-resin)

Protected peptide-resin 1b (0.11 g, 12.9 μmol) and 2b (0.28 g, 38.0 μmol) were swollen in DCM (3.0 mL) for 15 min, DIEA (2.5 equiv) was added, followed by adding thiophosgene (Cl₂-C=S, 1.5 equiv). The ninhydrin test was monitored until a negative result was obtained (2 h). The products, protected peptide 1c- and 2c-resins, were filtered, washed with DCM (5 x 2) and dried in vacuo.

Protected peptide-resin 1b (0.08 g, 9.89 μmol) and 2b (0.17 g, 23.0 μmol) were swollen in DCM (3.0 mL) for 15 min and chilled in an ice bath. DIEA (2 equiv) was added to the peptide-resin, followed by adding bromoacetyl bromide (BrCH₂COBr, 1.5 equiv). The mixture was stirred at 0°C for 1 h, and at room temperature until the negative ninhydrin test was obtained (24 h and 18 h for 1d and 2d, respectively). The peptide-resins 1d and 2d were filtered, washed with DCM (5 x 2) and dried in vacuo.

5.3.2.3 Cleavage reactions and purification

Protected peptide-resins 1a-1d and 2a-2d were treated with 10 mL of modified reagent K as described in chapter 4. Following cleavage of the peptides, the peptides were purified on a Rainin preparative gradient HPLC system and purity of peptides (> 98%) was
verified by analytical HPLC as described in chapter 4. The purified peptides were submitted for fast atom bombardment mass spectrometry.

5.3.3 Receptor binding assays

Opioid receptor binding studies were performed in Chinese hamster ovary (CHO) cells which express cloned μ, δ and κ opioid receptors. Wash-resistant inhibitions of binding CHO cell membranes will also be determined. The procedures for both assays are described in detail in chapter 4.

5.4 Results and discussion

5.4.1 Peptide syntheses

The protected peptides 1a and 2a were assembled on PAL-PEG-PS resin using standard Fmoc chemistry followed by removal of the Aloc protecting group using a Pd (0) catalyst [Kates et al., 1993] using the procedures previously described (see chapter 4). The Aloc deprotections of protected 1a- and 2a-resin were complete in 3 h and 2 h, respectively. Isothiocyanate and bromoacetamide reactive functionalities were subsequently introduced into protected 1b- and 2b-resin using thiophosgene and bromoacetyl bromide, respectively, under basic conditions. Conversion of the amines protected 1b- and 2b-resin to the isothiocyanates protected 1c- and 2c-resin was complete in 2 h, as indicated by quantitative ninhydrin test. The bromoacetamide analogues protected 1d- and 2d-resin, however, took 24 h and 18 h, respectively, for the reactions to go to completion. The N-terminal Boc
protecting group and side chain protecting groups were removed and the peptide amides then liberated from the resins using modified reagent K (ethanedithiol was eliminated to prevent reactions with the alkylating group, see chapter 4). In all cases, crude peptides in the \([\text{Lys}(X)_{8},\text{D-Pro}^{10}]\text{Dyn A}(1-11)\text{NH}_2\) series were obtained in excellent purity following cleavage from the resin (Fig. 5.2). Crude peptides in \([\text{Phe}(X)_{8},\text{D-Pro}^{10}]\text{Dyn A}(1-11)\text{NH}_2\) series, however, contained some minor side products (Fig. 5.1). Our results indicate that the incorporation of isothiocyanate or bromoacetamide functional groups into \([\text{Lys}(X)_{8},\text{D-Pro}^{10}]\text{Dyn A}(1-11)\text{NH}_2\) was faster and cleaner than into \([\text{Phe}(X)_{8},\text{D-Pro}^{10}]\text{Dyn A}(1-11)\text{NH}_2\). After purification by preparative reverse phase HPLC, all peptides were characterized by analytical reverse phase HPLC and FAB-MS (Table 5.1) and the parent peptides \(1b\) and \(2b\) by amino acid analysis.

5.4.2 Receptor affinity

The purified peptides were evaluated for their binding affinity to \(\kappa\), \(\mu\) and \(\delta\) opioid receptors using competition binding assays. The affinities for \(\kappa\), \(\mu\) and \(\delta\) were determined with cloned receptors expressed in Chinese hamster ovary (CHO) cell using radioligands \([^3\text{H}]\text{diprenorphine},[^3\text{H}]\text{DAMGO}\) and \([^3\text{H}]\text{DPDPE}\), respectively, and \(IC_{50}\) values (Table 5.2) were calculated from nonlinear regression analysis of competition curves.

Binding affinity of potential affinity label derivatives of dynorphin A for opioid receptors varied. Preliminary data show that all peptides had high affinity for both \(\kappa\) and \(\mu\) opioid receptors (\(IC_{50} = 0.16\text{-}2.89\text{ nM}\)) compared to the parent peptide \([\text{D-Pro}^{10}]\text{Dyn A}(1-11)\) (\(IC_{50} = 0.25\) and \(1.04\text{ nM}\) for \(\kappa\) and \(\mu\) opioid receptors, respectively). The isothiocyanate
Fig. 5.1 Chromatograms of crude peptides \( I_b \) (top), \( I_c \) (middle) and \( I_d \) (bottom). Column: Vydac (C\(_{18}\)). Solvent: A = aqueous 0.1% TFA, B = acetonitrile containing 0.1% TFA. Flow rate: 1.5 mL/min. Gradient: 0-75% B over 50 min.
Fig. 5.2 Chromatograms of crude peptides 2b (top), 2c (middle) and 2d (bottom). For chromatographic conditions see Fig 5.1.
Table 5.1 Analytical data of potential affinity label derivatives of dynorphin A.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>FAB-MS&lt;sup&gt;2&lt;/sup&gt; (M+H)&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Amino acid analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tyr (1)</td>
<td>Gly (2)</td>
</tr>
<tr>
<td>[Phe(X)&lt;sup&gt;8&lt;/sup&gt;,D-Pro&lt;sup&gt;10&lt;/sup&gt;]Dyn A(1-11)NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b, X= NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>19.9</td>
<td>1410.8</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1c, X= N=C=S</td>
<td>22.5</td>
<td>1454.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d, X= NHCOCH&lt;sub&gt;2&lt;/sub&gt;Br</td>
<td>22.0</td>
<td>1532.8</td>
<td></td>
</tr>
<tr>
<td>[Lys(X)&lt;sup&gt;8&lt;/sup&gt;,D-Pro&lt;sup&gt;10&lt;/sup&gt;]Dyn A(1-11)NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b, = -</td>
<td>19.0</td>
<td>1377.0</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2c, X= N=C=S</td>
<td>19.1</td>
<td>1419.0</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2d, X= NHCOCH&lt;sub&gt;2&lt;/sub&gt;Br</td>
<td>20.9</td>
<td>1498.8</td>
<td></td>
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</tbody>
</table>

<sup>1</sup>For chromatographic conditions see Fig. 5.1.
<sup>2</sup>Theoretical values are shown in parentheses.
<sup>3</sup>[Lys(X)<sup>8</sup>D-Pro<sup>10</sup>]Dyn A(1-11)NH<sub>2</sub> contains two Lys residues.
### Table 5.2 Preliminary binding assay data for potential affinity label derivatives of dynorphin A

<table>
<thead>
<tr>
<th>Compound name</th>
<th>IC₅₀ (nM) ±SE</th>
<th>IC₅₀ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>̅</td>
<td>̅</td>
</tr>
<tr>
<td><strong>κ</strong></td>
<td><strong>μ</strong></td>
<td><strong>δ</strong></td>
</tr>
<tr>
<td>[p-Phe(X)₈, D-Pro¹⁰]Dyn A(1-11)NH₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b, X = NH₂</td>
<td>1.54 ± 0.15</td>
<td>1.06 ± 0.29</td>
</tr>
<tr>
<td>1c, X = N=C=S</td>
<td>0.49 ± 0.09</td>
<td>1.71 ± 0.27</td>
</tr>
<tr>
<td>1d, X = NHCOCH₂Br</td>
<td>1.42 ± 0.06</td>
<td>2.89 ± 0.31</td>
</tr>
<tr>
<td>[Lys(X)₈, D-Pro¹⁰]Dyn A(1-11)NH₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b, X = -</td>
<td>0.16 ± 0.03</td>
<td>0.71 ± 0.13</td>
</tr>
<tr>
<td>2c, X = N=C=S</td>
<td>0.54 ± 0.03</td>
<td>1.04 ± 0.20</td>
</tr>
<tr>
<td>2d, X = NHCOCH₂Br</td>
<td>0.68 ± 0.10</td>
<td>1.16 ± 0.03</td>
</tr>
<tr>
<td>[D-Pro¹⁰]Dyn A(1-11)</td>
<td>0.25 ± 0.03</td>
<td>1.04 ± 0.15</td>
</tr>
</tbody>
</table>

