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Transport of Free and Peptide-Bound Glycated Amino Acids: Synthesis, Transepithelial Flux at Caco-2 Cell Monolayers, and Interaction with Apical Membrane Transport Proteins

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In glycation reactions, the side chains of protein-bound nucleophilic amino acids such as lysine and arginine are post-translationally modified to a variety of derivatives also known as Maillard reaction products (MRPs). Considerable amounts of MRPs are taken up in food. Here we have studied the interactions of free and dipeptide-bound MRPs with intestinal transport systems. Free and dipeptide-bound derivatives of N^6-(1-fructosyl)-lysine (FL), N^6-(carboxymethyl)lysine (CML), N^6-(1-carboxyethyl)-lysine (CEL), formyline, argpyrimidine, and methylglyoxal-derived hydroimidazolone 1 (MG-H1) were synthesized. The inhibition of L-[^{3}H]lysine and [^{14}C]glycylsarcosine uptakes was measured in Caco-2 cells which express the H^+/peptide transporter PEPT1 and lysine transport system(s). Glycated amino acids always displayed lower affinities than their unmodified analogues towards the L-[^{3}H]lysine transporter(s). In contrast, all glycated dipeptides except Ala-FL were medium- to high-affinity inhibitors of [^{14}C]Gly-Sar uptake. The transepithelial flux of the derivatives across Caco-2 cell monolayers was determined. Free amino acids and intact peptides derived from CML and CEL were translocated to very small extents. Application of peptide-bound MRPs, however, led to elevation (up to 80-fold) of the net flux and intracellular accumulation of glycated amino acids, which were hydrolyzed from the dipeptides inside the cells. We conclude 1) that free MRPs are not substrates for the intestinal lysine transporter(s), and 2) that dietary MRPs are absorbed into intestinal cells in the form of dipeptides, most likely by the peptide transporter PEPT1. After hydrolysis, hydrophobic glycated amino acids such as pyrraline, formyline, maltosine, and argpyrimidine undergo basolateral efflux, most likely by simple diffusion down their concentration gradients.

Introduction

Amino acids are targets for a variety of non-enzymatic chemical processes during food processing, such as oxidations or reactions with reducing sugars and their degradation products. This process is generally referred to as the "Maillard reaction" or "glycation".[1]

In the first stage of this process, lysine ε-amino groups react with reducing sugars such as glucose, lactose, and galactose to form the Amadori products N^6-(1-deoxy-1-fructosyl)lysine (fructoselysine, FL, **1**; Scheme 1), N^6-(1-deoxy-1-lactulosyl)lysine (lactuloselysine, **2**), and N^6-(1-deoxy-1-tagatosyl)lysine (tagatoselysine, **3**), respectively. Amadori products can degrade to highly reactive 1,2-dicarbonyl compounds, which again react with lysine ε-amino groups and arginine guanidino groups to form "advanced glycation end products" (AGEs). Lysine, for example, can be modified to N^6-(carboxymethyl)lysine (CML, **4**),[2] N^6-(1-carboxyethyl)lysine (CEL, **5**),[3] 6-(2-formyl-1-pyrrolyl)norleucine (formyline, Fom, **6**),[4] and 6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)norleucine (pyrraline, Pyrr, **7**).[5,6] 6-(3-Hydroxy-2-methyl-4-oxo-4(1*H*)-1-pyridinyl)-L-norleucine (maltosine, Mal, **8**) is formed during disaccharide degradation through reactions between lysine residues and isomaltol.[7] Reactions between arginine residues and methylglyoxal lead to N^5-(5-methyl-4-oxo-5-hydro-2-imidazolonyl)-L-ornithine (methylglyoxal-derived hydroimidazolone 1, MG-H1, **9**)[8] and N^5-(5-hydroxy-4,6-dimethyl-2-pyrimidinyl)-L-ornithine (argpyrimidine, Apy, **10**) as a fluorescent minor product.[9] Pentosidine (**11**) is an amino acid containing a lysine and an arginine residue.[10] In the late stages of the Maillard reaction, AGEs, proteins, sugars, and their degradation products react with one another to form colored high-molecular-weight networks, which is why the Maillard reaction is also termed "nonenzymatic browning".

Humans are exposed to these substances from heat-treated foods such as bread, cereals, cookies, and dairy products. The Amadori products, mainly those derived from glucose and di-

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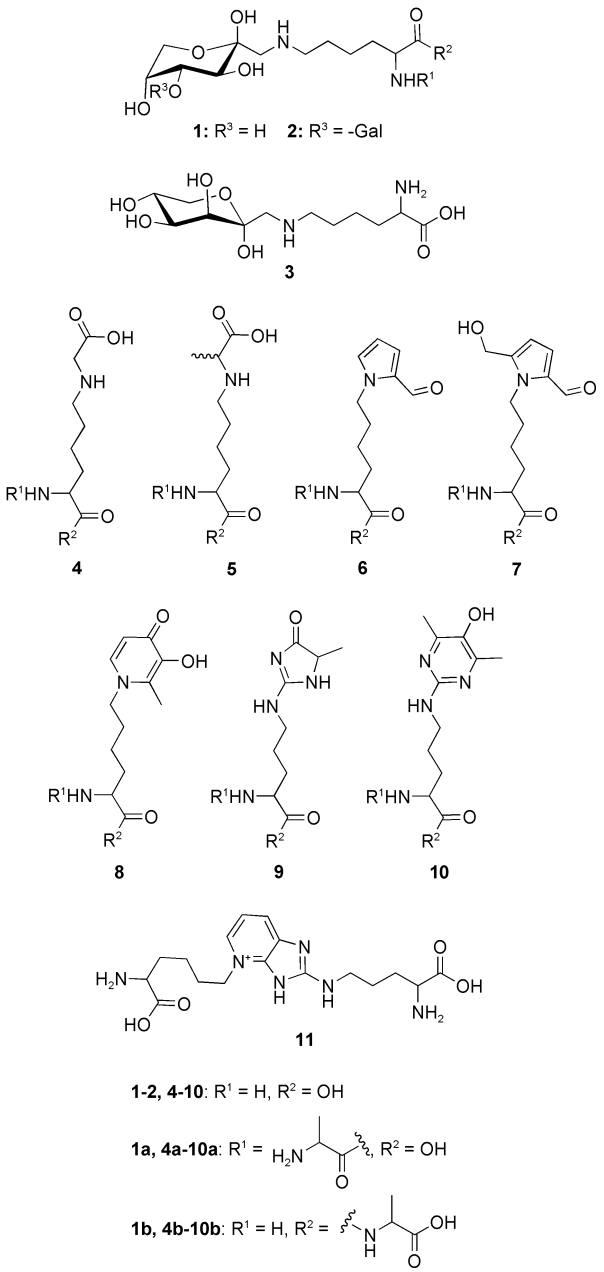


Scheme 1. Chemical structures of the investigated Maillard reaction products fructoselysine (**1**), lactuloselysine (**2**), tagatoselysine (**3**), CML (**4**), CEL (**5**), formyline (**6**), pyrraline (**7**), maltosine (**8**), MG-H1 (**9**), argpyrimidine (**10**), and pentosidine (**11**).

and oligosaccharides, are quantitatively the dominant Maillard reaction products (MRPs) in food. Amounts of between 0.5 and 1.2 g are ingested daily, together with 25 to 75 mg of AGEs (mainly **4** and **7**).[11] Recently, the daily CML intake from two standard diets used for clinical studies was determined to be 2.2–5.4 mg.[12] Because concentrations of other AGEs in most foods are not known, it is impossible to assess their daily intake.

