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Protease-catalysed synthesis of peptides containing histidine and lysine

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Abstract

The kinetically controlled α-chymotrypsin- and trypsin-catalysed syntheses of peptides starting from simple acyl donor esters containing histidine at the P_1-position (nomenclature according to Schechter and Berger[1]) and lysine derivatives as amino components were examined on the basis of their kinetic parameters. Despite higher specificity constants (k_{cat}/K_M) of trypsin-catalysed ester hydrolysis, α-chymotrypsin-catalysed acyl transfer to N^{ε}-unprotected lysine derivatives gave higher peptide yields as compared to trypsin-catalysed reactions, whereas in acyl transfer to N^{ε}-protected lysine derivatives the trypsin-catalysed reaction gave higher yields. α-Chymotrypsin-catalysed acyl transfer reactions in frozen systems demonstrated the yield-enhancing effect of freezing. Using specific ester leaving groups, both the amount of enzyme and the reaction time can be reduced. In frozen systems the ε-amino function of H–Lys–OH acts as an acyl acceptor at pH ≥9.

1. Introduction

The biological activity of peptides containing His-Lys sequences is based on their function as a carrier for ionic copper(II).[2] In particular, the tripeptide H–Gly-His-Lys–OH has been reported to have broad biological activity. This tripeptide is believed to influence the growth of a variety of differentiated cells and is suggested to have an important role in several physiological processes, e.g. wound healing, tissue repair or immunostimulation.[3]

Due to the stereo- and regiospecificity of enzymes there is no need for side chain protection and, furthermore, the danger of racemisation is excluded. The main goals in thermodynamically and kinetically controlled syntheses are the equilibrium shift towards the peptide products, and the prevention of undesired hydrolysis of the acyl donor and proteolysis of peptide products, respectively. A broad

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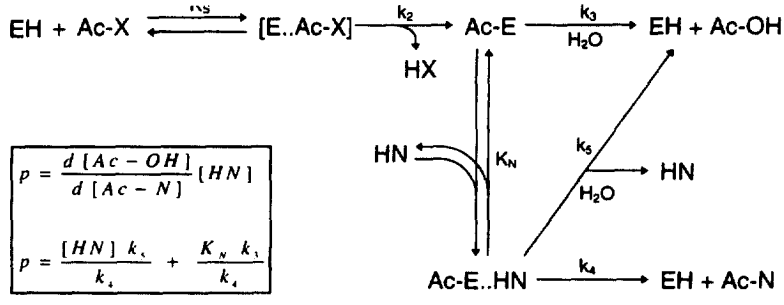
methodological repertoire of media-, substrate- and enzyme-engineering[4–11] has been published. In kinetically controlled processes, which are limited to serine and cysteine proteinases, less enzyme and shorter reaction times are required. Studies of the catalytic mechanism of serine and cysteine proteinases have revealed that the first step of an acyl transfer reaction is the acylation of the enzyme by a weakly activated amino acid or peptide ester, respectively. In the presence of a nucleophilic amino component, the acyl-enzyme intermediate is deacylated competitively by water and the added nucleophile, as shown in Scheme 1.[12]



Scheme 1. Kinetic scheme of protease-catalysed acyl transfer reactions: EH=free enzyme; Ac-X=acyl donor; E· · ·Ac-X=Michaelis complex; HX=leaving group; AcOH=hydrolysis product; Ac-N=aminolysis product; HN=nucleophile; Ac-E=acyl-enzyme; Ac-E· · ·HN=acyl-enzyme–nucleophile complex

The properties of acyl donors can be expressed by the kinetic parameters of enzymatic hydrolysis.[13] The efficiency of the nucleophilic amino component to an acyl-enzyme is expressed by the partition values (p). The p value was introduced as a parameter for estimating nucleophile reactivity and characterizing the S′ binding side of an enzyme. The partition value corresponds to the nucleophile concentration at which aminolysis and hydrolysis proceed with the same velocity.[14,15]

In the present paper we report on the results of α-chymotrypsin- and trypsin-catalysed formation of His-Lys bonds both in aqueous and frozen aqueous systems.

2. Results and discussion

2.1. α-Chymotrypsin- and trypsin-catalysed hydrolysis of acyl donor esters containing histidine in the P_1-position

Firstly, the α-chymotrypsin- and trypsin-catalysed hydrolysis of a series of acyl donors containing histidine in the P_1 position and various protecting and leaving groups was examined. The kinetic parameters of hydrolysis are compiled in Table 1. Comparing both enzymes, the acyl donor esters used were slightly better substrates for trypsin in comparison with α-chymotrypsin. In trypsin-catalysed hydrolysis, the specificity constants (k_{cat}/K_M) were about one order of magnitude higher compared with the corresponding data of α-chymotrypsin-catalysed reactions. The use of the 4-nitrobenzyl ester leaving group instead of the methyl ester moiety led to higher values of k_{cat}/K_M for both α-chymotrypsin- and trypsin-catalysed hydrolysis. These results are in agreement with literature data.[13] In the case of α-chymotrypsin-catalysed hydrolysis of Y–His–OR (Y=Mal, Z–Gly; R=Me, Nb),† K_M decreases with increasing hydrophobicity of the leaving group. On the other hand, there are increasing k_{cat} values.