¹Values without SE are from one experiment.
derivative of [Phe(X)^8,D-Pro^10]Dyn A(1-11)NH$_2$ (1c) bound to $\kappa$ and $\mu$ opioid receptors with greater affinity than the bromoacetamide derivative (1d) ($IC_{50} = 0.49$ and 1.71 vs 1.42 and 2.89 nM, respectively). For [Lys(X)^8,D-Pro^10]Dyn A(1-11)NH$_2$ series, both the isothiocyanate (2c) and bromoacetamide (2d) derivatives had comparable affinities for $\kappa$ and $\mu$ opioid receptors. Unexpectedly, the amine control peptide 2b had the greatest affinity for $\kappa$ binding sites ($IC_{50} = 0.16$ nM). Potential affinity label derivatives of dynorphin A also bound to $\delta$ opioid receptors with modest affinity ($IC_{50} = 4.92-13.9$ nM).

5.5 Conclusions

Isothiocyanate and bromoacetamide derivatives of [Phe(X)^8,D-Pro^10]Dyn A(1-11)NH$_2$ and [Lys(X)^8,D-Pro^10]Dyn A(1-11)NH$_2$ (where X = NH$_2$ (for amine compounds) or a reactive functional group (e.g.-N=C=S or -NCOCH$_2$Br)) have been prepared using the synthetic strategy described in chapter 4. Removal of Aloc group from the protected [Lys-(NHAlco)^8,D-Pro^10]Dyn A(1-11)NH-resin was faster than from the protected [Phe-(NH-Alco)^8,D-Pro^10]Dyn A(1-11)NH-resin (2 h vs 3 h). Conversion of the protected [Phe-(NH$_2$)^8,D-Pro^10]Dyn A(1-11)NH-resin and the protected [Lys^8,D-Pro^10]Dyn A(1-11)NH-resin to their isothiocyanate derivatives was complete in 2 h. Whereas, conversion of the protected [Lys^8,D-Pro^10]Dyn A(1-11)NH-resin to its bromoacetamide derivative took a shorter reaction time than that of the protected [Phe(NH$_2$)^8,D-Pro^10]Dyn A(1-11)NH-resin (18 h vs 24 h). Crude peptides in the [Lys(X)^8,D-Pro^10]Dyn A(1-11)NH$_2$ series were obtained in high purity, while crude peptides in the [Phe(X)^8,D-Pro^10]Dyn A(1-11)NH$_2$ series contained minor impurities which could be separated by reverse phase HPLC.
Preliminary binding assay data showed that the control compounds and affinity label derivatives of [D-Pro\textsuperscript{10}]Dyn A(1-11)NH\textsubscript{2} had affinity for all three opioid receptors (κ, μ and δ). Affinity for δ opioid receptors (IC\textsubscript{50} = 4.92 - 13.9 nM), however, was slightly lower than for κ (IC\textsubscript{50} = 0.16 - 1.42 nM) and μ (IC\textsubscript{50} = 1.06 - 2.89 nM) opioid receptors. Except for [Phe(NH\textsubscript{2})\textsuperscript{8},D-Pro\textsuperscript{10}]Dyn A(1-11)NH\textsubscript{2}, all peptides possessed higher affinity for κ opioid receptors than for μ opioid receptors. The highest affinity for κ opioid receptors was surprisingly found in the control compound [Lys\textsuperscript{8},D-Pro\textsuperscript{10}]Dyn A(1-11)NH\textsubscript{2} (IC\textsubscript{50} = 0.16 nM). Kappa selectivity of this peptide was comparable to the parent peptide [D-Pro\textsuperscript{10}] Dyn A(1-11)NH\textsubscript{2} (IC\textsubscript{50} ratio (κ/μ/δ) = 1/4/31 vs 1/4/22). In both [Phe(X)\textsuperscript{8},D-Pro\textsuperscript{10}]Dyn A(1-11)NH\textsubscript{2} and [Lys(X)\textsuperscript{8},D-Pro\textsuperscript{10}]Dyn A(1-11)NH\textsubscript{2} series, the isothiocyanate derivatives showed slightly higher affinity for κ opioid receptors than the bromoacetamide derivatives. Additional pharmacological experiments of these compounds are in progress.
CHAPTER 6

SOLID PHASE SYNTHESIS OF POTENTIAL AFFINITY LABEL DERIVATIVES OF DYNORPHIN A ASSEMBLED ON PAL-PEG-PS VS PAL-PS RESINS

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\textsuperscript{2}School of Pharmacy, University of Maryland at Baltimore
Abbreviations:

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature in *Biochem. J.* 1984, 219, 345-373. Amino acids are in the L-configuration except when indicated. Additional abbreviations used are as follows: Aloc, allyloxycarbonyl; Boc, t-butyloxycarbonyl; t-Bu, tert-butyl; DCM, dichloromethane, DIC, N,N'-diisopropylcarbodiimide; DIEA, N,N'-diisopropylethylamine; DMA, N,N-dimethylacetamide; Dyn A, dynorphin A; DMF, N,N-dimethylformamide; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (9-fluorenylmethoxy)carbonyl; HOBt, 1-hydroxy-benzotriazole; HPLC, high performance liquid chromatography; NMM, N-methylmorpholine; PAL, peptide amide linker, PEG, polyethylene glycol; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PS, polystyrene; TFA, trifluoroacetic acid, THF, tetrahydrofuran; Z, benzyloxy-carbonyl.
6.1 Abstract

The synthesis of affinity label-containing dynorphin A (Dyn A) analogues in our laboratory generally involves modification of a substituted phenylalanine residue at different positions. An appropriate protecting group is required for an amine functionality on the para position of Phe. Allyloxycarbonyl (Aloc) was used to provide orthogonal protection which was removed by Pd(PPh$_3$)$_4$ in DCM/5% AcOH/2.5% N-methylmorpholine (NMM). Rates of Aloc deprotection were affected by the resin type used for peptide assembly; the complete removal of Aloc was achieved in 3 h and 24 h for PAL-PEG-PS and PAL-PS resins, respectively. The reactive functionalities isothiocyanate (-N=C=S) and bromoacetamide (-NHCOCH$_2$Br) were introduced into the Aloc deprotected peptide-resins. Analyses by HPLC revealed the crude peptides assembled on the PAL-PS resin contained less impurities than those on the PAL-PEG-PS resin.
6.2 Introduction

One research project in our laboratory involves the preparation of electrophilic affinity labels for kappa opioid receptors based on dynorphin A. Affinity label-containing compounds can bind covalently to receptors and thus differ from reversible ligands. The irreversible binding of electrophilic affinity labels is due to the acylation or alkylation by the electrophilic group on the affinity label to a nucleophilic site on the receptors. This irreversible binding can increase the ligand's selectivity for one receptor type over another [Takemori et al 1985]. Therefore, affinity labels can be utilized to differentiate receptor types and even receptor subtypes. Dyn A analogues containing an affinity label are being synthesized to use as tools to study kappa opioid receptor structure and function. The reactive functionalities generally are being introduced at the p-amino group of a phenylalanine residue, for example at position 4 to give \[\text{[Phe}(X^4,\text{D-Pro}^{10})\text{Dyn A}(1-11)\text{NH}_2,\] and include isothiocyanate \((X = -\text{N}=\text{C}=\text{S})\) and bromoacetamide \((X = -\text{NHCOCH}_2\text{Br})\) derivatives.