The question of whether or not dietary AGEs play a causative role in the etiology of diseases such as diabetes and uremia is intensely debated.[13,14] Dietary AGEs are reported to enhance oxidative stress, to induce low-grade inflammation, and to promote atherosclerosis.[15] Many AGEs are strongly retained in end-stage renal disease.[16] Patients are encouraged to reduce their dietary AGE intakes in order to optimize their inflammatory status[17] and to prevent diseases. However, whether the accumulation of (dietary) AGEs in physiological fluids of patients detrimentally aggravates the diseases or whether it simply represents a side effect of uncontrolled sugar and carbonyl stress has not yet been shown.[18]

Food-borne AGEs can only affect physiological functions if they are absorbed from the diet. If it is assumed that MRPs, like other amino acids, are liberated from proteins, they arrive at the intestinal epithelial barrier bound in small peptides or, to a lesser extent, as the free amino acids.[19] Hypothetically, these products can cross the intestinal epithelium paracellularly by simple diffusion or transcellularly by diffusion, by endocytotic processes, or mediated by transport proteins. For the translocation of lysine and arginine through the apical membrane, enterocytes possess at least three different amino acid transporters: namely the systems $B^{0,+}$, $b^{0,+}$, and y^+.[20] Di- and tripeptides are transported by the proton-coupled peptide transporter 1 (PEPT1), which is driven by a transmembrane H^+ gradient and catalyzes the cotransport of its substrates with H^+ into intestinal and other cells (for a review, see ref. [21]). PEPT1 accepts many modified amino acids and dipeptides as substrates as long as the structural requirements for substrate binding and translocation are met.[22]

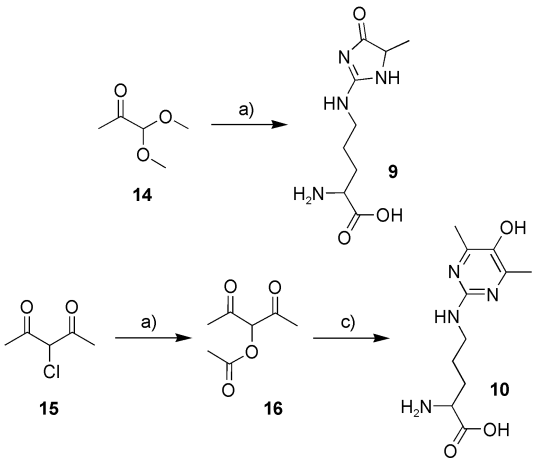
From the transit of immunoreactivity, Koschinsky et al.[13] estimated the absorption of AGEs in general to be about 10%, even though AGEs are a structurally quite heterogeneous group of substances (Scheme 1). We hypothesize that MRPs, due to their structural diversity, must interact quite differently with intestinal and renal amino acid and peptide transporters. Circumstantial evidence is provided by balance studies showing that less than 5% of ingested protein-bound fructoselysine (**1**) and pentosidine (**11**), but 50–100% of protein-bound pyrraline (**7**), appear in the urine.[23] In infants, the urinary excretion of CML (**4**) is dependent on the dietary intake.[24] The question that therefore arises is of how these compounds cross the intestinal barrier. Pyrraline (**7**) and maltosine (**8**) are actively transported by human PEPT1 in peptide form, but leave the cells as free **7** and **8** after peptidolysis.[25–27] Like fructoselysine (**1**) and CML (**4**),[28] free **7** and **8** are not transported through Caco-2 cells.

This study was directed towards characterizing the interaction of a broader range of glycated amino acids and dipeptides with membrane transport systems that might accept these types of compounds as substrates. Furthermore, the net transepithelial transport of these compounds across Caco-2 cell monolayers cultured on permeable filters in Transwell chambers was determined. Taken together, these techniques provide new information on the degree and mechanism of intestinal AGE absorption in vitro.

Results and Discussion

Synthesis and analysis of free and peptide-bound glycated amino acids

The Maillard reaction products investigated in this study are not commercially available, so sufficient amounts (30–200 mg) of ligands had to be synthesized. Dipeptides with the glycated amino acids both in the N-terminal and in the C-terminal positions were prepared, because the sequence of a peptide is crucial for its inhibitory and transport characteristics.[22,29] In this study, the synthesis of 12 glycated peptides is described for the first time. Moreover, the synthesis of MG-H1 (**9**, Scheme 2)



Scheme 2. Syntheses of N^5-(5-methyl-4-oxo-5-hydroimidazolon-2-yl)-L-ornithine (MG-H1, **9**) and N^5-(5-hydroxy-4,6-dimethylpyrimidin-2-yl)-L-ornithine (argpyrimidine, Apy, **10**). a) L-Arg, HCl (12 N), RT, 8 h. b) DMSO, NaOAc, RT, 3 h. c) L-Arg, HCl (12 N), RT, 20 h.

was significantly improved in terms of yield and ease of preparation by allowing unprotected arginine and the dimethylacetal **14** of methylglyoxal (which forms methylglyoxal in situ) to react in 12 M HCl. At arginine concentrations of about 10 mg mL^{-1}, compound **9** was obtained as the main product in one step in more than 40% molar yield. The synthesis could also be adapted for peptides incorporating **9**, but the incubation times were shortened in order to prevent peptide bond cleavage. We are currently investigating whether this can be applied as a general method for the synthesis of hydroimidazolones.

Another new strategy[30] was applied for the synthesis of argpyrimidine (**10**, Scheme 2). The 3-acetoxypentane-2,4-dione (**16**) precursor, however, was not synthesized from lead(IV) acetate,[30] but from 3-chloropentane-2,4-dione[31] (**15**), and was then incubated in 12 M HCl with unprotected arginine. Argpyrimidine (**10**) could be utilized as a "building block" for the synthesis of Ala-Apy and, after introduction of the Boc protecting group, for the synthesis of Apy-Ala. Taken together, the synthesis operations afforded all compounds in sufficient and partly unexpectedly high yields and purities as their formates, acetates, or hydrochlorides. Impurities, especially other amino acids or dipeptides, were absent, as verified by amino acid analysis (AAA).

The fluxes of UV-absorbing amino acids (**6**, **7**, **8**, **10**, **11**) and the corresponding dipeptides were measured by RP-HPLC after optimization of the gradients in terms of the best possible separation of the dipeptides from their constituent glycated amino acids. Other fluxes were measured by AAA with use of a lithium-based system with different gradient programs. The fluxes of **1a** and **1b**, however, additionally had to be measured with a sodium system, because fructoselysine (**1**) was not separated from the parent dipeptides in the lithium system.

Inhibition of L-[^{3}H]lysine uptake

The human intestinal cell line Caco-2 is a commonly used model for study of the intestinal transport of di- and tripeptides and amino acids.[32] Caco-2 cells express the PEPT1 and different amino acid transporters in their apical membranes. The three systems $B^{0,+}$, $b^{0,+}$, and y^+,[20] responsible for the transport of lysine and arginine, are also possible candidates for the transport of MRPs of lysine and arginine, and so the interaction of free and peptide-bound MRPs with the L-lysine transporter(s) was examined. Radiolabeled L-[^{3}H]lysine was used as the reference substrate for the lysine transporter(s). In competition assays, inhibition of the uptake of L-[^{3}H]lysine by each compound was first investigated with a concentration of 10 mM to determine the substrate specificity. If the compounds were able to inhibit the transport by at least 40%, increasing concentrations of them were applied for inhibition to allow calculation of their IC_{50} values. These values were converted into inhibition constants (K_i) as described earlier.[33]

At a concentration of 10 mM, free and dipeptide-bound MRPs inhibited the transport of L-[^{3}H]lysine by 20–75% and 0–38%, respectively (Table 1). For strongly inhibiting compounds, K_i values between 0.32 mM for **7**[25] and 4.6 mM for **10** were calculated. The unlabeled reference amino acid L-lysine itself inhibited L-[^{3}H]lysine transport with a K_i value of 0.11 ± 0.01 mM (Table 1). Gly-Sar and the other tested unlabeled unmodified dipeptides showed no affinity towards the lysine transporter(s).

In conclusion, a minor part of the free MRPs, but not the dipeptide-bound derivatives, interacts with the transporter(s) for the amino acids L-lysine and L-arginine, either as substrates or as inhibitors. Pyrraline (**7**) was the only compound to inhibit L-[^{3}H]lysine transport similarly to L-lysine, and it can be considered a high-affinity ligand (substrate or inhibitor).

