Three-Dimensional Quantitative Structure−Activity Relationship Analyses of β-Lactam Antibiotics and Tripeptides as Substrates of the Mammalian H^+/Peptide Cotransporter PEPT1

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The utilization of the membrane transport protein PEPT1 as a drug delivery system is a promising strategy to enhance the oral bioavailability of drugs. Since very little is known about the substrate binding site of PEPT1, computational methods are a meaningful tool to gain a more detailed insight into the structural requirements for substrates. Three-dimensional quantitative structure−activity relationship (3D-QSAR) studies using the comparative molecular similarity indices analysis (CoMSIA) method were performed on a training set of 98 compounds. Affinity constants of β-lactam antibiotics and tripeptides were determined at Caco-2 cells. A statistically reliable model of high predictive power was obtained ($q^2 = 0.828$, $r^2 = 0.937$). The results derived from CoMSIA were graphically interpreted using different field contribution maps. We identified those regions which are crucial for the interaction between peptidomimetics and PEPT1. The new 3D-QSAR model was used to design a new druglike compound mimicking a dipeptide. The predicted K_i value was confirmed experimentally.

Introduction

The mammalian transmembrane protein PEPT1 transports nutritional di- and tripeptides across the luminal membrane into small intestinal cells. The system is driven by an inward directed proton gradient. PEPT1 displays a very broad substrate specificity. Besides the physiological substrates, a variety of peptidomimetics such as β-lactam antibiotics, valacyclovir, δ-aminolevulinic acid, angiotensin-converting enzyme (ACE) inhibitors, bestatin, and other drugs are recognized and transported by PEPT1.[1−7] This ability of PEPT1 to transport nonnatural substrates allows the oral application of these drugs for the therapy of several deseases.[8−12]

The three-dimensional structure of the H^+/peptide cotransporter PEPT1 is still unknown and very little is known about the substrate binding site. Recently, we performed three-dimensional quantitative structure−activity relationship (3D-QSAR) studies for the dipeptide substrates[13] of PEPT1 by means of comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA).[14] This model was restricted to rather small molecules containing only one peptide bond. The molecule size of physiological substrates of PEPT1, however, ranges from Gly-Gly to Trp-Trp-Trp. Therefore, to obtain a deeper insight into the binding site of the carrier, it is mandatory to investigate larger substrates such as tripeptides containing voluminous side chains (e.g., Trp-Trp-Trp). Moreover, many structurally related drugs such as β-lactam antibiotics are known substrates for PEPT1 mimicking natural tripeptides (Figure 1). Their binding affinities are so far neither explainable nor predictable.

The prediction of the affinity of new compounds is one of the major challenges in drug design. A first attempt to map the binding site of PEPT1 using log *P* values of β-lactam antibiotics was performed by Swaan and coworkers.[15] CoMFA studies resulted in correlation values of $q^2 = 0.754$ and $r^2 = 0.993$, albeit the dataset contained only 10 compounds. Moreover, the interpretation of the CoMFA contour maps should be done with caution since some inherent deficits arise from the application of Lennard-Jones and Coulomb potentials.[16] Bailey and coworkers presented a template for PEPT1 substrates that was developed from structures and affinity constants of 42 compounds (dipeptides, tripeptides, β-lactam antibiotics, ACE inhibitors, and others) ranging from 0.019 to >50 mM.[9] However, the data collected from the literature were measured in different systems and might not be comparable. Furthermore, side chains of peptides were not considered. However, this study already established that interactions of substrates to the peptide transporter are a result of several characteristic features which cannot be reduced to one single parameter. This result agrees with our 3D-QSAR study. In contrast with Bailey, we used data obtained under identical experimental conditions with the same assay.

In the present paper, we employed the CoMSIA method to analyze and predict binding affinities of β-lactam antibiotics as well as di- and tripeptides measured in our laboratory. A simple and robust pharmacophore model was developed from a training set containing 98 dipeptides, tripeptides, and β-lactam antibiotics with binding affinities ranging from 0.01 to 100 mM. First, we measured the affinity constants of a

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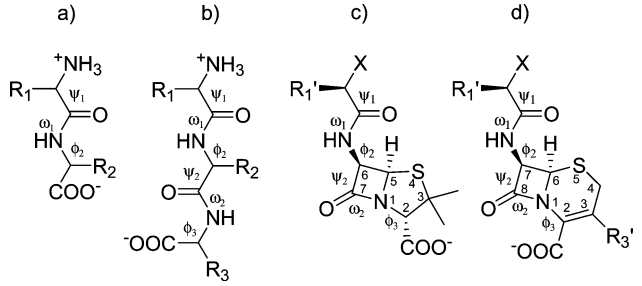


Figure 1. General structures of (a) dipeptides, (b) tripeptides, (c) penicillins, and (d) cephalosporins.

series of new tripeptides for PEPT1 in the Caco-2 competition assay.

Results and Discussion

Binding Studies. Competition assays using [^{14}C]Gly-Sar as the reference substrate were performed in Caco-2 cells to determine the binding affinities of 32 tripeptides, 6 β-lactam antibiotics, and 10 dipeptides. Affinity constants of 49 dipeptides and 25 β-lactam antibiotics were taken from our earlier studies.[14,17−19] The K_i values are shown in Tables 1−4.

Tripeptides in LLL configuration display a high affinity to PEPT1 with K_i values in the range of 0.1−0.5 mM, whereas tripeptides in DLL, LDL, and LLD configurations display low affinities with K_i values >5 mM, with a notable exception of D-Met-Met-Met (**119**, K_i = 0.52 mM). This stereochemical preference is commensurate with the characteristics of dipeptides.[6,20] In agreement with dipeptides, tripeptides with N-terminal Pro and Gly residues possess lower affinity to PEPT1, for example, Pro-Phe-Lys (**120**, K_i = 2.0 mM), Gly-His-Lys (**52**, K_i = 4.1 mM), and Pro-Gly-Gly (**67**, K_i = 16 mM).