Allyl-based protecting groups have been used extensively in organic synthesis and recently have been applied to peptide and DNA syntheses [Lyttle and Hudson, 1992]. Deprotection of allyl derivatives occurs smoothly and quantitatively with palladium (0) (Fig. 6.1). The mild conditions to deprotect allyl functionalities are compatible with classical Boc (butyloxycarbonyl)/Bzl (benzyloxycarbonyl) and Fmoc (9-fluorenylethoxycarbonyl)/t-Bu (t-butyl) chemistries [Albericio et al., 1992; Kates et al., 1994], and provide orthogonal protection schemes for the construction of complex peptides. An allyl-based protecting group such as allyloxycarbonyl or Aloc is easy to introduce using the readily available and
Fig. 6.1 Deprotection of allyl-based protecting groups.

Fig. 6.2 Structures of PAL-PEG-PS (top) and PAL-PS (bottom) resin.
inexpensive allyl chloroformate. Deprotection of Aloc can also be achieved using only a catalytic amount of catalyst (e.g. 125-250 mg of Pd(0) per 0.1 mmol peptide-resin) and the reaction tolerates a wide range of solvents (e.g. DCM and DMF). Thus, allyl-based protecting groups (e.g. Aloc) are suitable for use in the synthesis of dynorphin A analogues containing reactive functional groups which require different levels of protection. We used an Aloc protecting group for the amino acid residue where a reactive functionality was incorporated. The peptides were assembled on the resin and an affinity label group introduced after Aloc deprotection while the other amino acid side chains and N-terminus remained protected.

A novel polyethylene glycol-polystyrene resin with a peptide amide linker (PAL-PEG-PS) offers excellent coupling and deblocking efficiency [Kates et al., 1994; Barany et al., 1993; Auzanneau et al., 1995]. It has a high level of solvation due to the derivatized polyethylene glycol spacer (Fig. 6.2) between the functional group on the polystyrene gel bead and the handle or attachment point of the synthetic peptide. It also swells appreciably in a wide range of both organic and inorganic solvents (Table 6.1) enhancing rapid diffusion of reagents throughout the polymer network. Thus, the PAL-PEG-PS resin may be suitable for the synthesis of affinity label derivatives of Dyn A.

Previous studies (see chapter 4 and 5), however, showed that the crude peptides assembled on PAL-PEG-PS resin contained the desired peptides but that some impurities were also often present. Therefore, we synthesized potential affinity label Dyn A analogues, for example [Phe(X)⁴,D-Pro⁶]Dyn A(1-11)NH₂ (where X = -N=C=S or -NHCOC₂H₅Br), on a polystyrene resin, PAL (Peptide Amide Linker, 5-(4-Fmoc-aminomethyl-3,5-dimethoxy-
Table 6.1  Ratio of swollen/dry volumes for individual polyethylene glycol polystyrene (PEG-PS) graft beads and polystyrene (PS) support in different solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>PEG-PS</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCM</td>
<td>6.9</td>
<td>5.8</td>
</tr>
<tr>
<td>DMF</td>
<td>4.8</td>
<td>3.6</td>
</tr>
<tr>
<td>AcCN</td>
<td>3.5</td>
<td>1.4</td>
</tr>
<tr>
<td>TFE</td>
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<td>1.3</td>
</tr>
<tr>
<td>EtOAc</td>
<td>3.7</td>
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<td>THF</td>
<td>4.5</td>
<td>5.7</td>
</tr>
<tr>
<td>EtOH</td>
<td>2.3</td>
<td>1.2</td>
</tr>
<tr>
<td>H₂O</td>
<td>1.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

phenox) valeric acid-MBHA (methylbenzylhydrylamine))-PS resin. PAL-PS resin is normally used in our laboratory to obtain peptide amides, but preliminary studies using the qualitative ninhydrin test indicated that the Aloc deprotection of the protected peptide-PAL-PS resin required a longer reaction time (more than 12 h) than deprotection on the PAL-PEG-PS resin. In this study, we compared the effects of using PAL-PEG-PS vs PAL-PS resin on the Aloc deprotection rates and the purity of the final potential affinity label Dyn A analogues. The results from this study could be useful to improve the synthesis of potential affinity label derivatives of Dyn A as well as potential affinity label derivatives of other peptides.

6.3 Experimental section

6.3.1 Materials

The reagents and instruments used were those described previously (see chapters 4 and 5) with the following modification: PAL-PS resins (1% cross linked polyvinylstyrene, 0.38 mmol/g) was purchased from Perseptives (Bedford, MA).
6.3.2 Methods

6.3.2.1 Protected $[\text{Phe(NHAlloc)}^4, \text{D-Pro}^{10}]\text{Dyn A(1-11)NH-PAL-PEG-PS resin (protected 1a-resin)}$ and $[\text{Phe(NHAlloc)}^4, \text{D-Pro}^{10}]\text{Dyn A(1-11)NH-PAL-PS resin (protected 1b-resin)}$

Protected 1a- and 1b-resin were assembled on the PAL-PEG-PS resin (1% cross linked polyvinylstyrene, 0.17 mmol/g) and PAL-PS resin (1% cross linked polyvinylstyrene, 0.38 mmol/g), respectively, using the standard procedure described in chapter 4.

6.3.2.2 Time course studies of Aloc deprotection

A Biosearch 9500 automated peptide synthesizer was used for the Aloc deprotection. A time course study of Aloc deprotection was performed using 20 mg aliquots of protected 1a- and 1b-resin and the following times: 0.5, 2 and 3 h for protected 1a-resin, and 2, 6, 12 and 24 h for protected 1b-resin. The peptide-resin was swollen in DCM for 15 min and a solution of $\text{Pd(PPh}_3)_4$ (0.14 M, 3 equiv) in 92.5% DCM/5% AcOH/2.5% NMM ($N$-methylmorpholine) was manually added to the reaction vessel [Kates et al., 1993]. The reaction was mixed under $N_2$ and a sufficient amount of DCM was added occasionally due to evaporation during mixing. A freshly prepared palladium solution was added after prolonged mixing times (more than 6 h). Each aliquot of peptide-resin was washed with the following solvents: THF (3 x 2 min), DMF (3 x 2 min), DCM (3 x 2 min), 0.5% DIEA in DCM (3 x 2 min), 0.02 M sodium diethyldithiocarbamate in DMF (3 x 15 min) and DCM (3 x 2 min) [Kates et al., 1993]. The peptide-resin was dried in vacuo and the completeness of Aloc deprotection was determined by quantitative ninhydrin test [Stewart et al., 1984].
The amount of amine remaining was calculated based on the absorbance at 570 nm using the following formula:

\[
\text{amine (\(\mu\text{mol/g}\))} = \frac{A \times V \times 10^6}{\varepsilon_{570} \times \text{Wt}}
\]

\(A\) = absorbance at 570 nm

\(V\) = volume of final solution (mL)

\(\varepsilon_{570}\) = molar absorptivity at 570 nm (1.5 x 10^4 M⁻¹ cm⁻¹)

\(\text{Wt}\) = weight of peptide-resin used (mg)

6.3.2.3 [Phe(NH₂)⁴,D-Pro¹⁰]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 2a-resin) and [Phe (NH₂)⁴,D-Pro¹⁰]Dyn A(1-11)NH-PAL-PS resin (protected 2b-resin)

The time course study of Aloc deprotection described in the previous section indicated that the Aloc deprotection of the peptide assembled on the PAL-PEG-PS resin was complete in 3 h, while the reaction on PAL-PS resin required in 24 h. This data and the conditions described above were used on a larger scale Aloc deprotection of protected 1a- and 1b-resin (0.5 and 0.45 g, respectively) to yield protected 2a- and 2b-resin, respectively.

6.3.2.4 Protected [Phe(N=C=S)⁴,D-Pro¹⁰]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 3a-resin), [Phe(N=C=S)⁴,D-Pro¹⁰]Dyn A(1-11)NH-PAL-PS resin (protected 3b-resin), [Phe(NHCOCH₂Br)⁴,D-Pro¹⁰]Dyn A(1-11)NH-PAL-PEG-PS resin (protected 4a-resin) and [Phe(NHCOCH₂Br)⁴,D-Pro¹⁰] Dyn A(1-11)NH-PAL-PS resin (protected 4b-resin)

The reactive functionality groups isothiocyanate (-N=C=S) and bromoacetamide (-NHCOCH₂Br) were introduced into the protected peptide-resins 1a and 1b as described in chapter 4 to give the protected peptide-resins 3a, 3b, 4a and 4b. The products were
subsequently cleaved by modified reagent K (87.5% TFA/2.5% thioanisole/5% phenol/ 5% H₂O) [King et al., 1990] for 4 h and after filtering diluted with 10% acetic acid and extracted with ether (see chapter 4). Crude peptides 3a, 3b, 4a and 4b were analyzed by HPLC.