† *Abbreviations*: ACN, acetonitrile; Boc–, tert-butyloxycarbonyl; $BzSO_3H$, benzylsulfonic acid; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic) acid; Mal–, maleyl; Mca–,

Table 1
Kinetic constants of α-chymotrypsin- and trypsin-catalyzed hydolysis

Substrate	Protease	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
Mal-His-OMe	α-Chymotrypsin	32	1.9	$5.94 * 10^1$
Mal-His-ONb		1.1	8.2	$7.45 * 10^3$
Boc-His-OMe		10.1	4.6	$4.60 * 10^2$
Z-His-OMe		2.8	0.38	$1.36 * 10^2$
Z-Gly-His-OMe		4.3	1.50	$3.49 * 10^2$
Z-Gly-His-ONb		0.24	11.6	$4.75 * 10^4$
Mca-His-ONb		0.31	10.4	$3.35 * 10^4$
Mal-His-OMe	Trypsin	---	---	$3.4 * 10^2$
Mal-His-ONb		0.92	12.5	$1.36 * 10^4$
Mca-His-ONb		0.44	51	$1.16 * 10^5$

Conditions: pH 7.5, ϑ = 25 °C, I = 0.2 M (NaCl), 10 % (v/v) DMF, substrate concentration: 0.2 to 5 K_M; Mal, maleyl; Mca, monochloracetyl.

From these data an improved suitability of histidine subtrates bearing hydrophobic leaving groups can be concluded.

2.2. *α-Chymotrypsin- and trypsin-catalysed acyl transfer of histidine esters to lysine derivatives*

In order to compare both enzymes, we examined the α-chymotrypsin- and trypsin-catalysed acyl transfer of histidine esters (Mal–His–ONb, Z–His–OMe, Z–Gly-His–ONb) to H–Lys–NH_2, H–Lys–OMe and H–Lys(Z)–NH_2. The results are summarized in Table 2. From these data it can be established that due to the S_1'-specificity of α-chymotrypsin for basic amino acids[15], α-chymotrypsin favoured the N^{ε}-unprotected nucleophiles H–Lys–NH_2 and H–Lys–OMe, whereas trypsin-catalyzed acyl transfer to H–Lys(Z)–NH_2 provided the highest efficiency.

The results of chymotrypsin- and trypsin-catalysed syntheses of Mal–His-Lys–NH_2, Mal–His-Lys(Z)–NH_2 and Mal–His-Lys–OMe were reflected by their partition values for the corresponding acyl transfer reaction, as shown in Table 3.

According to the S_1' specificity of α-chymotrypsin, H–Lys–NH_2 acts as an efficient acyl acceptor whereas H–Lys(Z)–NH_2 is a less efficient nucleophile followed by H–Lys–OMe. The lower partition value for H–Lys–NH_2 is well documented in the literature[15] and is caused mainly by a hydrogen bond formed between the amide group at the P_2'-position and Phe-41 of α-chymotrypsin, which plays an important role in the orientation of the nucleophile.[16,17] Besides favourable electrostatic interactions,[15] a second hydrogen bond is formed between the side chain amino group of lysine and Cys-58 of the enzyme in α-chymotrypsin-catalysed reactions.[18] We propose that in our model reactions H–Lys–NH_2

monochloracetyl; –NH_2, amide; Nps–, 2-nitrophenylsulfenyl; –OCam, carboxamido methyl ester; –OMe, methyl ester; –ONb, 4-nitrobenzyl ester; TFA, trifluoroacetate; TPCK, N-tosyl-L-phenylalanine chloromethylketone; Z–, benzyloxycarbonyl.

Table 2
Protease-catalysed condensation of histidine esters with lysine derivatives

Nucleophile	Peptide	Leaving group	Peptide yield (%)	
			α-Chymotrypsin	Trypsin
H-Lys-NH_2	Mal-His-Lys-NH_2[1)]	-ONb	94[1)]	67[4)]
	Z-His-Lys-NH_2[2)]	-OMe	88[2)]	
	Z-Gly-His-Lys-NH_2[2)]	-ONb	77[3)]	
H-Lys-OMe	Mal-His-Lys-OMe[1)]	-ONb	23[1)]	8[4)]
H-Lys(Z)-NH_2	Mal-His-Lys(Z)-NH_2[1)]	-ONb	64[1)]	80[4)]

Conditions: [Acyl donor] = 2 mM, [Nucleophile] = 50 mM, [1)][α-Chymotrypsin] = 0.46 μM, [2)][α- Chymotrypsin] = 9.27 μM, [3)] [α-Chymotrypsin] = 0.37 μM, [4)] [Trypsin]= 0.2 μM, 10 % (v/v) DMSO, pH 9.5 (0.1M Carbonate buffer), I = 0.25 (NaCl), ϑ = 25°C; all results after full ester consumption.