Inhibition of [^{14}C]Gly-Sar uptake

Unlike amino acid transport, the uptake of peptides from the gut lumen is mediated by a single transport protein—PEPT1—that tolerates many side-chain-modified peptides without losing affinity.[22] Therefore, the interactions of glycated amino acids and dipeptides with PEPT1 in Caco-2 cells were studied. The radiolabeled dipeptide [^{14}C]Gly-Sar, which is relatively resistant to intra- or extracellular enzymatic hydrolysis, served as the reference substrate. Competition experiments were first

Table 1. Inhibition of L-[^{3}H]lysine (2 nM) and [^{14}C]Gly-Sar (10 μM) uptake in Caco-2 cells by Maillard reaction products and control substances. Uptakes were measured at pH 6.0 either for 5 min (L-[^{3}H]lysine) or for 10 min ([^{14}C]Gly-Sar) in the absence (control) or in the presence of the compounds, either at fixed concentrations (10 mM) for % uptake or at increasing concentrations (0.01–10 mM) of unlabeled compounds. K_i values were derived from competition curves. Data are means ± SEM. $n = 3, 4$.

Compound		L-[^{3}H]Lysine uptake [% of control]	K_i [mM]	[^{14}C]Gly-Sar uptake [% of control]	K_i [mM]
fructoselysine	**1**	41 ± 1	2.7 ± 0.1	32 ± 2[a]	8.7 ± 2.2[a]
Ala-FL	**1a**	100 ± 6	–	60 ± 1	> 10 (≈13)[b]
FL-Ala	**1b**	69 ± 11	–	36 ± 2	4.5 ± 0.2
lactuloselysine	**2**	80 ± 6	–	49 ± 2	8.7 ± 1.1
tagatoselysine	**3**	40 ± 1	3.1 ± 0.6	33 ± 3	3.5 ± 0.1
carboxymethyllysine	**4**	61 ± 1	–	93 ± 4	–
Ala-CML	**4a**	87 ± 3	–	9.9 ± 0.4	0.07 ± 0.01
CML-Ala	**4b**	94 ± 3	–	14 ± 1	0.9 ± 0.1
carboxyethyllysine	**5**	70 ± 4	–	90 ± 4	–
Ala-CEL	**5a**	97 ± 11	–	11 ± 1	0.22 ± 0.01
CEL-Ala	**5b**	96 ± 8	–	16 ± 1	1.1 ± 0.1
formyline	**6**	42 ± 1	2.2 ± 0.3	82 ± 2	–
Ala-Fom	**6a**	82 ± 5	–	10 ± 1	0.09 ± 0.01
Fom-Ala	**6b**	96 ± 7	–	9 ± 1	0.05 ± 0.01
pyrraline	**7**	25 ± 1[c]	0.32 ± 0.04[c]	77 ± 2[c]	> 10[c]
Ala-Pyrr	**7a**	66 ± 5[c]	> 10[c]	5.5 ± 0.4[c]	0.19 ± 0.01[c]
Pyrr-Ala	**7b**	78 ± 7[c]	> 10[c]	9.4 ± 0.2[c]	0.03 ± 0.01[c]
maltosine	**8**	48 ± 5	3.5 ± 0.4[d]	85 ± 2	> 10[d]
Ala-Mal	**8a**	95 ± 1	> 10[d]	17 ± 1	0.73 ± 0.05[d]
Mal-Ala	**8b**	92 ± 5	> 10[d]	10 ± 1	0.25 ± 0.02[d]
MG-H1	**9**	39 ± 4	1.6 ± 0.2	89 ± 1	–
Ala-(MG-H1)	**9a**	62 ± 11	–	17 ± 1	0.95 ± 0.02
(MG-H1)-Ala	**9b**	n.det.	–	n.det.	0.59 ± 0.05
argpyrimidine	**10**	55 ± 5	4.6 ± 1.1	39 ± 3	6.7 ± 0.6
Ala-Apy	**10a**	74 ± 2	–	13 ± 1	0.37 ± 0.04
Apy-Ala	**10b**	n.det.	–	n.det.	0.19 ± 0.03[e]
Gly-Sar		105 ± 5[c]	–	15 ± 1[c]	0.74 ± 0.01[c]
Lys		17 ± 2[c]	0.11 ± 0.01[c]	101 ± 4[c]	–
Arg		18 ± 1	0.027 ± 0.003	96 ± 3	–
Ala-Lys		85 ± 4	–	8.6 ± 0.2	0.23 ± 0.02
Lys-Ala		84 ± 6	–	13 ± 1	0.34 ± 0.02[f]
Ala-Arg	**12**	74 ± 3	–	12 ± 1	0.18 ± 0.01
Arg-Ala	**13**	65 ± 8	–	12 ± 1	0.29 ± 0.02

[a] Value from ref. [28]. [b] Extrapolated beyond measurement range. [c] Value from ref. [25]. [d] Value from ref. [27]. [e] $n = 2$. [f] Value from ref. [29]. n.det.: not determined.

performed at 10 mM concentrations of each compound to determine the substrate specificity. For ligands that inhibited [^{14}C]Gly-Sar uptake by at least 40%, competition assays were subsequently carried out with increasing concentrations of the compounds, to allow calculation of their K_i values. At a concentration of 10 mM, most of the free modified amino acids inhibited [^{14}C]Gly-Sar uptake only weakly, with the exceptions of fructoselysine (**1**), lactuloselysine (**2**), tagatoselysine (**3**), and argpyrimidine (**10**), for which K_i values between 3.5 and 8.7 mM were calculated (Table 1). According to Brandsch,[32] these compounds can be classified as medium- (**3**) and low-affinity ligands (**1**, **2**, **10**) for PEPT1. For purposes of comparison, L-lysine and L-arginine showed no affinity towards PEPT1.

The glycated dipeptides, however, inhibited [^{14}C]Gly-Sar uptake by 40 to 94%. K_i values between 34 μM (Pyrr-Ala) and > 10 mM (Ala-FL) were determined (Table 1). The affinities towards PEPT1 were strongly modulated, relative to those of the parent dipeptides, by the modifications. Peptides with pyrrole-modified side chains (**6a**/**6b**, **7a**/**7b**) displayed the highest affinities (34–190 μM). By Brandsch's classification,[32] Ala-CML, Ala-CEL, Mal-Ala, and both dipeptide derivatives of pyrraline, of formyline, and of argpyrimidine can be classified as high-affinity ligands ($K_i < 0.5$ mM). The other tested dipeptides can be considered medium-affinity ligands (0.5 mM $< K_i <$ 5 mM), except for Ala-FL, which is a low-affinity compound. Gly-Sar represents a medium-affinity PEPT1 substrate with a K_i value of 0.74 ± 0.01 mM (Table 1), whereas the unmodified lysine and arginine dipeptides are high-affinity substrates with K_i values between 0.18 and 0.34 mM. It is the hydrophobic peptides that show high affinity, which agrees with recent findings about the affinity-enhancing effects of hydrophobic side chain modifications.[29,34] Interestingly, the alanyl peptides **4a** and **5a**, which each have a negative charge in the side chain, bind to the carrier with affinities five and 13 times higher, respectively, than the corresponding lysyl dipeptides **4b** and **5b**. This could possibly be the result of unequal distribution of charges in the binding region of PEPT1. In contrast, the alanyl dipeptides of all other tested compounds had lower affinities towards PEPT1 (1.6 to 5.6 times) than the corresponding lysyl dipeptides. This is also consistent with recent studies on model compounds in which bulky hydrophobic groups were introduced onto the lysyl side chains of Ala-Lys and Lys-Ala, thereby strongly enhancing the affinities of lysyl peptides.[29]