6.4 Results and discussion

The synthesis of potential affinity label derivatives of Dyn A described previously in chapter 4 and 5 were achieved by utilizing the allyl/Fmoc/tBu protection scheme. The Aloc group provides orthogonal protection which can be selectively removed by Pd(0). Removal of Aloc followed by incorporation of a reactive functionality group could be achieved on a PAL-PEG-PS resin. Polyethylene glycol comprises 20-70 percent weight of the resin which makes the resin compatible with both organic and inorganic solvents [Kates et al., 1993]. Analyses by reverse phase HPLC, however, indicated that the crude products often contained some impurities in addition to the desired peptides. This prompted us to synthesize the potential affinity label derivatives of Dyn A on a different resin, PAL-PS resin, and to directly compare the Aloc deprotection rates and purity of the crude peptides on the two resins. Therefore, [Phe(NHAcloc)⁴,D-Pro¹⁰]Dyn A(1-11)NH₂ was assembled on the two resins, PAL-PEG-PS and PAL-PS resins. The reactive functional groups were introduced after removal of the Aloc group.

Deprotection of the Aloc group can be achieved using palladium (0) [Kates et al., 1993; Baeza et al, 1992]. The deprotection procedure, however, can be complicated by a number of factors including the insolubility of palladium, requirement for an inert atmosphere and the formation of side products (e.g. metal ions and allylamine) [Genet et al.,
A nucleophile (e.g. morpholine [Kunz, 1987; Kunz et al., 1984 and 1988], formic acid [Minami et al., 1985; Hayakawa et al., 1990], potassium 2-ethylhexanoate [Jeffery et al., 1982], dimedone [Guibe et al., 1981] or tributyltin hydride [Guibe et al., 1989; Dangles et al., 1987; Boullanger et al., 1986]) is required as an allylic scavenger to minimize the competitive formation of allylamine; palladium catalyzes the transfer of the allyl unit to these nucleophiles [Loffet et al., 1993] (Fig 6.1). The optimum conditions used in our study were Pd(PPh₃)₄ in 92.5% DCM/5% AcOH/2.5% NMM; DCM was used instead of chloroform [Kates et al., 1993] to avoid hepatic toxicities of the latter solvent. Organic acid (i.e. AcOH) was preferred to mineral acid (i.e. HCl) to prevent a heterogenous solvent mixture. Metal ions and side products were removed by washing with THF, DMF, DCM, 5% DIEA in DCM, 0.02 M sodium diethyldithiocarbamate in DMF, DMF and DCM; sodium diethyldithiocarbamate was required as a palladium scavenger [Kates et al., 1993].

Aloe deprotection rates of protected [Phe(NHAlc)₄,D-Pro₁⁰]Dyn A(1-11)NH₂ assembled on PAL-PEG-PS and PAL-PS resins are compared in Fig.6.3. Quantitative ninhydrin test was performed to measure amine levels. Quantitative ninhydrin data indicate that Aloc deprotection on the PAL-PEG-PS resin is significantly faster than on the PAL-PS resin. After 2 h, 73.8% and 11.1% of the expected maximum amine were obtained on PAL-PEG-PS and PAL-PS resins, respectively. The removal of Aloc was complete in 3 h on the PAL-PEG-PS resin, whereas a longer reaction time (24 h) was required for the PAL-PS resin. The polyethylene glycol spacer in PAL-PEG-PS resin probably enhances Aloc deprotection rates by allowing complete solvation of the reactive sites which resulted in a more efficient reaction. The protected peptides 2a and 2b on the resin were used as
Deprotection of Aloc on PAL-PEG-PS resin

![Graph comparing deprotection rates of Aloc on PAL-PEG-PS vs PAL-PS resins.](image)

**Fig. 6.3** Comparison of deprotection rates of Aloc from [Phe(NHAc)₄, D-Pro¹⁰] Dyn A(1-11)NH-resin synthesized on PAL-PEG-PS (top) vs PAL-PS (bottom) resins.
precursors for the preparation of affinity label-containing Dyn A analogues 3a, 3b, 4a and 4b. Crude peptides were obtained after the cleavage reactions and analyzed by reverse phase HPLC. The amine peptides 2a and 2b assembled on PAL-PEG-PS and PAL-PS resin, respectively, were obtained in excellent purity (>90%, Fig 6.4). The potential affinity label derivatives Dyn A assembled on PAL-PEG-PS, however, contained more side products than those assembled on the PAL-PS resin. The isothiocyanate 3a contained approximately 30% side products in addition to the desired peptide, whereas the isothiocyanate 3b was the only major product (>88%) assembled on the PAL-PS resin (Fig 6.5). Reverse phase HPLC analysis of the bromoacetamide derivatives assembled on PAL-PEG-PS and PAL-PS resin showed two major peptide products in approximately 2:1 and 1:3 ratios, respectively (Fig. 6.6). The less polar peptide (t<sub>R</sub> = 20.8 min) had a molecular ion (M+1 = 1498.5) which corresponded with the bromoacetamide derivative Dyn A, while the more polar peptide (t<sub>R</sub> = 19.7 min) had a molecular ion (M+1 = 1435.5) which was identified as the hydroxylated peptide [Phe(NHCOCH<sub>2</sub>OH)<sub>4</sub>]Dyn A(1-11)NH<sub>2</sub>. Therefore, although the Aloc deprotection was faster on the PAL-PEG-PS resin (complete reaction within 3 h), the crude potential Dyn A affinity label derivatives 3a and 4a contained more impurities compared to the crude peptides 3b and 4b assembled on the PAL-PS resin.

6.5 Conclusions

The allyloxycarbonyl (Aloc) group provides orthogonal protection of the para-amine on phenylalanine which can be selectively removed by palladium (0) without affecting the N-terminal Fmoc group or acid-labile side chain protecting groups. Deprotection rates of the
Fig. 6.4 Chromatograms of crude peptide [Phe(NH$_2$)$_4$,D-Pro$^{10}$]Dyn A(1-11)NH$_2$ from PAL- PEG-PS (left) and PAL-PS (right) resins. Column: Vydac ($C_{18}$). Solvent: A = aqueous 0.1% TFA, B = acetonitrile with 0.1% TFA. Flow rate: 1.5 mL/min. Gradient: 0-75% B over 50 min.
Fig. 6.5 Chromatograms of crude peptide [Phe(N=C=S)₄,D-Pro¹⁰]Dyn A(1-11)NH₂ from PAL-PEG-PS (left) and PAL-PS (right) resins. For chromatographic conditions see Fig. 6.4.
Fig. 6.6 Chromatograms of crude peptide [Phe(NHCOCH₂Br)₄,D-Pro¹₀]Dyn A(1-11)NH₂ from PAL-PEG-PS (left) and PAL-PS (right) resins. For chromatographic condition see Fig. 6.4.
Aloc group while the peptides are attached to the resin are dependent upon the resin used. Our study shows that the PAL-PEG-PS resin facilitates the Aloc removal from protected \([\text{Phe(NHAlc)}^4,\text{D-Pro}^{10}]\text{Dyn A(1-11)NH}_2\), presumably due to the greater accessibility of the catalyst reagent compared to the PAL-PS resin. The crude potential Dyn A affinity label derivatives obtained from the PAL-PS resin, however, contained fewer side products than the peptides from the PAL-PEG-PS resin.
CHAPTER 7
SYNTHESIS OF A DYNORPHIN A PRECURSOR
FOR RADIOLABELING

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Abbreviations:

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature in Biochem. J. 1984, 219, 345-373. Amino acids are in the L-configuration except when indicated. Additional abbreviations used are as follows: Boc, t-butyloxycarbonyl; t-Bu, tert-butyl; DCM, dichloromethane, DIC, N,N'-diisopropylcarbodiimide; DIEA, N,N'-diisopropylethylamine; DMA, N,N-dimethylacetamide; Dyn A, dynorphin A; DMF, N,N-dimethylformamide; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (9-fluorenylmethoxy)carbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; OPfp, pentafluorophenyl ester; PAL, peptide amide linker; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TFA, trifluoroacetic acid.
7.1 Abstract

