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The tandem chain extension aldol reaction used for synthesis of ketomethylene tripeptidomimetics targeting hPEPT1

Karina Thorn [a,*], Carsten Uhd Nielsen [b], Palle Jakobsen [c], Bente Steffansen [b], Charles K. Zercher [d], Mikael Begtrup [e]

[a] Protein Chemistry, Biogen Idec Hemophilia, 9 Fourth Avenue, Waltham, MA 02451, USA
[b] Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark
[c] Insulin Chemistry, Novo Nordisk A/S, Måløv Byvej 200, 2760 Måløv, Denmark
[d] Department of Chemistry, University of New Hampshire, 23 Academic Way, Durham, NH 03824, USA
[e] Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark

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ABSTRACT

The rationale for targeting the human di-/tripeptide transporter hPEPT1 for oral drug delivery has been well established by several drug and prodrug cases. The aim of this study was to synthesize novel ketomethylene modified tripeptidomimetics and to investigate their binding affinity for hPEPT1. Three related tripeptidomimetics of the structure H-Phe-ψ[$COCH_2$]-Ser(Bz)-X_{aa}-OH were synthesized applying the tandem chain extension aldol reaction, where amino acid derived β-keto imides were stereoselectively converted to α-substituted γ-keto imides. In addition, three corresponding tripeptides, composed of amide bonds, were synthesized for comparison of binding affinities. The six investigated compounds were all defined as high affinity ligands (K_i-values <0.5 mM) for hPEPT1 by measuring the concentration dependent inhibition of apical [^{14}C]Gly-Sar uptake in Caco-2 cells. Consequently, the ketomethylene replacement for the natural amide bond and α-side chain modifications appears to offer a promising strategy to modify tripeptidic structures while maintaining a high affinity for hPEPT1.

The human intestinal peptide transporter, hPEPT1, facilitates the transepithelial transport of dipeptides, tripeptides, and a number of peptidomimetic drugs.[1–4] The active transport via hPEPT1 over the apical membrane is mediated by an electrochemical proton gradient, and results in a co-transport of protons and substrates.[5] hPEPT1 is expressed in the luminal membrane of the small intestine, and is responsible for the natural absorption of digested protein products from the diet. Consequently, the substrate recognition of hPEPT1 is broad with mM affinities, and it possesses a high transport capacity. These features make the transporter an excellent target for exploitation as a transport system for drug delivery of small peptidomimetics and prodrugs.[6,7]

The main challenge with employing peptides as prodrugs or as potential drugs is their low stability after oral administration. Proteolytic enzymes and exopeptidases distributed in the gastrointestinal (GI) tract rapidly decompose the peptides before the therapeutic action has occurred. Fortunately, the oral bioavailability of peptidic drugs can be increased by chemical modifications.[8] Enzymatic degradation of peptides can be avoided by incorporation of D-configured amino acids, by introduction of unnatural α-side chains or by replacement of amide bonds with non-hydrolysable entities. *N*-Methylated amides, ketomethylene, hydroxyethylene, and thioamides are among the most commonly used amide bond bioisosteres.[9–12]

The choice of amide bond bioisostere in this study was based on known structural requirements necessary for ligands and substrates interacting with hPEPT1. A previous study of a series of H-Phe-Gly-OH dipeptidomimetics revealed that the ketomethylene analog and the native dipeptide possessed a similar affinity and translocation for hPEPT1, whereas the hydroxyethylene and hydroxyethylidene analogues were poor substrates.[13,14] Consequently, the carbonyl part of the amide bond mimetic appears to be significant for the hPEPT1 interaction.

We previously suggested H-Phe-Ser-Ala-OH as a lead promoiety targeting hPEPT1 due to high affinity and translocation.[15] In this tripeptide, the serine α-side chain is utilized to provide an ester linkage to a therapeutically active substance with low bioavailability containing a carboxylic acid functionality. Phenylalanine serves as a hydrophobic N-terminus and alanine as a small, uncharged C-terminal amino acid. The aim of this study was to evaluate if chemical modifications of the lead structure are tolerated in terms of hPEPT1 binding. We have thus synthesized the ketomethylene peptidomimetic H-Phe-ψ[$COCH_2$]-Ser(Bz)-Ala-OH (**14A**) in which both an amide bond replacement and a side chain modification

* Corresponding author at present address: Protein Chemistry, Biogen Idec Hemophilia, 9 Fourth Avenue, Waltham, MA 02451, USA.
E-mail address: karina.thorn@biogenidec.com (K. Thorn).