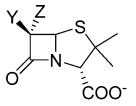
Conformational Analysis and Molecular Alignment. For the development of a pharmacophore model and CoMSIA studies, a training set consisting of 98 compounds (23 β-lactam antibiotics, 47 dipeptides and dipeptide derivatives, and 28 tripeptides) covering a broad range of affinity constants to PEPT1 (0.01−100 mM) was created. Most of these peptides and peptidomimetics are rather flexible molecules. This flexibility was taken into account in a systematic search for the relevant conformers of tripeptides and β-lactam antibiotics. On the basis of our previous pharmacophore model for dipeptides, the proposed bioactive conformation of dipeptides was used as an initial template for tripeptides.[14] For the development of a pharmacophore model for tripeptides, the rigidity of Val-Pro-Pro (**11**, K_i = 0.10 mM) proved to be useful. The systematic search of **11** yielded only three conformers in the trans arrangement. When these structures were aligned onto the peptide backbone atoms ($C_{\alpha1}-C-N-C_{\alpha2}$) of our dipeptide template, the conformation with the lowest

Table 1. Biological Data of Di- and Tripeptides of the Training Set

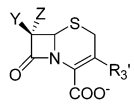
no.	amino acid 1	amino acid 2 (C-terminal residues)	amino acid 3	$K_{i,actual}$ (mM)	ref	no.	amino acid 1	amino acid 2 (C-terminal residues)	amino acid 3	$K_{i,actual}$ (mM)	ref
1	Bpa[a]	Ala		0.01	14	**39**	Ala	Orn		0.97	34
2	Bip[b]	Ala		0.02	14	**40**	Gly	Gly		1.0	14
3	Val	Phe		0.05	14	**41**	Gly	His		1.0	14
4	Ala	Pip[c]		0.06	19	**42**	Thr	Lys	Tyr	1.1	
5	Ala	Nle		0.09	14	**43**	Pro	Pro		1.2	19
6	Tyr	Ala		0.09	14	**44**	Lys	Glu		1.3	
7	Ser(OBzl)	Ala		0.09	18	**45**	D-Tyr(OBzl)	Ala		1.4	14
8	Tyr(OBzl)	Ala		0.09	18	**46**	Ala	Chx[d]		1.5	14
9	Leu	Pro	Arg	0.10		**47**	D-Ala	Ala		2.1	34
10	Met	Met	Met	0.10		**48**	Pro	Arg		2.5	19
11	Val	Pro	Pro	0.10		**49**	Ala	β-Ala		2.7	14
12	Val	Tyr		0.10	14	**50**	Ala	anilide		2.9	23
13	Ala	Phe	Pro	0.11		**51**	Ala	5-Fla[e]		3.1	23
14	Leu	Ala	Arg	0.11		**52**	Gly	His	Lys	4.1	
15	Leu	Thr	Leu	0.11		**53**	Ala	D-Ala		4.2	34
16	Phe	Ala		0.11	14	**54**	Val	Pro	D-Pro	4.3	
17	Phe	Phe		0.11	14	**55**	β-Ala	Ala		4.8	14
18	Ala	Ser		0.14	18	**56**	D-Ala	Pro		5.0	34
19	Tyr	Phe		0.14	14	**57**	Asp	Ala-NH_2		5.4	14
20	Val	Ala	Leu	0.14		**58**	Lys	Ala-NH_2		6.4	
21	Ser	Pro	Ile	0.17		**59**	D-Ala	Lys		7.0	34
22	Trp	Trp	Trp	0.17		**60**	D-Phe	Ala		7.0	14
23	Ala	Ala	Ala	0.18		**61**	β-Ala	Gly		7.3	14
24	Leu	Pro		0.18	19	**62**	D-Ala	Ala	Ala	7.9	
25	Cys	Gly		0.20	14	**63**	Ala	Ala	D-Ala	8.3	
26	Glu	Phe	Tyr	0.20		**64**	Pro	Asp		9.8	19
27	Ile	Val	Tyr	0.20		**65**	D-Tyr	Val	Gly	14	
28	Trp	Gly	Tyr	0.24		**66**	Tyr	D-Ala	Gly	14	
29	Glu	Ala		0.25	14	**67**	Pro	Gly	Gly	16	
30	Ile	Pro	Pro	0.28		**68**	Ala	D-Ala	Ala	17	
31	Gly	Pro		0.30	19	**69**	Ala	D-Phe	Ala	19	
32	Leu	Arg	Pro	0.30		**70**	Pro	Glu		20	19
33	Lys	Asp		0.33		**71**	D-Leu	Gly	Gly	25	
34	Tyr	Gly	Gly	0.35		**72**	Ala	D-Lys		>30 (∼49)[f]	34
35	Leu	Gly	Gly	0.39		**73**	D-Ala	D-Pro		>30 (∼65)[f]	34
36	Asp	Asp		0.41	14	**74**	D-Ala	D-Lys		>30 (∼72)[f]	34
37	Glu	Lys		0.51		**75**	D-Ala	D-Ala		>30 (∼100)[f]	34
38	Asp	Lys		0.86							

[a] Biphenylalanine. [b] Benzylphenylalanine. [c] Pipecolinic acid. [d] Cyclohexylamide. [e] 5-Fluoroanilide. [f] K_i values extrapolated beyond the measurement range.

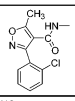
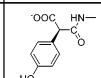
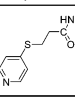
Table 2. Structures and Biological Data of β-Lactam Antibiotics of the Training Set. The Skeleton of the Particular Molecule Is Labeled with P for Penicillins and C for Cephalosporins



P



C

no.	compound	skeleton	Y	Z	R_3'	$K_{i,actual}$ [mM]	ref.
	Type I						
76	Ceftibuten	C		—H	—H	0.34	17
77	Cefixime	C		—H		12	17
78	Cefodizime	C		—H		22	17
79	Ceftriaxone	C		—H		> 30 (~40)[a]	17
80	Cefpirome	C		—H		> 30 (~45)[a]	17
81	Cefotaxime	C		—H		> 30 (~50)[a]	17
82	Cefepime	C		—H		> 30 (~70)[a]	17
	Type II						
83	Cyclacillin	P		—H		0.50	17
84	Cloxacillin	P		—H		2.5	35
85	Cefadroxil	C		—H	—H	7.2	17
86	Dicloxacillin	P		—H		7.2	35
	Type II (continued)						
87	Cefamandole	C		—H		8.1	17
88	Cephradine	C		—H	—CH_3	9.8	17
89	Cefoxitin[c]	C				10	
90	Cefaclor	C		—H	—Cl	> 10 (~11)[a]	17
91	Moxalactam[b]	C				12	35
92	Oxacillin	P		—H		12	35
93	Metampicillin	P		—H		13	35
94	Ampicillin	P		—H		15	17
95	Cephalothin	C		—H		> 10 (~14)[a]	17
96	Cefapirin	C		—H		> 10 (~14)[a]	17
97	Penicillin V	P		—H		21	17
98	Cefuroxime	C		—H		26	17

