DOI: 10.1002/cmdc.200600200

Design, Synthesis, and Evaluation of Tripeptidic Promoieties Targeting the Intestinal Peptide Transporter hPEPT1

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The human intestinal proton coupled di/tri-peptide transporter hPEPT1 promotes the oral bioavailability of several drug compounds. The strategy behind the present work is that by linking a suitable di- or tripeptidic promoiety to a drug substance, by a hydrolysable ester bond, it may give rise to a prodrug that targets hPEPT1. 29 tripeptides were designed based on known structural requirements for substrates binding hPEPT1. Serine, homoserine, or threonine was incorporated in the tripeptide as hydroxy group donors in order for them to be linked to carboxylic drug substances. Optimisation of the promoiety included a study of 14 unnatural tripeptides whose diversity was expressed by VolSurf descriptors. A total of 29 tripeptides was synthesised by solid phase peptide synthesis and a standard Fmoc protocol. The affinity of the tripeptides to hPEPT1 was determined by measuring the inhibition of [^{14}C]Gly-Sar in mature Caco-2 cell monolayers which resulted in K_i values ranging from 0.22 to 25 mM or above. Translocation through the intestinal membrane, mediated by hPEPT1, was measured by recording the membrane potential relative to that induced by the known substrate Gly-Sar. The change in membrane potential is caused by influx of protons due to the co-transport of substrates and protons by hPEPT1 and is, as such, an indication of translocation. A K_i value of 0.30 mM combined with efficient translocation indicated that H-Phe-Ser-Ala-OH is a suitable lead promoiety for targeted hPEPT1 prodrug design.

Introduction

The human intestinal proton coupled di/tri-peptide transporter, hPEPT1, is expressed in the luminal membrane of the small intestinal enterocytes. The transporter facilitates the cellular uptake of dipeptides and tripeptides arising from degradation of proteins present in the diet. Consequently, the recognition of di/tri-peptides by hPEPT1 leads to cellular uptake of the substrates. The transport of substrates by hPEPT1 across the luminal membrane of the intestine is dependent on the electrochemical proton gradient, and substrate translocation results in a co-transport of protons.[1] Interestingly, hPEPT1 seems to be the most important transporter for absorption of di-/tripeptides in the small intestine. The substrate recognition of the transporter is relatively broad, as it must accommodate the combination of dipeptides and tripeptides present in the normal diet.

Many small molecular weight, biologically active substances display low bioavailability due to low permeability or low solubility. If such drug substances are linked with a hydrolysable bond to a di- or tripeptidic promoiety, which is recognized by hPEPT1, the peptides may serve as a vehicle for the translocation of the assembled prodrug. As a result, the prodrug will enhance the oral bioavailability of the parent drug molecule. The concept of improving the bioavailability of a drug substance by using hPEPT1 as a promoter in the small intestine is promising because of the broad substrate specificity and high translocation capability of hPEPT1.[2]

The ability of hPEPT1 to translocate drug substances from the intestinal lumen to the systemic circulation has been investigated intensively. A number of drug substances like certain angiotensin converting enzyme (ACE) inhibitors and β-lactam antibiotics show affinity to and are translocated by hPEPT1.[3–6] A few prodrugs, for example, of acyclovir and L-dopa, are translocated by hPEPT1 as well.[7,8] However, very limited information on translocation of proteinogenic tripeptides is available.[9,10]

Several requirements for the promoiety have to be taken into account for a prodrug strategy targeting hPEPT1. The promoiety must have high affinity and translocation capability by hPEPT1. The promoiety must contain a functionality which enables creation of a bond to the drug substance which is stable in the intestines but hydrolyses in the blood stream with liberation of the drug substance.[11] It is essential that the peptide is

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stable to enzymatic and chemical hydrolysis in the gastro-intestinal (GI) tract. However, the present study only aims at developing a lead promoiety for hPEPT1 and does not deal with in vivo stability issues.

Design of hPEPT1 substrates, whether it is peptidomimetic drug substances or promoieties, entails information about the structural requirements for interaction with hPEPT1. The crystal structure of PEPT1 as a membrane protein is not known. Therefore, information about the binding cavity is based on affinity and translocation data from ligands or substrates, respectively. The effort for unravelling structural elements of the di-/tripeptides important for binding and translocation by hPEPT1 have resulted in a few quantitative structure–affinity relationship models.[12–15] It is generally accepted that the transporter has a preference for di- and tripeptides composed of L-configured amino acids with the peptide bonds in a *trans*-conformation. A free amino group in the N-terminal seems to be important whereas a free carboxylic acid in the C-terminal is not an absolute prerequisite. Peptides with uncharged hydrophobic side chains seem to be the best promoieties.[15,16] The stereoselectivity of hPEPT1 has been investigated using H-Val-Val-Val-OH in all combinations of D- and L-configurations. The L-L-L isomer displayed the highest affinity whereas the L-D-D, D-L-D, or D-D-D showed no affinity.[17]

The aim of the present study was to develop a tripeptidic promoiety for prodrugs targeting hPEPT1. Optimization of the promoiety was achieved by hPEPT1 affinity screenings of 29 related tripeptides, in combination with translocation studies of a selected series of peptides. Particularly, 14 tripeptides containing diverse nonproteinogenic N-terminal α-amino acids were expressed by VolSurf descriptors and examined for hPEPT1 binding affinity. In the following, affinity constants for ligands of hPEPT1 lower than 0.5 mM are considered as high, constants between 0.5 and 5.0 mM as medium, and constants above 5.0 mM as low.[16]

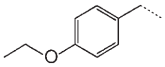
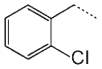
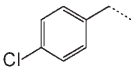
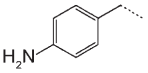
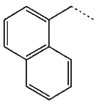
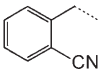
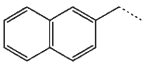
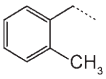
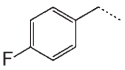
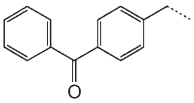
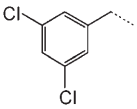
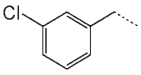
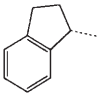
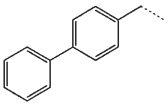
Results and Discussion

Structural design strategy

The hydrolysable ester linkage between the promoiety and the drug molecule was selected as previous studies have revealed that the stability of similar ester bonds are approximately 25 times higher in the small intestine (pH ~ 6.0) than in the blood stream (pH ~ 7.4). The small difference in pH constitutes the delicate balance between intact prodrug and release of the parent drug substance caused by specific base catalyzed hydrolysis.[11] Our previous studies have dealt with dipeptide based promoieties containing a carboxylic acid which can form an ester bond with a hydroxy group in the drug substance.[18,19] In the present study we applied a tripeptide based promoiety containing a hydroxy functionality for linking with a carboxylic acid in the drug substance. The hydroxy group of the promoiety originated from the proteinogenic serine (Ser) or threonine (Thr) or from the nonproteinogenic homoserine (hSer), which was placed in the centre or at the C-terminal of the tripeptide. Structural expansions seem to be allowed in the C-terminal as illustrated by the prodrug valacyclovir.[7] Both L- and D-configurations of these linker amino acids were incorporated to explore the specificity of hPEPT1 binding. A broad binding specificity of the linker amino acid and the remainder of the promoiety might indicate that attachment of a suitable drug molecule could be accepted by hPEPT1.