Transepithelial transport across intestinal epithelial cell monolayers

Interactions of compounds with transport systems do not necessarily mean that they are indeed transported. The molecular features necessary for uptake inhibition need not be the same as for translocation, so the inhibitors might be nontransported compounds with certain affinities towards the transporters. On the other hand, several other carriers at the intestinal epithelium can be responsible for the translocation of substances. Flux measurements were performed as in our previous studies;[25,27] [^{14}C]Gly-Sar and L-[^{3}H]lysine were again used as the reference substrates. The space marker [^{14}C]mannitol served as a reference compound for paracellular transport processes. Despite their relatively low inhibitions of L-[^{3}H]lysine uptake, all free amino acids were subjected to the transport experiment. No CML or CEL was found in the receiver compartment after 120 min (Table 2). The flux rates of other glycated amino acids ranged between 0.008 and 0.09% $cm^{-2}h^{-1}$ and were always lower than that of the space marker [^{14}C]mannitol (0.13 ± 0.03% $cm^{-2}h^{-1}$) and far lower than the flux of L-[^{3}H]lysine (6.86 ± 0.15% $cm^{-2}h^{-1}$). This argues against active transport of

Table 2. Transepithelial flux of Maillard reaction products across Caco-2 cells and cellular uptake after 120 min. Fluxes were determined in the presence of the compound in question (1 mM), [^{14}C]Gly-Sar (10 μM), L-[^{3}H]lysine (2 nM), and [^{14}C]mannitol (10 μM), at pH 6.0 (apical) and pH 7.5 (basolateral) over 2 h. Data are means ± SEM. $n = 3$.

Compound		Flux of the dipeptide [% cm^{-2} h^{-1}]	Cellular uptake[a] [%]	Flux of the amino acid [% cm^{-2} h^{-1}]	Cellular uptake[a] [%]
fructoselysine	**1**	–	–	0.008 ± 0.003[b]	0.03 ± 0.01[b]
Ala-FL	**1a**	n.d.	n.d.	n.d.	n.d.
FL-Ala	**1b**	n.d.	n.d.	n.d.	n.d.
lactuloselysine	**2**	–	–	n.d.	n.d.
tagatoselysine	**3**	–	–	n.d.	n.d.
carboxymethyllysine	**4**	–	–	n.d.[b]	0.62 ± 0.02[b]
Ala-CML	**4a**	0.02 ± 0.01	0.05 ± 0.01	0.11 ± 0.01	2.46 ± 0.08
CML-Ala	**4b**	0.02 ± 0.01	0.05 ± 0.01	0.17 ± 0.07	13 ± 2
carboxyethyllysine	**5**	–	–	n.d.	1.36 ± 0.07
Ala-CEL	**5a**	0.03 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	3.1 ± 1.5
CEL-Ala	**5b**	0.03 ± 0.01	0.17 ± 0.01	0.10 ± 0.05	12 ± 1
formyline	**6**	–	–	0.04 ± 0.01	0.08 ± 0.01
Ala-Fom	**6a**	n.d.	n.d.	3.37 ± 0.13	2.39 ± 0.08
Fom-Ala	**6b**	n.d.	n.d.	1.09 ± 0.16	0.86 ± 0.08
pyrraline	**7**	–	–	0.07 ± 0.02[c]	0.6 ± 0.1[c]
Ala-Pyrr	**7a**	n.d.[c]	n.d.[c]	1.06 ± 0.27[c]	8.8 ± 0.7[c]
Pyrr-Ala	**7b**	n.d.[c]	n.d.[c]	0.28 ± 0.08[c]	2.6 ± 0.2[c]
maltosine	**8**	–	–	0.02 ± 0.01[d]	0.07 ± 0.01[d]
Ala-Mal	**8a**	n.d.[d]	n.d.[d]	0.27 ± 0.08[d]	16 ± 2[d]
Mal-Ala	**8b**	n.d.[d]	n.d.[d]	0.16 ± 0.06[d]	2.7 ± 0.2[d]
MG-H1	**9**	–	–	0.09 ± 0.08	0.32 ± 0.04
Ala-(MG-H1)	**9a**	n.d.	n.d.	0.24 ± 0.11	10 ± 2
(MG-H1)-Ala	**9b**	n.d.	n.d.	0.22 ± 0.03	3.12 ± 0.17
argpyrimidine	**10**	–	–	0.01 ± 0.01	0.05 ± 0.02
Ala-Apy	**10a**	n.d.	n.d.	0.30 ± 0.02	1.79 ± 0.14
Apy-Ala	**10b**	n.d.	n.d.	0.14 ± 0.04	0.38 ± 0.03
pentosidine	**11**	–	–	0.03 ± 0.01	0.01 ± 0.01
[^{14}C]Gly-Sar		2.79 ± 0.47	7.67 ± 0.16	–	–
[^{14}C]mannitol		–	–	0.13 ± 0.03[c]	0.23 ± 0.02
L-[^{3}H]Lys		–	–	6.86 ± 0.15	4.01 ± 0.24

[a] Total cellular uptake at the end of the experiment (120 min). [b] Value from ref. [28]. [c] Value from ref. [25]. [d] Value from ref. [27]. n.d.: not detectable.

glycated amino acids by any of the Caco-2 amino acid transport systems.

The transport studies were then performed with glycated dipeptides. Caco-2 cells express membrane-bound peptidases, so the dipeptides were partly hydrolyzed in the donor compartment during the flux measurement. The fluxes and cellular uptakes reported in Table 2 therefore have to be regarded as minimum values. All MRPs except for fructoselysine appeared inside the cells and in the receiver compartment, but only the dipeptides of CML and CEL could be recovered, to small extents, in intact form. When calculated for the intact dipeptides, the flux rates of **4a**/**4b** and **5a**/**5b** are lower than that of the space marker. However, all dipeptides were hydrolyzed very quickly inside the cells, and the MRPs passed into the receiver compartment in the form of amino acids. Therefore, the fluxes are also calculated for the amino acids cleaved from the dipeptides (Table 2). Amino acids from the donor compartment cannot interfere with this calculation because they are not transported. The net flux rates of glycated amino acids, when applied as dipeptides, are increased by up to 80-fold relative to the free glycated amino acids (e.g., formyline **6**, 0.04 % cm^{-2} h^{-1}; **6** from **6a**, 3.37 % cm^{-2} h^{-1}). Most flux rates were higher than the flux of the [^{14}C]mannitol space marker. This means that glycated dipeptides are absorbed into the cells, most probably by PEPT1, and hydrolyzed to the free modified amino acids and alanine by intracellular peptidases. The amino acids reach the basolateral compartment either through the action of different amino acid transporters or possibly by simple diffusion.

Pronounced differences with regard to the tendencies of the glycated amino acids to leave the cells were found. The total proportions of glycated dipeptides and amino acids inside the cells and in the receiver compartment after 120 min are shown in Figure 1. More than 50 % of the glycated amino acids hydrolyzed from the dipeptides of CML, CEL, maltosine, and MG-H1 after absorption remain in the Caco-2 cells. This underlines the transport capacity of PEPT1 as the likely responsible carrier, capable even of transporting its substrates uphill against a concentration gradient.[21] The MRPs face the next barrier, the basolateral cell membrane, not as dipeptides, but as amino acids. Hydrophobic glycated amino acids such as **6**, **7**, and **10** can pass through the basolateral membrane more quickly than **4**, **5**, **8**, or **9**, which are strongly retained, if not trapped, inside the cells. We assume that the strong retention, especially of hydrophilic amino acids, inside the cells is due to the fact that they have to diffuse through the basolateral membrane, a process that is easier for hydrophobic amino acids. The hydrophobic amino acids permeate to the basolateral cell side to a greater extent if their side chain modifications lack the capacity to donate hydrogen bonds, as is the case for formyline **6**, but not for **7**, **8**, and **10**. No correlation between the affinity of a dipeptide towards PEPT1 and the actual transport was found.

