Development of Substance P 1–7 Related Peptides and Peptidomimetics

Targeting Neuropathic Pain

ANNA SKOGH
Abstract


The neuropeptide substance P 1–7 (SP<sub>1–7</sub>, H-Arg<sup>1</sup>-Pro<sup>2</sup>-Lys<sup>3</sup>-Pro<sup>4</sup>-Gln<sup>5</sup>-Gln<sup>6</sup>-Phe<sup>7</sup>-OH) and its amidated analogue SP<sub>1–7</sub>amide, have displayed intriguing effects in experimental models for neuropathic pain acting on a specific, yet unknown SP<sub>1–7</sub> target. The aim of this thesis was to design and synthesise SP<sub>1–7</sub> related peptides and peptidomimetics, to be used as research tools to study the SP<sub>1–7</sub> system, and to serve as drug leads in the neuropathic pain area.

The in vivo structure activity relationship (SAR) of the SP<sub>1–7</sub>amide was elucidated using Ala-substituted, N-terminally truncated and N-methylated variants. By evaluation of the anti-allodynic effect in spared nerve injury (SNI) mice and the pharmacokinetic properties it is suggested that Phe<sup>7</sup> acts as a message residue and Arg<sup>1</sup> as an address residue, both important for the overall anti-allodynic activity. In contrast, Lys<sup>3</sup> could be substituted by alanine, and the Pro<sup>2</sup>-Lys<sup>3</sup> and Pro<sup>4</sup>-Gln<sup>5</sup> bond could be N-methylated with retained anti-allodynic effect. The Pro<sup>2</sup>-Lys<sup>3</sup> bond was found most sensitive towards proteolysis and indeed, N-methylation of this bond delivered peptides completely inert in plasma. Conversely, prolonged plasma stability did not improve the overall in vivo activity for these peptides. Instead, the SP<sub>1–7</sub>amide remained the most potent peptide in vivo, despite fast degradation in plasma.

Besides peptide synthesis, the synthetic work included development of a Pd-catalysed aminocarbonylation protocol using an amino acid nucleophile, which was used for the synthesis of an imidazole-based peptidomimetic. This peptidomimetic was equipotent to the SP<sub>1–7</sub>amide, and more potent than the drug gabapentin, in regard to its anti-allodynic effect in SNI mice, and it is a promising drug lead for further development. The Pd-catalysed aminocarbonylation protocol was refined further, in regards to reaction scope and requirements for solid-phase peptide synthesis and has proven useful for N-capping, isotopic labelling, and intramolecular cyclisation of peptides.

In summary, the work presented herein resulted in an in vivo SAR for SP<sub>1–7</sub> related peptides, a novel small molecule SP<sub>1–7</sub> peptidomimetic, and methods expanding the toolbox for synthesising modified peptides and peptidomimetics – a field in drug discovery that presently gaining increasing attention.

Keywords: Substance P 1–7, Peptidomimetics, Solid-phase Peptide Synthesis (SPPS), Palladium catalysis, Carbonylation, Imidazole, Bioisostere, Neuropathic pain, Allodynia, Structure-activity relationships

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Till Johan och Erik
List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


† joint first author

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
</tr>
<tr>
<td>Ala</td>
<td>alanyl, alanine</td>
</tr>
<tr>
<td>Alloc</td>
<td>allyloxy carbonyl</td>
</tr>
<tr>
<td>Arg</td>
<td>arginyl, arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartyl, aspartic acid</td>
</tr>
<tr>
<td>AUC</td>
<td>area under curve</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>t-Bu</td>
<td>tert-butyl</td>
</tr>
<tr>
<td>Cl&lt;sub&gt;int&lt;/sub&gt;</td>
<td>intrinsic clearance</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CORM</td>
<td>CO-releasing molecules</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxyl-terminal</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diaza bicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMAP</td>
<td>N,N-dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>post-proline dipeptidyl (amino) peptidase IV</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>effective concentration giving 50% effect</td>
</tr>
<tr>
<td>EM-1/-2</td>
<td>endomorphine-1 or 2</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylethoxycarbonyl</td>
</tr>
<tr>
<td>Gln</td>
<td>glutaminyl, glutamine</td>
</tr>
<tr>
<td>Gly</td>
<td>glycyl, glycine</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HATU</td>
<td>N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylm ethanaminium hexafluorophosphate N-oxide</td>
</tr>
<tr>
<td>HBA</td>
<td>hydrogen bond acceptor</td>
</tr>
<tr>
<td>HBD</td>
<td>hydrogen bond donor</td>
</tr>
<tr>
<td>HBTU</td>
<td>N-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide</td>
</tr>
<tr>
<td>HOAt</td>
<td>1-hydroxy-7-azabenzotriazole</td>
</tr>
<tr>
<td>HOBt</td>
<td>1-hydroxybenzotriazole hydrate</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>inhibitor concentration giving 50% inhibition</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>Symbol/Abbreviation</td>
<td>Term/Description</td>
</tr>
<tr>
<td>---------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>i.t.</td>
<td>intrathecal</td>
</tr>
<tr>
<td>$K_i$</td>
<td>equilibrium dissociation constant for inhibitor binding</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>Leu</td>
<td>leucyl, leucine</td>
</tr>
<tr>
<td>Lys</td>
<td>lysyl, lysine</td>
</tr>
<tr>
<td>MBHA</td>
<td>4-methylbenzhydrylamine</td>
</tr>
<tr>
<td>MTBD</td>
<td>7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene</td>
</tr>
<tr>
<td>NEP</td>
<td>neutral endopeptidase</td>
</tr>
<tr>
<td>NIS</td>
<td>$N$-iodosuccinimide</td>
</tr>
<tr>
<td>NK-1/-2/-3</td>
<td>neurokinin receptor subtype 1, 2 or 3</td>
</tr>
<tr>
<td>NKA/B</td>
<td>neurokinin A or B</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>Mtt</td>
<td>para-methyltrityl</td>
</tr>
<tr>
<td>$o$-NBS-Cl</td>
<td>orto-nitrobenzenesulfonyl chloride</td>
</tr>
<tr>
<td>Pbf</td>
<td>2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfon</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>P-gp</td>
<td>permeability glycoprotein</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylalanin, phenylalanine</td>
</tr>
<tr>
<td>Pip</td>
<td>piperidine</td>
</tr>
<tr>
<td>PivOH</td>
<td>pivalic acid</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PPCE</td>
<td>post-proline-cleaving enzymes</td>
</tr>
<tr>
<td>Pro</td>
<td>prolyl, proline</td>
</tr>
<tr>
<td>PS</td>
<td>polystyrene</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reversed-phase high performance liquid chromatography</td>
</tr>
<tr>
<td>r.t.</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAR</td>
<td>structure–activity relationship</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SNI</td>
<td>spared nerve injury</td>
</tr>
<tr>
<td>SNRI</td>
<td>serotonin-noradrenaline reuptake inhibitor</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>SP$_{1-7}$</td>
<td>substance P 1–7</td>
</tr>
<tr>
<td>SPE</td>
<td>substance P endopeptidase</td>
</tr>
<tr>
<td>SPPS</td>
<td>solid-phase peptide synthesis</td>
</tr>
<tr>
<td>TCA</td>
<td>tricyclic antidepressant</td>
</tr>
<tr>
<td>TES</td>
<td>triethylsilane</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>Trt</td>
<td>trityl</td>
</tr>
</tbody>
</table>
Introduction

Peptides, defined as compounds consisting of 2–50 amino acids linked in a chain by amide bonds (i.e. peptide bonds), are often natural ligands for cell surface receptors (e.g. G-protein coupled receptors, GPCRs) and play vital roles in physiological processes in the human body. Thus, biologically active peptides like hormones and neuropeptides serve as interesting starting points in drug discovery programmes. The presence of basic, acidic, and hydrophobic amino acids containing numerous rotatable bonds make peptides highly complex with potentially a large degree of conformational freedom. Natural peptides frequently possess high biological activity, high specificity and low toxicity but are also commonly membrane impermeable and biologically unstable. Consequently, their inherent properties “define their strength and weaknesses as drug molecules”.1

In drug discovery programmes starting from a natural ligand, rational design strategies can be applied to reduce the peptide character of the peptide to ultimately generate orally bioavailable non-peptide compounds with the same biological response as the original peptide. Such analogues are known as peptidomimetics.2,3 However, due to the complexity of peptides as they are, they constitute important entities as potential future pharmaceuticals for addressing difficult targets and diseases with unmet medical need.4,5 Since 2000, 28 new peptide drugs have been approved worldwide and the total sales of peptide drugs were estimated to be 40 billion EUR in 2015, corresponding to 5% of the global drug market. This is expected to reach 60 billion EUR in 2019.1 Therapeutic use for peptide drugs are mainly found in areas such as metabolic diseases and oncology but they have also been explored in cardiovascular, urology, antiviral and antimicrobial, infertility, and pain management areas.1

The expanding market of peptide drugs has renewed interest in synthetic peptides. Solid-phase peptide synthesis (SPPS) was introduced by R. B. Merrifield back in 1963 and revolutionised the field of peptide synthesis.6 Since then, the method has been refined and many chemical approaches, compatible with SPPS, have emerged and expanded the toolbox for synthesising complex peptides. Numerous strategies are available to improve the pharmacokinetic properties of peptide drugs, such as N-methylation of the backbone, modification of the peptide terminus (e.g. N-capping), introducing constrained or unnatural amino acids and various cyclisation methods.7–10 In addition, although the preferred route of administration is through oral
delivery, new and more patient-friendly injection techniques as well as alternative formulation strategies for peptide drugs are emerging.4

Substance P 1–7 (SP1–7) is the major bioactive fragment of the neuropeptide substance P (SP) and is, together with peptidomimetics derived from this heptapeptide, the main focus of this thesis due to their intriguing effects on neuropathic pain (Figure 1).

![Figure 1. The bioactive neuropeptide substance P 1–7.](image)

Neuropathic Pain

Pain is a subjective experience, typically a direct response to tissue damage from an injury (nociceptive pain) or inflammation (inflammatory pain) but can also occur as a consequence of brain or nerve injury (neuropathic pain).11 Neuropathic pain is a maladaptive neural function as the pain experience is no longer a ‘normal’ response serving a protective function.

Neuropathic pain is defined as:

“Pain caused by a lesion or disease of the somatosensory nervous system”

– International Association for the Study of Pain

and by this definition separate from dysfunctional pain where there is no identifiable damage to the nervous system.11,12

Neuropathic pain can have either central or peripheral origin, however, the presence of mixed neuropathic pain is not unusual.13,14 Central neuropathic pain results from a lesion in the central nervous system (CNS) such as traumatic or non-traumatic spinal cord injury (SCI), stroke or multiple sclerosis (MS) while peripheral neuropathic pain results from a lesion in the peripheral nervous system (PNS) due to mechanical injury, metabolic disease (e.g. diabetes mellitus), virus infection (herpes zoster or human immunodeficiency virus, [HIV]), a neurotoxic chemical, or tumour invasion.11,13 The primary disease or injury (i.e. the etiological cause) and resulting neural damage to either the CNS or PNS initiates a cascade of changes in the nerves, that leads to maladaptive response in the nociceptive
pathways, giving rise to neuropathic pain (Figure 2). Neuropathic pain is characterised by spontaneous pain and sensory amplification like hyperalgesia (increased pain from a stimulus that usually provokes pain) and allodynia (pain due to a stimulus that usually does not provoke pain). The diversity of etiology causing the primary neural damages show how complex neuropathic pain can be in its clinical expression and patients with the same etiological cause can have very different pain distribution (i.e. phenotype profile). However, neural damage does not inevitably progress to neuropathic pain since recovery at an early stage is possible. Identifying the underlying pathophysiological mechanisms (i.e. pain mechanisms) as well as genetic, environmental and psychosocial factors that contribute to the risk of developing neuropathic pain is therefore of interest in preventing the damage from becoming chronic.

Figure 2. Possible outcome of neural damage in the central and peripheral nervous system.

Prevalence and General Health Status

Chronic pain is a heterogenic condition that can have either nociceptive or neuropathic origin. It is considered a major health problem with an average prevalence of 20% in the general population, where it is more common among women and people more than 50 years of age. The prevalence of chronic pain with neuropathic character in the general population has been estimated to be in the range of 7–10%.

Patients with chronic neuropathic pain exhibit higher mean pain severity scores than other chronic pain conditions and the condition is, as expected, associated with reduced function in all aspects of daily activities and a negative impact on family, social and professional life. In addition to the well-documented loss of quality of life when living with chronic pain, it has recently also been connected with a reduction in life expectancy.
Pharmacological Treatment

Neuropathic pain is associated with unsatisfactory pain management and possible undertreatment,\textsuperscript{25} due to the lack of efficacious safe pharmacological treatments.\textsuperscript{26} In addition, it is difficult to diagnose patients with neuropathic pain\textsuperscript{14,27} and identify those patients who will respond to available pharmacological treatment.\textsuperscript{28}

Patients with neuropathic pain respond less well to conventional analgesic drugs (e.g. opioids and NSAIDs). Considering the potential risk of abuse and overdose mortality, strong opioids (e.g. morphine and oxycodone) are only recommended as a third-line treatment.\textsuperscript{29} The current first-line treatment includes serotonin and noradrenaline reuptake inhibitors (SNRI), tricyclic antidepressants (TCA) and the anti-convulsing drugs gabapentin and pregabalin.\textsuperscript{29} Gabapentin is one of the most commonly used drugs, and is, despite its adverse effects, a relatively safe drug.\textsuperscript{30}

Neuropathic pain is historically treated based on its etiological cause. However, as the neuropathic pain progresses, the primary disease/injury becomes less relevant and the pain is no longer a symptom but becomes the disease itself.\textsuperscript{11} Treatment based on phenotype profiling (i.e. personalised pain treatment) is a promising alternative that suggests that each phenotype has its own pain mechanism and thereby its own specific molecular target.\textsuperscript{31} However, further research is needed to clarify underlying pain mechanisms that could be interesting new avenues for treatment of neuropathic pain.