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have been incorporated. With the purpose of optimizing the construct, two related peptidomimetics **14B–C** were synthesized. In these the methyl α-side chain of alanine was replaced with propyl or cyclohexyl. For comparison of binding affinities, the reference tripeptides **20A–C** were synthesized (Fig. 1).

Ketomethylene entities are usually obtained by complicated multi-step synthetic sequences, many of which do not facilitate stereoselective incorporation of the α-side chain mimic.[16,17] We used the recently introduced tandem chain extension aldol (TCEA) reaction for preparation of the ketomethylene entity.[18] By this approach β-keto imides were converted in one-pot to α-substituted γ-keto imides by the highly stereoselective zinc-mediated aldol reaction directed by chiral oxazolidinones.[19,20] Utilization of the TCEA reaction for stereoselective synthesis of α-substituted ketomethylene isosteres has been reported for proline as the stereodirecting element as well.[21]

A retrosynthetic analysis revealed that the H-Phe-ψ[$COCH_2$]-Ser(Bz)-X_{aa}-OH construct **14A–C** can be derived from the γ-keto imide **11**, which in turn can be prepared from the β-keto imide **9** using paraformaldehyde in the TCEA reaction (Fig. 1).

The amino acid used as starting material needed double protection of the terminal amine to prevent quenching of the enolate formed in the TCEA reaction.[19–21] We protected the amine group of L-phenylalanine **1** sequentially with *p*-methoxybenzyl (PMB) and benzyloxycarbonyl (Cbz) (Scheme 1). The PMB group was introduced by reductive amination by treating **1** with *p*-anisaldehyde and sodium borohydride in succession.[22] The Cbz group was inserted under standard conditions using benzyl chloroformate (Cbz-Cl). The overall yield of the double-protected L-phenylalanine **3** was 74%.

The 2-substituted oxazolidinone **7**, functioning as a chiral auxiliary, was prepared from D-phenylalanine (**4**).[23–25] Reduction of **4** to the β-amino alcohol **5** was performed by treatment with borane dimethylsulfide complex and boron trifluoride diethyl etherate. Subsequent exposure of **5** to triphosgene afforded the oxazolidinone **6**. Compound **6** was acetylated to give the chiral auxiliary **7** in 78% overall yield (Scheme 2).

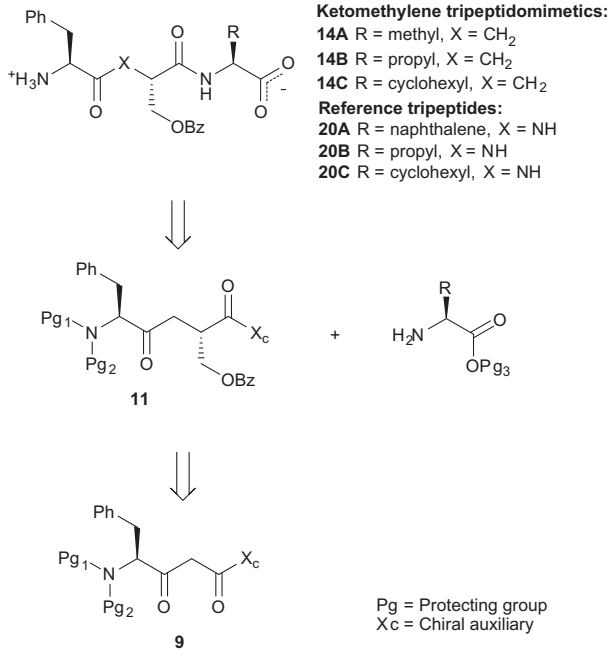
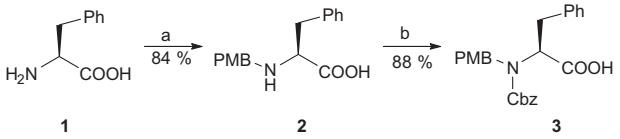
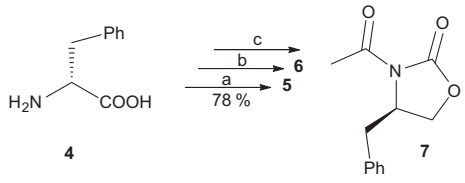


Figure 1. Retrosynthetic analysis of the preparation of H-Phe-ψ[$COCH_2$]-Ser(Bz)-X_{aa}-OH by the TCEA reaction.