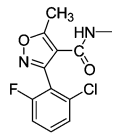
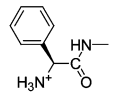
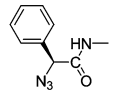
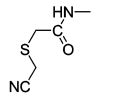
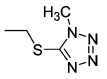
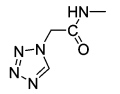
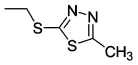
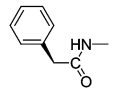
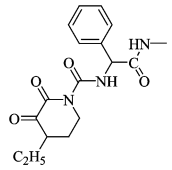
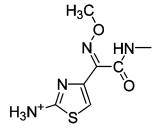
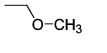
[a] K_i values extrapolated beyond measurement range because of limited solubility of compounds or low inhibition. [b] Moxalactam contains an oxygen atom instead of the sulfur atom in the penam ring. [c] Cefoxitin was modeled in 7*S* configuration.

root-mean-square (rms) deviation was selected. Subsequently, this structure was used as template for all of the tripeptides (Figure 2). It is characterized by torsion angles of $\psi_1 = 147°$, $\omega_1 = 180°$, $\varphi_2 = 286°$, $\psi_2 = 172°$, $\omega_2 = 180°$, and $\varphi_3 = 309°$. The distance between the N- and C-terminus averages 7.4 Å, which corresponds well to the distance of 7.8 Å suggested by Li and co-workers based on the conformational analysis of cephalexin (**100**) and Gly-Gly-Gly.[21]

When the structural alignment of di- and tripeptides is compared, the N-terminal amino group in L-configured amino acids is situated in the same region ($\psi_1 = 145°-165°$). One of the carboxylic oxygen atoms of dipeptides is superimposable to the carbonyl oxygen atom of the second peptide bond of tripeptides. Side chains of the first and second amino acid are oriented in the same direction.

Since β-lactam antibiotics sterically resemble tripeptides, they were initially aligned with their first amide bond along the first peptide bond of the tripeptide template disregarding the orientation of the amino group and other functional groups in R_1' position (Figure 2a). Bulky residues in the X position extend into the same area where the first side chain of the template is situated. The penam and cephem rings, respectively, are oriented with the C_5-C_6 bond of penicillins and the C_6-C_7 bond of cephalosporins along the backbone of the template Val-Pro-Pro. In this alignment, the carboxylic group of β-lactam antibiotics does not overlap with the C-terminal carboxylic group of the template. The result-

Table 3. Structures and Biological Data of β-Lactam Antibiotics of the Test Set

no.	compound	skeleton	Y	Z	R_3'	$K_{i,actual}$ [mM]	ref
99	Flucloxacillin	P		—H		3.2	
100	Cephalexin	C		—H	—CH_3	14[a]	17
101	Acidocillin	C		—H		15	
102	Cefmetazole[b]	C		—O—CH_3		28	17
103	Cefazoline	C		—H		> 30 (~31)	
104	Benzylpenicillin	P		—H		> 30 (~40)[a]	17
105	Piperacillin	P		—H		> 30 (~61)[a]	
106	Cefpodoxime	C		—H		> 30 (~110)[a]	

[a] K_i values extrapolated beyond measurement range because of of low inhibition. [b] Cefmetazole was modeled in 7*S* configuration.

Table 4. Biological Data of Di- and Tripeptides of the Test Set

no.	amino acid 1	amino acid 2	amino acid 3	$K_{i,actual}$ (mM)	ref
107	Asp(OBzl)	Ala		0.09	14
108	Ile	Tyr		0.12	14
109	Ala	Phe	Leu	0.10	
110	Ala	Ala		0.14	14
111	Ser	Ala		0.14	19
112	Phe	Leu	Leu	0.14	14
113	Ala	Pro	Leu	0.15	14
114	Trp	Ala		0.16	14
115	Ala	Lys		0.21	18
116	Tyr	Pro	Ile	0.25	
117	Asp	Ala		0.32	18
118	Lys	Ala		0.34	18
119	D-Met	Met	Met	0.52	
120	Pro	Phe	Lys	2.0	
121	Pro	Asp		9.8	14
122	Ala	D-Pro		15	19

ing model is characterized by torsion angles of $\psi_1 = 70°$, $\omega_1 = 180°$, and $\varphi_2 = 30°$ for penicillins and cephalosporins. However, analysis of the property fields obtained from CoMSIA showed that the high affinity of ceftibuten was attributed to the butenyl carboxylic acid side chain of ceftibuten (**76**, K_i = 0.34 mM). Since no other functional group of the training set was positioned in this region, the isopleths disappeared in this area when **76** was taken out. Moreover, these contour plots were in contrast to our previous results.[14] We hypothesized that the cephalosporins containing an aminothiazole ring such as ceftibuten (in the following referred to as type I β-lactam antibiotics) should be aligned differently. Thus, we superimposed the amino group onto the N-terminal amino group of the template Val-Pro-Pro and fitted the first amide bond onto the second peptide bond (Figure 2b). In this arrangement, the carbonyl group of the β-lactam ring is positioned in the same region as the C-terminal carboxylic group of the template so that these groups share the same acceptor functionality. The average torsion angles of this presumably biologically active conformation are $\psi_1 = 330°$, $\omega_1 = 176°$, and $\varphi_2 = 266°$. The average N···C=O_{lactam}

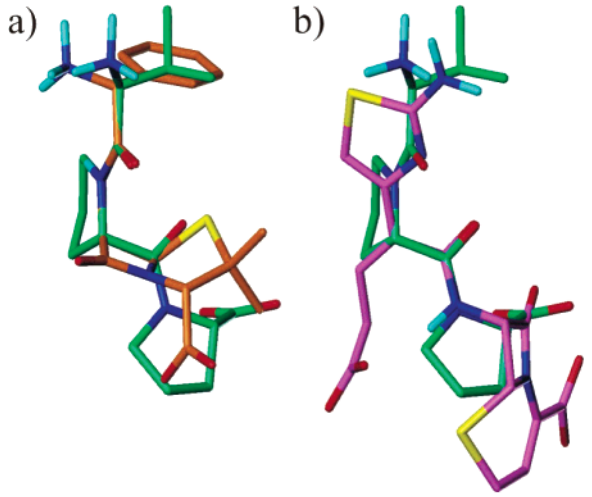


Figure 2. Structural alignment of (a) Val-Pro-Pro (**11**, green-blue) and ampicillin (**94**, orange) and (b) Val-Pro-Pro (**11**, green-blue) and ceftibuten (**76**, magenta). All of the nonpolar hydrogen atoms were omitted for clarity.