Table 3
p_0-Values of α-chymotrypsin- and trypsin-catalysed acyl transfer of Mal–His–ONb to lysine derivatives

Nucleophile	$p_0 = K_N k_3/k_4$ (mM)	
	α-Chymotrypsin	Trypsin
H-Lys-NH_2	5.6 ± 0.1	37.4 ± 0.6
H-Lys-OMe	37.0 ± 1	11.3 ± 0.4
H-Lys(Z)-NH_2	73.0 ± 4	464 ± 161

Conditions: [Mal-His-ONb] = 2mM, [Nucleophile] = 2-100mM, [α-Chymotrypsin] = 0.46 μM, [Trypsin] = 0.2 μM, 10 % (v/v) DMSO, pH 9.5 (0.1M, Carbonate buffer), I = 0.25 (NaCl), ϑ = 25°C.

and H–Lys(Z)–NH_2 have favourable orientations as a consequence of forming both of these hydrogen bonds. The comparatively low partition value of H–Lys–OMe, which is not typical for nucleophilic ester substrates, is based on these favourable interactions in the S_1' binding region. The partition value obtained for the α-chymotrypsin-catalysed acyl transfer of Mal–His–ONb to H–Lys–NH_2 corresponds to acyl transfer data of Mal–Phe–OMe to H–Arg–OMe.[17]

The lower efficiency of the N^{ε}-unsubstituted lysine derivatives H–Lys–NH_2 and H–Lys–OMe in trypsin-catalysed acyl transfer leads to higher partition values compared with α-chymotrypsin-catalysed reactions. Comparing both enzymes, the affinity of trypsin for H–Lys–NH_2 is 6.7 times less efficient than for α-chymotrypsin, while H–Lys–OMe differs by about one order of magnitude. The efficiency of H–Lys(Z)–NH_2 indicated for trypsin-catalysed reactions is higher than for unprotected H–Lys–NH_2 which corresponds to the partition values found in trypsin-catalysed acyl transfer of Nps–Arg–OMe to H–Lys-Leu–NH_2 and H–Lys(Ac)-Leu–NH_2,[19] respectively. According to published data[17] for nucleo-

Table 4
α-Chymotrypsin-catalysed dipeptide synthesis using Mal–His–OR (R=Nb, Me) and lysine derivatives as nucleophiles at 25°C compared with −25°C

Substrate	Nucleophile	[CT] (μM)	Time (min) +25°C	Time (h) -25°C	Yield (%) +25°C	Yield (%) -25°C
Mal-His-ONb	H-Lys-NH_2	15.2	5	3	94	97
	H-Lys(Z)-NH_2	15.2	60	47	64	93
	H-Lys-OMe	15.2	2	7	8	49
	H-Lys-OH	15.2	3	24	0	10
Mal-His-OMe	H-Lys-NH_2	59.7	120	50	92	98
	H-Lys(Z)-NH_2	120.7	120	240	53	75[a]

Reaction conditions: [Mal-His-OR] = 2 mM, [Nucleophile] = 50 mM, 10 % (v/v) DMSO; pH 9; (+25°C: 0.1 M Carbonate buffer, -25°C: adjusted with 1 N NaOH before freezing), [a] 82 % ester consumption.

philic ester substrates, the absence of this important hydrogen bond mentioned above in the case of H–Lys–OMe causes the drastic increase of the partition value.

2.3. *The effect of freezing in chymotrypsin-catalysed condensations of Mal–His–ONb with lysine derivatives*

It has been shown previously that freezing the reaction mixture can increase the peptide yield in protease-catalysed peptide syntheses also in the case of less efficient nucleophiles, e.g. free amino acids and amino acid esters (for a review, see the literature[20]).

We investigated the α-chymotrypsin-catalysed acyl transfer of Mal–His–OR (R: Me, Nb) to different lysine derivatives (H–Lys–NH_2, H–Lys(Z)–NH_2, H–Lys–OMe, H–Lys–OH). The results of α-chymotrypsin-catalysed reactions at room temperature compared with those in frozen aqueous systems are shown in Table 4. These data clearly demonstrate that freezing could also improve the peptide yield when less efficient nucleophiles were used. We found a slight increase of peptide yield when using a more specific acyl donor, Mal–His–ONb at room temperature compared with Mal–His–OMe. In frozen systems, the synthesis rate can be increased by the application of specific leaving groups. This effect is very important for practical purposes.