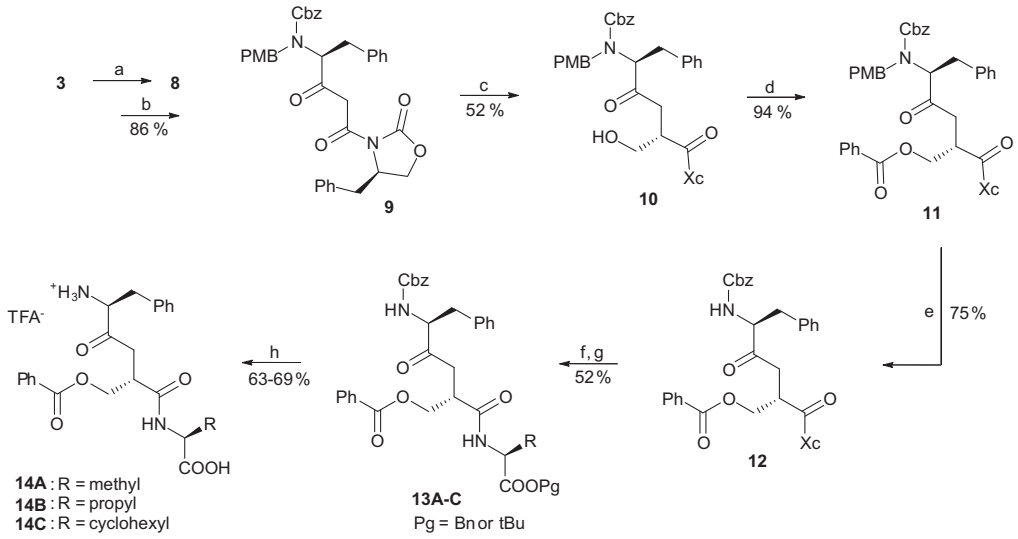
Scheme 1. Double protection of phenylalanine. Reagents and conditions: (a) (i) *p*-anisaldehyde, 2 M NaOH, rt, 30 min; (ii) $NaBH_4$, 0 °C, 30 min; (i + ii) repeated; (b) Cbz-Cl, 1 M NaOH, 0 °C, 1 h.



Scheme 2. Synthesis of the chiral auxiliary. Reagents and conditions: (a) (i) BF_3OEt_2, dry THF, 5 min, reflux; (ii) BH_3 Me_2S, overnight, reflux; (b) Et_3N, $(Cl_3CO)_2CO$, DCM, 2 h, <10 °C; (c) (i) *n*-BuLi, dry THF, 15 min, −40 °C; (ii) AcCl, 1 h, −40 °C.

The final steps of the ketomethylene synthesis are shown in Scheme 3. The double-protected L-phenylalanine (**3**) was activated with carbonyldiimidazole to form the intermediate **8**. Subsequently, **8** was treated with the lithium enolate of **7** which was obtained by deprotonation with lithium diisopropylamide. This gave the desired β-keto imide **9** in 86% purified yield. Utilizing the one-pot TCEA reaction, the β-keto imide **9** was converted to the α-substituted γ-keto imide **10** in 52% yield. The reaction was effected by treatment of **9** with the Furukawa reagent, $EtZnCH_2I$, to give the zinc enolate, which upon treatment in situ with paraformaldehyde provided the aldol product **10**. The L-stereochemistry of the α-side chain was controlled by the stereoconfiguration of the chiral auxiliary **7**. The facial selectivity in the TCEA reactions with oxazolidinones was established in earlier studies.[18,20] The product **10** existed as two rotamers in equilibrium with two hemiacetals giving rise to complex NMR spectra. The hemiacetal was particularly characterized by a distinctive ^{13}C NMR signal at 108 ppm and mass determination.