Conclusions

Free glycated amino acids are not inhibitors of the lysine transport system(s), nor are they transported in significant amounts across cell monolayers. In contrast, several glycated dipeptides are high-affinity inhibitors of [^{14}C]Gly-Sar uptake. In particular, the results for the carboxyalkylated peptides (**4a**/**4b** and **5a**/**5b**) show that not only side chain hydrophobization but also the introduction of hydrophilic and charged carboxyl groups can lead to strong inhibitors of [^{14}C]Gly-Sar uptake. Those derivatives that show high rates of [^{14}C]Gly-Sar uptake inhibition and high flux rates across Caco-2 monolayers are most likely substrates of the intestinal proton-coupled peptide transporter PEPT1. Depending on the kind of modification and the peptide sequence, glycated peptides can be transported by PEPT1 into the cells, where they are rapidly hydrolyzed. After addition of glycated dipeptides to the apical compartment, no dipeptide derivatives were detected in the basolateral compartment, but free glycated amino acids were found. In particular, hydropho-

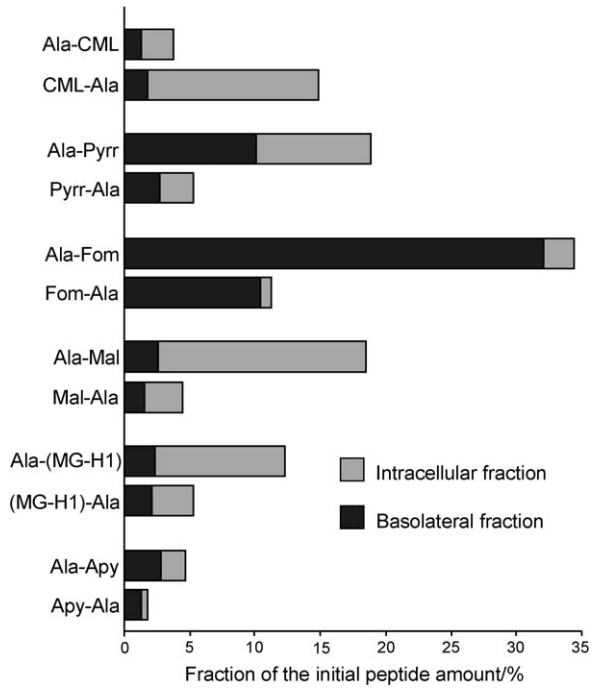


Figure 1. Total basolateral (■) and intracellular (▒) proportions of glycated peptides and amino acids at 120 min after apical application of glycated peptides (1 mM). Molar amounts of glycated peptides and the corresponding glycated amino acids were summed, divided by the initial peptide amount, and expressed in %.

bic glycated amino acids formed in the later stages of the Maillard reaction can quickly permeate the basolateral cell membrane either by simple diffusion or through the action of amino acid transporters, whereas hydrophilic amino acids are released much more slowly. Uptake rates of different dipeptides of the same MRP differ by factors of up to 4. It is highly relevant whether MRPs are in the N- or the C-terminal position. These findings are of nutritional and physiological relevance and should be discussed as part of "risk assessment". The data show that free and dipeptide-bound Amadori products, which represent more than 90% of the MRPs detected in foods, are not taken up into cells in vitro. Others such as CML (**4**), CEL (**5**), and MG-H1 (**9**) are strongly retained inside the cells. Further studies should show whether these products are released very slowly or are irretrievably trapped inside epithelial cells until desquamation. In particular, few hydrophobic MRPs such as pyrraline (**7**), maltosine (**8**), formyline (**6**), or argpyrimidine (**10**) are transported through the cells. Because dietary pyrraline (**7**), but not fructoselysine (**1**), is to a large extent excreted in the urine, and because **7**, but not **1**, is absorbable in its peptide form,[23] it can be assumed that maltosine (**8**), formyline (**6**), and argpyrimidine (**10**) can also be absorbed after the digestion of glycated food proteins. Further research should focus on hepatic metabolism and renal handling. Digestibility studies with proteins modified by the Maillard reaction are required in order to provide information as to what extent MRPs appear in absorbable peptide forms, and whether they are hydrolyzed by luminal or membrane-bound peptidases. The longer these MRPs are peptide-bound during intestinal digestion and the more hydrophobic they are, the higher is the chance of their appearance in the circulation.

Experimental Section

Materials: Boc-Ala-OSu, L-arginine, di-*tert*-butyl dicarbonate, glyoxylic acid monohydrate, and 1,1-dimethoxyacetone were obtained from Fluka. Pd on activated charcoal (Pd/C, 10%, *w/w*), 3-chloropentane-2,4-dione, glycylsarcosine (Gly-Sar), and Ala-Lys were purchased from Sigma–Aldrich. Boc-Ala-Lys, Boc-Lys-Ala, Boc-Ala-Arg, and Fmoc-Arg-Ala were from IRIS Biotech (Martinsried, Germany). Microcrystalline cellulose (particle size 20–160 μm), pyruvic acid, and *N*,*N*-diisopropyethylamine (DIPEA) from Merck were used. Ala-O*t*Bu and Lys-Ala were purchased from Bachem (Bubendorf, Switzerland) and *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TSTU) from Molekula (Taufkirchen, Germany). The following substances were synthesized by literature methods: 3-deoxyglucosone[6] (3-DG), fructoselysine[35] (**1**), 3-deoxypentosone[4] (3-DPs), lactuloselysine[4] (**2**), formyline[4] (**6**), and pentosidine[36] (**11**). The water used for the preparation of buffers and solutions was obtained by use of a Purelab plus purification system (USFilter, Ransbach-Baumbach, Germany). All other chemicals were purchased from standard suppliers and were of the highest purity available.

The human colon carcinoma cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell culture media, supplements, and trypsin solution were purchased from Invitrogen or PAA (Cölbe, Germany). Fetal bovine serum was from Biochrom (Berlin, Germany). [Glycine-1-^{14}C]Gly-Sar (specific radioactivity 56 mCi mmol^{-1}) and L-[4,5-^{3}H]lysine monohydrochloride (specific radioactivity 99 Ci mmol^{-1}) were synthesized by GE Healthcare. [^{14}C]Mannitol (specific radioactivity 53 mCi mmol^{-1}) was obtained from Hartmann Analytic (Braunschweig, Germany).

Thin layer chromatography: TLC was performed on Merck silica gel 60 plates with dichloromethane/methanol/25% aqueous NH_3 (2:2:1, *v/v/v*) as the mobile phase. Visualization was achieved by spraying the plates with a solution of ninhydrin in ethanol (0.1%) acidified with glacial acetic acid (3%, *v/v*) followed by heating until the appearance of spots. TLC plates were also used for the spotting test to identify target fractions after chromatographic separations. Each fraction (1 μL) was spotted onto the TLC plate and sprayed either with the ninhydrin reagent or with a solution of triphenyltetrazolium chloride (TTC, 1%) in NaOH (1 N).[4,35]

High-pressure liquid chromatography: All analytical HPLC analyses were performed with an Äkta 10XT high-pressure gradient system (Amersham Pharmacia Biotech, Uppsala, Sweden), consisting of a P-900 pump with an online degasser (Knauer, Berlin, Germany), a column oven, and a UV-900 UV detector. All separations were performed with a stainless steel column (150×4.6 mm) filled with Eurospher-100 RP-18 material of 5 μm particle size with an integrated guard column (5×4 mm) of the same material at a column temperature of 30 °C. The injection volume was 50 μL. A previously published solvent and gradient system permitted the separation of **6** from both its peptides; the measurements were performed at a wavelength of 293 nm.[4]

For the analyses of **10**, **11**, and the peptides **10a**/**10b**, the eluent was ammonium formate (pH 4.0, 10 mM), to which heptafluorobutyric acid (HFBA, 650 μL L^{-1}) was added (solvent A, final pH: 3.5). Solvent B consisted of a mixture of ammonium formate (pH 4.0, 50 mM, 200 mL) and methanol (800 mL), to which HFBA (650 μL L^{-1}) was added. A linear gradient from 3 to 40% B in 20 min and then to 80% B in 3 min was applied at a flow rate of

1.0 mL min^{-1}. Argpyrimidine and its dipeptides were quantified with use of a fluorescence detector (F-1050, Merck Hitachi) at excitation and emission wavelengths of 320 and 380 nm, respectively. For pentosidine, the wavelengths were set at 335 and 385 nm, respectively. External calibration was performed with the synthesized standards.