The Substance P System

The neuropeptide substance P (SP, H-Arg\textsuperscript{1}-Pro\textsuperscript{2}-Lys\textsuperscript{3}-Pro\textsuperscript{4}-Gln\textsuperscript{5}-Gln\textsuperscript{6}-Phe\textsuperscript{7}-Phe\textsuperscript{8}-Gly\textsuperscript{9}-Leu\textsuperscript{10}-Met\textsuperscript{11}-NH\textsubscript{2}) was discovered in 1931 by von Euler and Gaddum\textsuperscript{32} and was chemically characterised by Leeman and co-workers in 1971 (Table 1).\textsuperscript{33} In general, neuropeptides are widely distributed in the nervous system (i.e. CNS and PNS) and have their own unique distribution patterns. They coexist in the neurons with classical transmitters where they act as neurotransmitters and neuromodulators of various biological functions. The expression of specific neuropeptides is highly dynamic and is either constant at high levels under normal circumstances or upregulated after a pathological condition such as neural damage.\textsuperscript{34}

A member of the tachykinins,\textsuperscript{35,36} SP is the endogenous ligand for the G-protein-coupled neurokinin-1 (NK-1) receptor and is important in pain transmission in the spinal cord.\textsuperscript{37,38} Additionally, SP is involved in diverse processes in the CNS which include stress and anxiety,\textsuperscript{39} depression,\textsuperscript{40} drug addiction,\textsuperscript{41} migraine,\textsuperscript{42} nausea and vomiting\textsuperscript{43} as well as induction and progression of inflammatory responses.\textsuperscript{44,45}
Table 1. Mammalian tachykinins and their preferred neurokinin receptor subtype. Conserved amino acids are highlighted.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Amino acid sequence</th>
<th>Receptor subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂</td>
<td>NK-1</td>
</tr>
<tr>
<td>NKA</td>
<td>H-His-Lys-Thr-Asp-Ser-Val-Gly-Leu-Met-NH₂</td>
<td>NK-2</td>
</tr>
<tr>
<td>NKB</td>
<td>H-Asp-Met-His-Asp-Phe-Val-Gly-Leu-Met-NH₂</td>
<td>NK-3</td>
</tr>
</tbody>
</table>

H- denotes a free N-terminal α-amino group, -NH₂ a C-terminal α-carboxyamide.

Additional members in the tachykinin family are displayed in Table 1 together with their preferred neurokinin receptor subtype. The tachykinins are all characterised by the carboxyl-terminal (C-terminal) sequence Phe-X-Gly-Leu-Met-NH₂, where the X residue is either an aromatic or branched aliphatic amino acid.36 This common C-terminal region of the peptides accounts for the activity at the receptor (i.e. activates signal transduction) and hence acts as a message sequence. The divergent amino-terminal (N-terminal) region, represents an address sequence and accounts for receptor subtype selectivity.47 The message-address concept, initially defined for peptide hormones,48 has been shown to be of relevance also for opioid neuropeptides in their interactions with corresponding G-protein-coupled opioid receptors as well as the tachykinins in their interactions with the neurokinin receptors.

Due to the involvement of SP in various physiological conditions, antagonists to the NK-1 receptor have been considered as potential compounds for treatment of certain diseases, especially as pharmaceuticals for treatment of pain (Figure 3).49,50 Among the first selective non-peptide NK-1 receptor antagonists were CP96345, CP99994, and RP67580, identified through random screening, which showed promising results in various animal models of pain. RP67580 was equipotent with morphine regarding its anti-nociceptive activity. However, problems emerged with nonspecific effects at the L-type Ca²⁺ channels and the compounds displayed species differences in binding to the NK-1 receptor. PD154075 and CGP49823, in contrast, were identified through rational drug design strategies based on the natural ligand (i.e. SP), displayed promising anti-nociceptive effects in animal pain models. However, when tested in humans they displayed no analgesic activity. The only NK-1 receptor antagonist that has reached the market is aprepitant in 2003, developed by Merck and approved as an antiemetic agent (Figure 1).51

Although new NK-1 receptor antagonist pain drugs have failed to reached the market, the NK-1 receptor antagonist and agonists52 have served as important research tools to understand the SP system, including ligand–receptor interactions and receptor subtype selectivity (Figure 3). Additionally, ¹¹C-labelled NK-1 receptor antagonists, like [¹¹C]GR205171,53 have been valuable radiotracers in positron emission tomography (PET) to study brain uptake, distribution pattern, and binding selectivity of the tracer.
Figure 3. NK-1 receptor antagonists and agonist.

The Bioactive Metabolite SP$_{1-7}$ and its Binding Site

In contrast to what is seen for classic transmitters, the biological effect of neuropeptides are terminated by enzymatic degradation since neurons lack active uptake mechanisms of intact peptides. The enzymes responsible for the degradation of SP are the post-proline dipeptidyl (amino)peptidase IV (DPP-IV), the post-proline-cleaving enzymes (PPCE), the neutral endopeptidase (NEP), the angiotensin-converting enzyme (ACE), and the substance P endopeptidase (SPE) (Figure 4). Enzymatic cleavage of SP at the Phe$^7$-Phe$^8$ bond generates the biologically active N-terminal fragment substance P $1-7$ (SP$_{1-7}$, H-Arg$^1$-Pro$^2$-Lys$^3$-Pro$^4$-Gln$^5$-Gln$^6$-Phe$^7$-OH). The interest in this heptapeptide was raised in 1983 when Hall and Stewart observed that this N-terminal fragment had effects opposite to those shown by the C-terminal fragments which often mimic the parent peptide. Thus, SP$_{1-7}$ has shown ant-nociceptive, anti-inflammatory, and anxiolytic effects. It also attenuates several withdrawal symptoms in morphine-dependent rodents as well as the development of morphine tolerance. The anti-nociceptive effects demonstrated by SP$_{1-7}$ are the main focus in our research group. In particular, its positive effects in alleviating both central and peripheral neuropathic pain. Thus, central (intrathecal, i.t.) administration of the heptapeptide has shown potent anti-hyperalgesic effects in mice suffering...
from streptozotocin (STZ) induced diabetic neuropathy and peripheral administration (intraperitoneal, i.p.) of SP1‒7 produced anti-allodynic effects in both spared nerve injury (SNI) mice and spinal cord injury (SCI) rats.61–63

Although the actions of SP1‒7 have been known for a long time no macromolecular target has been identified and the mechanism of action is still unknown. As SP1‒7 is missing the message part important for binding to the NK-1 receptor, the heptapeptide consequently does not exert its biological effect through this receptor.64 Early observations suggest the presence of an SP1‒7 receptor65,66 and binding sites were found in brain and spinal cord of both mice and rats.67–69 To discriminate between the SP1‒7 target and other known receptors, a variety of neurokinin and opioid receptor ligands and non-peptides were screened towards the SP1‒7 target (Table 2).68

The C-terminal Phe7 residue seemed fundamental for specific binding because truncation to SP1‒6 or elongation to SP1‒8, SP1‒9 or to the full SP sequence weakened the binding when compared to SP1‒7. In contrast, D-SP1-7, an antagonist for the SP1‒7 target with retained aromaticity in position 7, has high affinity for the binding site. The selective ligands for NK-1, NK-2 and NK-3 receptor ([Sar9, Met(O2)11]SP, R-396, and senktide, respectively) show no affinity to the SP1‒7 binding site. Interestingly, the μ-receptor agonist endomorphine-2 (EM-2) and the synthetic opioid ligand DAMGO had high affinity for the SP1‒7 binding site. The observed difference for the binding between EM-1 and EM-2 suggests that the specific SP1‒7 target is also separated from the μ-receptor. The non-specific opioid receptor antagonist naloxone has been shown to attenuate several in vivo effects observed for SP1‒7, but had no affinity for the binding site (Table 2). It has been suggested that naloxone could also bind to a subtype of sigma receptors70 and in fact, the specific σ1-receptor agonist (+)-pentazocine reduces the anti-hyperalgesic effect of SP1‒7 in diabetic mice.61 However, SP1‒7 does not bind to the σ1-receptor63 which indicates that the heptapeptide influences the σ1 system in a downstream manner.
Table 2. Binding affinity of various neuropeptides and ligands as determined by inhibition of [3H]SP1‒7 binding to rat spinal cord membrane.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Amino acid sequence</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1‒7</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH</td>
<td>0.75</td>
</tr>
<tr>
<td>SP1‒6</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-OH</td>
<td>1830</td>
</tr>
<tr>
<td>SP1‒8</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-OH</td>
<td>74</td>
</tr>
<tr>
<td>SP1‒9</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Gly-OH</td>
<td>88</td>
</tr>
<tr>
<td>SP</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Gly-Leu-Met-NH$_2$</td>
<td>159</td>
</tr>
<tr>
<td>D-SP1‒7</td>
<td>H-Arg-D-Pro-Lys-Pro-Gln-Gln-d-Phe-OH</td>
<td>1.78</td>
</tr>
<tr>
<td>[Sar$^9$, Met(O$<em>2$)$</em>{11}$]SP</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Sar-Leu-Met(O$_2$)-NH$_2$</td>
<td>814</td>
</tr>
<tr>
<td>R-396</td>
<td>Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH$_2$</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Senktide</td>
<td>Suc-Asp-Phe-MePhe-Gly-Leu-Met-NH$_2$</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>DAMGO</td>
<td>Tyr-D-Ala-Gly-MePhe-Gly-ol</td>
<td>13.1</td>
</tr>
<tr>
<td>Endomorphin-1</td>
<td>H-Tyr-Pro-Trp-Phe-NH$_2$</td>
<td>1030</td>
</tr>
<tr>
<td>Endomorphin-2</td>
<td>H-Tyr-Pro-Phe-NH$_2$</td>
<td>7.5</td>
</tr>
<tr>
<td>Naloxone</td>
<td>Non-peptidergic</td>
<td>&gt;10000</td>
</tr>
</tbody>
</table>

$H$- denotes a free N-terminal α-amino group, $Ac$- an acetyl group. Suc- an succinyl group. -OH a C-terminal α-carboxylic acid and -NH$_2$ a C-terminal α-carboxamide, Sar- a sarcosine. Met(O$_2$) a methioninesulfon. Me an $N\alpha$-methyl, -Gly-ol a C-terminal glycinol.

Structure-Activity Relationship for SP1‒7 and EM-2

The pharmacological effects observed for SP1‒7 and the binding properties of the shorter peptide EM-2 motivated the use of these two peptides as starting points in a medicinal chemistry programme in our research group. The goal of the program was to develop reliable research tools to be used for in-depth pharmacological studies of the SP1‒7 system, which may, open up new avenues for finding better pharmaceutical alternatives for treatment of neuropathic pain.

Previously, a structure–activity relationship (SAR) study for binding to the SP1‒7 target was commenced in our research group with the aim of identifying important interactions as well as the minimal active sequence. It included an Ala-scan, truncation, and capping studies of SP1‒7 and EM-2 (for a summary of the SAR studies, see Table 3). From the Ala-scan, the C-terminal and in particular Phe$^7$ of SP1‒7 and Phe$^4$ of EM-2 were again identified as essential parts for binding affinity to the SP1‒7 target. Furthermore, the detailed SAR studies revealed that a C-terminal amide potentiates binding affinity and resulted in the identification of the SP1‒7 amide with improved binding affinity (SP1‒7 amide, $K_i = 0.3$ nM) as compared to the native heptapeptide (SP1‒7, $K_i = 1.6$ nM). This, together with the remarkable discovery from the truncation study that the dipeptide H-Phe-Phe-NH$_2$ (EM-23‒4, $K_i = 1.5$ nM, from now on referred to as the Phe-Phe amide) had the same affinity to the SP1‒7 target as SP1‒7, were the most striking outcomes of the SAR studies of SP1‒7 and EM-2.
Even more surprisingly, the Phe-Phe amide dipeptide possessed better anti-hyperalgesic and anti-allodynic effects in STZ induced diabetic mice in comparison to the full-length SP₁₋₇ peptide, after central (i.t.) administration. However, following peripheral (i.p.) administration, the dipeptide failed to show any anti-allodynic effect in SNI mice, which could be explained by the poor pharmacokinetic (PK) properties displayed by the compound, which will be discussed in the next section (Figure 5, p. 20). In addition, the Phe-Phe amide was screened against a number of receptors and enzymes that are associated with pain, as well as a series of common drug targets. No hit for the binding site was seen, which suggests interaction with a specific, yet unknown, SP₁₋₇ target. However, it cannot be excluded that the Phe-Phe amide binds at a site different from the conventional binding site for the tested drug targets.