Subsequently, the serine α-side chain mimic was derivatized by benzoylation with benzoyl chloride under standard conditions to give **11** in 94% yield. This esterification pushed the equilibrium in favor of the desired product **11** due to a higher reactivity of the primary alcohol group of the open aldol form of **10**. The PMB protecting group was removed by treatment with cerium ammonium nitrate in aqueous acetonitrile.[26] Epimerization of **12** during cleavage from the chiral auxiliary was suppressed by using the mildest possible conditions which were found to be 1 equiv lithium hydroxide in aqueous tetrahydrofuran at −10 °C for 15 min. Alternative cleavage of the chiral auxiliary using lithium peroxide[27] was abandoned since competitive Bayer–Villiger oxidation was observed in earlier studies.[20] When recycling the recovered auxiliary **7** diastereoselectivity was maintained indicating that the stereocenter of **7** does not epimerize under the conditions of the reaction. The free carboxylic acid obtained after deprotection of **12** was coupled to the C-terminal amino acid. Preactivation of the carboxylic acid with benzotriazol-1-yl-oxy-tris-dimethylaminophosphonium PF_4^- salt (BOP) was followed by treatment overnight with appropriate C-terminal protected amino acids to yield products **13A–C**. The protected tripeptidomimetics **13A–C** were purified by normal phase HPLC to remove small amounts (<10% determined by LC–MS) of the unwanted diastereomer formed by epimerization during the hydrolysis step using LiOH. Finally, the ketomethylene tripeptidomimetics **13A–C** were deprotected by hydrogenolysis



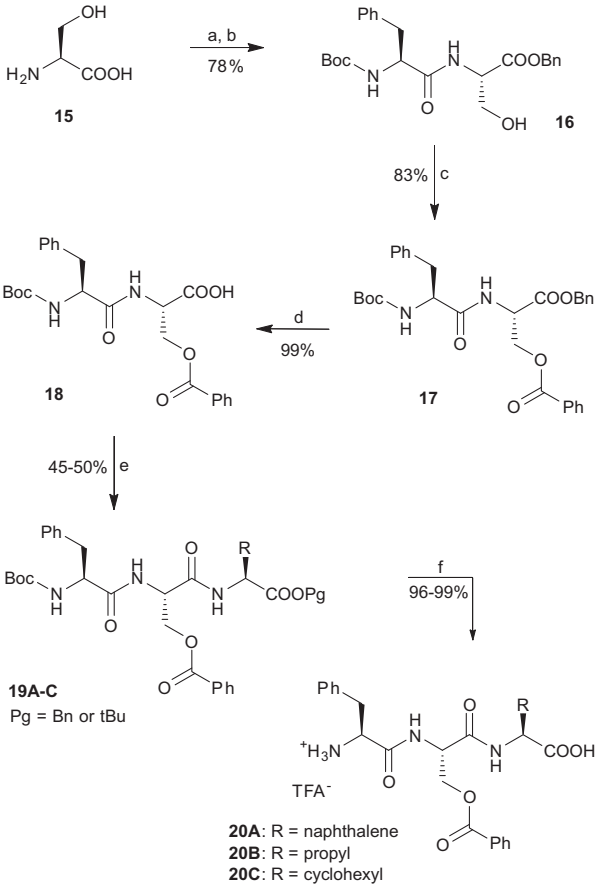
Scheme 3. Synthesis of ketomethylene tripeptidomimetics H-Phe-ψ[$COCH_2$]-Ser(Bz)-X_{aa}-OH. Reagents and conditions: (a) carbonyldiimidazole, dry THF, 30 min, rt; (b) (i) *i*-Pr_2NH, *n*-BuLi, dry THF, 15 min, −5 °C; (ii) compound **7**, 1 h, −78 °C; (iii) compound **8**, 1 h, −78 °C; (c) (i) Et_2Zn, CH_2I_2, dry DCM, 10 min, 0 °C; (ii) paraformaldehyde, compound **9**, 3 h, rt; (d) pyridine, DMAP, BzCl, DCM, 4.5 h, rt; (e) CAN, 4:1 MeCN H_2O, 35 min, rt; (f) LiOH·H_2O, 3:1 THF H_2O, 15 min, −10 °C; (g) (i) DIPEA, BOP, DMF, 3 min, rt; (ii) H-X_{aa}-OPg, overnight, rt; (h) 10% Pd on C, TFA, MeOH, overnight, rt, H_2.

affording the target compounds **14A–C**. An ultimate purification of each compound was carried out by reverse phase HPLC achieving a higher than 99% purity in each case as determined by LC–MS.

