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# Exploration and Pharmacokinetic Profiling of Phenylalanine Based Carbamates as Novel Substance P 1−7 Analogues

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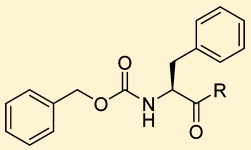
Supporting Information

**ABSTRACT:** The bioactive metabolite of Substance P, the heptapeptide $SP_{1-7}$ (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH), has been shown to attenuate signs of hyperalgesia in diabetic mice, which indicate a possible use of compounds targeting the $SP_{1-7}$ binding site as analgesics for neuropathic pain. Aiming at the development of drug-like $SP_{1-7}$ peptidomimetics we have previously reported on the discovery of H-Phe-Phe-$NH_2$ as a high affinity lead compound. Unfortunately, the pharmacophore of this compound was accompanied by a poor pharmacokinetic (PK) profile. Herein, further lead optimization of H-Phe-Phe-$NH_2$ by substituting the N-terminal phenylalanine for a benzylcarbamate group giving a new type of $SP_{1-7}$ analogues with good binding affinities is reported. Extensive *in vitro* as well as *in vivo* PK characterization is presented for this compound. Evaluation of different C-terminal functional groups, i.e., hydroxamic acid, acyl sulfonamide, acyl cyanamide, acyl hydrazine, and oxadiazole, suggested hydroxamic acid as a bioisosteric replacement for the original primary amide.




**KEYWORDS:** *Substance P 1−7, bioisostere, Caco-2 cells, neuropathic pain, $SP_{1-7}$*

Substance P (SP, H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$)[1] is the endogenous ligand to the neurokinin-1 (NK-1) receptor and acts as a neurotransmitter and neuromodulator in the central and peripheral nervous system.[2] SP is degraded into several bioactive fragments. In particular, the major N-terminal metabolite Substance P 1−7 ($SP_{1-7}$, H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH, **1**) has been extensively studied.[3−5] This heptapeptide is found in the cerebrospinal fluid in man and rodents[6] and exhibits high affinity to specific binding sites in the spinal cord and in the brain.[7−9] Several reports suggest that $SP_{1-7}$ has its own putative receptor, but no receptor has yet been cloned.

The SP metabolite $SP_{1-7}$ attracted our attention for several reasons; first, $SP_{1-7}$ counteracts the expression of opiate tolerance and withdrawal, in contrast to SP and its C-terminal fragments that instead augment the opioid withdrawal signs;[10,11] second, $SP_{1-7}$ attenuates the inflammatory[12] and nociceptive[13] effects exerted by SP; and third, our group recently demonstrated that $SP_{1-7}$ could induce a pronounced antihyperalgesia in diabetic mice.[14] The latter finding suggested to us that low molecular weight $SP_{1-7}$ peptidomimetics are desirable not only as pharmacological research tools for in-depth studies of this system but also as potential future therapeutic agents for the treatment of neuropathic pain.

We have commenced a medicinal chemistry program aimed at transforming $SP_{1-7}$ (**1**) into drug-like peptidomimetics. An alanine scan and subsequent N- and C-terminal modifications of $SP_{1-7}$ and endomorphin-2 followed by truncation afforded the lead compound H-Phe-Phe-$NH_2$ (compound **2**, see Figure 1).[15,16] Notably, the antihyperalgesic effect in diabetic mice of this compound was even more pronounced as compared to $SP_{1-7}$ itself, after intrathecal administration.[17] Structure−activity relationship (SAR) studies[16,18] of dipeptide analogues and a series of rigid analogues of H-Phe-Phe-$NH_2$ (exemplified by compound **3** in Figure 1) suggested that the two benzyl moieties in (*S*,*S*) configuration, a primary amine in the N-terminal in combination with an absolutely crucial primary amide function in the C-terminal, seemed optimal for high binding affinity. Unfortunately this pharmacophore was often correlated with high efflux of the compounds.[18] In order to improve the pharmacokinetic (PK) properties and especially to reduce problems with efflux, which is essential for compounds intended to pass the intestine as well as the blood−brain barrier (BBB), we felt prompted to challenge the pharmacophore and to investigate compounds with reduced hydrogen bond donors.[18] More precisely, we wanted to evaluate compounds lacking the relatively less important N-terminal primary amine, in accordance with prototype **A** (Figure 1). Initially, we decided to study compound **4** where the N-terminal phenylalanine in lead compound **2** was exchanged for a more lipophilic