Table 3. Binding affinity of SP₁₋₇ and EM-2 analogues as determined by inhibition of [³H]SP₁₋₇ binding to rat spinal cord membrane. Changes are highlighted.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Amino acid sequence</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP₁₋₇</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH</td>
<td>1.6</td>
</tr>
<tr>
<td>EM-2</td>
<td>H-Tyr-Pro-Phe-Phe-NH₂</td>
<td>8.7</td>
</tr>
<tr>
<td>[Ala₁]SP₁₋₇</td>
<td>H-Ala-Pro-Lys-Pro-Gln-Gln-Phe-OH</td>
<td>12.3</td>
</tr>
<tr>
<td>[Ala₂]SP₁₋₇</td>
<td>H-Arg-Ala-Lys-Pro-Gln-Gln-Phe-OH</td>
<td>1.7</td>
</tr>
<tr>
<td>[Ala₃]SP₁₋₇</td>
<td>H-Arg-Pro-Ala-Pro-Gln-Gln-Phe-OH</td>
<td>2.8</td>
</tr>
<tr>
<td>[Ala₄]SP₁₋₇</td>
<td>H-Arg-Pro-Lys-Ala-Gln-Gln-Phe-OH</td>
<td>2.8</td>
</tr>
<tr>
<td>[Ala₅]SP₁₋₇</td>
<td>H-Arg-Pro-Lys-Pro-Ala-Gln-Phe-OH</td>
<td>78.6</td>
</tr>
<tr>
<td>[Ala₆]SP₁₋₇</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Ala-Phe-OH</td>
<td>365.8</td>
</tr>
<tr>
<td>[Ala₇]SP₁₋₇</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gl-Ala-OH</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Ac-S₁₋₇</td>
<td>Ac-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH</td>
<td>7.1</td>
</tr>
<tr>
<td>SP₁₋₇ amide</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-NH₂</td>
<td>0.3</td>
</tr>
<tr>
<td>SP₂₋₇ amide</td>
<td>H-Pro-Lys-Pro-Gln-Gln-Phe-NH₂</td>
<td>2.8</td>
</tr>
<tr>
<td>SP₃₋₇ amide</td>
<td>H-Lys-Pro-Gln-Gln-Phe-NH₂</td>
<td>4.4</td>
</tr>
<tr>
<td>SP₄₋₇ amide</td>
<td>H-Pro-Gln-Gln-Phe-NH₂</td>
<td>4.5</td>
</tr>
<tr>
<td>SP₅₋₇ amide</td>
<td>H-Gln-Gln-Phe-NH₂</td>
<td>1.9</td>
</tr>
<tr>
<td>[Ala₁]EM-2</td>
<td>H-Ala-Pro-Phe-Phe-NH₂</td>
<td>11.5</td>
</tr>
<tr>
<td>[Ala₂]EM-2</td>
<td>H-Tyr-Ala-Phe-Phe-NH₂</td>
<td>10.2</td>
</tr>
<tr>
<td>[Ala₃]EM-2</td>
<td>H-Tyr-Pro-Ala-Phe-NH₂</td>
<td>9.4</td>
</tr>
<tr>
<td>[Ala₄]EM-2</td>
<td>H-Tyr-Pro-Phe-Ala-NH₂</td>
<td>1460</td>
</tr>
<tr>
<td>EM-2 acid</td>
<td>H-Tyr-Pro-Phe-Phe-OH</td>
<td>30.2</td>
</tr>
<tr>
<td>EM₂₋₄</td>
<td>H-Pro-Phe-Phe-NH₂</td>
<td>10.9</td>
</tr>
<tr>
<td>EM₂₋₄</td>
<td>H-Phe-Phe-NH₂</td>
<td>1.5</td>
</tr>
</tbody>
</table>

H- denotes a free N-terminal α-amino group, -OH a C-terminal α-carboxylic acid and -NH₂ a C-terminal α-carboxyamide. Ac- denotes an acetyl group.
Development of SP1-7 Peptidomimetics

The role of neuropeptides and their receptors in physiological processes can be studied using knockout techniques in transgenic animals. Unfortunately, such techniques are not possible in the study of their corresponding bioactive fragments. Therefore, development of peptidomimetics of such fragments are very important not only to serve as new pharmaceuticals but also to serve as essential research tools e.g. in imaging techniques such as PET. PET can via use of a positron ($\beta^+$) emitting radiotracer, visualise physiological processes in living organisms in real time. Thus, this technique has found increasing use as a fundamental tool in early drug development for various diseases of the CNS and other organs.75

Hence, the Phe-Phe amide was chosen as the lead compound for further optimisation and its uptake, permeability and metabolic stability were evaluated. It was not surprising that the in vitro studies revealed negligible Caco-2 permeability and low metabolic stability. Consequently, the dipeptide showed poor drug-like properties (Figure 5).

**Figure 5.** Optimisation of the Phe-Phe amide resulting in compounds A, B, and C, with pharmacophore features highlighted. Cut-off values for: metabolic stability; $47 < \text{Cl}_{\text{int}} < 92 \mu\text{l/min/mg}$ indicate moderate risk for first pass metabolism, for Caco-2 permeability; $0.2 < P_{\text{app}} < 1.6 \times 10^{-6} \text{cm/s}$ indicate moderate permeability. n.d. = not determined (no measurable permeability). R = benzyl (Bn) or H. When determined at the same occasion as the Phe-Phe amide, $K_i$ value for the Phe-Phe amide was 8.4 nM. When determined at the same occasion as the Phe-Phe amide, the efflux ratio value was 21. IC$_{50}$ value for the protected precursor (R = Bn) was 26 nM.
Through extensive research, synthesising N-methylated analogues of the Phe-Phe amide along with constrained variants, a pharmacophore hypothesis was proposed. The (S,S)-configuration of the side-chains, together with the free N-terminal amine and C-terminal amide functions are important for binding to the SP$_{1-7}$ target. From this, three main classes of Phe-Phe amide analogues, i.e. A, B, and C were designed (Figure 5). The compounds were evaluated with regard to their binding affinity towards the SP$_{1-7}$ target, pharmacokinetic properties and in vivo anti-allodynic effect in SNI mice.

Rigidification of the C-terminus, using a pyrrolidine, delivered a dipeptide analogue, A, with improved binding, high metabolic stability and permeability (Figure 5). Unfortunately, the compound suffered from high efflux over the Caco-2 cells monolayers in vitro and over the blood-brain barrier (BBB) in a rat pharmacokinetic study, presumably caused by the P-glycoprotein (P-gp) involvement in both cases. P-gp is expressed in the human intestine, and is also an important efflux transporter for limiting absorption of drugs into the CNS.

The basic amine in the N-terminal was substituted with a neutral carbamate moiety delivering compound B (Figure 5). This isosteric replacement led to decreased binding affinity, but interestingly the efflux was reduced significantly, and a further brain distribution study in rats revealed BBB penetration. Consequently, the primary amine in the N-terminal seems to be important for interacting with the target protein (i.e. SP$_{1-7}$ target) but also for the efflux (over the Caco-2 cells monolayers and the BBB) of the dipeptide analogues. A third series, compound C (R = H), containing an imidazole-based phenylalanine isostere was designed. Initial binding data of a precursor to this compound (R = benzyl [Bn]) indicated binding to the SP$_{1-7}$ target (Figure 5). However, this series needed further chemistry optimisation to secure sufficient amounts of material for further in vitro and in vivo evaluation, which is a part of the work presented in this thesis.

The anti-allodynic effect of A and B was evaluated in the SNI mouse model for neuropathic pain. Pleasingly, A had a potent anti-allodynic effect following i.p. administration at a low dose. The observed effect was strong but with a short duration presumably due to poor brain exposure (BBB efflux) of the compound. Compound B, on the other hand, showed no anti-allodynic effect in SNI mice, potentially caused by the hydrolytic instability of the carbamate function in B, in plasma.

Concurrently, the effect of SP$_{1-7}$ amide (Table 3, p. 19) on neuropathic pain was also evaluated in STZ induced diabetic mice, in SNI mice and in SCI rats. The peptide possessed strong anti-hyperalgesic effect in diabetic mice after i.t. administration and anti-allodynic effect in SNI mice and SCI rats following i.p. administration. In accordance with the binding affinity to spinal cord membrane shown in Table 3, the effect of the SP$_{1-7}$ amide was stronger than for the native SP$_{1-7}$ in all animal models for neuropathic pain. Surprisingly, when SP$_{1-7}$ amide was compared to the peptidomimetic A or to
N-terminally truncated versions of SP_{1-7} amide, which still possessed affinity for the specific SP_{1-7} target \textit{in vitro} (Table 3, p. 19), the full-length peptide was superior in producing a potent anti-allodynic effect \textit{in vivo}.\textsuperscript{62,63} Obviously, other key residues other than the C-terminal amino acids are important for producing the potent anti-allodynic effect \textit{in vivo}. This initiated a project to establish an \textit{in vivo} SAR for the full-length SP_{1-7} peptide, where the SP_{1-7} amide was chosen as a starting point in the project and this work is presented in this thesis. It was surmised that complete removal of the peptide character may not be necessary. Thus, SP_{1-7} peptidomimetics as well as SP_{1-7} related peptides, could serve as important research tools in the study of the SP_{1-7} system.
Aims

The overall aim of the work presented in this thesis was to develop drug-like SP1-7 related peptides and peptidomimetics that could be used as research tools to elucidate the physiological mechanism of the SP1-7 system, which can potentially lead to future treatments for neuropathic pain.

The specific aims were:

- To elucidate in vivo SAR of the SP1-7 amide by design and synthesis of a series of SP1-7 related peptides including Ala-substituted, N-terminally truncated, and N-methylated variants.
- To investigate the relationship between the in vivo pharmacological effect for the SP1-7 related peptides and the in vitro pharmacokinetic data i.e. metabolite identification, plasma stability and permeability.
- To develop a robust synthetic method for the Pd-catalysed aminocarbonylation of halide substituted imidazoles to generate SP1-7 peptidomimetics.
- To evaluate the imidazole-based SP1-7 peptidomimetic regarding its in vitro pharmacokinetic and in vivo pharmacological properties.

During the course of the work the following specific aim emerged:

- To develop a general and mild synthetic method combining Pd-catalysed aminocarbonylation and Fmoc SPPS in order to introduce various N-capping groups to a peptide chain which could also be used to enable isotope labelling and cyclisation of biologically active peptides (e.g. SP1-7).
In drug development programmes starting from a natural ligand, the medicinal chemist often initiates a stepwise alteration of the amino acid sequence (i.e. peptide scanning) to establish a SAR for the bioactive peptide. Thus, C- or N-terminal truncations and classic Ala-scans are important methods for identifying the minimum sequence and amino acid residues important for binding to the target. The importance of a basic N-terminal or an acidic C-terminal are determined by introduction of capping groups. In parallel with the identification of residues crucial for binding (i.e. the pharmacophore), pharmacokinetic experiments and metabolite detection are used to identify the amide bonds most sensitive to proteolytic cleavage. Possible sites for proteolytic stabilisation are identified by peptide backbone scan with D-amino acids, α-methyl amino acids, β-amino acids, peptoids or N-methyl amino acids. Besides, the SAR investigation will also give valuable information on residues that are not involved in the binding interaction and may suggest sites (“handles”) for cyclisation or conjugation such as functional groups containing $\beta^+$ emitting radionuclei (e.g. carbon-11 or fluorine-18) to enable PET studies.

All the above techniques can interfere with the conformational flexibility of the peptide and may disrupt important target interactions resulting in reduced biological activity. Hence, peptide lead optimisation is a delicate balance between maintaining or improving biological activity and improving pharmacokinetic properties.

**Background and Strategy**

Previous SAR studies successfully identified the C-terminal part of the SP$_{1-7}$, and in particular the Phe$^7$ element, as a key binding residue for target interaction (Table 3, p. 19). As mentioned before, the full-length SP$_{1-7}$ and SP$_{1-7}$ amide were shown to possess anti-hyperalgesic effect after i.t injection in STZ induced diabetic mice. In parallel, i.p administered SP$_{1-7}$ and its amidated analogue, along with truncated versions, were evaluated regarding their anti-allodynic effect in SCI rats (Table 4). The results showed that the...
Table 4. $K_i$ values for SP$_{1-7}$ analogues and effects of the respective peptides on mechanical allodynia in SCI rats and SNI mice.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Amino acid sequence</th>
<th>$K_i$ (nM)$^a$</th>
<th>Anti-allodynic effect$^b$ SCI rats</th>
<th>SNI mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP$_{1-7}$</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH</td>
<td>1.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SP$_{1-7}$ amide</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Phe-NH$_2$</td>
<td>0.3</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>SP$_{2-7}$ amide</td>
<td>H-Pro-Lys-Pro-Gln-Gln-Phe-NH$_2$</td>
<td>2.8</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>SP$_{3-7}$ amide</td>
<td>H-Lys-Pro-Gln-Gln-Phe-NH$_2$</td>
<td>4.4</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>SP$_{4-7}$ amide</td>
<td>H-Pro-Gln-Gln-Phe-NH$_2$</td>
<td>4.5</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>SP$_{5-7}$ amide</td>
<td>H-Gln-Gln-Phe-NH$_2$</td>
<td>1.9</td>
<td>-</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

$^a$Binding affinity for inhibition of [3H]SP$_{1-7}$ binding to rat spinal cord membrane. $^b$+ 185 nmol/kg (i.p.); ++ 18.5 nmol/kg (i.p.); – no effect. n.d. = not determined (not tested).

Full-length SP$_{1-7}$ amide was superior to the endogenous and truncated analogues (SP$_{1-7}$, SP$_{2-7}$ amide, SP$_{3-7}$ amide and SP$_{4-7}$ amide, Table 4). Further truncation resulted in complete loss of the in vivo anti-allodynic effect (SP$_{4-7}$ amide and SP$_{5-7}$ amide, Table 4). In addition, SP$_{1-7}$ and SP$_{1-7}$ amide were also potent in alleviating allodynia in SNI mice following i.p. administration.

According to the results in Table 4, most of the N-terminal sequence of the heptapeptide must be retained in order to keep the anti-allodynic effect in vivo. From this observation alternative hypotheses were formed. First, the full-length peptides could be more stable in plasma as compared to the smaller fragments. Thus, the full-length peptide could cause a “slow release” of the important C-terminal part by a relatively slow step-wise enzymatic degradation. A second reason for this observation might be sequence dependent cell permeability. The Arg$^1$ and Lys$^3$ residues could affect the permeability of the molecules through relevant tissue (e.g. BBB) and they could thereby be important for the peptide to reach its target. A third explanation might be a possible bivalent ligand approach, where the C-terminal message would thus be important for receptor transduction while the N-terminal part functions as an address part important for receptor selectivity and enhanced potency in vivo.