The corresponding tripeptides **20A–C** containing only amide bonds were synthesized as shown in Scheme 4. The dipeptide **6** was composed of Boc-Phe-OH and H-Ser-OBn as a result of peptide coupling under standard solution phase conditions using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI) and 1-hydroxybenzotriazole (HOBt) as coupling reagents. The serine α-side chain was benzoylated in a similar fashion to that used in the ketomethylene series to give **17** in 83% yield. The benzyl protecting group of **17** was removed quantitatively by hydrogenolysis to afford product **18**. The three C-terminal amino acids were introduced to give compound **19A–C** applying the peptide coupling protocol described above. Finally, the *tert*-butoxycarbonyl (Boc) and *tert*-butyl (^{t}Bu) protection groups were removed by exposure of **19A–C** to a 2:1 mixture of dichloromethane and trifluoroacetic acid to yield the desired tripeptides **20A–C** with a purity higher than 87% according to LC–MS.

The ketomethylene tripeptidomimetics **14A–C** and the control tripeptides **20A–C** were tested for hPEPT1 binding affinity by measuring the concentration dependent inhibition of apical [^{14}C]Gly-Sar uptake in Caco-2 cells (Table 1). The K_i values ranged between 0.05 and 0.32 mM which means that all of the compounds are defined as high affinity (K_i <0.5 mM) ligands for hPEPT1.

The C-terminal modified tripeptides **20A–C** were evaluated against the lead promoiety H-Phe-Ser-Ala-OH. Compounds **20A** and **20C** displayed similar affinity for hPEPT1 compared to H-Phe-Ser-Ala-OH. For **20B** a significantly higher affinity for hPEPT1 was observed compared to H-Phe-Ser-Ala-OH ($p < 0.05$, $N = 3$). In order to investigate whether larger C-terminal amino acids would have an effect on hPEPT1 affinity **20A** was synthesized. Compound **20A**, containing a naphthalene substituted C-terminal amino acid, demonstrated that hydrophobic modifications in the C-terminal part of the tripeptide did not seem to affect hPEPT1 affinity. These results indicate that even though the size of the tripeptidomimetic molecule was increased by the derivatized serine α-side chain and the altered C-terminal residue, this was not followed by a reduction of affinity for hPEPT1. This is partly in agreement with previous observations where hydrophobic modifications in the R^1 or R^2 position of dipeptide side chains seem to increase the hPEPT1 affinity.[28] In addition, a recent study illustrated that it is not only the hydrophobic character of the amino acid



Scheme 4. Synthesis of reference tripeptides. Reagents and conditions: (a) *p*-TsOH, 1:1 BnOH CCl_4, reflux, Dean–Stark conditions; (b) (i) Boc-Phe-OH, DIPEA, EDCI, HOBt, DMF, 5 min; (ii) H-Ser-Bn, overnight, rt; (c) pyridine, DMAP, BzCl, DCM, 4.5 h, rt; (d) 10% Pd on C, MeOH, overnight, rt, H_2; (e) (i) DIPEA, EDCI, HOBt, 3 min; (ii) H-X_{aa}-OPg, overnight, rt, N_2; (f) 2:1 DCM TFA, 6 h, rt.

Table 1
hPEPT1 affinity data[a]

Product	log K_i ± log SE	K_i (mM)
H-Phe-Ser-Ala-OH	−0.59 ± 0.09	0.26[b]
14A H-Phe-ψ[$COCH_2$]Ser(Bz)-Ala-OH	−0.49 ± 0.05	0.32
14B H-Phe-ψ[$COCH_2$]Ser(Bz)-Nva-OH	−0.60 ± 0.09	0.25
14C H-Phe-ψ[$COCH_2$]Ser(Bz)-Chg-OH	−1.33 ± 0.09	0.05
20A H-Phe-Ser(Bz)-Nal-OH		~0.3[c]
20B H-Phe-Ser(Bz)-Nva-OH	−0.91 ± 0.03	0.12
20C H-Phe-Ser(Bz)-Chg-OH	−0.61 ± 0.02	0.25

Nva, norvaline; Chg, cyclohexylglycine; Nal, naphthylalanine.
[a] The K_i-values are measured as the concentration dependent inhibition of 20 μM [^{14}C]Gly-Sar apical uptake in Caco-2 cells.
[b] Value obtained by refitting data presented in Thorn et al.[15]
[c] Value estimated due to limited solubility.

substitutions that influence hPEPT1 interaction but also the shape and orientation of the side chains.[29]