Previous dipeptide affinity studies have pointed out that a hydrophobic amino acid in the N-terminal is likely to increase affinity for hPEPT1. For instance H-Tyr-Gly-OH and H-Phe-Gly-OH displayed a two- and fourfold higher affinity than H-Gly-Gly-OH.[12,14] Modifications such as benzylations in the side-chain regions may increase the hPEPT1-affinity of dipeptides but also the likelihood of generating inhibitors, especially for hydrophobic modifications in the side chain of the N-terminal amino acid.[20] This has not been investigated for tripeptides. To get further information with regard to structural requirements in the side chain of the N-terminal amino acid, that may influence hPEPT1 binding and translocation, 14 R^1 substituted tripeptides based on H-Xaa$_1$-Ser-Ala-OH, were synthesised (Table 1). The substituted amino acids were structurally derived from phenylalanine but displayed variations in size and electronegativity. All three stereocentres comprised the natural L-configuration. Aside from the 14 R^1 substituted tripeptides, 15 tripeptides with hydrophobic proteinogenic N-terminal amino acids; that is, valine (Val), phenylalanine (Phe), or tyrosine (Tyr) in either their L- or D-configurations were synthesised with a hydroxy containing interchangeable linker amino acid and alanine (Ala) introduced in the centre or C-terminal of the tripeptides. Ala was selected to represent an uncharged and small

Table 1. Structural diversity of 14 nonproteinogenic tripeptides based on the core of H-Xaa$_1$-Ser-Ala-OH.

Compd	R^1	Compd	R^1
16		**23**	
17		**24**	
18		**25**	
19		**26**	
20		**27**	
21		**28**	
22		**29**	

hydrophobic residue. The reason not to insert glycine (Gly) and avoid any side chain was mainly based on the sixfold higher affinity for the dipeptide H-Ala-Ala-OH compared to H-Gly-Gly-OH.[10]

In addition to the obvious diversity between the selected 14 N-terminal substituted amino acid tripeptides (Table 1), a computational analysis was performed to reveal variations expressed by VolSurf descriptors. A previous study has shown the value of VolSurf descriptors to identify and rate structural features in tripeptides that are significant for their affinity to hPEPT1.[15] The VolSurf model takes into account descriptors, like molecular size and hydrophilic and hydrophobic properties, by applying GRID 3D molecular interaction fields (MIFs), and energy calculations of the interaction energy between the ligand and different probes. VolSurf descriptors for the 14, N-terminal amino acid substituted tripeptides shown in Table 1 were superimposed on the data set representing all possible 8000 proteinogenic tripeptides using the probes H_2O, DRY, and O (Figure 1).

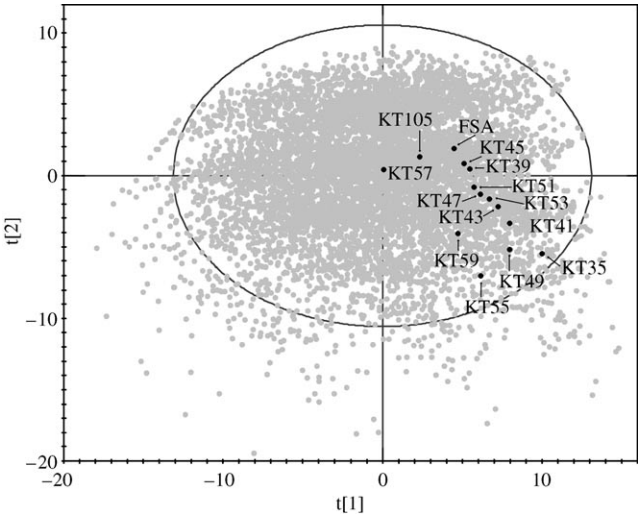
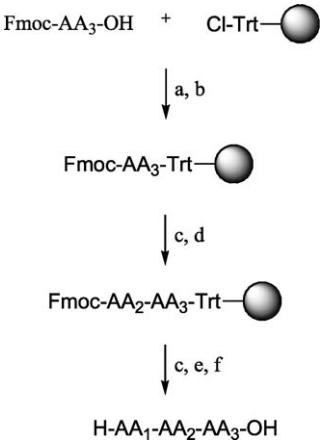


Figure 1. PCA score plot where t[1] and t[2] are the first and second principal components, respectively. The circle illustrates the 95% confidence interval. The dots represent all 8000 possible tripeptides constructed from the 20 proteinogenic amino acids. The high-lighted dots represent the 13 tripeptides H-Xaa$_1$-Ser-Ala-OH and H-Phe-Ser-Ala-OH (FSA). Tripeptides correspond to compound **16** (KT45), **17** (KT53), **18** (KT43), **19** (KT41), **20** (KT105), **21** (KT49), **22** (KT39), **23** (KT51), **24** (KT57), **25** (KT59), **27** (KT55), **28** (KT47), **29** (KT35).

In the VolSurf data two principal components, t[1] and t[2], provide detailed information about the structural features of the tripeptide affecting the affinity for hPEPT1.[15] Increase in the first principal component mainly reflects a decrease in the polarity of the peptide. Decrease in the second principal component predominantly reflects an increase in molecular weight. The PCA score plot indicated that the 14, N-terminal amino acid substituted tripeptides were structurally similar to H-Phe-Ser-Ala-OH (**1**) but displayed variations due to different size and polarity. Especially the variations of t[1] revealed that the principal component of the tripeptides' influence on hPEPT1 affinity was molecular size.

Synthesis

The 29 tripeptides were synthesised using solid phase peptide synthesis (SPPS) employing a cross-linked polystyrene resin.[21] 2-Chlorotrityl chloride was chosen as the linker for anchoring the first amino acid to the resin because of a simple loading procedure and negligible premature cleavage from the resin. As the chlorotrityl linker is acid labile the 9-fluorenylmethoxycarbonyl (Fmoc) group was used for protection of amino groups. The Fmoc group was routinely cleaved by treatment with 20% piperidine in dimethylformamide (DMF) at room temperature. (Scheme 1)



Scheme 1. General procedure for SPPS of tripeptides. a) 4.5 equiv DIPEA, dry DCM, 1 h, b) MeOH, 15 min 3×DCM, 3×DMF, 6×DCM, c) 20% piperidine in DMF, 2×10 min, d) 3 equiv Fmoc-AA$_2$-OH, 4 equiv DIPEA, 2.88 equiv TBTU, DMF, 2 h, e) 3 equiv Boc-AA$_1$-OH, 4 equiv DIPEA, 2.88 equiv TBTU, DMF, 2 h. f) TFA, DCM, H_2O, TIPS (70:20:5:5), 4 h.

The uronium salt *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium borontrifluoride (TBTU) was selected as the peptide coupling reagent because of the ease of activation, high yields, minimal racemisation, and limited side-reactions.[22] The activation of the acyl group was performed in a separate step, followed by immediate addition of the amino compound. Each coupling step was monitored either by a ninhydrine (Kaiser) or 2,4,6-trinitrobenzenesulfonic acid (TNBS) test.[23] As the N-terminal amino acid was protected with an acid labile *tert*-butoxycarbonyl (Boc) group the final deprotection step could be performed easily and concomitantly with cleavage from the solid support by treatment with acid.

The 29 tripeptides were purified by preparative high performance liquid chromatography (pHPLC) to provide yields above 70%. The peptides were characterised by ^{1}H and ^{13}C NMR spectra, and liquid chromatography–mass spectrometry (LC–MS). Additionally, the UV absorbance track at 214 nm from HPLC or the LC–MS analyses was applied for the purity determination. Purity was better than 90% unless otherwise stated in the experimental section.

Biological investigations

According to the design strategy L- and D-Phe, L- and D-Val, and L- and D-Tyr were investigated as the amino acid in the N-terminal position, whereas Ala and Ser were interchangeable in the centre or the C-terminal position.