To answer the questions raised from the above study, we felt prompted to return to the full-length peptides. A series of SP$_{1-7}$ related peptides were designed in which the basic amino acids Arg$^1$ and Lys$^3$ were removed by N-terminal truncation or substituted by a simple Ala function (marked in green and blue in Figure 6) in order to examine the impact of these side chains on the overall in vitro stability and permeability of the peptides. Furthermore, this change would indicate whether these amino acids have an in vivo message-address effect in the peptides.

In addition, results from Table 4 showed that the full-length SP$_{1-7}$ amide had a superior effect on neuropathic pain when administered peripherally and therefore should be re-investigated as a lead peptide in the project. Modification of the peptide backbone was next investigated to see if a simple
change could convey favourable pharmacokinetic properties and retain the pharmacological effect in vivo. An N-methylation scan was proposed to study backbone hydrogen bonding interactions and identify which positions are most sensitive to proteolytic degradation (marked in red in Figure 6).

To synthesise the designed peptides, the fluoren-9-ylmethoxy carbonyl solid-phase peptide synthesis (Fmoc SPPS)\(^8\) was chosen as the main method and previously reported procedures for site-selective N-methylation, compatible with Fmoc SPPS, were used.

![Image](image-url)

**Figure 6.** Study design to elucidate the observed anti-allodynic effect for the full-length SP1–7 amide peptide following peripheral administration.

**Solid-Phase Peptide Synthesis**

The SPPS allows anchoring of an amino acid to a functionalised solid support (i.e. resin bead). The main approach uses the acid stable but base labile Fmoc group for Na-protection whereas the protecting groups on the side chains are acid labile (e.g. tert-butyl [t-Bu]) (Figure 7). The SPPS technique starts at the C-terminus where the first building block is attached to the resin bead through a linker (Step 1). The linker can be tailored to provide a wide range of C-terminal functional groups in the final peptide. Several reactions (Step 2–4) can then be carried out in sequence on the attached amino acid, building the peptide towards the N-terminal. The final peptide is detached from the solid support by simultaneous cleavage of the linker and the removal of orthogonal protection groups using acid (Step 5).

The major advantages of this technique are speed, simplicity and the use of standard conditions for a wide range of couplings. The drawback, on the other hand, are that intermediates cannot be purified, which demands full conversion of each step using a large excess of reagents. However, excess of reagents and unbound by-products can easily be removed using filtration and washing of the resin. In addition, the repeated cycles of deprotection and coupling enable automation of SPPS, and automated peptide synthesisers are commercially available.
Figure 7. Principles of Fmoc/t-Bu SPPS.

Synthesis of SP1–7 Related Peptides

The SP1–7 (1), the SP1–7 amide (2), the Ala-substituted peptides 3–5, and N-terminal truncated peptides 6–9 were all synthesised with a peptide synthesiser using standard Fmoc SPPS chemistry (Scheme 1, for amino acid sequence see Table 5, p. 30). The starting polymer was preloaded H-Phe-Wang polystyrene (PS) resin or Rink Amide MBHA PS resin. Coupling (Step 1) was performed in N,N-dimethylformamide (DMF) using the coupling reagents HBTU and HOBT, and N,N-diisopropylethylamine (DIPEA) as base. The Fmoc group was removed (Step 2) by treatment with 20% piperidine (Pip) in DMF. After each coupling and deprotection step followed repeated washing with DMF. The final peptides were cleaved from the resin by the addition of triethylsilane (TES) and 95% aqueous trifluoroacetic acid (TFA) which also removed the Pbf, Boc, and Trt protecting groups from the Arg, Lys, and Gln side chains, respectively. The final peptides (1–9) were precipitated in diethyl ether and purified using reversed-phase high performance liquid chromatography (RP-HPLC) with isolated yields of 25-53% (crude purity for 3–5: 72–86%).
To obtain the *N*-methylated variants 10–18, the Fmoc-deprotected peptide-resin was removed from the peptide synthesiser when the site of *N*-methylation was reached. The site-selective *N*-methylation procedure was performed in a syringe fitted with a porous polyethylene filter following previously described methods.\textsuperscript{83,84} Thus, the aryl sulfonyl protecting group was introduce (Step 3) by treating the peptide-resin with *ortho*-nitrobensenesulfonyl chloride (*o*-NBSCl) and DIPEA in DMF. Deprotonation of the sulfonamide was accomplished with 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene (MTBD) and alkylation with methyl *para*-nitrobenzenesulfonate in DMF (step 4). For selective removal of the sulfonyl protecting group the peptide resin was treated with thiophenol sodium salt in DMF (Step 5). After each step, (Step 3‒5) a small portion of resin was removed for LC-MS analysis and the step was repeated if necessary to ensure full conversion.
Next, the peptide-resin was returned to the peptide synthesiser and the following amino acid was double coupled to the peptide-resin in DMF using HATU and HOAt as coupling reagents and DIPEA as base (Step 6). Remaining amino acids in the peptide were coupled with HBTU/HOBt/DIPEA in DMF and Fmoc removal using 20% Pip/DMF (Step 1 and 2). The peptides were cleaved from the resin with simultaneous removal of the orthogonal protecting groups using 95% TFA (aq) and TES. The final mono-, di-, or tri-methylated peptides (10–18) were precipitated in diethyl ether and purified using RP-HPLC. The multiple LC-MS analyses affected the overall yield for the \( N \)-methylated peptide (10–18) and gave an isolated yield of 5–18%. Pleasingly, the crude purity was high (65–84%), except for peptide 17 (43%). This was due to the incomplete removal of the sulfonyl protecting group (Step 5). However, the sulfonyl-protected side-product was easily separated from the desired product using RP-HPLC.

**Pharmacokinetic Properties**

_In vitro_ plasma stability and Caco-2 permeability were established for the synthesised peptides 1–18 (Table 5). From the plasma stability data two groups could be distinguished, namely peptides that were subject to fast degradation in plasma and those that were completely stable \( t_{1/2} > 180 \text{ min} \). Further, the data showed that the Arg\(^1\) and Lys\(^3\) residues are not important for the overall stability of the peptides, since the Ala-substituted peptides (3–5) had similar half-life times (0.5–7.1 min) as the endogenous SP\(_{1–7}\) (1) and its amidated analogue SP\(_{1–7}\) amide (2) (4.4 and 6.4 min, respectively). The plasma stability data for the N-terminally truncated fragments 6–9 showed that peptides 6 and 8 with an N-terminal Pro-residue were unaffected in plasma \( t_{1/2} > 180 \text{ min} \). This observation indicates that the post-proline dipeptidyl (amino)peptidase IV (DPP-IV) might be involved in the degradation of the peptides. In fact, this peptidase releases N-terminal dipeptides ending with Pro from the SP itself and thus hydrolyses SP at the Pro\(^2\)-Lys\(^3\) and Pro\(^4\)-Gln\(^5\) bonds (Figure 4, p. 17).\(^8^5\) Also, peptide 7 (SP\(_{3–7}\) amide), showed fast hydrolysis \( (t_{1/2} = 11.1 \text{ min}) \), while peptide 9, not expected to be susceptible to DPP-IV degradation, was completely inert in plasma \( t_{1/2} > 180 \text{ min} \). The possible involvement of DPP-IV is further strengthened by the plasma half-life measurements for the peptides 11, 15, 16, and 18, where the Pro\(^2\)-Lys\(^3\) bond is \( N \)-methylated and therefore resistant to DPP-IV degradation \( t_{1/2} > 180 \text{ min} \). Additional metabolite analysis of the SP\(_{1–7}\) amide (2) identified the Arg\(^1\)-Pro\(^2\) and SP\(_{3–7}\) amide (7) as the two major fragments formed in SNI mouse plasma. Thus, the Pro\(^2\)-Lys\(^3\) bond was identified as the most proteolytically labile amide bond in the SP\(_{1–7}\) amide (2) and indeed, \( N \)-methylation of this bond produced peptide analogues completely stable in plasma \( t_{1/2} > 180 \text{ min} \).
Table 5. Plasma stability and permeability data of the prepared peptides. Changes are highlighted.

<table>
<thead>
<tr>
<th>Ligand and amino acid sequence</th>
<th>Plasma stability</th>
<th>Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( t_{1/2} ) (min)</td>
<td>( P_{app} \times 10^{-6} \text{cm/s} )</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>human</td>
</tr>
<tr>
<td>1 H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>2 H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-NH₂</td>
<td>6.4</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Alanine-substituted peptides

<table>
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<th>Plasma stability</th>
<th>Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( t_{1/2} ) (min)</td>
<td>( P_{app} \times 10^{-6} \text{cm/s} )</td>
</tr>
<tr>
<td>3 H-Ala-Pro-Lys-Pro-Gln-Gln-Phe-NH₂</td>
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<td>n.d.</td>
</tr>
<tr>
<td>4 H-Arg-Pro-Ala-Pro-Gln-Gln-Phe-NH₂</td>
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</tr>
<tr>
<td>5 H-Ala-Pro-Ala-Pro-Gln-Gln-Phe-NH₂</td>
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</table>

Truncated peptides

<table>
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<th>Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( t_{1/2} ) (min)</td>
<td>( P_{app} \times 10^{-6} \text{cm/s} )</td>
</tr>
<tr>
<td>6 H-Pro-Lys-Pro-Gln-Gln-Phe-NH₂</td>
<td>&gt;180</td>
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</tr>
<tr>
<td>7 H-Lys-Pro-Gln-Gln-Phe-NH₂</td>
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<td>n.d.</td>
</tr>
<tr>
<td>8 H-Pro-Gln-Gln-Phe-NH₂</td>
<td>&gt;180</td>
<td></td>
</tr>
<tr>
<td>9 H-Gln-Gln-Phe-NH₂</td>
<td>&gt;180</td>
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\( N \)-methylated peptides

<table>
<thead>
<tr>
<th>Ligand and amino acid sequence</th>
<th>Plasma stability</th>
<th>Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( t_{1/2} ) (min)</td>
<td>( P_{app} \times 10^{-6} \text{cm/s} )</td>
</tr>
<tr>
<td>10 MeArg-Pro-Lys-Pro-Gln-Gln-Phe-NH₂</td>
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</tr>
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<td>11 H-Arg-Pro-MeLys-Pro-Gln-Gln-Phe-NH₂</td>
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<td>20.0</td>
<td>20.7</td>
</tr>
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<td>&gt;180</td>
<td>&gt;180</td>
</tr>
<tr>
<td>16 H-Arg-Pro-MeLys-Pro-Gln-MePhe-NH₂</td>
<td>&gt;180</td>
<td>&gt;180</td>
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<tr>
<td>17 H-Arg-Pro-Lys-Pro-MeGln-Gln-MePhe-NH₂</td>
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<td>27.5</td>
</tr>
<tr>
<td>18 H-Arg-Pro-MeLys-Pro-MeGln-Gln-MePhe-NH₂</td>
<td>&gt;180</td>
<td>&gt;180</td>
</tr>
</tbody>
</table>

\( ^a \)Me denotes a Nα-methyl. \( ^b \)\( t_{1/2} \) = half-life. \( ^c \)\( P_{app} \) = apparent permeability coefficient. Average of three filters. In presence of protease inhibitors (bestatin, diprotin A, and captopril). (a – b) = apical to basolateral. mb = mass balance. n.d. = not determined (no measurable permeability). \( P_{app} < 0.2 \times 10^{-6} \text{cm/s} \) indicates low permeability

Despite potential interspecies differences the \( N \)-methylated variants displayed similar half-lives in human and mouse, with 10 and 11, being less stable in human plasma, as exceptions.

An in vitro Caco-2 cell monolayer assay was used to give general information about the permeability of selected peptides (i.e. 2, 3, 7, and 11), and in particular the influence of Arg¹ and Lys³ on the permeability of the peptides. Good permeability is important for the peptides to reach their target and compound charge was previously shown to influence whether SP itself passes the BBB in vitro.⁸⁶

Unfortunately, three of the peptides (i.e. 2, 3, and 7) displayed concurrent poor stability in the Caco-2 cells monolayers (i.e. low mass balance) also in the presence of protease inhibitors. The peptides were therefore classified as low permeability compounds. In contrast, peptide 11 showed adequate mass
balance, suggesting that no degradation took place but, yet again, no measurable permeability was observed.

Pharmacological Evaluation

The peptides were evaluated for their effect on mechanical allodynia in SNI mice. The SP_1–7 amide (2) was administered i.p. in doses of 0.185 µmol/kg, 0.585 µmol/kg and 1.85 µmol/kg and selected peptides were administered i.p. in a single dose of 0.185 µmol/kg. The anti-allodynic effect of each peptide was assessed using the von Frey test.