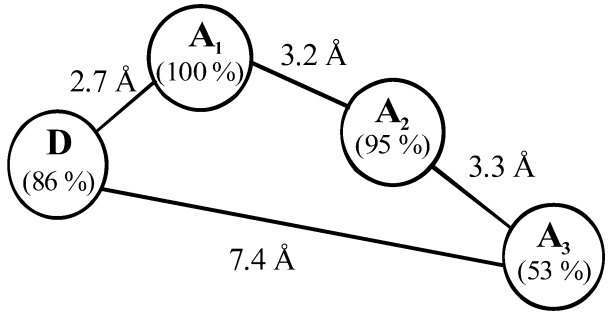


Figure 3. Pharmacophore model resulting from the training set: D = donor and A = acceptor. The distances between the features are average values. The percentage values denote the contributions of the corresponding functions in the compounds of the training set.

distance is again 7.4 Å. All of the other β-lactam antibiotics containing no aminothiazole ring, but bearing either a free amino or a hydroxyl group or completely different residues in the R_1' position, respectively, are referred to as type II β-lactam antibiotics (see Table 2). They were aligned as described above.

Figure 3 shows a sketch of the resulting pharmacophore model based on the training set. The donor site D as well as the acceptor sites A_1 and A_2 agree with our previous results, whereas the additional acceptor site A_3 is formed by the carboxylic group of tripeptides and the β-lactam carbonyl group of type I β-lactam antibiotics.[14] It is interesting to note that 86% of the compounds of the training set contain the donor group D. All of the molecules are alike in the acceptor site A_1. This position is occupied most often by the carbonyl function of the first peptide or amide bond but, alternatively, also by the thiazole nitrogen atom of type I β-lactam antibiotics. The acceptor site A_2, present in 95% of the compounds, accommodates the carboxylic groups of dipeptides as well as the second carbonyl group of tripeptides and the carbonyl group of the first amide bond of type I β-lactam antibiotics. About 53% of the compounds of the training set contain an acceptor group A_3.

CoMSIA Studies. The CoMSIA results summarized in Table 5 indicate the statistical significance of the

Table 5. Summary of CoMSIA Results

	CoMSIA results		CoMSIA results
q^2	0.828	F	274
S_{press}	0.420	predictive r^2	0.55
r^2	0.937	components	5
S	0.254	grid spacing (Å)	1
fraction	%	fraction	%
steric	11	hydrogen bond donor	29
electrostatic	22	hydrogen bond acceptor	15
hydrophobic	25		

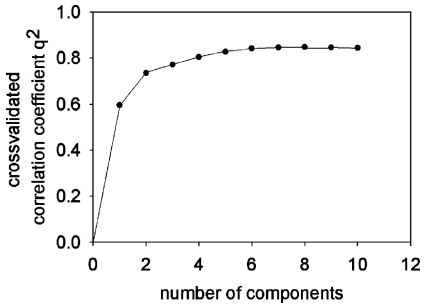


Figure 4. Plot of the cross-validated correlation coefficient q^2 vs the number of components.

model. This 3D-QSAR model is dominated by electrostatic properties when hydrogen bond donor and acceptor properties are considered electrostatic in nature, which agrees well with the pharmacophore model illustrated in Figure 3. Steric and hydrophobic properties contribute to a smaller extent (36%) to the model.

The partial least squares (PLS) analysis using the "leave-one-out" method yielded an optimal number of eight components. However, there is only a small difference in the statistical significance between 5 and 10 components (Figure 4). Within this range, the reduction of q^2 was less than 5%. Therefore, we selected an optimal number of five components, which simplifies the 3D-QSAR model.

The CoMSIA model provided a linear regression plot shown in Figure 5a. Variations between the measured and predicted affinities are less than 1 logarithmic unit, which indicates a sufficient correlation.[22] To verify the predictive power of the model, a test set containing 8 β-lactam antibiotics, 10 dipeptides, and 6 tripeptides was established, which are listed in Tables 3 and 4. The compounds of the test set were randomly selected covering a broad range of affinities. As depicted in Figure 5b, the predictions agreed well with the measured values and varied less than 0.8 logarithmic units, showing that our model is the first suitable to predict the affinities of di- and tripeptides as well as β-lactam antibiotics to PEPT1.

Graphical Interpretation of the Results. CoMSIA generates steric, electrostatic, hydrophobic, and hydrogen bond donor and acceptor fields enclosing specific regions of ligands with particular physicochemical properties, which are favored or disfavored for a high affinity. The structure−affinity information in the region of dipeptides is similar compared to our previous model. The analysis of the additional regions created by the tripeptides and β-lactam antibiotics expands our knowledge about binding specificities of PEPT1. Figures 6−9 illustrate the contour maps of property fields, which

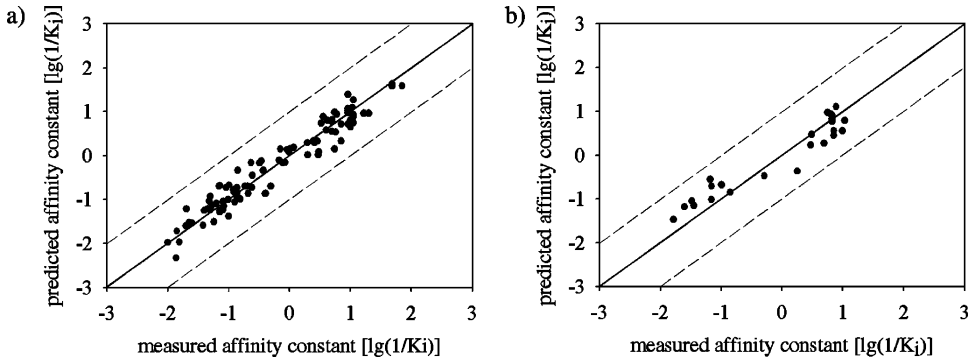


Figure 5. Predicted vs measured affinity constants (a) for the training set and (b) for the test set. The predicted values were obtained by PLS analysis using CoMSIA with a grid spacing of 1 Å. K_i values are expressed in mM. Dotted lines denote deviations of 1 logarithmic unit.