Amino acid analysis (AAA): Flux measurements were performed with an amino acid analyzer (S 433, Sykam, Fürstenfeldbruck, Germany) and a PEEK column (150×4.6 mm) filled with the cation exchange resin LCA K07/Li (particle size, 7 μm). Lithium buffers were purchased ready for use from Sykam and employed for different gradient programs according to the manufacturer's instructions. The lithium system was also used for the determination of the purities of synthesized substances, which were injected at a concentration of 40 μg mL^{-1}. Flux samples of fructoselysine dipeptides additionally had to be analyzed with the Alpha Plus amino acid analyzer (LKB Biochrom, Cambridge, UK) and a PEEK column (190× 4.6 mm) filled with a cation-exchange resin (particle size, 5 μm, K. Grüning, Olching, Germany). The conditions are available from the literature.[37] With both systems, post-column derivatization with ninhydrin was applied, and VIS detection was performed with integrated two-channel photometers working simultaneously at 440 nm and 570 nm. External calibration was performed with the synthesized standards. The injection volume was 50 μL.

Nuclear magnetic resonance spectroscopy, mass spectrometry, and elemental analysis (EA): Proton spectra were recorded with a Bruker DRX 500 instrument (Rheinstetten, Germany) at 500 MHz in D_2O as the solvent. Chemical shifts are given in parts per million (ppm) relative to the internal HOD signal (4.70 ppm). For ESI-MS, a PerSeptive Biosystems Mariner time-of-flight mass spectrometer fitted with an electrospray ionization source (ESI-TOF-MS, Applied Biosystems) working in the positive mode was used. Calibration of the mass scale was established with a mixture of bradykinin, angiotensin I, and neurotensin. After appropriate dilution with formic acid (1%) in aqueous acetonitrile (50%), the samples were injected into the ESI source by syringe pump at a flow rate of 5 μL min^{-1}. EA data were obtained with a Euro EA 3000 elemental analyzer (Eurovector, Milano, Italy). Elemental analysis was used to calculate the product contents of the preparations. The percentage of nitrogen in the preparation was divided by the theoretical percentage of nitrogen of the target substance and the content was expressed in per cent by weight. All data relating to the characterization of synthesis products are given in the Supporting Information.

Synthesis of ligands—general procedures: Purification of ligands was performed by ion-exchange chromatography (IEC) with the strongly acidic cation exchange resin DOWEX 50 WX-8 (200–400 mesh) unless otherwise stated. Before use, the resin was activated with three times its volume both of HCl (6 N) and of water. When the resin was to be used in the H^+ form, the material was placed in a suitable Econo glass column (BioRad, Munich, Germany) and equilibrated with three times its volume of HCl (0.01 N). When it was to be used in the Na^+ form, the activated resin was rinsed with three times its volume both of NaOH (1 N) and of water, placed in a suitable column, and equilibrated with three times its volume of sodium citrate buffer (pH 3.00, 0.1 N). When the resin was required in its pyridinium (Py^+) form, it was rinsed with three times its volume both of aqueous pyridine (2 M) and of water, placed in a suitable column, and equilibrated with three times its volume of pyridinium formate buffer (pH 3.00, 0.1 N). The synthesis mixtures were dissolved in equilibration buffer (30 mL) and applied to the column after adjustment of the pH to 3.0. After rinsing of the resin with a small volume of equilibration buffer, the products were eluted by gravity with the elution buffers stated below at flow rates of 0.2–0.4 mL min^{-1}. Fractions (10 mL) were collected with a fraction collector (RediFrac, Amersham Pharmacia Biotech) and the presence of the product was monitored by spotting of the fractions (1 μL) on TLC plates and spraying with the appropriate reagent(s). Fractions containing the target product were repeatedly concentrated to dryness and taken up in water until the smell of pyridine or HCl had become imperceptible. Combined fractions containing sodium citrate buffer were first desalted as described earlier.[27] All products were stored at −20 °C.

Ala-FL (1 a) and FL-Ala (1 b): Boc-Ala-Lys (507.3 mg, 1.6 mmol), and D-glucose (1.73 g, 9.6 mmol) were dissolved in dry methanol (90 mL) and heated under reflux for 4 h. The methanol was then evaporated in vacuo and the residue was taken up in water (150 mL). The pH was adjusted to 2.0 and the solution was transferred to a column (2.5×20 cm) filled with the cation exchanger Lewatit S100, previously equilibrated with HCl (6 M) and water (each 250 mL). The column was washed with water (300 mL) to remove the sugar, and the product was eluted with NH_3 (2 M , 300 mL) after overnight incubation.[35] Ammonia was removed with the aid of a rotary evaporator and the residue was subjected to IEC on a column (1.5×50 cm) in the Py^+ form. Compound **1 a** was eluted with pyridinium acetate buffer (pH 4.35, 0.4 N, 230–340 mL), as revealed by the spotting test (TTC and ninhydrin). The product was dissolved in methanol (2–3 mL) and precipitated in ice-cold butanone as described in the literature[4,35] to yield **1 a** as a white solid (384.7 mg, 53.2%).

For compound **1 b**, Boc-Lys-Ala (506 mg, 1.59 mmol) and D-glucose (1.72 g, 9.6 mmol) were dissolved in water (50 mL). After freeze-drying, the lyophilizate was incubated for 4 h at 70 °C in a sand bath in a drying oven. The synthesis mixture was worked up as described for **1 a**. Compound **1 b** was eluted with pyridinium acetate buffer (pH 4.35, 0.4 N, 350–550 mL) and was isolated as a white solid after precipitation in ice-cold butanone (309.3 mg, 49.4%).

Tagatoselysine (3): Boc-Lys-OH (621.7 mg, 2.5 mmol) and D-galactose (2.71 g, 15.1 mmol) were dissolved in *N*,*N*-dimethylformamide (DMF)/methanol (3:7, *v*/*v*, 100 mL) and heated under reflux for 2 h. The solvents were then evaporated in vacuo with repeated addition of water. Removal of the Boc protecting group and the excess of the sugar were performed by IEC as described for **1 a**. Ammonia was removed with the aid of a rotary evaporator and the residue was subjected to IEC on a column (1.5×50 cm) in the Py^+ form. The spotting test (ninhydrin, TTC) revealed that **3** eluted with pyridinium acetate buffer (pH 4.35, 0.4 N, 160–250 mL). The product was precipitated in ice-cold butanone as described above, to yield **3** as a white solid (420.6 mg, 43.4%).

CML (4), Ala-CML (4 a), CML-Ala (4 b), CEL (5), Ala-CEL (5 a), and CEL-Ala (5 b): These syntheses were performed by reductive alkylation of lysine derivatives with α-keto acids.[28] The amounts of reactants given below were dissolved in water (30 mL) and the pH of the solutions was adjusted to 8.75 with NaOH (1 M) prior to the addition of Pd catalyst. The mixture was hydrogenated at room temperature (RT) and atmospheric pressure for 24 h. During the syntheses of **5**, **5 a**, and **5 b**, the H_2 was renewed and hydrogenation was continued for further 24 h. The catalyst was then filtered off. Boc-CML and Boc-CEL were dissolved in HCl (3 M) to concentrations of 10–20 mg mL^{-1} and heated under reflux for 3 h to remove the Boc protecting group. The protected peptides were dissolved in aqueous acetic acid (10%, 1000 mL) and heated under reflux for 4 h at 70 °C.[6] After the removal of the acids with the aid of a rotary evaporator, the products were subjected to IEC on a column (1.5×50 cm) in the H^+ form. For the elution, a step gradient of ascending HCl concentrations (1, 1.5, 2 M HCl, each 300 to 600 mL) was applied. The spotting test (ninhydrin) showed that the products were well resolved from their educts and usually eluted with 1.5–2 M HCl. After the evaporation of HCl, the products were

lyophilized to yield the hydrochlorides as slightly yellow amorphous powders.