Protected \([\text{Phe}(3',5'-\text{I}_2,4'-\text{NH}_2)^4,\text{D-Pro}^{10}]\text{Dyn A}(1-11)\text{NH}_2, I\), was synthesized by solid phase synthesis using Fmoc-protected amino acids with \(t\)-butyl-type side chain protecting groups. The NovaSyn TG® resin was functionalized to bear a base-labile 4-hydroxymethylbenzoic acid linkage (substitution value 0.19 mmol/g) and used for the synthesis of the protected peptide \(I\). The peptide-resin linkage was cleaved by ammonolysis in isopropanol without removing other side chain protecting groups. Different ammonolysis times affected the recovery and purity of the protected peptide \(I\). The longest reaction time (5 days) gave the peptide with the highest purity (74.7%), but the lowest recovery (31.0%). Catalytic hydrogenation (or tritiation) of this peptide will yield the desired precursor for introduction of the affinity label group.
7.2 Introduction

Opiate drugs and opioid substances produce various pharmacological and physiological effects through a heterogenous opioid system. Both multiple opioid receptors (i.e. \(\mu\), \(\delta\) and \(\kappa\) opioid receptors) [Martin et al., 1976; Lord, 1977] and numerous endogenous opioid peptides (e.g. enkephalins, endorphins and dynorphins) [Holt et al., 1983] have been identified. Research in the opioid area involves the development of ligands with high selectivity for a particular receptor. Such ligands are useful as potential therapeutic agents and as tools for characterization of opioid drug-receptor interactions. Studies of an opioid receptor type can be greatly facilitated by using selective ligands.

Affinity labels are classified into electrophilic affinity labels and photoaffinity labels. Both are useful because of their complimentary applications. Electrophilic affinity labels can be utilized \textit{in vivo} for pharmacological studies, while photoaffinity labels are reversible until after photoactivation occurs [Glasel and Venn, 1981]. Non-peptide affinity labels have been used to irreversibly block selected receptor populations in tissues such as the guinea pig ileum and brain membranes which contain multiple receptor types, so that the remaining receptors can be studied in isolation [Rothman et al., 1990]. For example, BIT (2-(p-ethoxybenzyl)-1-diethylaminoethyl-5-isothioicyanobenzimidazole) selectively alkylates \(\mu\) opioid receptors [Rice et al., 1983], whereas FIT (N-phenyl-N-[1-(2-(p-isothiocyano)phenylethyl)-4-piperidinyl]propanamide) and superFIT selectively acylate \(\delta\) opioid receptors [Burke et al., 1983, 1986]. \(\beta\)-FNA (\(\beta\)-funaltrexamine) binds reversibly to both \(\mu\) and \(\kappa\) receptors, but only reacts irreversibly with \(\mu\) receptor. [Takemori and Portoghese, 1985]. Peptide-based affinity labels (e.g. DALECK (Tyr-D-Ala-Gly-Phe-LeuCH\(_2\)Cl), DALCE (Tyr-
D-Ala-Gly-Phe-leu-CysOH) and DAMK (Tyr-D-Ala-Gly-MePheCH₂Cl)) have also been prepared and used as biochemical tools to study μ and δ opioid receptors [Venn and Barnard, 1981; Szucs et al., 1987; Benhyhe et al., 1987; Bowen et al., 1987].

Affinity labels need to be labeled (i.e. with a radiolabel) for maximum utility. A radioligand should have very high affinity for the binding site to be labeled, be highly selective for that site and have high enough specific radioactivity to be usable at a low concentration in order to maximize its binding at the preferred site relative to that at the next-preferred site [Goldstein et al., 1988]. Radioisotopes with a high-energy mode of decay such as ³H and ¹²⁵I are normally incorporated into affinity label-containing ligands.

We are interested in studying κ opioid receptor structure and function, since selective agonists for κ receptor produce analgesia without the side effects associated with morphine or other μ receptor-selective analgesic agents [Millan, 1990; Rees, 1992]. Dyn A is a 17-amino acid endogenous peptide possessing high affinity and some selectivity for κ opioid receptors [Chavkin et al., 1982]. The [D-Pro¹⁰]Dyn A(1-11) analog, however, shows greater selectivity for κ receptors than the heptadecapeptide itself [Gairin et al., 1985; 1989]. The specific goal in this study was to synthesize potential affinity labels for κ opioid receptors using [D-Pro¹⁰]Dyn A(1-11) as a prototype. The peptide structure offers flexibility in the design of affinity labels and allows incorporation of other useful functionalities (e.g. radiolabel and/or biotin). Our goal is to identify modified Dyn A analogues which will retain high κ receptor affinity, exhibit κ receptor specificity and achieve irreversible attachment to κ opioid receptors. Peptides which show wash-resistant inhibition of binding to cloned wild type opioid receptors will subsequently be labeled using a radiolabel. The
labeled peptides will be reacted with cloned opioid receptors followed by isolation of the receptor complexes. The point of attachment of the affinity label can then be determined after proteolytic digestion of the receptor.

We designed a potential peptide affinity label with a radiolabel and an affinity labeling group incorporated in the same residue, so that the radiolabel will remain associated with the affinity label even after proteolytic digestion of affinity labeled receptors. Labeling methods for peptides depend upon the label incorporated (radiolabel and/or other label), sensitive groups on the peptide and the length of the peptide. This chapter describes the synthesis of a Dyn A precursor for radiolabeling. Since further modifications of the peptide is planned after assembly of the peptide chain, it is necessary to isolate the peptide in its protected form. Solid phase synthesis of the protected peptide requires an appropriate peptide-resin linkage and cleavage conditions which permit the peptide to be cleaved without removing the side-chain protecting groups. Thus, we functionalized the NovaSyn TG® resin and incorporated linker B (hydroxymethylbenzoic acid pentafluorophenyl ester) as a base-labile linkage. The NovaSyn TG® resin contains a polyethylene glycol polystyrene (PEG-PS) support which provides advantages over the commonly used polystyrene support. The PEG-PS support swells in a wide range of solvents and has greater solvation capacity than the PS support (chapter 6). The 9-fluorenylmethoxycarbonyl group (Fmoc) was utilized as the amine protecting group since it provides the flexibility of an orthogonal protection strategy and permits the use of mild acid for the final deprotection of side-chain protecting groups. We incorporated FmocPhe(3',5'-I,4'-NH₂) in position 4 of [D-Pro¹⁰]Dyn A(1-11) NH₂ peptide. After assembly of the peptide chain, the protected peptide was cleaved from
the functionalized NovaSyn TG® resin by ammonolysis: subsequent catalytic hydrogenation will yield protected peptide precursor of the affinity label. The conditions used for catalytic hydrogenation reaction will be optimized for small scale synthesis to give the unlabeled peptides. and similar conditions can later be applied to catalytic tritiation which will be performed by New England Nuclear. Following tritiation of the protected peptide, the affinity labeling group (e.g. isothiocyanate or bromoacetamide) can be attached to the p-amino group of the Phe(3',5'-3H₂,4'-NH₂) and the peptide deprotected (Fig. 7.1).

Fig. 7.1 Synthesis of tritium-labeled peptides bearing a reactive functionality.
7.3 Experimental section

7.3.1 Materials

The NovaSyn TG® resin was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Linker B-OPfp ester (hydroxymethylbenzoic acid-OPfp) and N,N-diisopropyl-ethylamine (DIEA) were purchased from Perseptives (Bedford, MA). FmocNle and FmocLys(Boc)NCA (N-carboxyanhydride) amino acids were obtained from BioResearch (San Diego, CA). Phe(3',5'-I₂,4'-NH₂) was from Bachem Bioscience (King of Prussia, PA), and it was converted to FmocPhe(3',5'-I₂,4'-NH₂) in this laboratory as described below. All other amino acids were obtained from Bachem Inc. (Torrance, CA). Reagents were obtained from Aldrich (Milwaukee, WI), and all solvents used (HPLC grade) were obtained from Burdick & Jackson (Muskegon, MI).