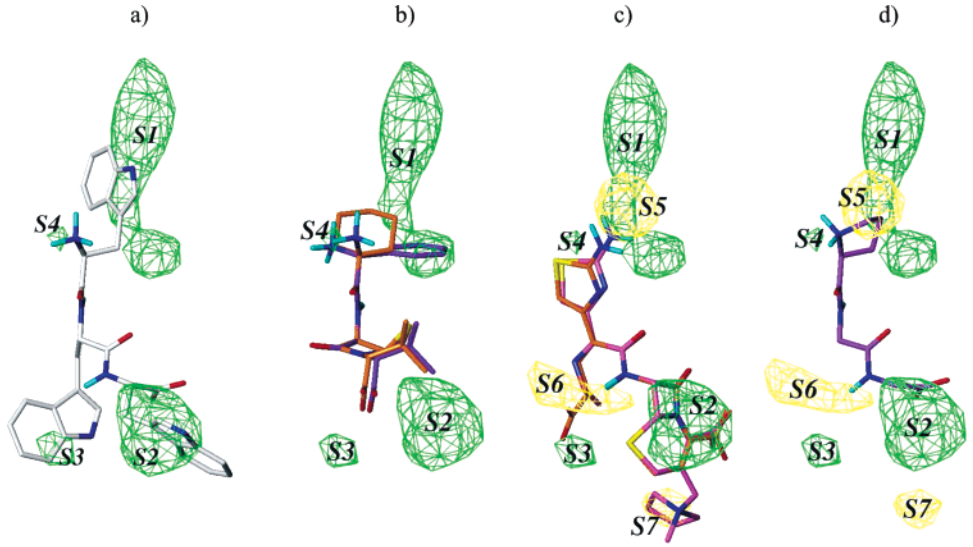


Figure 6. CoMSIA stdev*coeff contour plots for steric properties: (a) Trp-Trp-Trp (**22**), (b) cyclacillin (**83**, orange) and ampicillin (**94**, violet), (c) ceftibuten (**76**, orange) and cefepime (**82**, magenta), and (d) Pro-Gly-Gly (**67**). Sterically favorable regions are colored in green and disfavored regions are in yellow. The green isopleths **S1**−**S3** visualize three cavities in the substrate binding site occupied by bulky moieties of high affinity substrates. Yellow isopleths should be avoided. In (a) and (b), yellow isopleths are not displayed for clarity.

include exemplarily some substrates of PEPT1, for explaining affinity differences, especially for β-lactam antibiotics.

Contour maps for steric properties are displayed in Figure 6. Green contours are attributed to sterically favored regions occupied by bulky groups of the substrates. Areas indicated by yellow contours should be left unoccupied. Therefore, the presence of molecule parts in these areas is accompanied by a partial loss in affinity.

Three green regions (**S1**−**S3**) provide a first image of the cavity, which can be occupied by the substrates. These regions are mainly filled with side chains of high-affinity peptide substrates. The largest tripeptide Trp-Trp-Trp (**22**) is given as an example in Figure 6a. Molecule parts of β-lactam antibiotics are also placed there. The S1 region is occupied by all of the β-lactam antibiotics of type II carrying bulky residues in the R_1' position (Figure 6b). The large region **S2** is filled with bulky residues in R_3' such as tetrazole rings or ester groups of β-lactam antibiotics of type II (**87**, **89**, **91**, **95**, **96**, and **98**). Furthermore, because of the slightly different alignment of type I β-lactam antibiotics the cephem moiety is oriented into the same area (Figure 6c). On the other hand, these β-lactam antibiotics, except ceftibuten (**76**), also bearing bulky residues in R_3' position produce a sterically disfavored region **S7** next to **S2**, which indicates that the spatial extension of such compounds might lead to a loss of affinity. The small green area **S4** represents the N-terminal amino group of peptides and cyclacillin (**83**). Cyclacillin, a high-affinity PEPT1 substrate (**83**, $K_i = 0.50$ mM), is the only β-lactam antibiotic in our data set with an achiral C_α atom. The amino group is situated in the same favored region like the amino group of N-terminal L-amino acids (Figure 6b). The yellow isopleth **S5** results from the pyrrolidine ring of N-terminal proline containing di- and tripeptides, which display in general a lower affinity to PEPT1 (Figure 6d) or none at all.[19] The yellow isopleth **S6** reveals that parts of the aromatic ring of alanyl aryl amides extend into sterically disfavored regions, which is in agreement with our previous studies.[14,23]

Contour plots for electrostatic field properties are closely associated with hydrophilic (Figure 7) and hydrogen bond properties (Figures 8 and 9). Electrostatic fields identify regions where positively and negatively charged moieties are most favored for high affinity. Positively charged groups point into the blue

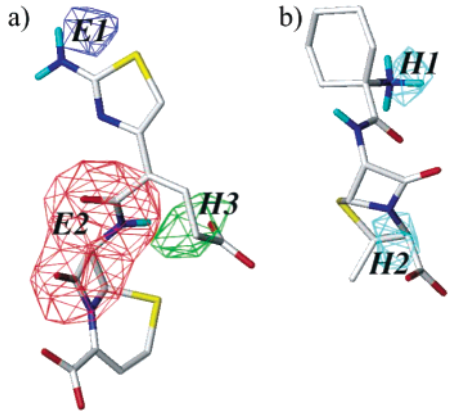


Figure 7. CoMSIA stdev*coeff contour plots for electrostatic and hydrophilic field properties: (a) ceftibuten (**76**) and (b) cyclacillin (**83**). The blue region **E1** favors a positively charged area, whereas the red region **E2** favors a negatively charged area. The green region **H3** indicates an area where lipophilic groups increase affinity. The cyan regions **H1** and **H2** denote favored hydrophilic areas.