The ketomethylene analogues **14A** and **14B** did not have significantly different affinities for hPEPT1 compared to the lead promoiety H-Phe-Ser-Ala-OH, whereas **14C** displayed a significantly higher affinity ($p < 0.01$, $N = 3$). Moreover, comparing **14B** with the corresponding tripeptide analogue **20B**, a twofold significantly lower affinity was observed for the ketomethylene product **14B** ($p < 0.05$, $N = 3$). Compounds **14C** and **20C** revealed the opposite pattern, where the affinity for hPEPT1 was significantly higher for the ketomethylene analogue ($p < 0.001$, $N = 3$). These observations provide no obvious explanation for the behavior of the favored ketomethylene analogue based on the knowledge about ligands interacting with hPEPT1. Perhaps the rotational flexibility of the ketomethylene part facilitates the adoption of a preferred conformation of the tripeptidomimetic **14C** thereby increasing the affinity for hPEPT1. The C-terminal α-side chain of **14C** is composed of a cyclohexyl group, which is a sterically large group compared to the α-side chain of **14A** or **14B**. This disparity could account for the differences between the ketomethylene compounds.

All products, **14A–C** and **20A–C**, displayed high affinity for hPEPT1 independent on whether the tripeptide skeleton was composed of amide or ketomethylene linkages. In other words, the nitrogen of the N-terminal amide group of the tripeptidomimetics did not influence hPEPT1 binding. This finding is supported by a series of dipeptidomimetics.[13,14] The fact that the ketomethylene compounds maintained the biological affinity for hPEPT1 demonstrates a promising stabilization strategy towards the environment in the GI-tract as the ketomethylene linkage is non-hydrolysable. Furthermore, introduction of the C-terminal non-proteinogenic amino acids in compound **14B** and **14C** would presumably increase the stability towards enzymatic degradations caused by carboxypeptidases.

For the reference tripeptides the stability of similar tripeptides during the affinity study in Caco-2 cell was investigated in a previous study. Less than 10% of the tripeptides were decomposed in the cell assay.[30]

Recently, a comprehensive investigation of translocation of tripeptides via hPEPT1 was published. The study found that 40 out of 55 tripeptides were translocated, 8 interacted with the assay used, and one (Met-Pro-Pro) was an inhibitor while one (Asp-Ile-Arg) was not recognized by hPEPT1.[31] A series of benzyl esters of the ketomethylene dipeptidomimetics Phe-ψ[$COCH_2$]Asp, Val-ψ[$COCH_2$]-Asp and Asp-ψ[$COCH_2$]Gly were also shown to display affinity for hPEPT1 and were transported through Caco-2 cell monolayers.[14] It could appear that most ligands are also substrates for the transporter, however, none of the antimicrobial di- and tripeptides tested in a recent study were substrates for hPEPT1 despite having moderate affinity towards the transporter for some of the peptides.[29] Thus, further studies are needed to prove unequivocally if **14A–C** and **20A–C** are substrates or inhibitors of hPEPT1. Such evidence could be acquired by measurements of translocation using hPEPT1 transfected cell models or oocytes expressing hPEPT1. Regardless of the translocation properties of **14A–C**, these structures are promising for either the design of substrates or inhibitors. Especially, the 50 μM affinity of **14C** could be a starting point for a chemical effort to find a higher affinity inhibitor which could be very useful for both in vitro and in vivo studies.

The present study of selected tripeptidic compounds targeting hPEPT1 has demonstrated that the tripeptidomimetics H-Phe-ψ[$COCH_2$]-Ser(Bz)-X_{aa}-OH maintain high affinity upon replacement of their N-terminal amide bonds with ketomethylene entities. The results signify that the amino nitrogen of the natural amide bond is not vital for hPEPT1 recognition of tripeptidic structures. Consequently, replacement of amide bonds with ketomethylene units is promising for stabilization against degradation of tripeptides targeting hPEPT1 in the GI tract. Derivatization of the serine α-side chain and incorporation of non-proteinogenic aliphatic amino acids at the C-terminus were also allowed indicating broad ligand recognition of the tripeptidomimetic core. Furthermore, the efficiency and application of the stereocontrolled tandem chain extension aldol (TCEA) reaction for generation of ketomethylene tripeptidomimetics using paraformaldehyde for construction of the α-side chain serine mimic was nicely demonstrated.

Supplementary data (Experimental procedures)

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.05.108.

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