Initially, a dose-response investigation for 2 revealed a dose-dependent increase in effect and no visible side-effects like drowsiness were observed even with a 10-fold higher dose (Figure 8). Although the effect was quite short-lived, the SP_1–7 amide (2) had a fast onset where the mechanical threshold was increased for 15–30 min whereupon it rapidly declined and no effect was observed at 60 min. Since the N-terminal residues Arg^1 and Lys^3 were expected to influence the in vivo effect, comparative studies were performed with [Ala^1]SP_1–7 amide (3), [Ala^3]SP_1–7 amide (4), and [Ala^1, Ala^3]SP_1–7 amide (5) (Figure 9). The investigation revealed that Ala-substitution of the Arg^1 residue was deleterious for the anti-allodynic effect since i.p. administration of neither peptide 3 nor 5 increases the mechanical threshold in SNI mice. In contrast, displacement of Lys^3 with Ala^3 resulted in analogue 4 with retained anti-allodynic effect. Indeed, an intact Arg^1 in SP_1–7 related peptides is fundamental for a strong in vivo effect, while Lys^3 is less important. From this, the Arg^1 residue was suggested to be an important address motif while the Phe^7 residue, as previou-

![Figure 8](image_url)

*Figure 8.* Time/dose-response curve and total anti-allodynic effect expressed as AUC of SP_1–7 amide (2) in SNI mice (doses in µmol/kg, i.p., n = 16). Statistical significance was marked as follows: *** p < 0.001; ** p < 0.01 (1.85 µmol/kg vs. control), ### p < 0.001 (0.585 µmol/kg vs. control) and & p < 0.05 (0.185 µmol/kg vs. control). Control mice were injected with 0.1 M acidified saline.
sly identified, functions as a message motif for the biological activity of SP1-7 related peptides. The message-address concept, briefly mentioned before (p. 15), proposes a bivalent ligand where different parts of the peptide ligand have separate functions for interacting with and activating the macromolecular target. The different components are responsible for receptor recognition (i.e. address) and receptor stimulation (i.e. message) and are close to one another in the peptide chain (either clearly separated or possibly overlapping). Consequently, the peptide ligand does not necessarily have to be rigid one-step interaction with its target molecule (in a “lock and key” manner). Instead, the synchronically organised peptide ligand is flexible and can adopt the correct conformation for activation while the address component has already formed a specific interaction with the target (in a “zipper-like” manner). Thus, the address component plays a vital role without being able to trigger the response itself.

To further elucidate the importance of the address-message residues for the anti-allodynic effect of SP1-7 related peptides, the mono-methylated analogues 10–12 and 14, and the di-methylated analogues 15–17 were evaluated in SNI mice using i.p. administration (Figure 10). Peptides [MeArg^1]SP1-7 amide (10) and [MePhe^7]SP1-7 amide (14) comprising a methyl in the address and message parts, respectively, had greatly diminished in vivo anti-allodynic effect. This observation implies that these backbone regions are, as expected, important for interaction with the target and therefore sensitive to minor modifications. Further support in this matter is given by the fact that also peptides 16 and 17, both containing a MePhe^7 residue and an additional N-methyl amino acid (MeLys^3 or MeGln^5) were essentially without anti-allodynic effect. On the other hand, the [MeLys^3]SP1-7 amide (11) and [MeGln^5]SP1-7 amide (12) had high anti-allodynic effects and introduction of an N-methyl amino acids in the
Figure 10. Anti-allodynic effect of SP1‒7 amide (2) and N-methylated variants (10‒12 and 14‒17) (0.185 µmol/kg i.p., n = 13‒17) and gabapentin (18.5 µmol/kg i.p., n = 10) in SNI mice. The results were presented as control subtracted AUC. Statistical significance was marked as follows: * p < 0.05; ** p < 0.01; *** p < 0.001 (vs. control), # p < 0.05; ## p < 0.01 (vs. gabapentin). Control mice were injected with 0.1 M acidified saline.

Figure 11. Time-course curve of von Frey mechanical threshold in SNI mice treated with 0.185 µmol/kg i.p. of 2, 11, and 12. Statistical significance was marked as follows: * p < 0.05; ** p < 0.01; *** p < 0.001 (vs. control). Control mice were injected with 0.1 M acidified saline.

Lys³ and Gln⁵ positions did not affect the activity. However, peptide 15 ([MeLys³, MeGln⁵]SP1‒7 amide) where both of these residues are N-methylated had no anti-allodynic effect. It is worth noting that the introduction of several N-methylated amino acids in a peptide chain can improve proteolytic stability but also hamper biological activity through, for example, conformational restrictions, yet again illustrating the tough balance between these two properties.

All in all, the N-methylation scan of the SP1‒7 amide identified two analogues with retained in vivo effect, i.e. [MeLys³]SP1‒7 amide (11) and [MeGln⁵]SP1‒7 amide (12). However, in comparison the SP1‒7 amide (2) itself was still superior both in potency and duration of the anti-allodynic activity (Figure 11). Thus, the anti-allodynic effects of the peptides did not correlate with their in vitro plasma stability since 2, 11, and 12 have plasma half-life.
times in SNI mouse plasma of 6.4 min, >180 min, and 14.8 min, respectively (Table 5). This intriguing observation prompted a re-evaluation of the major metabolite of the SP₁₋₇ amide (2) i.e. the SP₃₋₇ amide (7). Indeed, this metabolite demonstrated an anti-allodynic effect itself (Figure 9). However, the effect is considerably lower than 2, again stressing the importance of an intact Arg¹ residue. Taken together, the anti-allodynic effect for the SP₁₋₇ amide (2) seems to be partly attributed to the activities mediated by its metabolite (7). Thus, all peptide analogues that could not deliver this metabolite were completely inactive or showed decreased anti-allodynic effect compared to 2.

Finally, the SP₁₋₇ amide (2) and the active N-methylated peptides 11 and 12, were compared to gabapentin (Figure 10). This anti-convulsing agent is included in current first-line treatment of neuropathic pain (p. 14). All peptides were proven more effective than gabapentin in relieving SNI-induced allodynia, as mice injected with a 100-fold higher dose of gabapentin (18.5 µmol/kg) failed to reduce pain thresholds observed for mice injected with either 2, 11, or 12 at a dose of 0.185 µmol/kg. However, gabapentin displayed a comparable dose-response curve 2 when increasing the dose (185 µmol/kg and 585 µmol/kg, i.p.) with a peak in anti-allodynic effect after 60–90 min, thus being more long-lasting as compared to 2.⁹¹ These high dosages are in agreement with clinical data, where dosages of 2400 mg/day of gabapentin are required for successful treatment of peripheral neuropathic pain, which can give rise to burdensome side-effects like drowsiness.⁹²,⁹³

Summary and Future Outlook

Modulation of the SP₁₋₇ system is an interesting approach to the potential production of potent and safe peptide drugs for treatment of neuropathic pain. This work has shown that it is possible to fine-tune pharmacokinetic properties (e.g. plasma stability) while retaining the anti-allodynic effect for the SP₁₋₇ analogues. Interestingly, the active N-methylated variants were more potent than gabapentin following i.p. administration.

Three important conclusions were made that could be used to design future research tools for further studies of the SP₁₋₇ system. First, it is shown that the SP₁₋₇ peptide is a bivalent ligand. The Phe⁷ residue acts as a message motif while Arg¹ is an important address motif for producing the potent anti-allodynic effect. Second, the Pro²-Lys³ bond was identified as the bond most prone to proteolytic cleavage since N-methylation of this bond delivered a peptide completely stable in plasma and with potent anti-allodynic effect. Third, the Lys³ residue is not involved in binding interaction with the target and can serve as a “handle” for isotope labelled conjugates. However, further experiments are needed to see if it is possible to elongate the Lys³ side-chain without diminishing the in vivo anti-allodynic effect.
A lead compound with a desired pharmacological activity is commonly associated with undesirable side-effects, structural features that limit its bioavailability or characteristics that influences its metabolism and excretion from the body. Bioisosteres are used to replace a functional group that is important for activity but is problematic in one way or another, using related but different groups, heteroatoms or arrangements. Bioisosteres can mimic the spatial arrangement, electronic properties, and/or physicochemical properties of a functional group or ring while still conveying the desired biological properties (i.e. retaining the pharmacophore). Bioisostere replacement may convert a lead compound into a safer and more clinically effective agent.

Heterocycles can serve as replacement of various functional groups in lead compounds. Heterocycles are often found in pharmaceutically relevant compounds and nitrogen containing heterocycles are particularly prevalent. They have the ability to interact with targets through a variety of bonding forces and many are described as privileged scaffolds.

Background and Strategy

In parallel with elucidating the in vivo SAR for full-length SP\textsubscript{1−7} related peptides, extensive efforts have been devoted to the development of three main classes of traditional low-molecular drug-like compounds acting similarly to the neuropeptide SP\textsubscript{1−7} (i.e. SP\textsubscript{1−7} peptidomimetics, Figure 5, p. 20).

As previously discussed (p. 21), a pharmacophore hypothesis for the potent dipeptide Phe-Phe amide proposed that an \((S,S)\)-configuration of the side-chains, an N-terminal primary amine and a C-terminal primary amide were important features for interaction with the SP\textsubscript{1−7} target. However, pharmacokinetic and pharmacological evaluation of compound A and B revealed that the N-terminal primary amine may also be responsible for efflux (in the Caco-2 cells monolayers and at the BBB) of the dipeptide analogues, possibly due to the involvement of P-gp proteins. This together with fast
degradation in plasma reduced (A) or abolished (B) their anti-allodynic effect in SNI mice.\textsuperscript{63}

In cases where removing the NH is not possible, reducing the basicity of the amino group might be effective and serve as a compromise with sufficient hydrogen bond donor strength for binding to the target protein but too weak to induce P-gp efflux.\textsuperscript{78} Therefore, a bioisostere replacement was proposed where an aryl-substituted imidazole moiety was incorporated into the N-terminus of C (from now on referred to as compound 19, top picture, Figure 12). This imidazole-based phenylalanine bioisostere would be a less basic alternative to the primary amine in the Phe-Phe amide. Hopefully this would lead to improved plasma stability and increased selectivity between the target protein and efflux receptor.

![Figure 12](image)

Figure 12. Design of an N-terminal imidazole-based Phe-Phe amide analogue along with a retrosynthetic analysis.

Pharmacophore group alignment analysis of 19 suggested that the compound could display similar important interactions similar to the dipeptide Phe-Phe amide in low energy conformations (Figure 13). Indeed, the sterically congested imidazole-based 19 was able to maintain the spatial arrangement of the potential pharmacophore groups and a favourable orientation of key anchor points, i.e. three hydrogen bond donators (HBD), two hydrogen bond acceptors (HBA) and two aromatic rings, important for ligand–target interactions. Together with additional binding data to the SP\textsubscript{1-7} target for a precursor to compound 19 (Figure 5, p. 20), this supported the hypothesis that this compound could act as a potent Phe-Phe amide mimetic in relieving mechanical allodynia in SNI mice.

As outlined in the retrosynthetic analysis in Figure 12, a simple and fast route to functionalised imidazoles was explored to access the target compound 19. This involved the introduction of an aromatic amino acid side chain into the C5 position of the imidazole and an amino acid amide (i.e. peptide equivalent) connected to the C4 position through an amide coupling (i.e. forming a peptide bond). An expedient protocol for the C5 arylation was
developed, but unfortunately the following aminocarbonylation of the imidazole gave low yields. Hence, to move forward with this project a palladium-catalysed aminocarbonylation protocol needed to be developed to secure sufficient amounts of the target compound for pharmacokinetic and pharmacological evaluation.

![Figure 13. Potential pharmacophore group alignment of the Phe-Phe amide (green carbons) and imidazole-based compound 19 (orange carbons). Blue sphere = HBD; Red sphere = HBA; Orange torus = aromatic ring.](image)

**Palladium-Catalysed Aminocarbonylation**

Amides are important constituents in many pharmacologically compounds and are frequently attached to a heterocyclic core. While the traditional route of forming an amide bond uses *in situ* activation of a carboxylic group with a coupling reagent such as HBTU or HATU and subsequent reaction with an amine, the well-established palladium (Pd) catalysed aminocarbonylation reaction is sometimes an appealing alternative approach to accessing amides. This three-component Pd-catalysed coupling of an (hetero)aryl halide and an amine in the presence of carbon monoxide (CO) was introduced by Heck and co-workers in 1974. In addition, the reaction could give access to $^{11}$C-labelled analogues for PET studies by use of $[^{11}$C]CO. Such a process is referred to as *radiolabelling* or simply *labelling*.99

A proposed catalytic mechanism for the Pd-catalysed aminocarbonylation reaction is depicted in Figure 14. The reaction is initiated by the formation of the active palladium catalyst Pd(0) typically from a Pd(II) precatalyst salt. The *oxidative addition* of the (hetero)aryl halide generates the Pd(II) complex followed by *CO coordination* to the Pd(II) complex. 1,1-*insertion* of CO into the (hetero)aryl-palladium bond then forms the acylpalladium complex followed by *nucleophilic attack* directly on the acyl carbon thereby releasing
the carbonyl product. Finally, a reductive elimination step follows, which re-generates the Pd\(^0\) catalyst\(^{100,101}\).

The major drawback of the reaction has been the use of the toxic and flammable CO gas. Therefore, several solid \textit{in situ} CO-releasing molecules (CORMs) have been explored, such as Mo(CO)\(_6\) activated by DBU\(^{102}\). However, CORMs and the corresponding activator might interfere with the carbonylation reaction itself or hamper the purification of the final product when using a one-chamber reaction setup. To circumvent this, Skrydstrup and

![Proposed catalytic cycle of aminocarbonylation.](https://example.com/catalytic_cycle)

\begin{align*}
\text{Pd}^{II} \text{ precatalyst} & \quad \text{Base-HX} \\
\text{Pd}^{II}-\text{L} & \quad \text{Oxidative addition} \\
\text{X} & \quad \text{CO coordination} \\
\text{H-Pd}^{II}-\text{L} & \quad \text{Nucleophilic attack} \\
\text{(Het)Ar} & \quad \text{1,1-insertion} \\
\end{align*}

\textit{Figure 14.} Proposed catalytic cycle of aminocarbonylation. (Het)Ar = (Hetero)Aryl; X = Leaving group (LG); L = Ligand.