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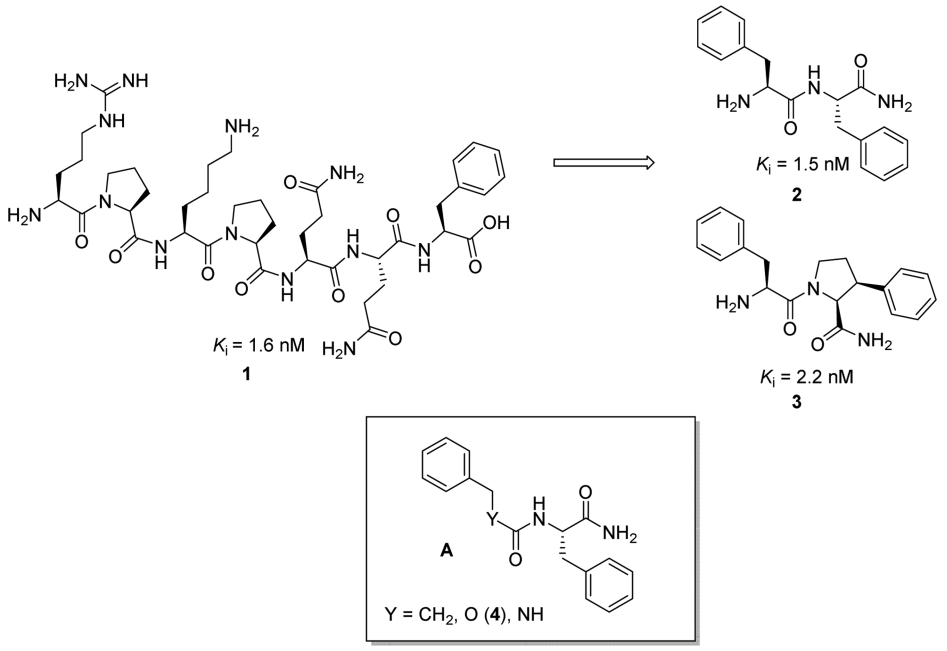
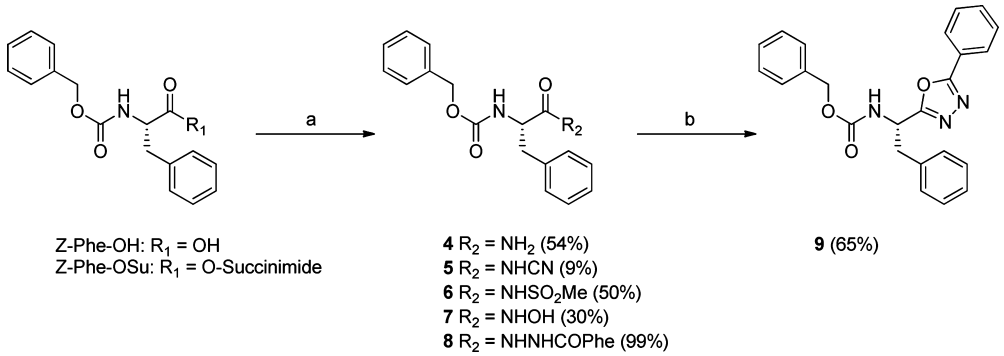



**Figure 1.** $SP_{1-7}$ (**1**) and two of its most active dipeptide analogues (**2** and **3**) identified so far. Prototype structure **A**.