Affinity data for the first series of tripeptides implied that H-Phe-Ser-Ala-OH (**1**), H-Phe-Ala-Ser-H (**2**), H-Val-Ser-Ala-OH (**5**), and H-Tyr-Ser-Ala-OH (**8**) had comparable high affinities for hPEPT1, which were not significantly different compared to H-Phe-Ser-Ala-OH (**1**) (Table 2). However, shifting to the D-configuration of the N-terminal amino acid to get H-D-Phe-Ala-Ser-OH (**4**) and H-D-Val-Ser-Ala-OH (**7**), resulted in an approximately 60-fold decrease in affinity compared to the L-L-L isomers. In contrast, the affinity for H-D-Phe-Ser-Ala-OH (**3**) only decreased by tenfold compared to its corresponding L-configured tripeptide. Thus, the initial affinity screening identified H-Phe-Ser-Ala-OH (**1**) as a possible lead promoiety targeting hPEPT1. The affinity investigations were followed by translocation studies (Table 3), which showed that H-Phe-Ser-Ala-OH (**1**) and H-Phe-Ala-Ser-OH (**2**) were able to induce changes in membrane potentials in hPEPT1-transfected HeLa cells, comparable to changes induced by the prototypic hPEPT1 substrate H-Gly-Sar-OH. This indicates that these tripeptides, in addition to binding to hPEPT1, are translocated into the cell cytosol by an hPEPT1 mediated process. Surprisingly, H-Val-Ser-Ala-OH (**5**) caused a significantly lower change in membrane potential compared to H-Gly-Sar-OH. This further supports the identification of H-Phe-Ser-Ala-OH (**1**) as a tripeptidic lead promoiety.

To investigate the linker amino acid, Ser, Thr, and hSer were inserted in the H-Phe-Xaa$_2$-Ala-OH frame. Affinities for hPEPT1 revealed that introduction of hSer and Thr did not significantly change the affinity compared to H-Phe-Ser-Ala-OH (**1**) (Table 4). Shifting Ser to the D-amino acid caused a 20-fold decrease, whereas the affinity for the tripeptides containing D-Thr or D-hSer decreased more than 80-fold. Thus, H-Phe-Ser-Ala-OH (**1**) remained the most promising lead promoiety.

Table 2. hPEPT1 affinity data of a selection of tripeptidic promoieties.

Tripeptide	K_i [mM ± SE][a]
1 H-Phe-Ser-Ala-OH	0.30 ± 0.04
2 H-Phe-Ala-Ser-OH	0.22 ± 0.01
3 H-D-Phe-Ser-Ala-OH	2.5 ± 0.3[b,d]
4 H-D-Phe-Ala-Ser-OH	~12[c,d]
5 H-Val-Ser-Ala-OH	0.22 ± 0.01
6 H-D-Val-Ala-Ser-OH	~25[c,d]
7 H-D-Val-Ser-Ala-OH	~14[c,d]
8 H-Tyr-Ser-Ala-OH	0.42 ± 0.03
9 H-D-Tyr-Ala-Ser-OH	~18[c,d]
10 H-D-Tyr-Ser-Ala-OH	2.8 ± 0.4[d]

[a] The K_i-values are measured as the concentration dependent inhibition of 20 μM [^{14}C]Gly-Sar uptake in Caco-2 cells. [b] $P < 0.05$ compared to the affinity determined for H-Phe-Ser-Ala-OH. [c] Estimated from an inhibitory effect at 5 mM tripeptide. [d] K_i values published in our previous study.[15]

Table 3. Changes in fluorescence induced by tripeptides.

Tripeptide	K_i [mM ± SE]	Ratio[b]	GS_{max} [% ± SD][a]
1 H-Phe-Ser-Ala-OH	0.30 ± 0.04	12.5	112 ± 21
2 H-Phe-Ala-Ser-OH	0.22 ± 0.01	14.3	115 ± 24
5 H-Val-Ser-Ala-OH	0.22 ± 0.01	17.3	62 ± 18[c]
11 H-Phe-D-Ser-Ala-OH	5.6 ± 1.6	0.8	54 ± 26[c]
17 H-4-Cl-Phe-Ser-Ala-OH	0.25 ± 0.02	12.0	105 ± 16
19 H-2-NaphthylAla-Ser-Ala-OH	0.29 ± 0.02	9.7	0 ± 17[c]
27 H-4-PhCO-Phe-Ser-Ala-OH	0.43 ± 0.04	7.4	1 ± 14[d]

[a] HeLa cells transiently transfected with hPepT1 were washed once with HBSS (pH 6.0) and then incubated with 50 μL Blue Membrane Potential dye pr. well for 60 min at 37 °C. Tripeptides were added to each preloaded well, and changes in fluorescence were recorded continuously. Fluorescence was measured on a NOVOStar at wavelengths of 544 nm (excitation) and 590 nm (emission) at 37 °C. [b] The ratio is the concentration of tripeptide used in the assay divided with the K_i-value of the tripeptide. [c] $P < 0.05$, [d] $P < 0.001$ compared to the maximal response measured for 5 mM Gly-Sar (100 ± 19). The maximal change in fluorescence measured is reported as the average ± SD for 4–5 hPepT1 transfected HeLa cell monolayers.

Table 4. hPEPT1 affinity data of a selection of tripeptidic promoieties containing various linker amino acids.

Tripeptide	K_i [mM ± SE][a]
1 H-Phe-Ser-Ala-OH	0.30 ± 0.04
11 H-Phe-D-Ser-Ala-OH	5.6 ± 1.6
12 H-Phe-hSer-Ala-OH	0.34 ± 0.04
13 H-Phe-D-hSer-Ala-OH	> 25
14 H-Phe-Thr-Ala-OH	0.33 ± 0.04
15 H-Phe-D-Thr-Ala-OH	> 25

[a] The K_i-values are measured as the concentration dependent inhibition of 20 μM [^{14}C]Gly-Sar uptake in Caco-2 cells.

The longer distance between the peptide backbone and the drug substance, with hSer as the linker amino acid, could nevertheless be important for a prodrug strategy. These speculations are based on the observation that the recognition of the promoiety by hPEPT1 should not conflict with the drug substance attached to the linker amino acid. Correspondingly, the methyl group of Thr could shield the drug molecule and thereby increase the translocation of the prodrug conjugate through the hPEPT1 transporter. To fully reveal the value of different linker amino acids, by investigating translocation, these should be based on assembled prodrugs. As preparing assembled prodrugs was out of the scope of the present study this was not investigated further. Notably, optimization of linker amino acids could vary and be influenced by the selection of drug substances.

To investigate whether the affinity of H-Phe-Ser-Ala-OH (**1**) could be increased by modifying the R^1 side chain, 14 tripeptides containing substituted N-terminal amino acids were synthesized. Affinity determinations for these are given in Table 5 and structures are given in Table 1. The K_i-values were determined to be in the range of 0.24 to 0.45 mM. However, none of the tripeptides investigated were significantly different from the parent H-Phe-Ser-Ala-OH (**1**) tripeptide.

Table 5. hPEPT1 affinity data of a selection of tripeptides based on the H-Xaa$_1$-Ser-Ala-OH core.

Tripeptide	K_i [mM ± SE][a]
1 H-Phe-Ser-Ala-OH	0.30 ± 0.04
16 H-4-OEt-Phe-Ser-Ala-OH	0.24 ± 0.03
17 H-4-Cl-Phe-Ser-Ala-OH	0.25 ± 0.02
18 H-1-NaphthylAla-Ser-Ala-OH	0.28 ± 0.03
19 H-2-NaphthylAla-Ser-Ala-OH	0.29 ± 0.02
20 H-4-F-Phe-Ser-Ala-OH	0.30 ± 0.03
21 H-3,5-diCl-Phe-Ser-Ala-OH	0.32 ± 0.01
22 H-Indan-1-ylGly-Ser-Ala-OH	0.33 ± 0.04
23 H-2-Cl-Phe-Ser-Ala-OH	0.34 ± 0.03
24 H-4-NH$_2$-Phe-Ser-Ala-OH	0.37 ± 0.05
25 H-2-CN-Phe-Ser-Ala-OH	0.38 ± 0.02
26 H-2-Me-Phe-Ser-Ala-OH	0.38 ± 0.02
27 H-4-PhCO-Phe-Ser-Ala-OH	0.43 ± 0.04
28 H-3-Cl-Phe-Ser-Ala-OH	0.45 ± 0.06
29 H-4-Ph-Phe-Ser-Ala-OH	N/A[b]

[a] The K_i-values are measured as the concentration dependent inhibition of 20 μM [^{14}C]Gly-Sar uptake in Caco-2 cells. [b] The compound could not be tested due to solubility problems.