![The two-chamber system consisting of chamber A (CO-releasing) and chamber B (CO-accepting) allows \textit{ex situ} generation of CO from a solid CORM.](https://example.com/two_chamber_system)

\textit{Figure 15.} The two-chamber system consisting of chamber A (CO-releasing) and chamber B (CO-accepting) allows \textit{ex situ} generation of CO from a solid CORM.
co-workers developed a two-chamber system (Figure 15) utilising \textit{ex situ} generation of CO from a solid source in one chamber (A) and diffusion to the second chamber (B) where the reaction takes place. In this way several Pd-catalysed carboxylations can be conducted in a simple manner.\textsuperscript{105–108}

**Synthesis of Imidazole-Based SP\textsubscript{1–7} Peptidomimetics**

As mentioned above, previous attempts to connect the halogenated imidazole with an amino acid amide through Pd-catalysed aminocarbonylation used a one-chamber setup. The approach with \textit{in situ} generation of CO from Mo(CO)\textsubscript{6} was unsuccessful mainly due to the presence of molybdenum in the reaction mixture that hampered the purification.\textsuperscript{79}

Investigation of the Pd-catalysed aminocarbonylation step used a two-chamber setup where CO was generated \textit{ex situ} from Mo(CO)\textsubscript{6} (Table 6).\textsuperscript{105} The Pd(PPh\textsubscript{3})\textsubscript{4} was selected as the catalysis since it previously has served as a general catalyst for aminocarbonylation of aryl iodides.\textsuperscript{105} The choice of temperature, \textit{N}1-protecting group (PG), base and acylation catalyst were all factors that affected the isolated yield. In particular, the nature of the base was crucial. Triethylamine (TEA) favoured urea formation\textsuperscript{109,110} from two amino acid amide molecules, and nothing of the desired product was isolated (entry 1).

**Table 6.** \textit{Pd-catalysed aminocarbonylation of \textit{N}1-protected 4-iodo-1\textit{H}-imidazoles using \textit{ex situ} generation of CO.}

<table>
<thead>
<tr>
<th>Entry</th>
<th>PG</th>
<th>Temp. (°C)</th>
<th>Base</th>
<th>Acylation catalyst</th>
<th>Product</th>
<th>Yield (%)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SO\textsubscript{2}N(CH\textsubscript{3})\textsubscript{2}</td>
<td>85</td>
<td>TEA</td>
<td></td>
<td>22a</td>
<td>n.d.\textsuperscript{b}</td>
</tr>
<tr>
<td>2</td>
<td>SO\textsubscript{2}N(CH\textsubscript{3})\textsubscript{2}</td>
<td>85</td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td></td>
<td>22a</td>
<td>44%\textsuperscript{c}</td>
</tr>
<tr>
<td>3</td>
<td>SO\textsubscript{2}N(CH\textsubscript{3})\textsubscript{2}</td>
<td>100</td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td></td>
<td>22a</td>
<td>57%\textsuperscript{d}</td>
</tr>
<tr>
<td>4</td>
<td>SO\textsubscript{2}N(CH\textsubscript{3})\textsubscript{2}</td>
<td>100</td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td>DMAP</td>
<td>22a</td>
<td>64%\textsuperscript{d}</td>
</tr>
<tr>
<td>5</td>
<td>CH\textsubscript{2}C\textsubscript{6}H\textsubscript{5}</td>
<td>100</td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td>DMAP</td>
<td>22b</td>
<td>90%</td>
</tr>
<tr>
<td>6</td>
<td>CH\textsubscript{2}C\textsubscript{6}H\textsubscript{5}</td>
<td>85</td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td>DMAP</td>
<td>22b</td>
<td>92%</td>
</tr>
<tr>
<td>7</td>
<td>CH\textsubscript{2}C\textsubscript{6}H\textsubscript{5}</td>
<td>85</td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td></td>
<td>22b</td>
<td>42%</td>
</tr>
</tbody>
</table>

Reaction conditions: Chamber A: Mo(CO)\textsubscript{6} (0.5 equiv), DBU (1.5 equiv) in 1,4-dioxane (3 mL). Chamber B: \textit{N}1-protected 4-iodo-1\textit{H}-imidazoles \textbf{20a–b} (0.4 mmol, 1 equiv), H-Phe-NH\textsubscript{2} \textbf{21} (2 equiv), Pd(PPh\textsubscript{3})\textsubscript{4} (5 mol %), base (2 equiv) and DMAP (2 equiv, entry 4-6) in 1,4-dioxane (3 mL). \textsuperscript{a}Isolated yield. \textsuperscript{b}n.d. = not determined (No isolated yield since urea-formation originating from two amino acid amide nucleophiles was identified as major product). \textsuperscript{c}Incomplete consumption of the imidazole substrate. \textsuperscript{d}Competing side-product originating from homo-coupling of the imidazole substrate was observed.
Changing the base to K$_2$CO$_3$ gave an isolated yield of the desired product of 44% (entry 2). Attempts to increase the conversion by raising the temperature (entry 3), however, led to formation of a competing bis-imidazole homo-coupling$^{111,112}$ while adding DMAP (entry 4) increased product formation slightly (64% vs. 57%). Since the sulfonamide protecting group was suspected to be responsible for the enhanced dimerization of the imidazole substrate, the $N$1-protecting group was changed to a benzyl group (entry 5). The change of protecting group resulted in an isolated yield of 90%. A modest improvement (92%) was obtained when decreasing the reaction temperature (entry 6). The acylation catalyst DMAP$^{113}$ was crucial for product formation since removal resulted in an isolated yield of only 42% (entry 7).

Next, focus was shifted to the synthesis of the disubstituted imidazole substrate through C5 arylation and C4 halogenation as outlined in the retrosynthetic analysis (Figure 12, p. 36). The Pd-catalysed C5 arylation of 1-benzyl-1$H$-imidazole (23) was conducted under microwave irradiation for 1 h at 160 °C using aryl bromides (24a–e), Pd(OAc)$_2$, P(2-furyl)$_3$, pivalic acid (PivOH) and K$_2$CO$_3$ in DMF (Scheme 2, step 1).$^{79}$ Although the competing formation of a 2,5-diarylation side-product hampered the isolated yield, 25a–e were isolated in yields from 46–65%. Next, introduction of iodine in C4 position of compounds 25 were accomplished using N-iodosuccinimide (NIS) with catalytic amounts of TFA in acetonitrile (Step 2).$^{114}$

Scheme 2.

PivOH = pivalic acid. *Maximum expected CO pressure in the system is 2.3 bar.
The starting material was fully consumed and despite formation of trace amounts of di-iodinated side-product, the isolated yield for the iodinated compounds 26a–e was of 73–84%. The following aminocarbonylation was performed according to the previously optimised conditions (Table 6) to yield the constrained imidazole-based Phe-Phe amide analogues 27a–e (Scheme 2, step 3). The steric congestion of the C5 aryl substituent on the imidazole was expected to hamper the coupling and, indeed the initial attempt run at 100 °C for 15 h (overnight) led to incomplete conversion of the substrate (isolated yield 45%, aryl = C₆H₅). Prolonging the reaction time and increasing the temperature resulted in full consumption of the substrate and good isolated yields of 27 (72–85%). However, traces of dehalogenated starting material lowered the yield compared to compound 22b (Table 6, entry 5 and 6), but the two-chamber set-up allowed separation of the Mo(CO)₆ from the reaction mixture and simplified the work-up of the desired products. In addition, to determine whether these conditions caused any racemisation, the corresponding D-isomer of 27e was synthesised using D-Phe-NH₂ as nucleophile. Specific optical rotation measurement and chiral HPLC showed that racemisation did not occurred.

The final Pd-catalysed N-debenzylation (Step 4) was difficult, however performed in an autoclave using Pd/C and H₂ (10 bar) yielded the target compound 19 in an overall isolated yield of 28% (4 steps).

Pharmacokinetic and Neurotoxicity Evaluation

Evaluation of compound 19 in plasma stability and Caco-2 permeability assays (Table 7) showed that the introduction of an imidazole-based phenylalanine mimetic in the N-terminal group improved the plasma stability. Compound 19 was completely inert in both mouse and human plasma (t½ > 180 min).

The permeability data classified 19 as a compound with moderate permeability. However, introducing the less basic imidazole in the N-terminal did not reduce the propensity for efflux. Due to the promiscuous nature of the P-gp efflux protein, the compound is most likely still a P-gp substrate. Furthermore, it is possible that the high concentration (100 µM) used here may saturate the P-gp protein and thereby result in a higher apical to basolateral (a – b) value than what would have been seen if a lower concentration was used. In addition, drug plasma concentrations rarely reach saturating levels at the BBB, so compound 19 would probably have problems accessing the CNS.⁷⁸

Next, the potential neurotoxicity of compound 19 was evaluated (Figure 16). Primary cortical cell cultures were treated with increasing concentrations of 19 (10⁻⁹–10⁻⁵ M) and lactate dehydrogenase (LDH) release
Table 7. Plasma stability and permeability data for compound 19.  

<table>
<thead>
<tr>
<th>Plasma stability&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Caco-2 permeability&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td>P&lt;sub&gt;app&lt;/sub&gt; (× 10&lt;sup&gt;-6&lt;/sup&gt; cm/s)</td>
</tr>
<tr>
<td>mouse human</td>
<td>(a – b) mb (%) (b – a) mb (%) efflux ratio</td>
</tr>
<tr>
<td>&gt; 180</td>
<td>0.84 ± 0.11 65 16.0 ± 1.50 67 19</td>
</tr>
</tbody>
</table>

<sup>a</sup> t<sub>1/2</sub> = half-life. <sup>b</sup>P<sub>app</sub> = apparent permeability coefficient. (a – b) = apical to basolateral. mb = mass balance. (b – a) = basolateral to apical. 0.2 < P<sub>app</sub> < 1.6 × 10<sup>-6</sup> cm/s indicates moderate permeability. efflux ratio = (b – a) / (a – b)

Figure 16. Primary cortical cell culture cytotoxic death (A) and viability (B) measured in the LDH and MTT assay, respectively, after treatment with 19 (10<sup>-9</sup>-10<sup>-5</sup> M). Triton X-100 served as a positive control. The assays were performed two times in triplicate. Primary cortical cells from Sprague-Dawley rats were used.

was monitored with colorimetric detection. No cytotoxicity was detected for the cells even at the highest concentration of compound 19 (Figure 16A). Furthermore, to monitor viability of the primary cortical cells the MTT assay was used. The cells were treated with increasing concentrations of 19 (as for the LDH assay) and no MTT cleavage was observed (Figure 16B). Triton X-100 served as a positive control in both assays. Consequently, compound 19 displayed a favourable safety profile compared to control.

Pharmacological Evaluation

Compound 19 was evaluated for its effect on mechanical allodynia in SNI mice<sup>87</sup> following i.p. administration at doses of 1.85, 18.5, 185 and 1850 nmol/kg. The anti-allodynic effect was assessed using the von Frey test.<sup>88</sup> A dose-response curve for 19 was established and a clear dose-dependent increase, as compared to control, of the mechanical threshold for mice injected with 185 nmol/kg or 1850 nmol/kg i.p. was obtained (Figure 17). Despite the improved plasma stability for 19 compared to the SP<sub>1-7</sub> amide (2)
The anti-allodynic effect was not prolonged. For **19**, a short-onset with a decline after 30 min was observed following the trend of **2** and the N-methylated variants **11** and **12** ([Figure 11, p. 33]). The pharmacological response of **19** was comparable to that of the full-length SP₁₋₇ amide (**2**) and revealed no statistically significant difference between the two groups (Figure 18). As mentioned, it should be noted that the previously examined SP₁₋₇ peptidomimetics (**A and B**, Figure 5, p. 20) had weaker anti-allodynic effect in SNI mice (**A**) than **2** or showed complete inactivity (**B**). Here, the imidazole-based SP₁₋₇ peptidomimetic **19** gratifyingly demonstrated, at the same 185 nmol/kg i.p. dose, an anti-allodynic effect equipotent to that of the SP₁₋₇ amide (**2**) (Figure 18).

In an attempt to further elucidate if the small peptidomimetic compound **19** really acts and binds at the same site as the parent peptide SP₁₋₇, the SNI mice were pre-treated with an antagonist to the SP₁₋₇ target, [D-Pro²,D-Phe⁷]SP₁₋₇ (D-SP₁₋₇, \(K_i = 1.8\ \text{nM}\))⁶⁸ (Figure 19). This antagonist has previously been documented to reduce several *in vivo* effects displayed by SP₁₋₇. For example,
i.t. administration of SP1-7 was shown to decrease withdrawal jumping behaviors in morphine-dependent mice, an effect that was extinguished by i.t. pre-treatment with the antagonist D-SP1-7. A similar antagonistic effect was observed after i.p. administration in morphine-dependent mice, where D-SP1-7 extinguished the effect of SP1-7. With compound 19, the antagonist reduced the anti-allodynic effect. Mice pre-treated with D-SP1-7 showed a lower peak mechanical threshold than mice injected with 19 alone suggesting that 19 and the D-SP1-7 interact at the same target.

Finally, dose-response values of 19, gabapentin, and morphine were compared and ED50 values were calculated (Figure 20). For 19 a half-maximum peak anti-allodynic effect was observed at a dose of 0.21 µmol/kg whereas gabapentin and morphine required 336 µmol/kg and 6.5 µmol/kg, respectively.

![Figure 19](image1.jpg)

*Figure 19.* Effect of D-SP1–7 antagonist (1850 nmol/kg, i.p.) on the anti-allodynic effect of 19 (185 nmol/kg, i.p.) in SNI mice (n = 6–9). Total anti-allodynic effect expressed as AUC. Statistical significance was marked as follows: ** p < 0.01 (vs. control). Control mice were injected with 0.1 M acidified saline.