**Scheme 1**[a]




[a]Reagents and conditions: (a)(i) Z-Phe-Osu, $NH_3$, THF, 0 °C to r.t., or (ii) Z-Phe-OH, cyanamide, HATU, DIEA, DMF, 3 days at 40 °C, or (iii) Z-Phe-OH, methylsulfonamide, CDI, DBU, THF, 3 h, or (iv) Z-Phe-OH, hydroxylamine hydrochloride, *N*-methylmorpholine, isobutylchloroformate, $Et_3N$, THF, DMF, 2 h, or (v) Z-Phe-OH, benzhydrazide, HATU, DIEA, DCM, r.t.; (b) **8**, Burgess reagent, THF, 75 °C.

benzyloxycarbonyl moiety. To start with, the binding affinity of compound **4** toward the $SP_{1-7}$ binding site and its *in vitro* PK data were to be determined and compared to the previously developed lead compounds **2** and **3**.

We herein report that replacement of the basic N-terminal amine group for a neutral moiety can deliver a new class of more drug-like ligands with retained binding affinity and more favorable *in vitro* PK data. In addition, the C-terminal requirement for optimal binding affinity of this new compound class has been elucidated by evaluation of a set of C-terminal modifications.

The general synthesis toward the carbamate based derivatives is outlined in Scheme 1. The primary amide **4** was prepared through the reaction of C-terminal activated benzyloxycarbonyl protected phenylalanine (Z-Phe-OSu) with ammonia. The acyl cyanamide **5** was obtained by activation of Z-Phe-OH by *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridine-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU). Although the reaction was allowed to run for 3 days at 40 °C, the acyl cyanamide was obtained in low yield. The synthesis of acyl sulfonamide **6** was done by preactivation of Z-Phe-OH with 1,1′-carbonyldiimidazole (CDI) at 60 °C before the methylsulfonamide was added. The reaction was run in 3 h at room temperature, giving the desired compound in 50% yield. Activation of Z-Phe-OH with isobutylchloroformate at low temperature and coupling with hydroxylamine hydrochloride gave the hydroxamic acid **7** in reasonable yield. The synthesis of the 1,3,4-oxadiazole **9** was prepared via the diacylhydrazine compound **8**, which was further dehydrated using Burgess reagent in order to give the heterocyclic compound **9**.[19]

First, compound **4** was evaluated in the $SP_{1-7}$ binding assay. Despite discarding the basic *N*-terminal amine, the new carbamate based derivative possessed reasonable good binding affinity ($K_i$ = 5.2 nM), demonstrating that this new ligand still maintains significant binding interactions with the target. Together with the other two lead compounds (**2** and **3**),[16,18] compound **4** were evaluated in an extensive *in vitro* profile program (Table 1).[20−23] Several parameters of importance for oral bioavailability were assessed, such as solubility, plasma


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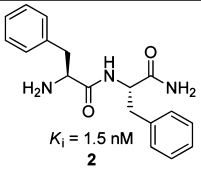
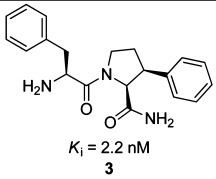
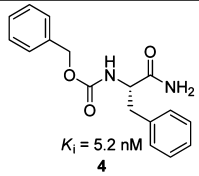
**Table 1. *In Vitro* Pharmacokinetic Profiles of Compounds 2, 3, and 4**