2.4. *Regiospecificity of chymotrypsin-catalysed coupling of Mal–His–ONb with N^{ε}-unprotected lysine derivatives*

It is well established that freezing can induce a dramatic change in the specificity of enzymes.[20] Due to this shift of specificity, we investigated the effect of freezing and pH on α-chymotrypsin-catalysed coupling of Mal–His–ONb with N^{α},N^{ε}-unprotected lysine derivatives H–Lys–NH_2 and H–Lys–OH. As shown in Fig. 1, we found that α-chymotrypsin-catalysed synthesis of Mal–His-Lys–NH_2 gave one product in high yield independent of the temperature and pH. In the acyl transfer reaction from

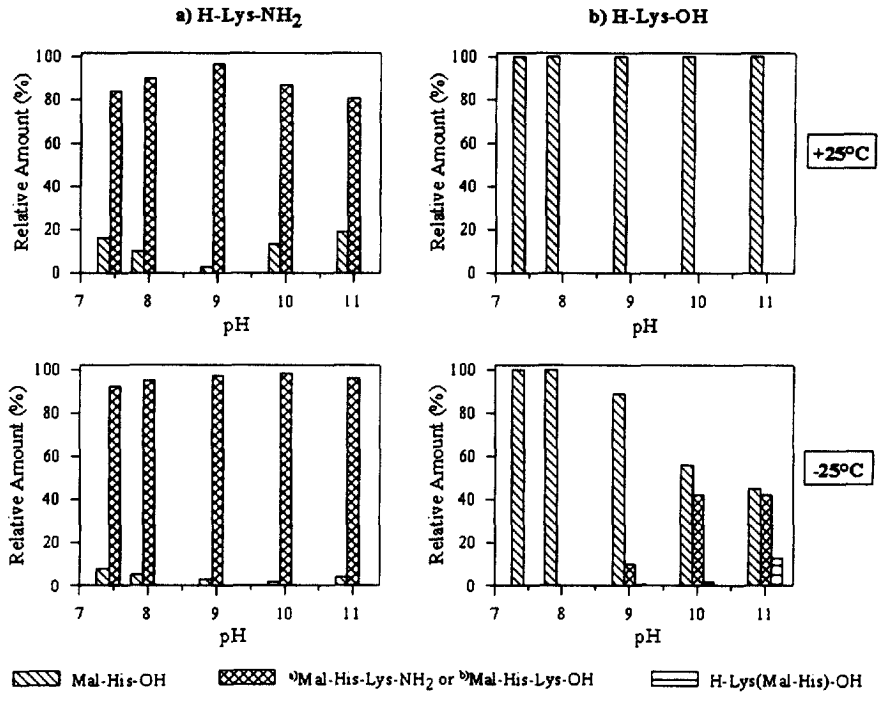


Fig. 1. Dependence of regiospecificity of acyl transfer from Mal–His–ONb to H–Lys–NH_2 and H–Lys–OH on temperature and pH. Reaction conditions: [Mal–His–ONb]=2 mM, [nucleophile]=50 M, 10% (v/v) DMSO; pH 9; (+25°C: 0.1 M carbonate buffer; −25°C: adjusted with 1 N NaOH before freezing); [α-chymotrypsin]=9.27 μM

Table 5
^{1}H-NMR chemical shift data of α-methine and ε-methylene protons in lysine (solvent D_2O)

Compound	Chemical shift (ppm)	
	α-Methine	ε-Methylene
H-Lys-NH_2.2 HCl	4.00	2.98
Mal-His-Lys-NH_2.2 HCl	4.34	3.05
H-Lys-OH.2 HCl	3.93	3.08
Mal-His-Lys-OH.2 HCl	4.42	3.04
H-Lys(Mal-His)-OH.2 TFA	4.00	3.26

Mal–His–ONb to H–Lys–OH, no product was detected at room temperature. In contrast, we observed the formation of two products at pH ≥9 in frozen reaction mixtures.

Comparing the ^{1}H-NMR chemical shift data of α-methine and ε-methylene protons of the lysine moiety of isolated products with those of lysine compounds, a significant downfield shift was observed for signals of α-methine protons of the product of acyl transfer to lysine amide and of the main product of acyl transfer to lysine, while chemical shifts of ε-methylene protons showed small differences. The proton signals of the α-methine and ε-methylene groups of the by-product in acyl transfer reactions to lysine indicated a strong downfield shift of ε-methylene protons and no significant change in α-methine signals (see Table 5).

These data verify the formation of α-peptide bonds using H–Lys–NH_2 as the amino component. The main product of acyl transfer reactions to H–Lys–OH in a frozen system was Mal–His-Lys–OH. The formation of the ε-isomer H–Lys(Mal–His)–OH as a by-product was observed at pH ≥9. The amount of the ε-isomer in the reaction mixture increased with increasing pH and deprotonation of the ε-amino group, respectively. We assume that this undesired side reaction is caused by an unfavourable orientation of H–Lys–OH in the active site since no hydrogen bond can be formed between this substrate and Phe-41 of α-chymotrypsin. In addition, unfavourable ionic interactions between the carboxyl group of lysine and the S′ subsite of α-chymotrypsin may occur.