![Figure 20](image2.jpg)

*Figure 20.* Dose-response curves of 19 (1.85–1850 nmol/kg, i.p.), gabapentin (18.5-585 µmol/kg, i.p.) and morphine (0.185–12 µmol/kg, i.p.) and the control subtracted total anti-allodynic effect (AUC) in SNI mice (n = 10–18). Statistical significance was marked as follows: ** p < 0.001 (vs. gabapentin), # p < 0.05 (vs. morphine).
Summary and Future Outlook

The introduction of an imidazole-based phenylalanine mimetic into the Phe-Phe amide was achieved and indeed proved to be a potent bioisostere. The target compound 19 demonstrated a clear improvement in plasma stability. I.p. administration of low doses of 19 showed an anti-allodynic effect in SNI mice, equal to that of the SP1–7 amide (2) and significantly higher than gabapentin, the current first-line treatment for neuropathic pain. Competition experiments indicate that 19 interacts with the SP1–7 target.

However, the bioisostere replacement in compound 19, did not improve selectivity between binding to the target and to the efflux protein (in Caco-2 cell monolayer and presumably at the BBB). Regardless of this, 19 produced a potent anti-allodynic effect in vivo which indicates target engagement centrally or peripherally. Despite the improved plasma stability of 19 this neither increased nor prolonged the anti-allodynic effect. This observation could be due to several factors, e.g. low brain exposure, low hepatic stability, low central metabolic stability, discrepancy between in vitro and in vivo plasma stability and/or poor affinity to the SP1–7 target. Consequently, in vivo testing does not give a clear-cut result.94,116

Finally, a synthetic route to 19 has been developed containing a Pd-catalysed aminocarbonylation step which could, by isotopic substitution, deliver 11C-labelled 19 suitable for PET studies. For this, further optimisation is required to meet the demands for 11C-labelling chemistry, e.g. the radiolabelling of the compound is performed as late as possible in the synthesis with the time used for labelling and ensuing deprotection and purification compatible with the half-life of the $\beta^+$ emitting radionuclei ($t_{1/2} = 20.4$ min for carbon-11).117
Combining Pd-catalysed Aminocarbonylation with Fmoc SPPS (Paper V)

The increasing importance in recent years of peptide therapeutics, as mentioned in the introduction, has renewed interest in synthetic peptides where recent advances in Fmoc SPPS enable the medicinal chemist to synthesise complex peptides and peptidomimetics.\textsuperscript{118} In addition, methods for radiolabelling peptides for applications in PET are presently gaining a lot of interest.\textsuperscript{117,119}

The desired peptide is synthesised through Fmoc SPPS followed by cleavage from the solid support and simultaneous deprotection of side-chain protecting groups (Figure 21). Before labelling, the peptide is purified, analysed and lyophilised. Radiolabelling methods can be broadly divided into three categories: labelling of unprotected peptides (\textit{direct labelling}), conjugation with a labelled prosthetic group (\textit{indirect labelling}) or labelling of a pre-modified peptide (\textit{a chelating agent or “tag” is linked to the peptide and then labelled}).\textsuperscript{120} The peptides can be labelled with radiometals (e.g. gallium-68) or with non-metal isotopes (e.g. fluorine-18 or carbon-11). Members of the latter group is used for PET imaging of compounds intended to reach CNS.

\textit{Figure 21.} A general scheme for the preparation of radiolabelled peptides by Fmoc SPPS. Inspired by Okarvi.\textsuperscript{120}

Recently, \textsuperscript{11}C-labelling methods of bioactive small peptides (linear and cyclic) have attracted considerable attention.\textsuperscript{121–124} Ideally, radiolabelling a small lead peptide should introduce as small a change as possible into the compound and thus preserve its biological behaviour. The natural abundance of carbon in peptides makes direct \textsuperscript{11}C-labelling methods especially attractive.\textsuperscript{119} However,
direct labelling of a peptide in solution can be troublesome due to competing side-reactions from unprotected nucleophilic amino acid residues, resulting in product mixtures that can be difficult to purify.

Background and Strategy

At the onset of this project, several unsuccessful attempts had been made to $^{11}$C-label the peptidomimetic compound 19 (data not shown). Together with the observation that full-length SP$_{1-7}$ related peptides could serve as useful tools (e.g. radiotracers) for further research on the SP$_{1-7}$ system, the following proposal took form: Would it be possible to combine the Pd-catalysed aminocarbonylation with Fmoc SPPS and thereby access a method for $^{11}$C-labelling of peptides while still attached to a solid support? Furthermore, could this method be applied to label SP$_{1-7}$ related peptides with carbon-11 for use in PET studies?

Inspiration for developing a method that could be used to $^{11}$C-label biologically active peptides (Figure 22) came from a paper published by Sutcliffe-Goulden and co-workers, in which a Arg-Gly-Asp (RGD) peptide was indirectly $^{18}$F-labelled while still attached to a solid support. In addition, our previous work (Paper III), where Pd-catalysed aminocarbonylation of 4-iodo-1H-imidazole introduced an N-capping group into a peptide equivalent, added further support to the feasibility of this approach.

Figure 22. Inspirations and future ambition to develop a method for $^{11}$C-label peptides attached on a solid support followed by detachment using acid.

With this future ambition, the first step would be to find suitable reaction conditions for combining Pd-catalysed aminocarbonylation with Fmoc SPPS. The method would not only enable introduction of various $N$-capping groups to a peptide chain, but hopefully also be used for side-chain conjugation and
side-chain to side-chain cyclisation of biologically relevant peptides, thereby expanding the toolbox for SPPS. In addition, solid-phase methods have been used in Pd-catalysed carbon-carbon reactions such as Stille, Suzuki, Heck, and Sonogashira couplings, all well documented robust methods.\textsuperscript{126–129}

**Development of a Mild and Easy-to-Execute Reaction**

The combination of Pd-catalysed aminocarbonylation and SPPS required careful planning and consideration. Five different factors were identified as important for development of an easy-to-execute method compatible with the solid support. The first factor was the reaction temperature. A low reaction temperature was preferable to avoid racemization of the asymmetric centre in the amino acids. The second factor was mixing. Could an alternative way of mixing the reaction as to the standard magnetic stirring used in palladium coupling, be employed? Mechanical stirring is not recommended in SPPS because of the risk of grinding up the resin. Traditionally, in solid-phase synthesis, mixing is usually performed by rotation, tilting or bubbling an inert gas through the suspension. Furthermore, the third and fourth factors concerned the resin and the solvent. A suitable resin for this method should have a good swelling profile in a solvent which is compatible with aminocarbonylation. The fifth factor concerned the CO pressure to be used in the experiment. To ensure high CO incorporation, a stoichiometric amount of CO should preferably be used. The pressure necessary to ensure optimal penetration of the CO into the swollen resin bed therefore required investigation.

Recently, Skrydstrup and co-workers demonstrated the use of a palladacycle precatalyst (29, Scheme 3)\textsuperscript{130} for promoting aminocarbonylations at 45°C using (hetero)aryl bromides to obtain a range of amide products.\textsuperscript{131} It was envisioned that the palladacycle precatalyst (29) in combination with (hetero)aryliodides could allow reactions at room temperature (r.t.). Additionally, this would simplify the mixing since it would be possible to agitation the reaction vessel at r.t. Regarding the choice of CORM, the CO source should preferably provide CO-release at r.t., be easy to remove from the resin, and have an activator compatible with the aminocarbonylation reaction. Preferably, the CORM selected would also be suitable for isotope labelling. Thus, a silacarboxylic acid, MePh\textsubscript{2}SiCOOH, that can be activated with KF was chosen as the solid CO source.\textsuperscript{132} Since the solid-phase bound amine was the limiting reagent, pre-loaded Wang resin was used as the solid support. This PS-based resin is a commonly used, cheap resin with a high loading capacity and good swelling properties. Furthermore, preloaded versions are commercially available from many suppliers. PS has a good swelling profile in DMF, which therefore was used as the solvent.
In line with the reasoning above, the reaction of 4-iodoanisole (4 equiv) with solid-phase bound phenylalanine in dry DMF and in the presence of two mol % of palladacycle precatalyst 29 and 1.5 equiv of MePh₂SiCOOH (30) was explored (Scheme 3). The mixture was agitated in a closed vial at r.t. overnight, and resulted in incomplete conversion of the amine 28 as shown by ¹H-NMR analysis. To get the greatest benefit from the use of a solid-phase bound nucleophile, the reaction needs to reach full conversion so that the product can be easily isolated. Thus, an increase to two equivalents of CO resulted in full conversion of the amine. Efficient cleavage from the resin in 95% aqueous TFA followed by precipitation in cold diethyl ether gave 31 in 95% isolated yield.

Scheme 3.

1. Palladacycle precatalyst 29 (2 mol %)
MePh₂SiCOOH (30) (2 equiv)
KF (2 equiv), TEA (4 equiv)
DMF, r.t., 15 h

Agitated in a closed 8 mL vial

2. 95% TFA (aq), TES
r.t., 2 h

○0.3 mmol, 0.6 mmol/g, 0.075M. *Isolated yield after precipitation.

Reaction Scope for N-capping Solid-Phase Bound Nucleophiles

The optimised reaction conditions were expanded to a variety of solid phase bound amino acid nucleophiles and, as expected, proved to be well tolerated (Table 8, 32a–f). The conversion of the secondary amine (Pro) to the corresponding amide proceeded efficiently and afforded 32a in 94% yield. Furthermore, the use of β-branched amino acids (Val and Thr) was also allowed and their corresponding amide product were isolated in 90% and 95% yield, respectively (32b and 32c).

In the previously reported Pd-catalysed aminocarbonylation using an amino acid nucleophile (Paper III), racemisation was investigated by measuring the optical rotation compared to that of the corresponding D-product, synthesised from the D-amino acid nucleophile using the same conditions. The optical rotation of the respective analogue was measured and compared. In addition, chiral HPLC analyses of 27a–e (Scheme 2, p. 40) were also performed. Here, selected products in Table 8 were synthesised from para-anisic acid, HBTU, and DIPEA and optical rotation of each product was
Table 8. Pd-catalysed aminocarbonylation of aryl and heteroaryl iodides with a solid-phase bound amino acid nucleophile.

<table>
<thead>
<tr>
<th>(Het)Aryl</th>
<th>H₂N-Aaa</th>
<th>(Het)Aryl-N-Aaa-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>32a-f</td>
</tr>
</tbody>
</table>

1. Palladacycle precatalyst 29
   MePh₂SiCOOH (30)
   KF, TEA
   DMF; r.t., 15 h
   (Agitated in a closed 8 mL vial)
2. 95% TFA (aq), TES
   r.t., 2 h

(Het)Aryl = Preloaded Wang PS resin (0.56-0.70 mmol/g)

<table>
<thead>
<tr>
<th>32a</th>
<th>94%</th>
<th>32b</th>
<th>90%</th>
<th>32c</th>
<th>95%</th>
<th>32d</th>
<th>93%</th>
<th>32e</th>
<th>90%</th>
<th>32f</th>
<th>91%</th>
</tr>
</thead>
</table>

| 33a | 93% | 33b | 90% | 33c | 82% | 33d | 83% | 33e | 85% | 33f | 86% | 33g | 85% | 33h | 86% | 33i | 82% |

Reaction conditions: (Hetero)aryl iodide (4 equiv), solid-phase bound amine (0.3 mmol, 1 equiv), palladacycle precatalyst 29 (2 mol %), MePh₂SiCOOH 30 (2 equiv), KF (2 equiv), TEA (4 equiv) in DMF (4 mL). Isolated yields after precipitation. Following side-chain protection was used: Thr(tBu), Asn(Trt), Asp(OrBu), Lys(Boc). Optical rotation of products was compared with the corresponding product synthesised from p-anisic acid, HBTU, and DIPEA. Five mol % of 29 was used. Three equiv of 30 and KF were used. 45 °C, under ultrasonication. Synthesised from corresponding heteroaryl bromide. Synthesised from Trt protected 4-iodo-1H-imidazole. Synthesised from Boc protected 3-iodo-1H-anisole.
measured and compared with the corresponding product obtained from the aminocarbonylation method. Equivalent values were obtained in all cases, indicating that the aminocarbonylation method is performing equally well as the conventional coupling in this aspect.

Adapting the reaction conditions to other aryl iodides was somewhat troublesome (selected examples in Table 8, compounds 33a–d). In fact, each entry had to be optimised separately to reach full conversion of the amine and therefore it is difficult to compare the isolated yields for the compounds. In general, the method was easily adapted to other electron-rich aryl iodides (33a) while electron-poor aryl iodides required a higher catalytic loading (5 mol %) and increased amounts of CO (3 equiv) to reach full conversion (33b and 33c). For the ortho-substituted aryl iodides, full conversion was reached when the reaction temperature was increased to 45 °C, under ultrasonication (33d).

Next, products 33e and 33f, were synthesised from the 4- or 5-substituted 2-bromothiazoles. The aminocarbonylation of 2,4-bromothiazole displayed complete selectivity for the bromide in the more electron poor 2-position, thus securing 33e as the only product. Moreover, the ethyl ester in product 33f was unaffected when treated with aqueous TFA and TES to cleave the product from the solid-phase resin.

The acid labile Trt- and Boc-protecting groups, respectively, were used for the imidazole and indole substrates and were rapidly cleaved upon treatment with 95% TFA and TES, to give products 33g and 33h.

Applications: Labelling, Side-Chain Conjugation, and Cyclisation

The applicability of the Pd-catalysed aminocarbonylation method in Fmoc SPPS was demonstrated in the preparation of biologically active peptides (Scheme 4).133,134 The peptides were synthesised using standard Fmoc SPPS on Rink Amide MBHA PS resin on a 0.1 mmol scale. The N-capping group in the bortezomib analogue 34 was incorporated as a last step before final cleavage from the resin. The optimised aminocarbonylation protocol resulted in complete conversion of the starting peptide with an isolated yield of 78% after preparative RP-HPLC. The corresponding 13C-labelled analogue, [13C]34*, was secured using silacarboxylic acid [13C]30* with an isolated yield of 73%.