| Compound structure / In vitro profile |  $K_i$ = 1.5 nM **2** |  $K_i$ = 2.2 nM **3** |  $K_i$ = 5.2 nM **4** |
|---|---|---|---|
| **Physiochemical properties** | | | |
| ClogP | 1.02 | 1.67 | 2.15 |
| Polar surface area (PSA) | 107 | 94 | 88 |
| Soluble Dried DMSO (μM) | > 400 | 257 | 97 |
| **Plasma protein binding** | | | |
| Human | NV | 60 | 19 |
| PPB Fu % 10 μM | | | |
| Mean recovery (%) | 22 | >95 | >95 |
| Rat | NV | 67 | 18[a] |
| PPB Fu % 10 μM | | | |
| Mean recovery (%) | 1 | >95 | >70[a] |
| **Brain tissue binding** | | | |
| Mean Fu % | NV | 29 | 17 |
| Mean $V_u$ (mL/g brain) | NV | 3.4 | 6.0 |
| Mean recovery (%) | NV | >95 | >95 |
| **Permeability and efflux** | | | |
| $P_{app}$ ($10^{-6}$ cm/s) (apical to basolateral) | NV | 2.8 | 31 |
| Efflux ratio | NV | 4.2 | 1 |
| **Metabolism** | | | |
| $Cl_{int}$ (Rat Mic) | 102.8 | 8.3 | 21.2 |
| $Cl_{int}$ (Human Mic) | 97.3 | 13.9 | 22.6 |
| $Cl_{int}$ (Rat Hep) | NV | 7.1 | 39.4 |
| **Cytochrome P450 inhibition[b]** | | | |
| CYP2D6 | Weak | Weak | Weak |
| CYP3A4 | Weak | Moderate | Weak |
| CYP1A2 | Weak | Weak | Weak |
| CYP2C9 | Weak | Weak | Weak |

[a]Using protease inhibitors. [b]Weak: $IC_{50}$ > 20 μM. Moderate: $IC_{50}$ 2−20 μM. Abbrevations: PSA = polar surface area; PPB = plasma protein binding; NV = no value (below level of quantification); Fu = fraction unbound; $V_u$ = unbound brain volume of distribution; $Cl_{int}$ = clearance intrinsic (μL/min/mg); Mic = microsomes; Hep = hepatocytes.

protein binding (PPB), permeability including efflux, metabolic stability, and their influence on the CYP enzymes, in order to obtain a profound PK profile of each compound.

The physicochemical parameters in Table 1 clearly demonstrate, as one might expect, that with reduced peptide character the lipophilicity increases (**4** > **3** > **2**) and the polar surface area (PSA) decreases. Looking at our first lead compound H-Phe-Phe-$NH_2$ (**2**) it is clear that this dipeptide has poor drug-like properties due to low membrane permeability (below level of quantification), which probably is a result of brush-border peptidase degradation.[18] Furthermore, **2** shows high *in vitro* clearance and no measurable PPB (fraction unbound), probably due to rapid degradation in plasma, which altogether diminish the use of this compound in advanced *in vivo* studies. Evaluation of compound **3** shows that rigidification of the C-terminal phenylalanine results in a concomitant reduction of PSA, an increased clogP, and an improved permeability ($P_{app}$ = 2.8 × $10^{-6}$ cm/s). However, the efflux ratio of **3** was still 4.2. The rigidification of the side chain to the peptide backbone also gave a significant reduction in *in vitro* clearance in comparison to compound **2** (compare human $Cl_{int}$ 13.9 and 97 μL/min/mg for compounds **3** and **2**, respectively). In fact, in comparison to both **2** and **4**, compound **3** had the lowest *in vitro* clearance in both human and rat microsomes. Replacing the N-terminal basic amine with the carbamate function as in our prototype, compound **4** resulted in significant improvement of the permeability (31 × $10^{-6}$ cm/s) and also notably in less efflux propensity (efflux ratio 1). Moreover, all three compounds showed no or very week inhibition of the tested Cytochrome P450 isoenzymes (CYP2D6, CYP3A4, CYP1A2, and CYP2C9).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Taken together, both compounds **3** and **4** have improved *in vitro* PK profiles as compared to H-Phe-Phe-$NH_2$ (**2**). The constrained analogue **3** was metabolically stable but with a high efflux ratio, whereas the new carbamate based compound **4** demonstrated excellent membrane permeability, low efflux, and moderate *in vitro* clearance.