Despite the VolSurf analysis predicting diversity between the 14 unnatural tripeptides, the structural diversity in the N-terminal side chain did not affect the hPEPT1 binding affinity. The influence of side chain modifications in the N-terminal of tripeptides seems to be inferior for the binding affinity for hPEPT1. The findings indicate that the structural characteristics of a free N- and C-terminal, two carbonyl functionalities in the backbone of the peptide, and L-configured amino acids appear to have a greater importance for hPEPT1 binding than side chain modifications in the R^1 position. Thus, H-Phe-Ser-Ala-OH (**1**) continues to be the lead promoiety.

The hPEPT1 translocation study of selected tripeptidic promoieties revealed, however, some differences between the unnatural tripeptides (Table 3). H-4-Cl-Phe-Ser-Ala-OH (**17**) caused a comparable translocation as for H-Phe-Ser-Ala-OH (**1**), whereas H-2-NaphthylAla-Ser-Ala-OH (**19**) and H-4-PhCO-Phe-Ser-Ala-OH (**27**) were not translocated. These results are in agreement with previous studies showing that increasing the hydrophobicity of the side chains will increase the likelihood of generating inhibitors for hPEPT1.

The prodrug strategy has been designed to link the promoiety to the drug substance by an ester bond. The ester group allows for smooth release of the drug substance after translocation. In addition, the hydroxy group of the promoiety could be attached to the drug substance by a sulfonic ester or an ether bond. The three functionalities which can be linked to the hydroxy group of the promoiety are present in about 40% of all drug substances[24] and in a substantial fraction of drug candidates which does not pass to sales because of low bioavailability. Therefore, H-Phe-Ser-Ala-OH (**1**) has good potential to be a promoiety for hPEPT1 targeted prodrug delivery.

Conclusions

The present study suggests that H-Phe-Ser-Ala-OH is a promising lead promoiety for tripeptide based prodrugs targeted at hPEPT1 delivery. H-Phe-Ser-Ala-OH possesses a high affinity for hPEPT1 and is translocated by the transporter. The prodrug approach is based on using Ser as a hydroxy group donor to enable ester linkage of carboxylic drug substances. The basis for selecting H-Phe-Ser-Ala-OH as a lead promoiety is based on investigations of Ser, Thr, and hSer as linker amino acids and on exploration hPEPT1 specificity to bind various individual configured amino acids in a series of 15 related tripeptides. The selection of the lead promoiety is supported by an investigation of 14 structurally diverse tripeptides, containing substituted N-terminal amino acids. These promoieties displayed statistically similar affinities to hPEPT1 as H-Phe-Ser-Ala-OH but also tended to cause a lower translocation by hPEPT1.

Future studies will aim at synthesising stabilised tripeptidomimetics based on the lead promoiety presented herein, H-Phe-Ser-Ala-OH. One approach may be to develop amide bond bioisosteres to avoid hydrolytic and enzymatic degradation in the GI-tract.

Experimental Section

General procedures. All reactions were performed in standard glassware or Teflon equipment suitable for solid-phase peptide synthesis. Starting materials and solvents of HPLC grade were commercially available and used without further purification. The resin and the protected amino acids were purchased from Novabiochem, Sigma–Aldrich, and Iris Biotech.

General procedure for loading of the first amino acid to the resin. Dry 2-chlorotrityl chloride resin was swelled with dry dichloromethane (DCM) and washed three times with *N*,*N*-dimethylformamide (DMF) or 1-methyl-2-pyrrolidone (NMP). A solution of 1.5 equiv Fmoc protected C-terminal amino acid and 1.5 equiv of diisopropylethylamine (DIPEA) in dry DCM (10 mL per g resin) was added to the resin. After 5 min, a further 3 equiv of DIPEA was added, and the reaction mixture was shaken for 1 h at room temperature (RT). The reaction was quenched with methanol (MeOH) and shaken for a further 15 min. Subsequently, the mixture was filtrated, the resin washed with 3×DCM, 3×DMF, 6×DCM, and then dried in vacuo (about 1 mmHg).

General SPPS coupling procedure. The Fmoc group, from protected amino acid or peptide attached to the resin, was removed by treatment with 20% piperidine in DMF for 10 min. The procedure was repeated. Then, the resin was washed six times with DMF. A Kaiser or TNBS test verified the presence of a free N-terminal amino group.

Prior to the coupling procedure, 3 equiv of the next N-protected amino acid was preactivated by treatment with TBTU (2.88 equiv) and DIPEA (4 equiv) in DMF (2 mL) for 2 min. This mixture was added to the resin and the coupling mixture was shaken for 2 h. Subsequently, the resin with attached peptide was dried in vacuo and washed six times with DMF. A negative Kaiser or TNBS test implied a complete coupling.

General cleavage procedure. The resin and attached peptide was washed an additional ten times with DCM, and dried in vacuo for 1 h. Deprotection from the N-terminal Boc group and side chain protection groups like trityl (Trt) and *tert*-butyl (*t*Bu) was performed concomitantly with cleavage from the resin by treatment with trifluoroacetic acid (TFA)-DCM-water-triisopropylsilane (TIPS) (70:20:5:5) for 4 h. Filtration and further extraction with acetonitrile

gave a filtrate which was evaporated to dryness in vacuo to give the unprotected peptide which was purified as described below.

Purification procedure. Reverse-phase pHPLC purifications were performed on Daiso ODDMS C18 column (10μ, 200 Å, 25 cm × 22 mm) applying a gradient elution of 20–90% acetonitrile with 0.1% TFA in MQ-water. The gradient processed over 20 min with a flow of 8 mL min^{-1}. The HPLC system consisted of a Gilson 215 liquid handler, a Gilson 322 pump, and a Gilson 155 UV detector. The HPLC system was operated from Unipoint software. The tripeptides were freeze-dried in a Christ Alpha 2–4 LSC freeze-drier.

Characterisation and purity determination. ^{1}H and ^{13}C characterisations of the peptides were either performed on an Oxford 400/100 MHz or a 300/75 MHz apparatus and the data were recorded on a Mercury Plus Varian or Gemini 2000 Varian. Chemical shifts (δ) are given in parts per million (ppm) and are relative to dioxane as internal standard. Coupling constants (J) are given in Hertz (Hz) and the multiplicities are described by following abbreviations: Singlet (s), doublet (d), triplet (t), octet (o), multiplet (m), double of doublets (dd).

Purity determination and further characterisation were obtained by LC–MS analyses. A binary pump system employed a gradient of 10–90% acetonitrile with 0.1% TFA in MQ-water over 10 min. The Waters LC–MS instrument was equipped with a Phenomenex Jupiter C4 column (5 μ, 300 Å, 50 mm × 4.60 mm), quadrupole mass spectrometer detector (2–4000 amu), Waters 2795 alliance separation module, and a Waters 2996 photodiode array detector. The mass determination were achieved by electrospray ionization in positive mode. The purity resolution was determined by measuring the absorbance of UV light at 214 nm. The purity was confirmed by analytical HPLC for selected tripeptides applying a C18 column, an isocratic solvent system of 79.5% MQ-water, 20% methanol, and 0.5% TFA, and detecting the UV absorbance at 214 and 254 nm.

Cell culture and transfections. Caco-2 cells were cultured as previously described.[25] Briefly, cells were seeded in culture flasks and passaged in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U mL^{-1} and 100 μg mL^{-1}, respectively), 1% L-glutamine, and 1% nonessential amino acids. Caco-2 cells were seeded onto tissue culture treated 12-well Transwell plates (1.13 cm^2, 0.4 μm pore size) at a density of 10^5 cells cm^{-2}. Monolayers were grown in an atmosphere of 5% CO_2–95% O_2 at 37 °C. Growth media were replaced every other day. TEER was measured at RT before each experiment. The monolayers used had TEER's > 300 Ω cm^2. Experiments were performed on day 14–28 after seeding. Caco-2 cell monolayers from passages between 25 and 50 were used for the experiments.