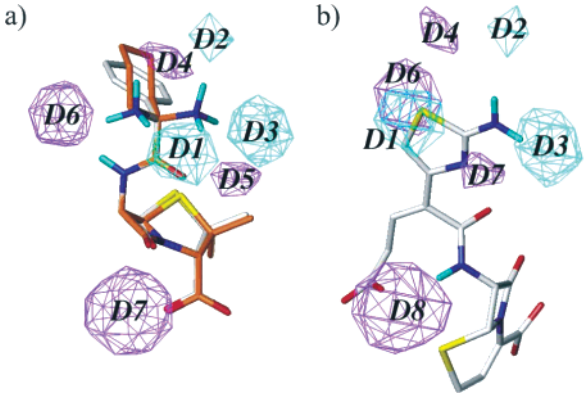


Figure 8. CoMSIA stdev*coeff contour plots for hydrogen bond donor fields: (a) cyclacillin (**83**, orange) and cefadroxil (**85**, gray) and (b) ceftibuten (**76**). The cyan isopleths indicate areas where hydrogen bond acceptor groups of the substrate binding site of PEPT1 are situated to form hydrogen bonds with the ligand. Violet areas characterize regions, which are unfavorable for hydrogen bonding.

contoured region (**E1**), which is triggered by amino groups of peptides, type I cephalosporins, and cyclacillin (**83**). Favored negatively charged molecule parts are favorably oriented into the red region (**E2**). The latter region results from carboxylic and carbonyl groups of di- and tripeptides and all of the type I β-lactam antibiotics. Both isopleths, **E1** and **E2** (Figure 7a), coincide with areas favorable for hydrophilic properties, **H1** and **H2**, indicated in cyan color (Figure 7b). However, the latter region is also occupied by hydrophobic molecule parts of the penam and cephem ring of type II β-lactam antibiotics, which is unfavorable in this area (Figure 7b). The green region **H3** represents an area where hydrophobicity increases affinity. This can be attributed to hydrophobic side chains in R_2 position of high-affinity peptide substrates. The butenyl chain of ceftibuten (**76**) is also placed in this area, whereas the carboxylic group sticks out of this contour plot (Figure 7a). In contrast to other type I β-lactam antibiotics, the more hydrophilic character of the oxime residue in this position leads to a reduced affinity. As expected, the electrostatic fields **E1** and **E2** correspond to those

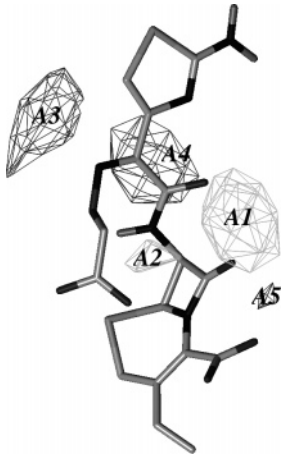


Figure 9. CoMSIA stdev*coeff contour plots for hydrogen bond acceptor fields. The light gray isopleths visualize areas favored by acceptors, while dark gray isopleths denote areas in which acceptor groups have no counterparts at the carrier site. Cefixime (**77**) has been chosen to illustrate this fact.

observed in our earlier study,[14] only rendering a larger field **E2** since it accommodates carboxylic groups of di- and tripeptides as well as carbonyl groups of type I β-lactam antibiotics.

The maps for hydrogen bond properties highlight those areas where residues of the protein could form hydrogen bonds with the substrate, which significantly influence binding affinity (Figures 8 and 9). The cyan isopleths **D1**−**D3** in Figure 8 encompass areas where acceptor groups of the substrate binding site of PEPT1 are expected to form hydrogen bonds with donor substituents of the substrate. These areas originate from amino groups of peptide substrates and cyclacillin (**83**) (Figure 8a). Interestingly, under physiological conditions type I β-lactam antibiotics are also able to form appropriate hydrogen bonds to PEPT1 (Figure 8b).

Because of the different orientation of the amino group of cefadroxil, cephradine, cefaclor, and ampicillin (**85**, **88**, **90**, **94**) the formation of hydrogen bonds to the protein site is restricted. Only one hydrogen atom of the protonated amino group is oriented in a favored area (**D1**), which agrees with the low affinities between 7 and 14 mM (Figure 8a, Table 2).

Cefamandole (**87**) contains a hydroxyl group in the X position and shows unexpectedly an affinity of 8.1 mM. The analysis of the molecular fields shows that this hydroxyl group points into the region **D1** and should be able to form hydrogen bonds. Tests revealed, however, that HO−CH(CH_3)−CO−Ala has no affinity to PEPT1 ($K_i \sim 46$ mM).[24] Thus, we conclude that the recognition of cefamandole results from other interactions with PEPT1 such as favorable steric properties. The absence of a donor group in X position as in benzylpenicillin (**104**, $K_i \sim 40$ mM) results in a complete loss of affinity. When a contribution of hydrogen bond donor properties of 28% is taken into account, this result underlines the importance of a donor group placed in the right position. The violet isopleths **D4**−**D7** mark areas where the presence of hydrogen bond donors decreases affinity (Figure 8). The isocontours **D4** and **D6** are generated by the protonated amino group of N-terminal D-configured amino acids. **D5** is produced by the imino group of N-terminal proline-containing

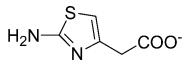


Figure 10. Structure of 2-aminothiazole-4-acetic acid.

peptides. The violet contour plot **D7** results from the nitrogen atom of the second peptide bond of tripeptides, which may indicate why tripeptides and also dipeptide amides (**57** and **58**) show a slightly reduced affinity compared to the higher affinity of dipeptides.

The light gray isopleths **A1** and **A2** in Figure 9 enclose areas favored by hydrogen bond acceptors. They are occupied by carboxylic groups of high affinity substrates. The dark gray isopleths **A3**−**A5** denote areas in which acceptor groups have no counterparts at the protein side. This region results from carboxylic groups of DD or LD dipeptides and LDL or LLD tripeptides as well as from the oxime residue of type I β-lactam antibiotics. An additional region should be expected around the first carbonyl group of peptide substrates or type II β-lactam antibiotics. Moreover, type I β-lactam antibiotics carrying an aminothiazole ring should also be able to form a hydrogen bond in the same region, because the nitrogen atom of the thiazole ring might act as a weak acceptor.[25] However, since all of the compounds of the training set possess hydrogen bond acceptor properties in this position, there is no significant difference affecting the affinity. Nevertheless, the need of an acceptor group in this area is undisputable.