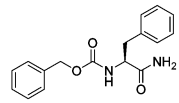
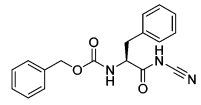
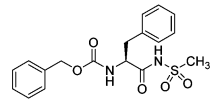
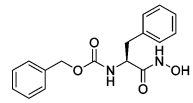
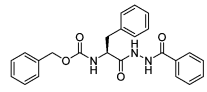
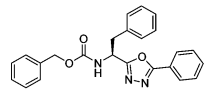
Compounds **3** and **4** were further characterized in *in vivo* rat PK studies.[23] A subcutaneous administration (dose 10 $\mu$mol/kg) of compound **3** revealed drug concentrations of 1.2 and 0.09 $\mu$M after 1 h in plasma and brain, respectively, and 0.8 and 0.09 $\mu$M after 3 h in plasma and brain, respectively. At the 3 h time-point and after correlations of free fractions in both brain and plasma, the free brain-free plasma ratio was determined to be around 0.05. This *in vivo* value suggests that compound **3** is subjected to BBB efflux.[23] This is in agreement with the propensity for *in vitro* efflux in the Caco-2 measurements observed for **3** (Table 1). Similar protein transporters, such as the PgP efflux protein, can indeed be found in both these membrane types. The plasma stability of compound **3** was found to be high in both human and rat plasma (mean recovery > 95% in both human and rat plasma; Table 1).

An intravenous administration (dose 3 $\mu$mol/kg) of compound **4** revealed a clearance of 225 mL/min/kg, which is considerably higher than the rat liver blood flow, suggesting extensive metabolic degradation. Oral administration (dose 10 $\mu$mol/kg) generated no measurable plasma exposure. These *in vivo* characteristics are not fully in agreement with the *in vitro* PK stability data shown in Table 1. The reason for this discrepancy is likely due to an additional hydrolytic instability of the carbamate function in **4** in rat plasma, which was further confirmed with a rat plasma stability experiment revealing a $t_{1/2}$ of 0.4 h. Plasma protein binding experiments also revealed apparent hydrolytic instability of **4** that eventually was prevented by hydrolase inhibitors. A brain distribution study[21−23] (dose 2 $\mu$mol/kg bolus + 2 $\mu$mol/kg/h infusion) revealed a brain concentration of 0.17 $\mu$M after 2 h, whereas no drug concentration could be detected in plasma, suggesting a rapid exposure (including BBB penetration) and compound stability in brain, which also agrees with the *in vitro* permeability data in Table 1. Despite the blood/plasma instability in rat, compound **4** showed a remarkable stability in human plasma, with a $t_{1/2}$ of 14.6 h. Additionally, equilibrium dialysis experiments using human plasma showed good mass recovery (>95%), further indicating that plasma instability will not be an issue in humans.

The promising binding affinity and improved permeability properties of the prototype compound **4** prompted us to further explore this type of compound. In a first series of compounds we decided to evaluate different C-terminal groups. The use of bioisosteres is a common strategy in drug discovery in order to optimize a lead structure. A successful isosteric replacement can result in improved potency, selectivity, or PK profile.[24,25] Properties such as acidity and hydrogen bond accepting and donating capacity were considered when choosing the functional groups. In this study we chose to retain the carbonyl moiety of the amide. The target compounds **5**−**9** were evaluated in the binding assay displacing the [$^3$H]-$SP_{1-7}$ analogue (Table 2).

The benzyl carbamate based compounds showed overall moderate to good binding affinities toward the $SP_{1-7}$ binding site ($IC_{50}$ ranging from 4 to 60 nM; Table 2), suggesting that large structural modifications in this area are allowed. When the primary amide in compound **4** was exchanged for an acyl cyanamide (**5**) or to an acyl sulfonamide (**6**), the affinity decreased 3 and 11 times, respectively. This reduced affinity can be assigned the acidity of these compounds, which are in the same range as a carboxylic acid. This is in agreement with earlier studies on peptide based $SP_{1-7}$ analogues, which proved primary amides to be around 5-fold more potent than carboxylic acids.[16] Possibly the negative charge of compounds **5** and **6** is less favorable as compared to a corresponding polar but neutral amide group with preserved hydrogen bonding abilities. Substitution of the primary amide for a hydroxamic acid slightly improved the binding affinity and resulted in the most potent compound (**7**) concerning binding affinity as well as the compound with the highest lipophilic lead efficiency (LLE); Table 2. The preserved binding affinity highlights the importance of the neutral character and hydrogen bonding abilities similar to the primary amide. The hydrazine-based C-terminal group in **8** and the 1,3,4-oxadiazole in **9** retained good binding affinities (cf. **4** with **8** and **9**) against the $SP_{1-7}$ binding site. Both **8** and **9** encompass an aryl extension of their C-terminal parts, which despite its larger size appears to be acceptable; however, the LLE-values are considerably lower for these analogues.