The peptides were synthesized on a Biosearch 9500 automated peptide synthesizer (Novato, CA). FmocPhe(3',5'-I₂,4'-NH₂) and crude peptides were analyzed on a Beckman System Gold high performance liquid chromatography (HPLC) system consisting of a model 126 solvent module, model 168 detector and model 507 autosampler. The HPLC column was a Vydac analytical column (C₁₈, 300 Å, 5 μ, 4.6 x 250 mm) equipped with a Vydac guard cartridge. The samples were eluted using a linear gradient of 0%-75% solvent B over 50 min with a flow rate of 1.5 mL/min (except where indicated) and detected at 214 nm; solvent A was aqueous 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA. Preparative reversed phase HPLC was performed on a Rainin gradient HPLC system using a Vydac semipreparative column (C₁₈, 300 Å, 10 μ, 21 x 250 mm) equipped with a Vydac
guard cartridge. The amino acid analysis was done by the Protein and Nucleic Acid Facility, Beckman Center, Stanford University. The samples were hydrolyzed for 24 h at 110 °C with 6 N HCl plus 1% phenol (peptide samples) or 1/1 propionic acid/conc HCl (peptide-resin samples) and analyzed on a Beckman Model 6300 amino acid analyzer using ninhydrin detection. Molecular weights of modified amino acids and synthetic peptides were determined by fast atom bombardment (FAB) mass spectrometry in the positive mode on a Kratos MS 50RFTC in the Department of Agricultural Chemistry at Oregon State University, Corvallis, OR. Elemental analyses were performed by MWH Laboratories, Phoenix, AZ.

7.3.2 Methods

7.3.2.1 NovaSyn TG* resin functionalization (Fig. 7.2)

NovaSyn TG* resin (1.01 g, reported substitution value: 0.29 mmol/g) was swollen with DCM (20 mL) for 15 min and then washed with DCM/DMA (1:1) (3 x 20 mL). The resin was neutralized with a 10% DIEA in DCM (3 x 15 mL) and washed with DCM (6 x 20 mL) and DCM/DMA (1:1) (3 x 20 mL). FmocNle was incorporated as an internal standard. FmocNle (0.51 g, 1.4 mmol) and HOBt (0.22 g, 1.4 mmol) in DMA (3.6 mL) were coupled to the resin with 3.2 mL of DIC (1.4 mmol) in DCM. The ninhydrin test was negative (yellow) after 2 h. An aliquot of peptide-resin was taken for Fmoc analysis (calcd. substitution value: 0.22 mmol/g). The Fmoc protecting group was removed using piperidine/toluene/DMA (30%/35%/35%), and the resin then washed with DCM/DMA and DCM (3 x 20 mL each). A positive blue color was obtained from the ninhydrin test. 4-
Fig. 7.2 NovaSyn TG® resin functionalization.
Hydroxymethylbenzoic acid pentafluorophenyl ester (linker B, 0.37 g, 1.2 mmol, 4-fold excess) was coupled to the resin with HOAt (0.16 g, 1.2 mmol) in DCM (5 mL), and the resin was washed with DCM/DMA (1:1) and DCM (5 x 20 mL each). The coupling, monitored by the ninhydrin test, was complete after 5 h. FmocLys(Boc)-NCA (0.43 g, 0.92 mmol, 3-fold excess) in a mixture of toluene (4.37 mL) and NMM (9.26 µL, 84.3 µmol) was coupled to the resin, and the resin then washed with toluene (5 x 10 mL) and MeOH (3 x 10 mL). Fmoc analysis after 1, 3 and 4 h showed substitution values of 0.22, 0.28 and 0.28 mmol/g, respectively. The resin was treated with 0.30 M 1-acetylimidazole in DCM (20 mL) for 30 min, washed with DCM/DMA (1:1) and DCM (3 x 20 mL each) and then dried in vacuo. Quantitative amino acid analysis after 4 h coupling showed a substitution of 0.19 and 0.16 mmol/g of Nle and Lys, respectively.

7.3.2.2 Synthesis of FmocPhe(3',5'-I₂,4'-NH₂)

Fmoc-Cl (0.38 g, 1.46 mmol) in dioxane (3 mL) was added to a solution of Phe(3',5'-I₂,4'-NH₂) (0.64 g, 1.47 mmol) in 10% Na₂CO₃/dioxane (1/1), 5 mL) and the mixture was stirred at 0 °C for 1 h and at room temperature for 12 h. The mixture was then poured into ice water (20 mL) and extracted with ether (2 x 20 mL). The aqueous layer was chilled in a ice bath and acidified with 1 N HCl to pH 2, the white precipitate collected by filtration, washed with 0.1 N HCl, H₂O and dried in vacuo. The residue was dissolved in EtOAc and the solution washed with 0.1N HCl and H₂O, and dried with MgSO₄. Fmoc Phe(3',5'-I₂,4'-NH₂) was recrystallized from THF (0.90 g, 93.7% yield): mp 132-134°C; TLC (silica gel, DCM/MeOH, 1:1) Rₜ = 0.62; FAB-MS m/z 655 (M+1); [α]D +27.6 (c 0.4, DCM).
Anal. calcd for C$_{24}$H$_{30}$I$_2$N$_2$O$_4$·1.5THF: C 47.24, H 4.2, N 3.67%. Found: C 47.64, H 3.97, N 3.73%.

7.3.2.3 Synthesis of protected [Phe(3',5'-I$_2$4',4'-NH$_2$)$_4$,D-Pro]$^{10}$Dyn A(1-11)NH-functionalized NovaSyn TG® resin (I) (Fig. 7.3)

The potential affinity label precursor protected [Phe(3',5'-I$_2$4',4'-NH$_2$)$_4$,D-Pro]$^{10}$Dyn A(1-11)NH$_2$, I, was assembled on the functionalized NovaSyn TG® resin (0.80 g, substitution value from quantitative amino acid analysis: 0.16 mmol/g). All amino acids used were Fmoc-amino acids, except for BocTyr(t-Bu) which was incorporated at the N-terminus. Side chain protecting groups used were Pmc for Arg, t-Bu for Tyr and Boc for Lys. The resin was swollen in DCM/CMA for 15 min before beginning the synthesis. The solid-phase synthesis cycle was started by washing the resin with 4 x 10 mL DCM/DMA and removing the Fmoc protecting group from the C-terminal amino acid using a piperidine/toluene/DMA (30%/35%/35%) solution. Fmoc-amino acids (4.0-fold excess in DMA) were coupled to the peptide with an equimolar amount of HOBt and an equal volume of 0.40 M DIC in DCM for 2 h, followed by washes with DCM/DMA (4 x 5 mL). After coupling the N-terminal amino acid, the peptide-resin was washed with DCM/DMA (10 x 5 mL), DCM (5 x 10 mL) and MeOH (4 x 5 mL) and dried in vacuo. The protected peptide-resin was submitted for quantitative amino acid analysis (%peptide found compared to Nle internal standard: 93.0%).
Fig. 7.3 Synthesis of dynorphin A precursor for radiolabeling.
7.3.2.4 Ammonolysis of protected peptide-resin 1

Aliquots of protected peptide-resin 1 (0.61, 0.52 and 0.95 g) were treated with ammonia in i-PrOH in a heavy-walled pressure bottle (250 mL, 6' long, 2.5' outer diameter) for 1, 3 and 5 days, respectively. At the end of the reaction time, the resins were filtered and washed with i-PrOH (3 x 10 mL). The i-PrOH filtrates were combined and evaporated to yield the crude protected peptide amide. The crude protected peptides were characterized by an analytical RP-HPLC column (C₁₈, 300 Å, 5 μ, 4.6 x 250 mm), where solvent A was aqueous 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA. A linear gradient of 55 to 100% acetonitrile over 30 min was used at a flow rate of 1.5 mL/min and peptides were detected at 214 nm. Aliquots of the resins left after ammonolysis were sent for amino acid analysis to determine percentages of cleavage. The crude protected peptide amide after 5 days ammonolysis was purified on a preparative RP-HPLC column (C₁₈, 300 Å, 5 μ, 22 x 250 mm) using the conditions described above except that the wavelength monitored was 280 nm. The purified peptide was analyzed by analytical RP-HPLC (tᵣ = 22.7 min) and FAB-MS ((M+1) = 2683).