To validate our pharmacophore and CoMSIA models with regard to ceftibuten, we asked whether a small compound containing an aminothiazole residue, which is a component of many drugs, would bind to PEPT1. The amino group features donor properties (D) whereas the ring nitrogen atom may have acceptor functionality (A_1). We selected 2-aminothiazole-4-acetic acid (Figure 10) whose carboxylic group is properly positioned in the region A_2 of our pharmacophore model.

At Caco-2 cells, a K_i value of 4.9 ± 0.6 mM was measured. This indicates that this dipeptide mimetic, which contains neither an amide bond nor a protonated amino group, is recognized by PEPT1. The affinity constant predicted by CoMSIA was 2.0 mM, which, considering the very broad range of affinity constants of substrates at PEPT1 from 2 μM to 15 mM,[6] proved the predictive power of the model. The interaction of this small compound with PEPT1 reveals an exciting aspect concerning its utilization as part of a potential prodrug.

The 2-aminothiazole-4-acetic acid resembles ceftibuten (**76**) in the N-terminal part but lacks the butenyl side chain and the β-lactam skeleton. Similar compounds were described by Meredith and collaborators, who have shown that 4-aminomethylbenzoic acid and 4-aminophenylacetic acid are recognized by PEPT1.[26] Interestingly, 4-aminophenylacetic acid is a bioisostere to 2-aminothiazole-4-acetic acid.

Conclusions

A simple and robust 3D-QSAR model to explain binding affinity constants of di- and tripeptides as well as β-lactam antibiotics at PEPT1 was developed. It can now be applied for the prediction of affinity constants for new substrates, for example, prodrugs. This model expands our previous model to the entire range of compounds known to be substrates of PEPT1. We found that the amino group of β-lactam antibiotics containing an aminothiazole ring (type I) has donor properties just as the N-terminal amino group of high affinity peptides and β-lactam antibiotics, which might explain the surprisingly high affinity of ceftibuten (**76**). The alignment of these structures is distinct from that of type II β-lactam antibiotics. The present CoMSIA study highlights the regions which are significant for the binding of tripeptides and β-lactam antibiotics. A spatial impression of the binding site of PEPT1 is given by steric field properties, which indicate that the size of the side chain R_3' of type I β-lactam antibiotics is crucial for the binding affinity. Whereas only a hydrogen atom or a very short side chain in this position are tolerated by PEPT1, type II β-lactam antibiotics can bear bulky residues in R_3'.

The CoMSIA results show that the presence of a hydrogen bond donor group in X position regardless of its configuration is essential for high affinity. Donor groups in the *R* configuration are placed differently, which results in medium affinities such as those of most type II β-lactam antibiotics. This reduction of the affinity can be partially compensated by bulky residues in the R_1' position such as aromatic rings.

The dipeptide mimetic 2-aminothiazole-4-acetic acid containing neither an amide bond nor a protonated amino group was found to be recognized by PEPT1. Such small substrates or inhibitors of PEPT1, which are not amenable to hydrolysis by proteases, may be used for the attachment of drugs.

Experimental Section

Biological Data. Materials. The human colon carcinoma cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). [^{14}C]Gly-Sar (specific radioactivity, 53 mCi mmol^{-1}) was supplied from Amersham Biosciences U.K. (Limited Little Chalfont, U.K.). Flucloxacillin was a gift from GlaxoSmithKline (SmithKline Beecham Pharmaceuticals, Worthing, U.K.), acidocillin was generously given by InfectoPharm (Heppenheim, Germany), and cefpodoxime was obtained from Sanofi-Aventis (Frankfurt am Main). Cefazoline and cefoxitin were purchased from Sigma-Aldrich Chemie GmbH (Germany). The 2-aminothiazole-4-acetic acid was from Acros Organics (Germany). The peptides AAA, D-AAA, AA-D-A, A-D-AA, AFP, A-D-FA, APL, DK, EFY, EK, GHK, KA-NH_2, KD, KE, LGG, D-LGG, LRP, MMM, D-MMM, PFK, PGG, TKY, VAL, WGY, WWW, Y-D-AG, YGG, and D-YVG were purchased from Bachem (Germany). AFL, FLL, IPP, IVY, LAR, LPR, LTL, SPI, VPP, VP-D-P, and YPI were synthesized in our laboratory according to standard procedures in peptide chemistry.

General Method for Peptide Synthesis. Peptide synthesis was performed on a Symphony/Multiplex peptides synthesizer (Protein Technologies Inc., USA). Peptides were synthesized according to standard solid-phase (fluorenylmethoxy)carbonyl (Fmoc) chemistry with 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as the coupling reagent using 5 equiv of HBTU/HOBt/Fmoc-Xaa-OH/NMM (1:1:1:2). The coupling time was 1 h. Fmoc deprotonation was performed with 20% piperidine in *N*,*N*-dimethylformamide (DMF) and cleavage/side chain deprotection with trifluoroacetic acid (TFA)/H_2O/TIS (95:2.5:2.5 (v/v/v)).

The resins used were Fmoc-Amino-Acid-Wang resins or, in the case of tripeptides with proline in the sequence, 2-chlorotrityl-resin loaded with the C-terminal amino acid (Calbiochem-Novabiochem AG). Fmoc-protected amino acids were

obtained from Calbiochem-Novabiochem AG. Side chain protecting groups were Boc (Lys), Pbf (Arg), and Bu^t (Thr, Ser and Tyr).

Purification and Analysis of Peptides. The crude peptides were purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) (Merck-Hitachi consisting of a pump L-6250 and a L-4000 UV detector module) applying gradient elution on a Merck Hibar column (RT 250-25, LiChrosorb RP-8, 7 μm). On the basis of the degree of separation and polarity of the compounds, variable concentrations (20−80%) of acetonitrile containing 0.1% TFA in water were used over 40 min at a flow rate of 6 mL/min. The absorbance was monitored at 220 nm.