**Table 2. Binding Affinity and Lipophilic Lead Efficiency (LLE) of C-Terminal Modified Compounds**

| Compound | Structure | $IC_{50}$ (nM) | LLE[a] |
|---|---|---|---|
| **4** |  | 6.2[b] | 5.72 |
| **5** |  | 16.5 | 4.81 |
| **6** |  | 60.6 | 4.09 |
| **7** |  | 4.2 | 6.47 |
| **8** |  | 6.5 | 2.81 |
| **9** |  | 9.0 | 2.39 |

[a]LLE = $pIC_{50}$ − cLogP (cLogP derived from QikProp, version 3.3; Schrödinger, LLC: New York, 2010). [b]$IC_{50}$ value of compound **4**, corresponds to the $K_i$ value = 5.2 nM.

The C-terminally modified compounds (**5**−**9**) were further evaluated with regard to their uptake, permeability, and metabolic stability (see Table 3) in order to study the influence of the isosteric replacement on the PK properties. *In vitro* intrinsic clearance ($Cl_{int}$, $\mu$L/min/mg) in human microsomes were determined using previous published models.[26,27] Three out of the five benzyl carbamate based derivatives (**5**, **6**, and **7**) showed a low risk of oxidative metabolism and significantly reduced $Cl_{int}$ in comparison to H-Phe-Phe-$NH_2$ (**2**) itself and comparable $Cl_{int}$ with the prototype compound **4**. In this series,


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**Table 3. Metabolic Stability, Uptake, and Permeability Data of the Compounds Comprising Different C-Terminal Groups**

| | stability | | | uptake (pmol/mg protein/min) | | | Caco-2 permeability $P_{app}$[c] ($10^{-6}$ cm/s) | | |
|---|---|---|---|---|---|---|---|---|---|
| compd[a] | $Cl_{int}$[b] ($\mu$L/min/mg) | rat plasma $t_{1/2}$ (h) | human plasma $t_{1/2}$ (h) | CHO-PepT1 | CHO-K1 | ratio PepT1/K1 | a–b[d] | b–a[e] | efflux (ratio ba/ab) |
| **5** | 11 ± 4 | 8.7 | 6.6 | 4 ± 1 | 4 ± 2 | 1.0 | 52 ± 2 | 62 ± 1 | 1.2 |
| **6** | 17 ± 4 | 6.9 | 26.7 | 90 ± 1 | 77 ± 4 | 1.2 | 6 ± 0 | 3 ± 0 | 0.5 |
| **7** | 23 ± 1 | 0.06 | 7.9 | 5 ± 0 | 4 ± 1 | 1.2 | 60 ± 2 | 45 ± 0 | 0.7 |
| **8** | 65 ± 13 | 0.31 | 9.9 | 127 ± 7 | 156 ± 10 | 0.8 | 11 ± 0 | 14 ± 1 | 1.3 |
| **9** | 287 ± 4 | 8.7 | 11.2 | 1082 ± 53 | 1128 ± 74 | 1.0 | 13 ± 0 | 7 ± 1 | 0.6 |

[a]Results are expressed as mean ± SD. For uptake and permeability experiments, compound **6** was analyzed at 100 $\mu$M, and **5** and **7**–**9** were further diluted to 25 $\mu$M due to poor aqueous solubility. See Supporting Information for experimental conditions. [b]$Cl_{int}$ = *in vitro* intrinsic clearance in human liver microsomes (HLM). [c]$P_{app}$ = apparent permeability coefficient. [d]a–b = apical to basolateral. [e]b–a = basolateral to apical.