HeLa cells were obtained from American Type Culture Collection. HeLa cells were grown at 37 °C, in an atmosphere of 5% CO_2 and 90% relative humidity in standard growth media: Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1% NEAA, 2 mM L-glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Cells were seeded at a density of 5×10^3 cells per well onto polycarbonate 96 wells (BD Falcon Black/Clear Bottom) with a growth area of 0.32 cm^2. HeLa cells were transfected as previously described for HEK 293 cells.[26] When the HeLa cells reached approximately 50–60% confluency they were transfected using Jet-PEI (Qbiogene, Carlsbad, CA) and pcDNA 3.1 plasmids carrying hPepT1.[26] 24 h after transfection, the cell monolayers were used for experiments.

Affinity experiments. Affinity of tripeptides for hPEPT1 were measured as concentration-dependent inhibition of the apical uptake of [^{14}C]Gly-Sar (20 μM) in Caco-2 cell monolayers using a proton gradient across the monolayer with extracellular pH 6.0 (apical) and pH 7.4 (basolateral), as described by Nielsen et al.[18] In brief, Caco-2 cells were equilibrated for 15 min with 0.5 mL buffer pH 6.0 (10 mM MES and 0.05% BSA) on the apical side and 1 mL buffer pH 7.4 (10 mM HEPES and 0.05% BSA) on the basolateral side. After 15 min, buffers were removed and solutions of 20 μM (0.5 μCi) [^{14}C]Gly-Sar and varying concentrations of tripeptides in buffer pH 6.0 was added to the apical side, and buffer pH 7.4 was added to the basolateral side of the monolayers. After 5 min, the uptake of [^{14}C]Gly-Sar was terminated by removal of solutions and washing of cells with ice cold HBSS buffer. The filter supports were cut out and the amount of [^{14}C]Gly-Sar was measured by liquid scintillation spectrometry. Experiments with Caco-2 cell monolayers were performed using at least three separate monolayers. The aqueous solubility of the unnatural tripeptides was increased by addition of 3% *N,N*-dimethylacetamide, which was shown to have no influence on the uptake of [^{14}C]Gly-Sar (data not shown).

The IC_{50}-values were calculated as described in Nielsen et al. and the conversion to K_i as described by Cheng and Prusoff[18,27] using the K_m-value obtained for Gly-Sar. K_i-values are expressed as means ± standard error (SE).

Fluorescence-based measurements of membrane potential. HeLa cells transiently transfected with hPEPT1 were washed once with HBSS (pH 6.0) and then incubated with 50 μL Blue Membrane Potential (MP) dye (Molecular Devices Corporation Sunnyvale, USA) per well for 60 min at 37 °C. The protocol is slightly modified from the protocol previously described by Faria et al.[28] Tripeptides or Gly-Sar were dissolved in 3% dimethylacetamide and MP dye (the final diethylacetamide concentration was 1.5%). A solution of HBSS with 1.5% dimethylacetamide was used as a blank control. 5 mM Gly-Sar was used as a positive control. The experiments were initiated by adding 50 μL solution to each preloaded well giving a total sample volume of 100 μL. Fluorescence was measured for 72 seconds post compound addition on a NOVOStar (BMG Labtech Offenburg, Germany) at wavelengths of 544 nm (excitation) and 590 nm (emission) at 37 °C. Quantifications of the changes in membrane potential caused by the addition of tripeptides were performed by subtracting the changes caused by addition of the blank (values were less than 1%), furthermore, the responses caused by addition of tripeptides were corrected for the responses obtained in mock-transfected cells and subsequently normalised with respect to the changes in membrane potential caused by 5 mM Gly-Sar. Experiments on Gly-Sar were also performed in mock-transfected and nontransfected HeLa cells. Gly-Sar did not cause any changes in fluorescence when added to mock-transfected or nontransfected HeLa cells.

Computational procedures—molecular descriptors and multivariate data analysis. VolSurf descriptors were calculated using the VolSurf (v4.1.3) program implemented in SYBYL (v7.1). Cruciani and co-workers have extensively described the methodology behind the VolSurf descriptors.[29] According to standard procedures, the VolSurf descriptors were based on GRID 3D MIFs resulting from interaction energies between the ligand and different probes. The generation of VolSurf descriptors was performed with default settings. VolSurf descriptor definitions can be found in the software manual.[30]

The VolSurf descriptors were analyzed by PCA (Principal Component Analysis) in SIMCA-P (v10.0). Default settings were applied. The variables were centred and scaled to unit variance. Cross validation (7 rounds) was performed to test the significance of the model.

Statistical analysis. Affinity values are given as means ± SE ($n = 3$). Data on changes in membrane potential are given as means ± SD ($n = 4$–5). The statistical significance of the results was determined using two-tailed unpaired Students t-test. When means with different variances were compared, the Welch t-test was used. $P < 0.05$ was considered significant.

Characterisation of products.

*H-Phe-Ser-Ala-OH (**1**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.35$ (d, 3H, $J =$ 7.3 Hz), 3.14 (m, 2H), 3.68 (dd, 1H, $J = 7.4$, 11.6 Hz), 3.74 (dd, 1H, $J = 5.2$, 11.6 Hz), 4.28 (m, 2H), 4.39 (t, 1H, $J = 5.8$ Hz), 7.23 (m, 2H), 7.34 ppm (m, 3H). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.4$, 37.0, 48.9, 54.8, 55.2, 61.3, 128.4, 129.8 (2C), 130.0 (2C), 134.8, 168.9, 170.2, 176.3 ppm. LC–MS *m/z*: 324.11 [*M*+1]. Purity > 90%.

*H-Phe-Ala-Ser-OH (**2**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.35$ (d, 3H, $J =$ 7.2 Hz), 3.13 (dd, 1H, $J = 7.9$, 14.2 Hz), 3.24 (dd, 1H, $J = 6.8$, 14.2 Hz), 3.86 (dd, 1H, $J = 4.1$, 12.1 Hz), 3.97 (dd, 1H, $J = 4.5$, 12.1 Hz), 4.23 (t, 1H, $J = 7.4$ Hz), 4.39 (q, 1H, $J = 7.2$ Hz), 4.46 (t, 1H, $J = 4.2$ Hz), 7.26 (m, 2H), 7.36 ppm (m, 3H). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.4$, 36.4, 49.4, 53.9, 54.8, 60.9, 127.9, 129.1 (2C), 129.5 (2C), 133.7, 168.6, 170.6, 174.1 ppm. LC–MS m/z: 324.04 [M+1]. Purity > 90%.

*H-D-Phe-Ser-Ala-OH (**3**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 1.34$ (d, 3H, $J =$ 7.3 Hz), 3.14 (dd, 2H, $J = 2.6$, 7.4 Hz), 3.52 (dd, 1H, $J = 4.9$, 11.6 Hz), 3.62 (dd, 1H, $J = 5.7$, 11.6 Hz), 4.19–4.34 (m, 3H), 7.19 (m, 2H), 7.25–7.35 ppm (m, 3H). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.8$, 37.5, 49.4, 55.1, 56.0, 61.5, 128.5, 129.7 (2C), 129.9 (2C), 134.2, 169.8, 171.5, 176.6 ppm. LC–MS *m/z*: 324.4 [*M*+1]. Purity > 90%.