CML (4): Boc-Lys (1000 mg, 4.1 mmol), glyoxylic acid (480 mg, 5.2 mmol), Pd/C (50 mg). Yield 517 mg (46.4%).

Ala-CML (4a): Boc-Ala-Lys (500 mg, 1.6 mmol), glyoxylic acid (187 mg, 2.0 mmol), Pd/C (38 mg). Yield 369 mg (60.7%).

CML-Ala (4b): Boc-Lys-Ala (500.3 mg, 1.6 mmol), glyoxylic acid (710.8 mg, 7.5 mmol), Pd/C (50.6 mg). Yield 183.4 mg (31.2%).

CEL (5): Boc-Lys (1243 mg, 5.1 mmol), pyruvic acid (586 mg, 6.5 mmol), Pd/C (84.0 mg). Yield 980 mg (61.5%).

Ala-CEL (5a): Boc-Ala-Lys (500.0 mg, 1.6 mmol), pyruvic acid (357 mg, 4.0 mmol), Pd/C (34 mg). Yield 430.0 mg (66.9%).

CEL-Ala (5b): Boc-Ala-Lys (500 mg, 1.6 mmol), pyruvic acid (695 mg, 7.7 mmol), Pd/C (35 mg). Yield 377 mg (58.4%).

Ala-Fom (6a) and Fom-Ala (6b): For **6a**, Boc-Ala-Lys (486 mg, 1.5 mmol) and 3-DPs (1000 mg) were dissolved in water (5.6 mL). The solution was mixed with cellulose (4.5 g), and after lyophilization the mixture was incubated at 70 °C in a drying oven. The brown cake was extracted with water (3×100 mL). The pH of the combined aqueous phases was adjusted to 1.0, and the solution was extracted with ethyl acetate (3×100 mL). The pH of the solution was adjusted to 4.5, and the extraction was repeated (3× 100 mL ethyl acetate). The combined organic layers were evaporated to dryness with the aid of a rotary evaporator. Boc-protected intermediates were deprotected as described above for carboxyalkylated peptides. After evaporation of acetic acid, the residue was subjected to IEC on a column (1.5×20 cm) in the Py^+ form. Elution was performed first with pyridinium formate buffer (pH 3.75, 0.3 N, 250 mL) and then with pyridinium formate buffer (pH 4.05, 0.3 N). Compound **6a** eluted between 20 and 140 mL of the second buffer. After evaporation, the residue was precipitated in butanone as described above, to yield **6a** as a light yellow powder (73.4 mg, 15.2%).

The synthesis of Fom-Ala (**6b**) was performed in the same way, starting from Boc-Lys-Ala (452.7 mg, 1.4 mmol) and 3-DPs (750 mg) dissolved in water (4.8 mL) and deposited on cellulose (3.8 g). During IEC, compound **6b** eluted with 15–200 mL of the second elution buffer. Precipitation in butanone provided **6b** as a light yellow powder (56.8 mg, 12.4%).

MG-H1 (9), Ala-(MG-H1) (9a), and (MG-H1)-Ala (9b): L-Arginine (1.009 g, 5.8 mmol) and 1,1-dimethoxyacetone (**14**, 678 μL, 5.7 mmol) were dissolved in HCl (12 M, 110 mL). After the system had been stirred at RT for 8 h, water (200 mL) was added and the solution was concentrated to dryness in vacuo. The dried residue was taken up in water (150 mL), and the pH was adjusted to 2.0. The solution was transferred to a column (2.5×20 cm) filled with the cation exchanger Lewatit S100, previously equilibrated with HCl (6 M) and water (each 250 mL). The column was washed with water (300 mL) to remove uncharged byproducts, and the product was eluted immediately with HCl (4 M, 300 mL). After evaporation of the acid, the residue was subjected to IEC on a column (1.5× 50 cm) in the Na^+ form. Elution was first performed with sodium citrate buffer (pH 4.50, 0.2 N, 300 mL) and then with sodium citrate buffer (pH 5.28, 0.3 N). MG-H1 (**9**) eluted with the second buffer (180–330 mL). After desalting and lyophilization, off-white amorphous **9** was obtained (794.4 mg, 41.5%).

Ala-(MG-H1) (**9a**) was prepared accordingly, from a mixture of **12** (78.5 mg, 0.23 mmol) and **14** (28.0 μL, 0.23 mmol) in HCl (12 M, 9 mL), which was stirred at RT for 4 h. After the removal of byproducts as described for **9**, the residue was subjected to IEC on a column (1.5×50 cm) in the Py^+ form. Elution was performed first with pyridinium acetate buffer (pH 4.35, 0.4 N, 400 mL), and then with pyridinium acetate buffer (pH 5.00, 0.4 N). Compound **9a** eluted with the second buffer (140–200 mL). After evaporation of the buffer, the residue was lyophilized, to yield **9a** as a light brownish powder (24.7 mg, 27.2%).

(MG-H1)-Ala (**9b**) was prepared accordingly, from a mixture of **13** (81.6 mg, 0.23 mmol) and **14** (26.6 μL, 0.22 mmol) in HCl (12 M, 9 mL), which was stirred at RT for 4 h. During IEC, **9b** eluted with the second elution buffer (270–400 mL). After the removal of the buffer, the product was lyophilized, to yield **9b** as a light yellow powder (27.1 mg, 31.9%).

Argpyrimidine (10): 3-Chloropentane-2,4-dione (**15**, 1.68 mL, 14.9 mmol) was dissolved in DMSO (25 mL), and anhydrous sodium acetate (2.44 g, 29.7 mmol) was added.[31] After the mixture had been stirred for 3 h at RT, water was added (200 mL). The mixture was extracted with diethyl ether (5×100 mL) after cooling. The extracts were dried ($MgSO_4$) and concentrated to dryness. The remaining brownish oil was subjected to flash chromatography (FC) on silica gel (20 g) with petroleum ether (40–60 °C)/ethyl acetate (8:2, *v/v*). Target fractions of 3-acetoxypentane-2,4-dione **16** eluted between 80–170 mL as revealed by the spotting test (TTC). The slightly red oil that remained after evaporation of the solvents was immediately added to a solution of L-arginine (1.047 g, 6.0 mmol) in HCl (12 M, 35 mL).[30] A second portion of **16** was added after 3 h and the mixture was stirred at RT for 20 h. The solution was then diluted with water (200 mL) and extracted with diethyl ether (3× 100 mL). The aqueous phase was concentrated to dryness and the residue was subjected to FC on silica gel (30 g) with methanol/ethyl acetate (2:1, *v/v*).[9] TLC of the fractions revealed that **10** eluted between 60–180 mL (R_f = 0.77). After evaporation of the solvents, argpyrimidine was isolated by IEC on a column (1.5×50 cm) in the Py^+ form. Elution was performed first with pyridinium formate buffer (pH 4.05, 0.3 N, 200 mL), and then with pyridinium acetate buffer (pH 4.35, 0.4 N). Compound **10** eluted with the second buffer (380–600 mL). After buffer removal, the residue was precipitated in butanone as described for **1a**, to yield **10** as a white powder (468.0 mg, 29.7%).