3. Conclusions

The results of the present study show that the stereo- and regiospecificity of proteases can be favourably exploited for the synthesis of peptides containing His-Lys sequences. α-Chymotrypsin and trypsin are capable of hydrolysing esters containing a histidine moiety at the P_1-position and catalyse acyl transfer to N^{ε}-protected and N^{ε}-unprotected lysine amides. In frozen aqueous reaction mixtures even inefficient amino components, such as H–Lys–OMe and H–Lys–OH, were accepted by α-chymotrypsin, demonstrating the yield-enhancing effect of freezing. Rates of peptide synthesis could be markedly increased using specific hydrophobic leaving groups. This finding is very important for practical purposes, since side reactions can be suppressed and the reaction time can be dramatically shortened. In frozen aqueous systems, unprotected N^{ε}-amino functions of inefficient lysine derivatives provide ε-isomers at pH ≥9 as by-products.

4. Experimental

4.1. Apparatus, materials and methods

Melting points were determined on a Boetius heating block and are uncorrected. The NMR spectra were obtained with a Varian Genius 300 spectrometer using $CDCl_3$, D_2O or DMSO-d_6 as internal standards. Mass spectra were obtained with a Hewlett–Packard VG ZAB-HSQ (FAB method), a 5989 A (thermospray method) and a Kratos Kompakt MALDI 5 V5.1.2 (MALDI method), respectively. Elementary analyses were performed on a Heraeos Analyser CHN-O Rapid.

4.2. Chemicals and enzymes

α-Chymotrypsin (EC 3.4.21.1) was a product of Serva (Germany), trypsin (EC 3.4.21.2, TLCK-treated) was purchased from Fluka (Germany). Both enzymes were used without further purification. Normalities of enzyme-stock solutions were determined by active site titration according to general procedures described to be 61% (α-chymotrypsin, 4-nitrophenylacetate) and 50% (trypsin, 4-nitrophenyl-4′-guanidinobenzoate), respectively. Amino acids, amino acid amides and Boc–His–OMe were used from Bachem (Switzerland). H–Lys(Z)–OH was a generous gift from Degussa AG Hanau (Germany). Z–His–OH was a product of Rexim (France). All other substances were synthesized as described below.

4.3. Specificity studies

The specificity constants of substrates were determined potentiometrically using a pH-stat video titrator VIT 90 (Radiometer, Copenhagen). The reactions were carried out at 25°C and pH 7.5. The total volume was adjusted to 2 ml, containing 0.2 M NaCl and 10% (v/v) DMF. Substrate concentrations were varied from 0.2 K_M to 5 K_M. Reactions were started by addition of the enzyme solution. A final pH of 7.5 was adjusted by addition of 0.02 N NaOH. The kinetic parameters K_M and V_{max} were obtained by nonlinear curve fitting of the data (ENZFITTER, Elsevier Biosoft, Cambridge, UK). The k_{cat} values were determined as a value of $V_{max}/[E_0]$.

4.4. HPLC

The samples were analysed by RP-HPLC. Analyses were performed using a gradient system (Shimadzu LC 6A, Shimadzu, Japan) equipped with an autoinjector SIL-6A, 2 pumps LC-6A, UV-detector SPD-6AV, a system controller SCL-6A and an integrator S-R4A chromatopac. A Lichrosorb RP-18 column with 7 μm particle size (250×4 mm^2; E. Merck, Germany) and an Impaq RP 18 column with 10 μm particle size (250×4 mm^2; Bischoff, Germany) were used. Samples were eluted at a flow rate of 1 ml/min by a gradient elution system using mixures of 2–60% acetonitrile in 0.1% aqueous trifluoroacetic acid (Mal–His-Lys(Z)–NH_2) and 2–35% methanol in 0.1% aqueous trifluoroacetic acid (all other peptides) respectively. The substrate and peptide ratio was monitored at 254 nm. When the hydrolysis and aminolysis products contained the same chromophoric group, the molar extinction coefficients were assumed to be equal. When the acyl donor contained the 4-nitrobenzyl leaving group, the ratio of absorbtion coefficients was determined.

Preparative RP-HPLC was perfomed on the HPLC system described above using an Impaq RG 1010 C-18 column with 10 μm particle size (250×12.5 mm^2; Bischoff, Germany) with a precolumn at a flow rate of 2.5–5 ml/min. Absorbance was monitored at 254 nm.