the acyl cyanamide (**5**) turned out to be the most stable C-terminal group. In contrast, a C-terminal hydrazine (**8**) and in particular a C-terminal oxadiazole (**9**) significantly decreased the metabolic stability. The increased $Cl_{int}$ of these derivatives might be attributed to the incorporation of an aromatic group in the C-terminal, susceptible to additional oxidative metabolism together with their significantly higher LogP. The stability in both rat and human plasma were further investigated for compounds **5**–**9**. In comparison to compound **4**, the rat plasma stabilities were improved for all bioisosteric compounds except for compounds **7** and **8** (Table 3). However, in agreement with compound **4**, the stabilities in human plasma were high for all compounds.

The intestinal epithelial permeability, expressed as apparent permeability coefficients ($P_{app}$), was determined from transport rates across Caco-2 cell monolayers, as described previously.[28,29] Since these compounds (**5**–**9**) are amino acid derivatives, uptake studies with CHO-K1 and CHO-PepT1 cells (control and PepT1 stably transfected cells, respectively) were performed to explore if they are substrates for peptide transporter PepT1. In order to be classified as a substrate for the peptide transporter PepT1 and to be actively transported in to the cell, the PepT1/K1 ratio should be above 1. The ratio for the compounds in this series (**5**–**9**) ranged from 0.8 (**8**) to 1.2 (**6** and **7**), which indicates that these compounds are not actively transported by this transport protein. However, the Caco-2 permeability data in the a–b direction ranged from 6 × $10^{-6}$ cm/s (**6**) to 60 × $10^{-6}$ cm/s (**7**), and the efflux ratios were close to 1, which in contrast to H-Phe-Phe-$NH_2$ (**2**) indicates that these compounds are highly cell permeable. Compound **7**, with the highest binding affinity ($IC_{50}$ = 4.2 nM), also turned out to have the highest permeability (60 × $10^{-6}$ cm/s) and a low efflux (ratio = 0.7).

In summary, a new class of $SP_{1-7}$ analogues with good binding affinity and improved *in vitro* PK profile has been developed by substituting the N-terminal phenylalanine of H-Phe-Phe-$NH_2$ (**2**) with a benzylcarbamate group as in prototype compound **4**. *In vitro* and *in vivo* profiles of rigidified compounds **3** and **4** showed that **3** displayed improved *in vivo* stability compared to compound **2** but exhibited poor brain exposure, which is consistent with its high *in vitro* efflux properties, and that compound **4** showed good permeability *in vitro* and was able to enter the CNS according to the *in vivo* infusion study. However, extrahepatic clearance, due to hydrolytic cleavage in rat blood/plasma of compound **4**, prevented good exposure, after single dosing in rat. In contrast, the human plasma stability data clearly indicates that this is not a concern in humans. The observed divergent plasma stability between species suggests that this should be addressed in further optimization of the benzylcarbamate series in order to be able to evaluate compounds in future rodent pharmacology and toxicity studies. Indeed, it is herein shown that bioisosteric replacement give options to optimize this compound class regarding potency and/or PK properties, including plasma stability in rat. Thus, structural optimizations combining the complementary profiles of the rigidified H-Phe-Phe-$NH_2$ analogue **3**, the prototype compound **4**, and isosteres can generate compounds with increased permeability, low efflux, and good metabolic stability.

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental procedures including compound characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

SP, substance P; SAR, structure−activity relationship; BBB, blood−brain barrier; PK, pharmacokinetic; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridine-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; CDI, 1,1′-carbonyldiimidazole; THF, tetrahydrofuran; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; PPB, plasma protein binding; PSA, polar surface area; $Cl_{int}$, intrinsic clearance; $P_{app}$, apparent permeability coefficient; DMSO, dimethyl sulfoxide; Fu, fraction unbound; Mic, microsomes; Hep, hepatocytes; LLE, lipophilic lead efficiency

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