*H-D-Phe-Ala-Ser-OH (**4**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 1.35$ (d, 3H, $J =$ 7.2 Hz), 3.30 (dd, 1H, $J = 8.5$, 13.9), 3.40 (dd, 1H, $J = 6.2$, 13.5 Hz), 4.03 (dd, 1H, $J = 3.8$, 11.7 Hz), 4.13 (dd, 1H, $J = 4.8$, 11.8 Hz), 4.35 (t, 1H, $J = 7.3$ Hz), 4.42 (q, 1H, $J = 7.1$ Hz), 4.67 (t, 1H, $J = 5.1$), 7.42 (m, 2H), 7.49–7.60 ppm (m, 3H). ^{13}C NMR (75 MHz, D_2O): $\delta = 17.1$, 37.6, 50.1, 55.1, 55.4, 61.5, 128.5, 129.6 (2C), 129.9 (2C), 134.3, 169.3, 173.5, 175.1 ppm. LC–MS *m/z*: 324.4 [*M*+1]. Purity > 90%.

*H-Val-Ser-Ala-OH (**5**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 0.98$ (d, 3H, $J =$ 3.8 Hz), 0.99 (d, 3H, $J = 3.9$ Hz), 1.38 (d, 3H, $J = 7.2$ Hz), 2.20 (m, 1H), 3.77 (dd, 1H, $J = 7.4$, 11.7 Hz), 3.84 (m, 1H), 3.87 (dd, 1H, $J =$ 7.6, 11.6 Hz), 4.34 (q, 1H, $J = 7.1$ Hz), 4.49 ppm (m, 1H). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.2$, 16.7, 17.6, 29.8, 48.8, 55.4, 58.3, 61.0, 169.5, 170.7, 176.1 ppm. LC–MS *m/z*: 275.91 [*M*+1]. Purity > 90%.

*H-D-Val-Ala-Ser-OH (**6**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 0.95$ (d, 6H, $J =$ 6.9 Hz), 1.37 (d, 3H, $J = 7.2$ Hz), 2.13 (o, 1H, $J = 6.8$ Hz), 3.72 (d, 1H, $J = 6.1$ Hz), 3.81 (dd, 1H, $J = 4.0$, 11.8 Hz), 3.90 (dd, 1H, $J = 4.9$, 11.7 Hz), 4.36 (q, 1H, $J = 7.1$ Hz), 4.44 ppm (t, 1H, $J = 4.3$ Hz). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.4$, 16.7, 17.4, 29.6, 49.3, 54.8, 58.2, 60.7, 168.7, 172.9, 174.2 ppm. LC–MS *m/z*: 276.4 [*M*+1]. Purity > 90%.

*H-D-Val-Ser-Ala-OH (**7**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 0.96$ (d, 3H, $J =$ 6.9 Hz), 0.97 (d, 3H, $J = 6.9$ Hz), 1.37 (d, 3H, $J = 7.4$ Hz), 2.25 (o, 1H, $J = 6.8$ Hz), 3.82–3.95 (m, 3H), 4.41 (q, 1H, $J = 7.2$ Hz), 4.52 ppm (dd, 1H, $J = 4.8$, 6.2 Hz). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.5$, 17.3, 18.1, 30.3, 49.1, 55.9, 59.0, 61.4, 169.9, 171.2, 176.4 ppm. LC–MS *m/z*: 276.4 [*M*+1]. Purity > 90%.

*H-Tyr-Ser-Ala-OH (**8**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 1.35$ (d, 3H, $J =$ 7.3 Hz), 3.04 (m, 2H), 3.63 (dd, 1H, $J = 6.4$, 12.1 Hz), 3.74 (dd, 1H, $J = 4.6$, 11.9 Hz), 4.10–4.25 (m, 2H), 4.35 (m, 1H), 6.73 (d, 2H, $J =$ 8.5 Hz), 7.02 ppm (d, 2H, $J = 8.4$ Hz). ^{13}C NMR (75 MHz, D_2O): $\delta =$ 16.5, 36.3, 49.1, 54.7, 55.3, 61.5, 116.0 (2C), 125.6, 131.0 (2C), 155.4, 169.4, 170.3, 176.6 ppm. LC–MS *m/z*: 340.21 [*M*+1]. Purity = 60%.

*H-D-Tyr-Ala-Ser-OH (**9**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 1.34$ (d, 3H, $J =$ 7.1 Hz), 3.20 (dd, 1H, $J = 7.6$, 13.0), 3.34 (dd, 1H, $J = 7.3$, 13.1 Hz), 4.03 (dd, 1H, $J = 6.9$, 11.7 Hz), 4.14 (dd, 1H, $J = 6.8$, 11.8 Hz), 4.28 (t, 1H, $J = 7.0$ Hz), 4.42 (q, 1H, $J = 7.1$ Hz), 4.67 (t, 1H, $J = 7.4$ Hz), 7.04 (d, 2H, $J = 8.6$ Hz), 7.29 ppm (d, 2H, $J = 8.6$ Hz). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.1$, 35.8, 49.1, 54.2, 54.5, 60.5, 115.4 (2C), 125.1, 130.3 (2C), 154.6, 168.4, 172.6, 174.1 ppm. LC–MS *m/z*: 340.5 [*M*+1]. Purity = 82%.

*H-D-Tyr-Ser-Ala-OH (**10**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 1.37$ (d, 3H, $J =$ 7.3 Hz), 3.01–3.15 (m, 2H), 3.56 (dd, 1H, $J = 5.1$, 11.8 Hz), 3.66 (dd, 1H, $J = 5.5$, 11.9 Hz), 4.19 (t, 1H, $J = 6.7$ Hz), 4.25–4.36 (m, 2H), 6.82 (d, 2H, $J = 8.2$ Hz), 7.15 ppm (d, 2H, $J = 8.3$ Hz). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.8$, 36.7, 49.4, 55.2, 56.0, 61.6, 116.4 (2C), 126.0, 131.3 (2C), 155.6, 169.9, 171.6, 176.6 ppm. LC–MS *m/z*: 340.5 [*M*+1]. Purity = 80%.

*H-Phe-D-Ser-Ala-OH (**11**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 1.35$ (d, 3H, $J =$ 7.3 Hz), 3.44 (m, 2H), 3.76 (dd, 1H, $J = 5.2$, 11.5 Hz), 3.89 (dd, 1H, $J = 5.3$, 11.5 Hz), 4.52 (m, 2H), 4.60 (t, 1H, $J = 5.3$ Hz), 7.51 (m, 2H), 7.61 ppm (m, 3H). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.2$, 36.5, 49.3, 54.1, 54.7, 60.7, 127.5, 128.7 (2C), 128.9 (2C), 133.2, 168.7, 170.2, 173.3 ppm. LC–MS *m/z*: 324.22 [*M*+1]. Purity > 90%.

*H-Phe-hSer-Ala-OH (**12**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 1.38$ (d, 3H, $J =$ 7.4 Hz), 1.72–1.91 (m, 2H), 3.12 (m, 2H), 3.55 (dd, 2H, $J = 5.8$, 10.1 Hz), 4.21 (m, 2H), 4.37 (t, 1H, $J = 8.2$ Hz), 7.17 (m, 2H), 7.30 ppm (m, 3H). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.7$, 34.3, 37.5, 49.4, 51.3, 54.8, 58.2, 128.6, 129.8 (2C), 130.0 (2C), 134.2, 169.4, 172.8, 176.8 ppm. LC–MS *m/z*: 338.20 [*M*+1]. Purity > 90%.

*H-Phe-D-hSer-Ala-OH (**13**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 1.37$ (d, 3H, $J =$ 7.3 Hz), 1.37–1.67 (m, 2H), 2.87–3.08 (m, 4H), 3.96–4.14 (m, 3H), 7.06 (m, 2H), 7.16 ppm (m, 3H). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.7$, 34.0, 37.6, 49.5, 51.5, 55.2, 58.2, 128.7, 129.8 (2C), 130.0 (2C), 134.5, 169.7, 173.7, 176.9 ppm. LC–MS *m/z*: 338.20 [*M*+1]. Purity > 90%.