4.5. Peptide syntheses on an analytical scale

Peptide synthesis reactions at −25°C were performed in 1.5 ml polypropylene tubes at a total sample volume of 0.1 ml. The nucleophile (5 μmol) dissolved in 80 μl of water, was adjusted with 1 N NaOH to give final pHs of 7.5, 8.0, 9.0, 10.0 and 11.0, respectively. After addition of the reaction components in the order enzyme (10 μl; concentrations see tables) and acyl donor (0.2 μmol of acyl donor dissolved in 10 μl of DMSO), the tubes were shaken and placed in liquid nitrogen for 20 s to achieve shock-freezing. Then they were transferred for the time of reaction into a constant temperature cryostat (HAAKE, Germany). After a predefined time, the reactions were stopped by adding 0.8 ml of 0.7 N acetic acid in water. The reactions at room temperature were performed in the same manner as described without freezing using a total volume of 1 ml, containing 0.1 M phosphate buffer and carbonate buffer, respectively.

4.6. Determination of partition values

Acyl transfer reactions were carried out at 25°C in 1 ml of solution containing 0.1 M carbonate buffer (pH 9.5) and 0.25 M NaCl. The stock solutions of acyl donor (20 mM in DMSO), nucleophile (100 mM in water) and enzyme (46 μM α-chymotrypsin and 20 μM trypsin, respectively) were prepared daily. After mixing the stock solutions of the nucleophile (5–250 μl) and enzyme (10 μl), the reactions were started by addition of 100 μl of acyl donor stock solution to give a final acyl donor concentration of 2 mM and

nucleophile concentrations of 2 mM, 4 mM, 10 mM, 20 mM, 50 mM and 100 mM, respectively. When H–Lys–OMe was used as nucleophile, the reaction was started by addition of 10 μl of enzyme stock solution. After 5–15% ester consumption, a sample was diluted in 0.7 N acetic acid and analysed by RP-HPLC as described above. In order to determine non-enzymatic ester hydrolysis, a blank experiment without the enzyme was perfomed. Three independent reactions with equal starting concentrations were carried out. Calculation of partition values p_0 was perfomed using the method of Schellenberger et al.[21]

4.7. *Mal–His–OMe*

Gaseous NH_3 (dried over NaOH) was bubbled through a suspension of 6.03 g (25 mmol) of H–His-OMe·2HCl in 145 ml of dry $CHCl_3$ for 15 min at 0°C. The precipitated NH_4Cl was filtered off and washed three times with 10 ml of dry $CHCl_3$. The filtrate was evaporated, the obtained oily residue was dissolved in 10 ml of dry methanol and evaporated again to remove all NH_3. The clear oil was dissolved in 50 ml of dry $CHCl_3$, then a solution of 2.5 g (25 mmol) of maleic anhydride in 20 ml of dry ether was added. The reaction mixture was stirred for 2.5 h at room temperature, then 70 ml of ether was added and the precipitate formed was filtered off. The product was washed twice with 10 ml of ether, dried over KOH/P_2O_5 and crystallized twice from methanol containing 1% water, $C_{11}H_{13}N_3O_5$=267.24. Yield: 4.27 g (64%); m.p.: 136–138°C; m/z (FAB): 268 $(M+H)^+$; ^{1}H-NMR (400 MHz; D_2O): 3.12–3.26 (m, 2H, His–$C^\beta H_2$), 3.67 (s, 3H, O–CH_3), 4.70–4.76 (m, 1H, His–C^αH), 5.87 (d, 1H, HOOC–C*H*=), 6.25 (d, 1H, =C*H*–CO–NH–), 7.19 (s, 1H, >C=C*H*–NH–), 8.47 (s, 1H –N=C*H*–NH–). Anal. calcd: C, 49.44; H, 4.90; N, 15.72; found: C, 48.83; H, 4.99; N, 15.32.

4.8. *Mal–His–ONb*

The preparation of Mal–His–ONb was performed analogously to the procedure described for Mal–His–OMe starting from 6.06 g (10 mmol) of H–His–ONb·2BzSO_3H and 1.0 g (25 mmol) of maleic anhydride to give Mal–His–ONb. The product was crystallized from methanol, $C_{17}H_{16}N_4O_7$=388.34. Yield: 2.82 g (67%); m.p.: 119–121°C; m/z (FAB): 389 $(M+H)^+$; ^{1}H-NMR (400 MHz; DMSO-d_6): 2.93–3.08 (m, 2H, His–$C^\beta H_2$), 4.68 (t, 1H, His–C^αH), 5.25 (s, 2H, –OCH_2–), 6.28 (s, 2H, HOOC–C*H*=C*H*–CONH–), 6.89 (s, 1H, >C=C*H*–NH–), 7.53 (d, 2H, =C*H*–C(CH_2–)=C*H*–), 7.68 (s, 1H –N=C*H*–NH–), 8.21 (d, 2H, =CH–C(NO_2)=CH–), 9.50 (1H, brs, –NH–). Anal. calcd: C, 50.25; H, 4.64; N, 13.75; found: C, 50.94; H, 4.58; N, 14.09.