To enable the introduction of conjugates to nucleophilic lysines present in the precursor peptide of 35 (peptide part of the anticancer agent CR1166) the Alloc group was used for orthogonal protection and was then selectively removed using Pd(PPh3)4 and PhSiH3 in dry DCM. To ensure complete conversion of both amines in the precursor peptide in the following aminocarbonylation reaction, 8 equivalents of the aryl iodide were used toge-
Scheme 4.

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction Conditions</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>20% Pip/DMF, Fmoc-Aaa-OH HBTU/DIPEA DMF</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>20% Pip/DMF, Fmoc-Aaa-OH HBTU/DIPEA DMF</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>20% Pip/DMF, Fmoc-Aaa-OH HBTU/DIPEA DMF</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>20% Pip/DMF, Fmoc-Aaa-OH HBTU/DIPEA DMF</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>20% Pip/DMF, Fmoc-Aaa-OH HBTU/DIPEA DMF</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>20% Pip/DMF, Fmoc-Aaa-OH HBTU/DIPEA DMF</td>
<td></td>
</tr>
</tbody>
</table>

*a* Reaction conditions: (Het)aryl iodide (4 equiv), Palladacycle precatalyst *29* (5 mol%), MePh2SiCOOH (30, 3 equiv), KF (3 equiv), TEA (4 equiv) in DMF (3 mL). Isolated yields after preparative RP-HPLC.

*b* MePh2SiCOOH (30) or MePh2Si13COOH ([13C]30*) was used for the isotopic labelling.

*c* Following side-chain protection was used: Ser(tBu), Gln(Tn).

*d* 8 equiv of 1-bromo-4-iodobenzene and 4 equiv of 30 and KF were used in the aminocarbonylation.

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ther with an increased amount of CORM. The final peptide was obtained in an isolated yield of 61%. The yield was affected by multiple LC-MS samples withdrawn to follow each step in the syntheses and by poor solubility of the crude peptide.

Finally, the aminocarbonylation protocol was extended to intramolecular cyclisation of a model peptide (Scheme 5). To accomplish the side-chain to side-chain cyclisation the following changes to the previous protocol were necessary in order to obtain the cyclised products. First, the highly acid labile $N$-methyltrityl (Mtt) protecting group was used instead of Alloc since the procedure for removing Alloc also reductively cleaved the iodine in the phenylalanine residue. Second, the intramolecular cyclisation required a slightly elevated temperature (45 °C, under ultrasonication) where the N-terminal Fmoc group proved to be unstable. Third, to circumvent this the N-terminal amino acid was therefore introduced as the Boc-derivative in the precursor peptide using HBTU/DIPEA in DMF.

Scheme 5.

\[ \text{Scheme 5.} \]

\[ \text{Rink Amide MBHA} \]

\[ \text{PS resin (0.69 mmol/g)} \]

\[ \text{1. 20\% Pip/DMF} \]

\[ \text{2. Fmoc-Aaa-OH} \]

\[ \text{HBTU/DIPEA} \]

\[ \text{DMF} \]

\[ \text{Repeated until desired} \]

\[ \text{sequence was reached} \]

\[ \text{(0.1 mmol scale)} \]

\[ \text{3. Boc-Phe-OH} \]

\[ \text{HBTU, DlPEA} \]

\[ \text{DMF} \]

\[ \text{4. 1.8\% TFA in DCM} \]

\[ \text{3 min/2 mL, wash} \]

\[ \text{10 washes} \]

\[ \text{5. Palladacycle precatalyst (29)} \]

\[ \text{MePh}_2\text{SiCOOH (30, 3 equiv), KF (3 equiv), TEA (4 equiv) in} \]

\[ \text{DMF (3 mL). Isolated yields after preparative RP-HPLC.} \]

\[ \text{6. 95\% TFA (aq), TES} \]

\[ \text{\textsuperscript{13}C} \]

\[ \text{36*} \]

\[ \text{Isolated as} \]

\[ \text{cis/trans rotamers and contains traces of side product formed from intermolecular cyclisation.} \]
Thus, 4-iodophenylalanine and lysine protected Mtt were incorporated into the N-terminally Boc-protected linear model precursor peptide to 36 and [13C]36*. The Mtt protecting group was selectively removed by a short treatment with 1.8% TFA in DCM to give the cyclisation precursor. Running the intramolecular aminocarbonylation using the standard protocol developed, but at 45 °C gave the desired product together with a contaminating intermolecular dimer product, identified by LC/MS on the crude product. This was later confirmed by 13C-NMR and HRMS analysis.

Increasing the reaction temperature further, to 65 °C favoured formation of this competing side-product. This observation indicated that the dimerization actually took place during the reaction and not something occurring under LC-MS/HRMS analysis. It is possible that a fourth modification to the protocol, lowering the loading of the resin would have decreased intermolecular side reactions, but this was not investigated further.

Overall, the intermolecular aminocarbonylation afforded 36 and [13C]36* in isolated yields of 11% and 6%, respectively. The yields were reduced by the multiple samples for LC-MS analysis to follow each step of the synthesis, minor cleavage of the precursor peptide from the resin during Mtt deprotection and by the successive HPLC runs required for separation of the desired product from the co-eluting side product.

Summary and Future Outlook

Protocols for direct 11C-labelling of SP were published as early as in the late 1980s.135,136 The peptide was synthesised in solution and the radiolabelling was accomplished by 11C-methylation of a homocysteine residue in the C-terminal part of SP (i.e. position 11). Even though the method had problems with cleavage of protecting groups on other amino acid residues and the fact that labelling could only be conducted if a methionine residue was present, the [11C-Met11]SP was intended for thorough investigation in biological studies. Although 11C-labelling of the SP1-7 peptide remains a distant prospect, the methodology presented herein might be a suitable onset for further research to develop such a method.

The main advantage of the solid-phase strategy are the use of excess reagents to ensure completion of the reaction due to easy workup using filtration, improved yields, and simplicity of Fmoc SPPS chemistry. The new methodology presented here, where the Pd-catalysed aminocarbonylation was combined with Fmoc SPPS, proved to be easy-to-execute, mild and versatile. Indeed, the method could be tailored to enable incorporation of various N-capping groups to solid-phase bound amino acid/peptide nucleophiles, selective [13C]acyl labelling, conjugation of orthogonal lysine residues, and intramolecular cyclisation. This will be a very useful addition to the available toolbox of methods for synthesising complex peptides and peptidomimetics.
Concluding Remarks

This thesis describes the design and synthesis of SP1–7 related peptides and peptidomimetics where the *in vivo* pharmacological anti-allodynic activity of the designed analogues has been correlated to important *in vitro* pharmacokinetic properties. The main conclusions are presented below.

- The basic Arg¹ and Lys³ residues are not important for the overall *in vitro* plasma stability or permeability of the full-length peptides.

- However, a basic amino acid residue (Arg¹) at the N-terminal with an unblocked amino function is proposed to be a key address residue while an unmodified phenylalanine amide (Phe⁷-NH₂) residue in the C-terminal functions as a message carrier. Both residues are important for potent *in vivo* activity.

- The lysine residue (Lys³) is not important for the *in vivo* activity for the peptides.

- *N*-methylation of the Pro²-Lys³ and Pro⁴-Gln⁵ bonds were possible without losing the anti-allodynic activity. In addition, the Pro²-Lys³ bond was identified as the most sensitive bond to proteolytic degradation. *N*-methylation of this bond delivered peptides completely stable in mouse plasma.
The major metabolite of the SP\textsubscript{1–7} amide (2), i.e. the SP\textsubscript{3–7} amide (7), possessed in vivo anti-allodynic activity itself. Formation of this metabolite seems to contribute to the overall anti-allodynic activity of 2 since peptides that did not deliver this metabolite in vivo displayed reduced or no activity.

The active peptides 2, 11, and 12 were more potent as compared to gabapentin in reducing allodynia in SNI mice in a single dose.

Two Pd-catalysed aminocarbonylation protocols, utilizing ex situ or in situ generation of CO from a solid CORM, have been developed to access an imidazole containing phenylalanine based peptidomimetics and biologically relevant modified peptides.

a) A convenient and robust process for the synthesis of imidazole-based SP\textsubscript{1–7} peptidomimetics by the Pd-catalysed aminocarbonylation of 5-aryl-4-iodo-1\textsubscript{H}-imidazoles and an amino acid amide nucleophile using ex situ generated CO from Mo(CO)\textsubscript{6} in a two-chamber system has been developed.

b) A mild and easy-to-execute Pd-catalysed aminocarbonylation reaction using in situ generation of CO from MePh\textsubscript{2}SiCOOH, was applied to Fmoc SPPS for introduction of various N-capping groups to a peptide chain. The protocol was used for isotopic labelling (\textsuperscript{13}C) and side-chain conjugation of biologically active peptides useful for intramolecular side-chain to side-chain cyclisation.

The imidazole-based SP\textsubscript{1–7} peptidomimetic 19 was completely inert in both human and mouse plasma. However, the bioisosteric replacement of phenylalanine did not improve the selectivity between the SP\textsubscript{1–7} target protein and the efflux receptor.

Compound 19 displayed a clear dose-dependent in vivo anti-allodynic effect. The activity was equal to that of the SP\textsubscript{1–7} amide (2) and was also significantly more potent than gabapentin.

A competition experiment indicates that 19 interacts at the SP\textsubscript{1–7} target.

The findings presented in this thesis have resulted in the development of an understanding of the in vivo SAR for SP\textsubscript{1–7} related peptides, a novel small molecule SP\textsubscript{1–7} peptidomimetic, and methods expanding the toolbox for synthesising modified peptides and peptidomimetics – a field that presently is gaining a lot of interest in drug discovery.
Populärvetenskaplig sammanfattning


Figur 1. SP signalerar smärta via NK-1 receptorn. Mekanismen för hur SP₁₋₇ och dess syntetiska analoger 2 och 19 utövar sin smärtlindrande effekt är okänd.

Substans P (SP) är en neuropeptid som via neurokinin-1 (NK-1) receptorn förmedlar smärta via nervsystemet (Figur 1). När denna peptid bryts ner i kroppen bildas flera fragment, varav substans P 1–7 (SP₁₋₇) är det bioaktiva fragmentet som bildas i störst mängd. SP₁₋₇ har dokumenterad smärtlindrande effekt i flera djurmodeller för nervsmärta och utgör därmed en intressant utgångspunkt för att hitta nya läkemedel för behandling av nervsmärta. Dock är det okänt hur den utövar sin smärtlindrande effekt och ingen målmolekyl (dvs receptor) är identifierad. Det behövs således mer kunskap om hur SP₁₋₇ systemet fungerar och utveckling av tillförlitliga forsknings-verktyg som exempelvis peptidanalogen 2 och den syntetiska peptidhärmande föreningen 19 (Figur 1) kan vara till stor hjälp för att identifiera SP₁₋₇ receptorn.
I denna avhandling har nya SP₁⁻₇ peptidanaloger designats och syntetiserats för att vidare utvärderas in vivo i möss som lider av nervsmärta. Genom detta har följande slutsatser kunnat dras: 1) Fenylalanin (Phe⁷) fungerar som en meddelande-del och Argininen (Arg¹) som en adress-del (blåa rutor i figur 2). Meddelande-address konceptet bygger på att peptider i kroppen innehåller en adress-del som ser till att peptiden hittar rätt receptor i kroppen medan meddelande-delen ser till att rätt signal skickas vid aktivering av receptorn (ex. smärta/smärtlindring). 2) Lysin (Lys³) kan tas bort helt utan att den smärtlindrande effekten påverkas (röd ruta). 3) Två positioner i skelettet på SP₁⁻⁷ amiden (2) har identifierats där förändringar kan göras utan att den smärtlindrande effekten försvinner (gröns rutor).

Mest anmärkningsvärt är att SP₁⁻⁷ amiden (2) och 19 har en större smärtlindrande effekt vid lägre dos än gabapentin, ett läkemedel som för närvarande används för behandling av nervsmärta. Intressant är också att föreningarna inte uppvisar synliga biverkningar såsom dåsighet som annars är en vanlig effekt hos läkemedel som ingår i standardbehandling. Figur 2. Sammanfattning av de viktigaste forskningsresultaten som visar vilka delar av SP₁⁻⁷ amiden (2) som kan modifieras för bibehållen smärtlindrande effekt men också vilka delar som måste vara intakta för att peptiden fortfarande ska vara aktiv.

Parallellt har två syntesmetoder utvecklats som kan användas för att syntetisera komplexa peptider och peptidomimetika föreningar. Den ena metoden kan användas för isotopmärkning av peptider (eller syntetiska småmolekyler som 19) och kommer att vidareutvecklas och förfinas för en radioaktiv märkning av SP₁⁻⁷ peptidanaloger eller 19 med kol-11 (¹¹C). Detta skulle möjliggöra att föreningarna kan spåras in vivo (i möss) med den medicinska avbildningstekniken positronemissionstomografi (PET).

Sammantaget kommer forskningsresultaten i denna avhandling att ligga till grund för design av tillförlitliga SP₁⁻⁷ peptidanaloger eller småmolekyler (dvs forsknings-verktyg) för vidare studier av det komplexa SP₁⁻⁷ systemet. Förhoppningsvis kan detta leda till identifiering av SP₁⁻⁷ receptorn och i förlängningen utveckling av nya och effektivare kandidater för behandling av nervsmärta.
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Anna, 28 February 2018
References


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