*H-Phe-Thr-Ala-OH (**14**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 1.11$ (d, 3H, $J =$ 6.4 Hz), 1.36 (d, 3H, $J = 7.4$ Hz), 3.09 (dd, 1H, $J = 5.0$, 12.6 Hz), 3.12 (dd, 1H, $J = 5.9$, 12.6 Hz), 3.88 (p, 1H, $J = 6.6$ Hz), 4.11–4.24 (m, 3H), 7.13 (m, 2H), 7.26 ppm (m, 3H). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.4$, 19.0, 37.1, 49.1, 54.6, 59.3, 67.7, 128.2, 129.3 (2C), 129.6 (2C), 133.8, 169.2, 170.4, 176.4 ppm. LC–MS *m/z*: 338.20 [*M*+1]. Purity > 90%.

*H-Phe-D-Thr-Ala-OH (**15**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 0.92$ (d, 3H, $J =$ 6.4 Hz), 1.40 (d, 3H, $J = 7.3$ Hz), 3.20 (d, 2H, $J = 7.5$ Hz), 3.80–3.89 (m, 1H), 4.07 (d, 1H, $J = 4.3$ Hz), 4.13–4.21 (m, 2H), 7.10 (m, 2H), 7.14–7.24 ppm (m, 3H). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.4$, 18.8, 37.2, 49.2, 54.7, 59.1, 67.3, 128.3, 129.5 (2C), 129.6 (2C), 134.0, 169.7, 171.4, 176.6 ppm. LC–MS *m/z*: 338.20 [*M*+1]. Purity > 90%.

*H-4-OEt-Phe-Ser-Ala-OH (**16**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.13$ (t, 3H, $J = 7.3$ Hz), 1.38 (d, 3H, $J = 7.1$ Hz), 3.10 (m, 2H), 3.67 (dd, 1H, $J = 7.2$, 12.6 Hz), 3.76 (dd, 1H, $J = 7.1$, 12.8 Hz), 4.06 (q, 2H, $J =$ 7.3 Hz), 4.21 (m, 1H), 4.25 (m, 1H), 4.39 (m, 1H), 6.90 (d, 2H, $J =$ 9.3 Hz), 7.15 ppm (d, 2H, $J = 9.2$ Hz). ^{13}C NMR (100 MHz, D_2O): $\delta =$ 13.9, 16.0, 35.9, 48.6, 54.5, 55.1, 61.3, 64.5, 115.1 (2C), 126.1, 130.7 (2C), 157.7, 169.0, 170.0, 176.0 ppm. LC–MS *m/z*: 368.01 [*M*+1]. Purity = 87%.

*H-4-Cl-Phe-Ser-Ala-OH (**17**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.41$ (d, 3H, $J =$ 7.4 Hz), 3.12 (m, 1H), 3.18 (m, 1H), 3.66 (dd, 1H, $J = 7.1$, 11.3 Hz), 3.76 (dd, 1H, $J = 7.3$, 11.4 Hz), 4.23 (m, 2H), 4.39 (m, 1H), 7.18 (d, 2H, $J = 8.9$ Hz), 7.34 ppm (d, 2H, $J = 8.8$ Hz). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.1$, 36.2, 48.7, 54.1, 54.9, 61.3, 129.0 (2C), 130.9 (2C), 132.4, 133.2, 168.7, 170.0, 176.2 ppm. LC–MS *m/z*: 358.14 [*M*+1]. Purity > 90%.

*H-1-NaphthylAla-Ser-Ala-OH (**18**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.35$ (d, 3H, $J = 7.4$ Hz), 3.48 (dd, 1H, $J = 7.7$, 11.3 Hz), 3.60–3.65 (m, 3H), 3.98 (q, 1H, $J = 7.5$ Hz), 4.08 (t, 1H, $J = 7.7$ Hz), 4.33 (t, 1H, $J = 7.6$ Hz), 7.46 (m, 2H), 7.56 (m, 2H), 7.87 (d, 1H, $J = 8.0$ Hz), 7.95 ppm (m, 2H). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.0$, 33.8, 48.6, 54.0, 54.5, 61.5, 122.8, 125.7, 126.2, 126.7, 128.4, 128.6, 129.0, 130.0, 131.5, 133.7, 168.7, 168.9, 176.0 ppm. LC–MS *m/z*: 373.96 [*M*+1]. Purity = 81%.

*H-2-NaphthylAla-Ser-Ala-OH (**19**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.36$ (d, 3H, $J = 7.5$ Hz), 3.43 (dd, 1H, $J = 8.8$, 13.1 Hz), 3.59 (dd, 1H, $J = 6.9$, 13.8 Hz), 3.77 (dd, 1H, $J = 6.7$, 11.8 Hz), 3.79 (q, 1H, $J = 7.8$ Hz), 3.87 (dd, 1H, $J = 5.1$, 11.3 Hz), 4.49 (t, 1H, $J = 5.6$ Hz), 4.53 (dd, 1H, $J = 6.7$, 8.9 Hz), 7.55 (d, 1H, $J = 8.3$ Hz), 7.72 (m, 2H), 8.05 ppm (m, 4H). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.0$, 37.0, 48.3, 54.3, 54.8, 61.3, 126.5, 126.7, 126.8, 127.6, 127.7, 128.2, 128.8, 131.4, 132.4, 133.2, 168.7, 169.4, 176.1 ppm. LC–MS *m/z*: 374.00 [*M*+1]. Purity > 90%.

*H-4-F-Phe-Ser-Ala-OH (**20**).* ^{1}H NMR (300 MHz, D_2O): $\delta = 1.42$ (d, 3H, $J = 7.2$ Hz), 3.20 (d, 2H, $J = 7.2$ Hz), 3.69–3.85 (m, 2H), 4.19–4.30 (m, 2H), 4.45 (t, 1H, $J = 5.7$ Hz), 7.10 (t, 2H, $J = 9.0$ Hz), 7.26 ppm (dd, 2H, $J = 5.8$, 8.9 Hz). ^{13}C NMR (75 MHz, D_2O): $\delta = 16.8$, 36.3, 49.8, 54.5, 55.4, 61.6, 116.0 (2C, $^2J_F = 21.8$ Hz), 129.8, 131.4 (2C, $^3J_F = 8.5$ Hz), 162.4 ($^1J_F = 243.8$ Hz), 169.2, 170.2, 177.5 ppm. LC–MS *m/z*: 342.17 [*M*+1]. Purity > 90%.

*H-3,5-diCl-Phe-Ser-Ala-OH (**21**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.42$ (d, 3H, $J = 7.4$ Hz), 3.21 (dd, 1H, $J = 7.7$, 11.4 Hz), 3.34 (dd, 1H, $J = 7.3$, 11.2 Hz), 3.62 (dd, 1H, $J = 7.8$, 11.7 Hz), 3.72 (dd, 1H, $J = 7.4$, 11.7 Hz), 4.26 (m, 2H), 4.40 (m, 1H), 7.20 (d, 1H, $J = 8.4$ Hz), 7.28 (d, 1H, $J = 8.3$ Hz), 7.50 ppm (s, 1H). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.2$, 34.0, 48.8, 52.7, 54.8, 61.5, 127.7, 129.5, 130.4, 132.6, 134.1, 134.7, 168.3, 169.5, 176.3 ppm. LC–MS *m/z*: 392.11 [*M*+1]. Purity > 90%.

*H-Indan-1-ylGly-Ser-Ala-OH (**22**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.38$ (d, 3H, $J = 7.3$ Hz), 2.91 (m, 3H), 3.04 (t, 1H, $J = 7.7$ Hz), 3.16 (m, 1H), 3.73 (dd, 1H, $J = 7.5$, 12.1 Hz), 3.82 (dd, 1H, $J = 7.6$, 12.2 Hz), 4.10 (d, 1H, $J = 7.2$ Hz), 4.33 (m, 2H), 7.20 ppm (m, 4H). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.3$, 34.1, 35.1, 41.1, 48.9, 55.3, 56.4, 61.3, 124.3 (2C), 127.0 (2C), 141.2, 141.4, 169.0, 170.3, 176.0 ppm. LC–MS *m/z*: 349.99 [*M*+1]. Purity > 90%.