4.9. *Mca–His–ONb*

The synthesis of Mca–His–ONb was similar to the preparation of Mal–His–OMe starting from 3.03 g (5 mmol) of H–His–ONb·2BzSO_3H and 0.85 g (5 mmol) of monochloracetic acid anhydride to give Mca–His–ONb. The crude product was crystallized from acetyl acetate/methanol/n-hexane, $C_{15}H_{15}N_4O_5Cl$=366.45. Yield: 0.68 g (42%); m.p.: 120–124°C; m/z (TS-POS): 367 $(M+H)^+$; ^{1}H-NMR (300 MHz; DMSO-d_6): 3.00 (d, 2H, His–C^βH), 4.13 (s, 2H, Cl–CH_2–), 4.65 (q, 1H, His–C^αH), 5.25 (s, 2H, –OCH_2–), 6.87 (s, 1H, >C=C*H*–NH–), 7.53 (d, 2H, =C*H*–C(CH_2–)=C*H*–), 7.67 (s, 1H –N=C*H*–NH–), 8.21 (d, 2H, =C*H*–C(NO_2)=C*H*–), 8.78 (1H, d, –NH–). Anal. calcd: C, 49.12; H, 4.09; N, 15.28; found: C, 48.5; H, 4.33; N, 14.56.

4.10. Z–His–OMe

Z–His–OH (1 g, 3.46 mmol) was suspended in 50 ml of dry methanol at 0°C. Then 0.93 ml (12.7 mmol) of $SOCl_2$ were added slowly. The reaction mixture was incubated under stirring for 1 h at 0°C, then 20 h at room temperature. The solvent was evaporated, the oily residue was dissolved in 5 ml of methanol and the pH was adjusted to 7 with triethylamine. After adding 150 ml of dry ether, triethylamine hydrochloride precipitated. The salt was filtered off and washed twice with 10 ml of dry ether. The filtrate and the washings were evaporated and the oily residue was washed several times with ether containing n-hexane to give solid Z–His–OMe, $C_{15}H_{17}N_3O_4$=303.32. Yield: 0.84 g (42%); m.p.: 72–73°C; m/z (TS-POS): 304 $(M+H)^+$; ^{1}H-NMR (300 MHz; DMSO-d_6): 2.80–8.98 (m, 2H, His–C$^\beta$H), 3.60 (s, 3H, O–CH_3), 4.28–4.38 (dd, 1H, His–C$^\alpha$H), 5.01 (s, 2H, –OCH_2–), 6.83 (s, 1H, >C=C*H*–NH–), 7.28–7.41 (m, 5H, –C_6H_5), 7.59 (s, 1H –N=C*H*–NH–), 7.67 (d, 1H, –NH–). Anal. calcd: C, 59.40; H, 5.65; N, 13.85; found: C, 59.22; H, 5.07; N, 14.09.

4.11. Z–Gly-His–OMe

Z–Gly–OCam (0.222 g, 0.836 mmol; prepared using a modified procedure according to Martinez et al.[22] by reaction of N$^\alpha$-benzyloxycarbonylglycine with iodoacetamide in the presence of dicyclohexylamine in dry dioxan) was suspended in 125 μl of HEPES/Na^+-buffer (1 M, pH 7.9), containing 0.202 g (0.835 mmol) of H–His–OMe·2HCl. Concentrated aqueous ammonium hydroxide solution (225 μl) was used to release the nucleophile from its salt and to adjust the pH. After addition of 12 μl of Triton X-100 the reaction was started by addition of a solution of 40 mg papain and 8 mg DTE in 125 μl HEPES/Na^+ (1 M, pH 7.9) and 18 μl EDTA (4.67 mM). The reaction mixture was intensively mixed. After complete ester consumption, the reaction mixture was dissolved in 20 ml of methanol and heated to inactivate the enzyme. After filtration, the product was isolated by column chromatography (silica gel 60, 70–230 mesh ASTM; E. Merck, Germany) using $CHCl_3$ containing 5–15% (v/v) methanol as eluents to give Z–Gly-His–OMe, $C_{17}H_{20}N_4O_5$=360.35. Yield: 0.178 g (59%); m/z (MALDI): 361.89 $(M+H)^+$; ^{1}H-NMR (300 MHz; $CDCl_3$): 3.05 (d, 2H, His–C$^\beta$H), 3.62 (s, 3H, O–CH_3), 3.81 (d, 2H, Gly–CH_2), 4.74 (dd, 1H, His–C$^\alpha$H), 5.06 (s, 2H, –OCH_2–), 6.28 (brs, 1H, –NH–), 6.72 (s, 1H, >C=C*H*–NH–), 7.21–7.37 (brs, 5H, –C_6H_5), 7.21–7.37 (d, 1H, –N=C*H*–NH–), 7.75 (d, 1H, –NH–), 8.29 (brs, 1H, NH).