7.4 Results and discussion

The dynorphin A precursor for labeling was prepared by solid phase peptide synthesis (SPPS) (Fig.7.3) and the protected peptide cleaved from the resin in preparation for further modification in solution. Thus N²-amino and side chain protecting groups and a peptide-resin linkage of differing labilities were needed. We utilized the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group to protect the N²-amine, and acid-labile tert-butyl (t-Bu)
type groups to protect the side chains of amino acids. A resin with a linker that yields protected peptide amides was preferred, since the amide derivatives of dynorphin A possess higher chemical and metabolic stability [Leslie and Goldstein, 1982] than the acid derivatives. Commercially available resins such as the PAL® (peptide amide linker) resin, an MBHA (4-methylbenzhydrylamine) resin with a 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid linker [Albericio et al., 1990], and the Samba resin (4-succinylamino-2,2',4'-trimethoxyphenyl-propionic acid) [Penke et al., 1989] yield peptide amides upon treatment with acids. Final cleavage requires concentrations of trifluoroacetic acid (TFA) which also remove the t-butyl-type side chain protecting groups used in Fmoc syntheses. We, therefore, developed a base-labile resin that yields protected peptide amides following ammonolysis.

7.4.1 NovaSyn TG® resin functionalization

The NovaSyn TG® resin was functionalized to incorporate the base-labile 4-hydroxy-methylbenzoic acid linkage (Fig. 7.2). The NovaSyn TG® resin contains a polyethylene glycol spacer which enhances its compatibility with various solvents. First, FmocNle was incorporated as an internal standard using FmocNle, DIC and HOBt; after removal of the Fmoc group by piperidine, 4-hydroxymethylbenzoic acid (linker B) was incorporated by its pentafluoro-phenyl ester and HOAt. The functionalized NovaSyn TG® resin was coupled to the intramolecularly activated FmocLys(Boc)-NCA, and subsequently capped with 1-acetyl-imidazole to acylate any unreacted sites. Coupling of FmocNle to the resin, as monitored by a negative result from the ninhydrin test, was complete within 2 h, and the
substitution value calculated from the Fmoc analysis was comparable to the reported value (0.22 vs 0.29 mmol/g, respectively). Coupling of 4-hydroxymethylbenzoic acid (linker B) to the Nle-NovaSyn TG® resin was complete after 5 h. The resin was then esterified with FmocLys(Boc)-NCA. Urethane-protected amino acid N-carboxy anhydrides (UNCAs) are very reactive amino acid derivatives, and thus are efficient reagents for peptide synthesis [Wakselman et al., 1994]. They are soluble in various solvents and can be used at high concentrations for difficult coupling reactions. UNCA's are activated internal mixed anhydrides which form peptide bonds directly, quickly and cleanly with carbon dioxide as the only byproduct [Fuller et al., 1990]. Our study shows that the FmocLys(Boc)-NCA was successfully esterified to the functionalized resin after 3 h; the completeness of the reaction was monitored by quantitative Fmoc analysis at 1, 3 and 4 h coupling and showed loadings of 0.22, 0.28 and 0.28 mmol/g, respectively. Quantitative amino acid analysis after Fmoc removal showed a substitution of 0.19 and 0.16 mmol/g resin for Nle and Lys, respectively.

7.4.2 Peptide syntheses

The synthesis of dynorphin A analogues in this model study involves modifications of phenylalanine in position 4 of [D-Pro10]Dyn A(1-11)NH2. The affinity label (e.g. isothiocyanate and bromoacetamide) and the radiolabel (e.g. tritium) group will be incorporated on the same residue. Tritiation of the model peptide will be accomplished by incorporation of Phe(3',5'-I2,4'-NH2) into the peptide (Fig. 7.1), followed by catalytic tritiation of a protected peptide precursor. We prepared FmocPhe(3',5'-I2,4'-NH2) from Phe(3',5'-I2,4'-NH2) using Fmoc-Cl under basic conditions. The product was obtained in a
good yield (93.7%) after recrystallization from THF (Fig. 7.4). Because of the bulk of the 3',5'-bisiodine functionality, the p-amino group was left unprotected during the assembly of the peptide chain.

The functionalized NovaSyn TG® resin described in the previous section was used for solid phase peptide chain assembly of the model dynorphin A precursor for labeling. The Fmoc protecting group was removed by piperidine/toluene/DMA (30%/35%/35%) and the next Fmoc-amino acid was coupled to the growing peptide chain using DIC and HOBt in DCM/DMA (1/1). Completeness of individual coupling reactions was monitored by the ninhydrin test. Coupling of each amino acid was achieved after 2 h, except for the D-Pro¹⁰ which required a recoupling. After peptide chain assembly, the peptide on the resin was submitted for amino acid analysis. The results shows a substitution value of 0.12 mmol/g, 93.1% of the expected value based on the internal standard, and the expected ratios of individual amino acids.

Protected peptide I was cleaved from the functionalized NovaSyn TG® resin by ammonolysis using isopropanol instead of methanol to avoid the methyl ester side product. Previous studies [Story and Aldrich, 1992] in our laboratory indicated that the yield and purity of the crude peptide Boc-Tyr-Gly-Gly-Phe-Leu-Arg(Pmc)NH₂ from ammonolysis depended upon reaction times. A negligible recovery of the peptide was obtained after 4 h ammonolysis, but the yield was increased to 83% when the reaction time was extended to 4 days. A 2.5" x 6", 250 mL heavy-walled pressure bottle was found to be optimum in these studies. In the current study, we compared reaction times of 1, 3 and 5 days for ammonolysis of protected peptide I using the same dimension bottle with a teflon lined cap. Cleavage of
Fig. 7.4 Chromatograms of Phe(3',5'-I-,4'-NH₂) (dotted line) and FmocPhe(3',5'-I-,4'-NH₂) (solid line). Column: Vydac (C₁₈). Solvent: A = aqueous 0.1%TFA, B = 0.1%TFA/AcCN. Flow rate: 1.5 mL/min. Gradient: 0-75%B over 50 min.
the peptide was determined by amino acid analysis by comparison of the amino acid content of peptide vs the internal standard Nle on the resin (Table 7.1). The majority of the protected peptide was cleaved from the resin after 1 day ammonolysis (87.4% cleavage), while the recovery of peptide for different reaction times varied (%recovery: 54.8, 67.0 and 31.0%, respectively). The crude protected peptide after ammonolysis showed a major peak by analytical RP-HPLC ($t_R = 22.7$ min, Fig. 7.5) plus several side products. The longer reaction time (5 days) gave the crude protected peptide of the highest purity (74.7%) with an acceptable recovery (31.0%). The crude protected peptide with lower purities, 27.6 and 22.9%, was obtained after 1 and 3 days ammonolysis (Fig. 7.5), respectively. The crude protected peptide after 5 days ammonolysis was purified by reverse phase preparative HPLC and further characterized by analytical reverse phase HPLC and FAB-MS. The pure peptide obtained showed a single peak (96%) by reverse phase HPLC and the expected mass by FAB-MS ($\text{[(M+H)$^+$]} = 2683$).

The ultimate goal of our study is to prepare dynorphin A analogues containing a radiolabel and an affinity labeling group on the same residue. These labeled peptides, hopefully, will assist in the isolation and characterization of $\kappa$ opioid receptors. The procedures described in this chapter will be used as guidelines for the synthesis of such analogues. The optimum conditions will be determined for the catalytic hydrogenation and subsequently applied to the catalytic tritiation (to be performed at New England Nuclear). An affinity labeling group (e.g. isothiocyanate or bromoacetamide) will then be incorporated into the p-amino group of the Phe(3',5'-3$^3$H$_2$4'-NH$_2$), followed by deprotection of the peptide. The purified tritiated affinity label peptides will be characterized by analytical RP-HPLC.
Table 7.1 Characterization of [Phe(3',5'-I,4'-NH₂)₄, D-Pro¹⁰]Dyn A(1-11)NH₂ before and after ammonolysis¹.