The purity of the final peptides was verified by analytical HPLC and capillary electrophoresis (CE). The analytical HPLC (LaChrom, Merck-Hitachi comprising of an autosampler L-7200 and a diode array detector L-7450) was performed on a Merck LiChroCART 125-4, 5 μm, LiChrospher 100 RP-18 column using a stepwise gradient starting with 100% of solvent A (acetonitrile/water (5:95%), 0.1% TFA), reaching 40% of solvent B (acetonitrile/water (80:20%), 0.1% TFA) at 10 min and ending with 100% of solvent B at 15 min at a flow rate of 0.8 mL/min. Elution of the peptides was measured at 220 nm. CE data (BioFocus 3000, Bio-Rad Laboratories) were achieved using an uncoated capillary (50 cm × 50 μm) and 0.1 M phosphate buffer at pH 2.5. Electrophoretic separation was performed by applying a positive voltage at 20 kV. The absorbance of peptides was monitored at 200 nm. Final purity of all of the peptides by both analytical systems was >98%. Molecular weights of the compounds were determined by direct injection into an Esquire-LC electrospray ionization mass spectrometer. The analytical data for each synthesized compound are presented in the Supporting Information.

Transport Studies and HPLC. Affinity data of various substances were measured in Caco-2 cells. The procedure of cell culture has been described in detail previously.[23,27] The uptake of [^{14}C]Gly-Sar was measured at room temperature on the 6th day after the cells reached confluence.[2,18,19,23,28,29] The uptake buffer (1 mL) contained 25 mM Tris/Mes (pH 6.0), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.0 mM glucose, 10 μM [^{14}C]Gly-Sar, and increasing concentrations of unlabeled inhibitors. After incubation for 10 min, the cells were quickly washed, solubilized, and prepared for liquid scintillation spectrometry. All of the data are given as the mean of four to six independent experiments. Inhibition constants (K_i) were calculated from IC_{50} values as described previously.[23,29] To determine the stability of tripeptides in the uptake buffer, recovery rates of representative tripeptides during uptake were measured by HPLC. Similar to the transport studies, cells were washed with buffer pH 6.0 and incubated for 0 (control) or 10 min with the particular tripeptide (1 mM). HPLC analysis was performed using a Merck-Hitachi HPLC-system equipped with a diode array detector (L 7455). A Supersphere 100 RP 18 column (endcapped, 125 × 2) from Merck (Darmstadt, Germany) was used as the stationary phase. HPLC grade water and acetonitrile from Merck (Darmstadt, Germany) were used as the solvents. The eluent contained 2−40% acetonitrile in water adjusted with TFA to pH 2.0. The retention times varied from 1.30 to 6.45 min. Twenty microliters ($n = 2$) of the 1:10 diluted probes was injected and detected at 207 nm. The flow rate was 0.2 mL/min. The compounds were recovered from the uptake medium intact to 86−98%.

Computational Methods. Data Set. The training set for CoMSIA calculations contained 98 compounds comprising 35 dipeptides, 28 tripeptides, 12 dipeptide derivatives, and 23 β-lactam antibiotics. Twenty six compounds were already included in our earlier training set.[14] Di- and tripeptides carrying diverse side chains (e.g., positively and negatively charged, neutral, and aromatic side chains) covering an evenly spread range of binding affinities from 0.01 to 100 mM (Tables 1 and 2) were chosen for the training set. We also paid attention to a uniform distribution of side chains in all of the feasible positions of di- and tripeptides. All of the K_i values were measured in our laboratory using the same assay. In the CoMSIA studies, log $1/K_i$ values were used. The validation of the model was carried out using a test set of 24 randomly selected compounds, again tested in our laboratory for their affinity toward PEPT1.

Conformational Search and Molecular Alignment. All of the molecular modeling and 3D-QSAR studies were performed on a SGI Octane2 R12000 workstation using the SYBYL 6.9 and 7.0 programs.[30] Molecular structures were constructed as described previously.[14] N-Terminal amino groups were considered protonated except the amino group of thiazole containing β-lactam antibiotics. All of the C-terminal carboxylic groups were considered to be deprotonated. β-Lactam antibiotics were modeled in 2*S*, 5*R*, and 6*R* configurations for penicillins and 6*R* and 7*R* for cephalosporins (Figure 1) except cefoxitin and cefmetazole with 6*R* and 7*S* configurations.[31] The chiral carbon atom bearing X and R_1' residues was regarded to be *R* configured. Corresponding fragments of β-lactam antibiotics were taken from the Cambridge Structural Database.[32] R_1' residues of aminothiazole containing β-lactam antibiotics (butenyl carboxylic and oxime groups) were modeled as *Z* isomers.[33] The computational procedure for conformational searching and energy minimization was performed as described previously.[14]

Conformer databases of the β-lactam antibiotics were superimposed onto the backbone ($C_{\alpha1}-C-N-C_{\alpha2}$) of the tripeptide template using the match option in SYBYL. The aligned structures were stored in a molecule database for subsequent 3D-QSAR analyses.

3D-QSAR Analysis. The program CoMSIA was used to perform a 3D-QSAR. All of the CoMSIA calculations were carried out as described in detail previously.[14] Steric, electrostatic, hydrophobic, hydrogen-bond donor, and acceptor fields were calculated at grid lattice points using a sp^3 carbon atom as a probe atom with a radius of 1 Å. Charge, hydrophobicity, and hydrogen bond properties were set to +1. An attenuation factor of 0.3 was used. A lattice with a grid spacing of 1 Å and a sufficiently large margin was applied.

The SYBYL standard protocol was used to perform the PLS analysis by means of the SAMPLS method. The optimal number of components (highest q^2 and lowest standard error of prediction S_{press}) was obtained by the cross-validation method "leave-one-out". The column filtering was set to 2.5 kcal/mol considering about 10% of the variables in the PLS analyses.

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Appendix

Abbreviations used for amino acids follow the rules of the IUPAC−IUB Joint Commission of Biochemical Nomenclature (JCBN) in *Eur. J. Biochem.* 1984, *138*, 9−37.

Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; NMM, *N*-methylmorpholine; DMF, *N*,*N*-dimethylformamide; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Boc, *tert*-butyloxycarbonyl; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl; Bu^t, *tert*-butyl. Amino acid symbols denote the L-configuration unless otherwise indicated.

Supporting Information Available: Analytical data of synthesized tripeptides and RP-HPLC analyses of selected tripeptides (determination of stability of tripeptides in uptake

buffer). This material is available free of charge via the Internet at http://pubs.acs.org.

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