Ala-Apy (10a) and Apy-Ala (10b): Boc-Ala-OSu (840.9 mg, 2.94 mmol), compound **10** (199.1 mg, 0.78 mmol), and DIPEA (330 μL, 1.9 mmol) were dissolved in a mixture of DCM (20 mL) and methanol (10 mL). After overnight stirring at RT, the solvents were removed under reduced pressure. The residue was dissolved in $NaHCO_3$ solution (5%, 50 mL) and extracted with ethyl acetate (3× 50 mL). The pH was adjusted to 1.0 with HCl (6 M), and the aqueous phase was extracted with ethyl acetate (3×100 mL). The combined organic layers were dried (Na_2SO_4) and concentrated to dryness. The protected derivative was dissolved in HCl (6 M)/tetrahydrofuran (1:1, *v/v*, 30 mL) and stirred at RT for 1 h.[38] The solvents were removed with the aid of a rotary evaporator. Compound **10a** was then isolated by IEC on a column (1.5×50 cm) in the Py^+ form. Elution was performed first with pyridinium acetate buffer (pH 4.35, 0.4 N, 300 mL) and then with pyridinium acetate buffer (pH 5.00, 0.4 N). Compound **10a** eluted with the second buffer (440–650 mL). After the removal of the buffer, the residue was lyophilized, to yield **10a** as a white powder (53.3 mg, 17.9%).

For **10b**, compound **10** (162.7 mg, 0.64 mmol) was dissolved at 2 °C in a mixture of tetrahydrofuran (10 mL) and Na_2CO_3 solution (0.5 M, 10 mL). Di-*tert*-butyl dicarbonate (560 mg, 1.28 mmol) was then added slowly. The cooling bath was removed and the mixture was stirred for 2 h at RT. After removal of the solvents, the residue was partitioned between water (30 mL) and ethyl acetate (10 mL). The pH was adjusted to 2.4 and the aqueous phase was extracted with ethyl acetate (3×30 mL). The combined organic layers were extracted with water (2×20 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The residue, consisting of Boc-argpyrimidine, was taken up in DCM (5 mL), and DIPEA (329 μL, 1.9 mmol) and TSTU (232 mg, 0.77 mmol) were added. The mixture was

stirred at RT for 20 min. A portion of Ala-O*t*Bu hydrochloride (261.0 mg, 1.4 mmol) in DCM (5 mL) was then added, and the solution was stirred for 30 min at RT. DCM was removed and the residue was dissolved in diethyl ether (100 mL) and extracted with HCl (1 M, 2×50 mL), $NaHCO_3$ solution (5%, 2×50 mL), and water (2× 50 mL). The organic layer was concentrated under reduced pressure and the residue was deprotected and isolated as described for **10a**. Compound **10b** eluted with the second elution buffer (630–880 mL). After the removal of the buffer, the residue was lyophilized, to yield **10b** as a white powder (32.7 mg, 11.9%).

Ala-Arg (12) and Arg-Ala (13): Boc-Ala-Arg (509.7 mg, 1.4 mmol) was deprotected as described for **10a**. The dried residue was subjected to IEC on a column (1.5×50 cm) in the Na^+ form. The column was first rinsed with sodium citrate buffer (pH 5.35, 0.3 N, 400 mL), and then with sodium citrate buffer (pH 6.00, 0.3 N, 400 mL). The product was then eluted with sodium citrate buffer (pH 6.00, 0.5 N, 300 mL). After desalting, off-white amorphous **12** was obtained (452.6 mg, 92.7%).

For **13**, Fmoc-Arg-Ala (704.8 mg, 1.5 mmol) was dissolved in DMF/methanol/morpholine (72:8:20, *v/v/v*, 100 mL). The mixture was stirred at RT for 1 h and then concentrated to dryness. The residue was partitioned between water (100 mL) and diethyl ether (50 mL). The aqueous phase was extracted with diethyl ether (2×50 mL) and then concentrated to dryness. The residue was subjected to IEC on a column (1.5×50 cm) in the Na^+ form. After rinsing of the column with sodium citrate buffer (pH 6.00, 0.3 N, 500 mL), the product was eluted with sodium citrate buffer (pH 6.00, 0.5 N). TLC showed a chromatographically pure fraction eluting between 180–310 mL (R_f = 0.27). After desalting, off-white amorphous **13** was obtained (270.4 mg, 49.4%).

Cell culture: Caco-2 cells were routinely cultured in culture flasks (75 cm^2) with minimum essential medium supplemented with fetal bovine serum (10%), gentamicin (50 μg mL^{-1}), and nonessential amino acid solution (1%) at 37 °C under a humidified atmosphere [CO_2 (5%), O_2 (95%)].[25,28,33] Cultures with a confluence of 80% were treated for 5 min with Dulbecco's phosphate-buffered saline followed by a 2 min incubation with trypsin solution. For uptake experiments, the cells were seeded in 35 mm disposable Petri dishes (Sarstedt, Nümbrecht, Germany) at a density of 0.8×10^6 cells per dish. The uptake measurements were performed on the seventh day after seeding. Protein content per dish was determined by a Pierce Protein Assay (660 nm, Thermo Fisher Scientific) by the manufacturer's protocol.

For the flux measurements, Caco-2 cells were cultured on permeable polycarbonate Transwell cell culture inserts (diameter 24 mm, pore size 3 μm, Costar, Bodenheim, Germany) with a cell density of 0.2×10^6 cells per filter for 21 days.[25,28] The lower (receiver) compartment contained medium (2.6 mL) and the upper (donor) compartment medium (1.5 mL). The transepithelial electrical resistance was measured at day 21 with a Millicell ERS (Millipore Intertech).

Transport studies: Uptake of [^{14}C]Gly-Sar and L-[^{3}H]lysine into Caco-2 cells cultured on plastic dishes was measured at RT as described earlier.[25,28,33] The uptake buffer contained Mes/Tris (pH 6.0, 25 mM), NaCl (140 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgSO_4$ (0.8 mM), glucose (5 mM), [^{14}C]Gly-Sar (10 μM), or L-[^{3}H]lysine (2 nM), together with unlabeled compounds at increasing concentrations (0–10 mM). After incubation either for 10 min ([^{14}C]Gly-Sar uptake) or for 5 min (L-[^{3}H]lysine uptake), the cells were quickly washed four times, dissolved in Igepal CA-630 buffer, and prepared for liquid scintillation spectrometry. The nonsaturable component of [^{14}C]Gly-Sar and L-[^{3}H]lysine uptake (diffusion, adherent radioactivity) determined by measuring the uptake of the labeled compound in the presence of the unlabeled compound [Gly-Sar (50 mM) or L-lysine (20 mM)] represented 8% and 21% of the total uptake, respectively. This value was used during nonlinear regression analysis of inhibition constants.[25]

Transepithelial flux experiments at Caco-2 cell monolayers were performed at day 21 after seeding at 37 °C in a shaking table incubator.[25,28,33] In brief, after washing of the inserts with buffer [Hepes/Tris (pH 7.5, 25 mM), NaCl (140 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgSO_4$ (0.8 mM), glucose (5 mM)], uptake was started by addition of uptake buffer (pH 6.0, 1.5 mL) containing compounds (1 mM) to the donor side. After 10, 30, 60, and 120 min, samples (200 μL) were taken from the receiver compartment (2.6 mL) and replaced with fresh buffer (pH 7.5). Samples were stored at −20 °C until analysis by HPLC. After 2 h, the filters were quickly washed four times with ice-cold uptake buffer, cut out of the plastic inserts and stored in TCA solution (10%, 1 mL), which was frozen and defrosted three times. Before HPLC and AAA, the samples were diluted appropriately with the solvent A and loading buffers, respectively.

Data analysis: Results are given as means ±SEM (n = 4 to 9). IC_{50} values (that is, concentration of unlabeled compounds necessary to inhibit 50% of [^{14}C]Gly-Sar or L-[^{3}H]lysine carrier-mediated uptake) were determined by nonlinear regression by using the logistic Equation (1) for an asymmetric sigmoid (allosteric Hill kinetics):

$$Y = \mathrm{Min} + \frac{\mathrm{Max}-\mathrm{Min}}{1 + (X/\mathrm{IC}_{50})^P} \quad (1)$$

where Max is the initial Y value, Min the final Y value, and the power P represents Hill's coefficient (SigmaPlot program, Systat, Erkrath, Germany), and converted into inhibition constants (K_i).[33] Flux data were calculated after correction for the removed amounts by linear regression of appearance in the receiver well versus time.

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