<table>
<thead>
<tr>
<th></th>
<th>Amino acid analysis</th>
<th>Peptide substitution (mmol/g)</th>
<th>Nle internal standard (mmol/g)</th>
<th>% Peptide on resin</th>
<th>% Cleavage (% Recovery)</th>
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<tbody>
<tr>
<td>Before cleavage</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Y² (1) G (2) F (1)</td>
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<td></td>
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<tr>
<td></td>
<td>L (3) R (1) I (1)</td>
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<td></td>
<td>P (1) K (1)</td>
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<tr>
<td></td>
<td></td>
<td>1.53 0.88 0.97 3.35 0.98 0.96</td>
<td>1.33</td>
<td>0.12</td>
<td>0.13</td>
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<tr>
<td>After cleavage</td>
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<td>5 d</td>
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</table>

¹Theoretical values are shown in parentheses.
²Tyr was destroyed during hydrolysis and was excluded from the calculation.
Fig. 7.5 Chromatograms of crude protected peptide I after ammonolysis; 1 (top), 3 (middle) and 5 (bottom) days. Column: Vydac (C18). Solvent: A = aqueous 0.1%TFA, B = 0.1%TFA/AcCN. Flow rate: 1.5 mL/min. Gradient: 55-100% B over 30 min.
7.5. Conclusions

The Fmoc protection strategy provides orthogonality for the synthesis of protected peptide amides. A proper peptide-resin linkage which permits cleavage without removing the side chain protecting groups is required. The NovaSyn TG® resin containing a base-labile 4-hydroxymethylbenzoic acid linkage was successfully developed in our laboratory and used for peptide synthesis. The resin gave the expected model dynorphin A precursor I, [Phe(3',5'-I₂,4'-NH₂)₄,D-Pro₁⁰]Dyn A(1-11)NH₂, needed for labeling with 93.1% of the expected peptide substitution, calculated from amino acid analysis of peptide-resin. The protected peptide I was liberated from the resin by ammonolysis in isopropanol, and greater than 85% of peptide was successfully cleaved from the resin. The longest reaction time (5 days) gave the crude peptide with the highest purity (74.7%) compared to those from 1 (27.6%) or 3 (22.9%) days ammonolysis.
Potential affinity label derivatives of dynorphin A based on [D-Pro$^{10}$]Dyn A(1-11)NH$_2$ were synthesized for use as pharmacological probes for $\kappa$ opioid receptors. A reactive functionality (isothiocyanate or bromoacetamide) was incorporated into the N-terminal "message" or C-terminal "address" sequences of the parent peptide [D-Pro$^{10}$]Dyn A(1-11)NH$_2$. Modifications in the "message" sequence (chapter 4) involved the replacements of tyrosine in position 1 with the isothiocyanate derivative of phenylalanine, and phenylalanine in position 4 with the isothiocyanate or bromoacetamide derivatives of $p$-aminophenylalanine. Structural changes in the "address" sequence (chapter 5) were performed at isoleucine in position 8; Ile$^8$ was replaced with either the isothiocyanate or bromoacetamide derivatives of $p$-aminophenylalanine or lysine. The peptides containing an amine (i.e. without a reactive functional group) were also prepared and used as reversible controls for the pharmacological assays.

We have developed synthetic strategies for the preparation of these potential affinity label derivatives of dynorphin A. Initially, all protected peptides were assembled on a polyethylene glycol (PEG)-polystyrene (PS) support with a peptide amide linker (PAL) using standard Fmoc chemistry. The allyloxycarbonyl (Aloc) group was introduced into the side chain of the amino acid residue where a reactive functional group was to be incorporated in order to provide orthogonal protection. The Aloc group can be selectively removed by palladium (0) in DCM/5% AcOH/2.5% $N$-methylmorpholine, and the reactive functional
group then introduced. Finally, peptides were liberated from the support and other side chain protecting groups were removed simultaneously using modified reagent K (87.5% TFA/5% phenol/5% H2O/2.5% thioanisole); ethanedithiol was excluded from the cleavage cocktail to prevent reaction with the alkylating group. Reverse phase HPLC analysis of the crude peptides after cleavage indicated the purity of the peptides. Crude peptides from the [Phe(X)4,D-Pro10]Dyn A(1-11)NH2 series, where X = -NH2 (amine compound) or -N=C=S (for the potential affinity label derivative), and [Lys(X)8,D-Pro10]Dyn A(1-11)NH2 series, where X = -H (amine compound), or -N=C=S and -NHCOC2H2Br (for potential affinity label derivatives), were obtained in excellent purity. Affinity label derivatives from the other series, namely [Phe(X)4,D-Pro10]Dyn A(1-11)NH2] and [Phe(X)8,D-Pro10]Dyn A(1-11)NH2 where X = -N=C=S and = -NHCOC2H2Br, however, contained side products, including the starting amine and other side products, (e.g. the hydroxyl derivative (-NHCOC2H2OH)) as well.

The peptides' affinities (IC50 values) for different opioid receptors were determined in radioligand binding assays using cloned opioid receptors stably expressed in Chinese hamster ovary cells. Preliminary binding assay data (Table 4.3 and 5.2) indicated that, except for the bromoacetamide derivative of [Phe(NH2)4,D-Pro10]Dyn A(1-11)NH2, all peptides had high affinity for κ opioid receptors. Surprisingly, [Lys8,D-Pro10]Dyn A(1-11)NH2 exhibited the highest affinity for κ opioid receptors (IC = 0.16 nM) with comparable κ selectivity to the parent peptide. The isothiocyanate derivatives generally showed higher affinity for κ opioid receptors than did the bromoacetamide derivatives (the bromoacetamide group was not introduced into Phe4). Incorporation of a reactive functional
group in the N-terminal "message" and C-terminal "address" sequences of Dyn A yielded peptides with varying selectivity for opioid receptors: $\kappa > \mu >> \delta$ (for the "message" sequence modifications) and $\kappa = \mu > \delta$ (for the "address" sequence modifications).

Since some of the potential affinity label derivatives of Dyn A assembled on PAL-PEG-PS resin contained side products, we investigated a different resin, PAL-PS resin, using [Phe(X)\(^4\),D-Pro\(^{10}\)]Dyn A(1-11)NH\(_2\) as a model (chapter 6). Rates of Aloc deprotection and purity of crude peptides from the two resins were compared. Aloc deprotection was greatly enhanced on the polyethylene glycol polystyrene support; removal of Aloc from peptides assembled on this support was complete within 3 h compared to 24 h from peptides assembled on a polystyrene support. The polyethylene glycol spacer in the PAL-PEG-PS resin may play an important role in enhancing Aloc deprotection rates by allowing complete solvation of the reactive sites which would result in a more efficient reaction. Crude peptides assembled on both resins were characterized by reverse phase HPLC. The amine compounds assembled on either the PAL-PEG-PS or the PAL-PS resin both showed excellent purity, but the potential affinity label derivatives assembled on the PAL-PEG-PS resin contained more side products than those synthesized on the PAL-PS resin.

The last part of this research focused on the synthesis of a dynorphin A precursor for radiolabeling using protected [Phe(3',5'-I\(_2\),4'-NH\(_2\),D-Pro\(^{10}\)]Dyn A(1-11)NH\(_2\) as a prototype. NovaSyn TG\(^\circ\) resin was functionalized by incorporating a base-labile 4-hydroxymethylbenzoic acid linkage (linker B) (substitution value 0.19 mmol/g) and used for the synthesis. The protected peptide was obtained with 93.1% of the expected peptide substitution, calculated from comparison of amino acid analysis of the peptide on the resin vs. an internal
Almost 90% of the protected peptide was liberated from the resin after 1 day ammonolysis in isopropanol. An extended reaction time (5 day), however, gave the product with higher purity. This peptide can then be subjected to catalytic tritiation and the affinity label group then introduced. This strategy allows us to introduce a radiolabel and affinity labeling group on the same residue. The radiolabel will then remain associated with the affinity label even after proteolytic digestion of affinity labeled receptors. The radiolabel affinity label peptides will hopefully be useful for studies of interactions of peptide ligands with κ opioid receptors.

Additional pharmacological assays will be performed to identify affinity label derivatives of dynorphin A which bind irreversibly to opioid receptors. The synthetic strategies described in the previous section will then be applied to these lead peptides, optimum conditions will then be determined for catalytic tritiation (to be performed at New England Nuclear), and a reactive functionality (e.g. isothiocyanate or bromoacetamide) will be incorporated into the tritiated protected peptide. Deprotection will then give the desired peptides containing both an affinity label and tritium label which can be used in future pharmacological studies.


Fournie-Zaluski, M.-C.; Chaillet, C.; Bouboutou, R.; Couland, A.; Cherot, P.; Wakaman, G.; Costentin, J.; Roques, B.P. Analgesic Effects of Kelatorphan a New Highly


Schiller, P.W.; Nguyen, T.M.-D.; Lemuex, C. New Types of Opioid Peptide Analogues Showing High μ-Receptor Selectivity and Preference for Either Central or Peripheral