*H-2-Cl-Phe-Ser-Ala-OH (**23**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.41$ (d, 3H, $J = 7.2$ Hz), 3.27 (dd, 1H, $J = 7.5$, 12.5 Hz), 3.34 (dd, 1H, $J = 7.6$, 12.3 Hz), 3.63 (dd, 1H, $J = 7.1$, 13.2 Hz), 3.74 (dd, 1H, $J = 7.4$, 12.9 Hz), 4.25 (dd, 1H, $J = 7.3$, 11.6 Hz), 4.29 (dd, 1H, $J = 7.4$, 11.7 Hz), 4.43 (dd, 1H, $J = 7.8$, 8.1 Hz), 7.25–7.33 (m, 3H), 7.43 ppm (d, 1H, $J = 8.4$ Hz). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.2$, 34.5, 48.6, 53.0, 55.0, 61.4, 127.6, 129.8, 129.9, 131.5, 131.7, 133.9, 168.6, 169.7, 176.0 ppm. LC–MS *m/z*: 358.17 [*M*+1]. Purity > 90%.

*H-4-NH$_2$-Phe-Ser-Ala-OH (**24**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.40$ (d, 3H, $J = 7.4$ Hz), 3.23 (d, 2H, $J = 7.8$ Hz), 3.70 (dd, 1H, $J = 7.5$, 11.8 Hz), 3.80 (dd, 1H, $J = 7.3$, 11.7 Hz), 4.29 (m, 2H), 4.45 (dd, 1H, $J = 7.3$, 8.2 Hz), 7.32–7.39 ppm (m, 4H). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.2$, 36.1, 48.8, 54.0, 55.1, 61.2, 123.6 (2C), 129.6, 131.1 (2C), 134.9, 168.8, 170.2, 176.1 ppm. LC–MS *m/z*: 339.19 [*M*+1]. Purity > 90%.

*H-2-CN-Phe-Ser-Ala-OH (**25**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.40$ (d, 3H, $J = 7.5$ Hz), 3.33 (dd, 1H, $J = 7.3$, 13.1 Hz), 3.43 (dd, 1H, $J = 7.5$, 13.3), 3.60 (dd, 1H, $J = 7.4$, 12.4 Hz), 3.71 (dd, 1H, $J = 7.7$, 12.6 Hz), 4.25 (m, 2H), 4.43 (m, 1H), 7.41 (d, 1H, $J = 8.4$ Hz), 7.46 (t, 1H, $J = 8.5$ Hz), 7.63 (t, 1H, $J = 8.3$ Hz), 7.73 ppm (d, 1H, $J = 8.4$ Hz). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.2$, 35.3, 48.6, 53.5, 54.9, 61.4, 112.0, 118.1, 128.7, 130.7, 133.7, 134.0, 137.3, 168.0, 169.5, 176.0 ppm. LC–MS *m/z*: 349.16 [*M*+1]. Purity > 90%.

*H-2-Me-Phe-Ser-Ala-OH (**26**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.39$ (d, 3H, $J = 7.3$ Hz), 2.26 (s, 3H), 3.17 (m, 2H), 3.62 (dd, 1H, $J = 7.4$, 10.9 Hz), 3.71 (dd, 1H, $J = 7.5$, 11.1 Hz), 4.20 (m, 2H), 4.39 (m, 1H), 7.09–7.23 ppm (m, 4H). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.2$, 18.4, 34.0, 48.6, 53.4, 54.9, 61.3, 126.5, 128.1, 130.1, 130.8, 132.1, 137.1, 169.0, 169.7, 175.9 ppm. LC–MS *m/z*: 338.19 [*M*+1]. Purity > 90%.

*H-4-PhCO-Phe-Ser-Ala-OH (**27**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.35$ (d, 3H, $J = 7.4$ Hz), 3.13 (dd, 1H, $J = 7.3$, 12.5 Hz), 3.40 (dd, 1H, $J = 7.6$, 12.3 Hz), 3.73 (dd, 1H, $J = 7.2$, 11.8 Hz), 3.83 (dd, 1H, $J = 7.4$, 12.2 Hz), 4.20 (dd, 1H, $J = 7.5$, 12.3 Hz), 4.38 (dd, 1H, $J = 7.1$, 8.4 Hz), 4.49 (dd, 1H, $J = 7.2$, 8.3 Hz), 7.45 (d, 2H, $J = 10.3$ Hz), 7.62 (t, 2H, $J = 8.7$ Hz), 7.74–7.87 ppm (m, 5H). ^{13}C NMR (100 MHz, D_2O): $\delta =$ 16.0, 36.8, 48.6, 54.1, 54.8, 61.4, 128.6 (2C), 129.5 (2C), 130.2 (2C), 131.0 (2C), 133.6, 136.2, 136.7, 139.5, 168.6, 169.8, 175.9, 200.2 ppm. LC–MS *m/z*: 428.21 [*M*+1]. Purity > 90%.

*H-3-Cl-Phe-Ser-Ala-OH (**28**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.40$ (d, 3H, $J = 7.4$ Hz), 3.12 (dd, 1H, $J = 7.5$, 13.1 Hz), 3.19 (dd, 1H, $J = 7.1$, 12.8 Hz), 3.67 (dd, 1H, $J = 7.2$, 11.3 Hz), 3.77 (dd, 1H, $J = 7.4$, 11.5 Hz), 4.28 (m, 2H), 4.42 (m, 1H), 7.15 (d, 1H, $J = 8.2$ Hz), 7.22 (s, 1H), 7.31 ppm (m, 2H). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.3$, 36.2, 48.6, 54.0, 55.1, 61.3, 127.8, 128.0, 129.3, 130.5, 134.0, 135.7, 168.7, 170.0, 176.1 ppm. LC–MS *m/z*: 358.14 [*M*+1]. Purity = 79%.

*H-4-Ph-Phe-Ser-Ala-OH (**29**).* ^{1}H NMR (400 MHz, D_2O): $\delta = 1.36$ (d, 3H, $J = 7.4$ Hz), 3.34 (dd, 1H, $J = 7.3$, 11.4 Hz), 3.47 (dd, 1H, $J = 7.4$, 11.6 Hz), 3.84 (dd, 1H, $J = 6.9$, 12.1 Hz), 3.94 (dd, 1H, $J = 7.9$, 12.1 Hz), 4.18 (q, 1H, $J = 7.5$ Hz), 4.48 (t, 1H, $J = 7.4$ Hz), 4.56 (t, 1H, $J = 7.6$ Hz), 7.50 (d, 2H, $J = 7.4$ Hz), 7.59 (t, 1H, $J = 7.0$ Hz), 7.69 (t, 2H, $J = 7.8$ Hz), 7.83 (d, 2H, $J = 7.2$ Hz), 7.87 ppm (d, 2H, $J = 7.1$ Hz). ^{13}C NMR (100 MHz, D_2O): $\delta = 16.5$, 37.2, 49.6, 55.0, 55.8, 62.5, 127.8 (2C), 128.3 (2C), 129.0, 130.2 (2C), 130.9 (2C), 134.0, 140.8, 141.0, 169.9, 170.8, 176.9 ppm. LC–MS *m/z*: 399.95 [*M*+1]. Purity > 90%.

Acknowledgements

The Danish Medicinal Council supported this work (Project grant # 22-03-0274). Dr. Carsten Uhd Nielsen acknowledges the financial support from the Carlsberg Foundation which enabled the purchase of the FluoroStar system. Dr. Birger Brodin is acknowledged for guidance on the fluorescence based measurements. Furthermore, the authors appreciate the work of technicians Betina Dinitzen and Susanne N. Sorensen.

Keywords: binding affinity · hPEPT1 · promoiety · solid-phase synthesis · translocation

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Received: August 11, 2006
Revised: November 6, 2006