4.12. Z–Gly-His–ONb

The dipeptide was prepared as described for Z–Gly-His–OMe starting from 400.8 mg (1.67 mmol) of Z–Gly–OCam, 1.063 g (1.67 mmol) of H–His–ONb·2BzSO_3H and 80 mg of papain. After filtration of denaturated enzyme, the product was precipitated by addition of three volumes of distilled water. After completing the precipitation at 4°C overnight, the peptide product was filtered off and dried over P_2O_5, $C_{23}H_{23}N_5O_7$=481.47. Yield: 1.26 g (78%); m.p.: 125–130°C; m/z (TS-POS): 482 $(M+H)^+$; ^{1}H-NMR (300 MHz; DMSO-d_6): 2.98 (d, 2H, His–C$^\beta$$H_2$), 3.67 (d, 2H, Gly–$CH_2$), 4.58–4.67 (m, 1H, His–C$^\alpha$H), 5.05 (s, 2H, O–C*H*$_2$–CO–NH–), 5.22 (s, 2H, O–C*H*$_2$–C_6H_4–NO_2), 6.83 (s, 1H, >C=C*H*–NH–), 7.22–7.43 (m, 5H, –C_6H_5), 7.44–7.60 (brd, 4H, =C*H*–CH=C(NO_2)–CH=C*H*–, –N=C*H*–NH– and –NH–), 8.20 (d, 2H, –C*H*=C(NO_2)–C*H*), 8.44 (d, 1H, –NH–). Anal. calcd: C, 57.38; H, 4.82; N, 14.55; O, 23.26; found: C, 57.69; H, 5.14; N, 15.37; O, 22.90.

4.13. H–Lys(Z)–NH_2·HCl

H–Lys(Z)–OH (1.4 g, 5 mmol) in 15 ml of dry methanol was reacted with 1.3 ml of $SOCl_2$ as described for Z–His–OMe. After removal of methanol and $SOCl_2$, the product was fractionally crystallized from methanol/ether. Yield: 1.15 g (77%) H–Lys(Z)–OMe·HCl [m.p.: 110–113°C, ^{1}H-NMR (400 MHz, DMSO): 1.22–1.32 (m, 2H, Lys–C$^{\gamma}$H$_2$), 1.32–1.46 (m, 2H, Lys–C$^{\delta}$H$_2$), 1.78 (q, 2H, Lys–C$^{\beta}$H$_2$), 2.97 (q, 2H, Lys–C$^{\varepsilon}$H$_2$), 3.72 (s, 3H, –OCH$_3$), 3.96 (t, 1H, Lys–C$^{\alpha}$H), 4.99 (s, 2H, –C*H*$_2$–O–CO–NH–), 7.26–7.38 (m, 5H, –C$_6$H$_5$), 8.62 (brs, 2H, –NH$_2$)]. H–Lys(Z)–OMe·HCl (1 g, 3.45 mmol) was dissolved in 50 ml of methanol (saturated at 0°C with dry NH_3) and the mixture was allowed to stand for 8 days at room temperature. After removal of methanol and NH_3 the solid product was crystallized twice from methanol:ether (1:5), $C_{14}H_{22}N_3O_3Cl$=315.78. Yield: 0.75 g (77%); m.p.=144–152°C, m/z (FAB): 280 $(M+1)^+$, ^{1}H-NMR (300 MHz, DMSO): 1.25–1.35 (m, 2H, Lys–C$^{\gamma}$H$_2$), 1.35–1.50 (m, 2H, Lys–C$^{\delta}$H$_2$), 1.67–1.78 (q, 2H, Lys–C$^{\beta}$H$_2$), 2.92–3.04 (q, 2H, Lys–C$^{\varepsilon}$H$_2$), 3.68 (t, 1H, Lys–C$^{\alpha}$H), 5.00 (s, 2H, –C*H*$_2$–O–CO–NH–), 7.23–7.41 (m, 5H, –C$_6$H$_5$), 7.50 (s, 1H, –NH–), 7.93 (s, 2H, –NH$_2$), 8.1 (brs, 2H, –NH$_2$).

4.14. Mal–His-Lys–NH_2

H–Lys–NH_2 (109 mg, 0.5 mmol) and 4.7 mg of α-chymotrypsin were dissolved in 2.5 ml of carbonate buffer (0.2 M, pH 9). The reaction was started by addition of 101.5 mg (0.25 mmol) of Mal–His–ONb in 1 ml DMSO. The mixture was stirred for 1 h at 25°C. After complete ester consumption, 7 ml of 0.7 N acetic acid were added to inactivate the enzyme. The enzyme was filtered through a polyester membrane (pore size 0.4 μm, Reichelt Chemietechnik, Germany) and the filtrate was freeze-dried. Elution from silica gel 60 (70–230 mesh ASTM; E. Merck, Germany) using methanol:$CHCl_3$:8% aqueous NH_3 (5.1:3.4:1.5) provided the pure dipeptide, $C_{16}H_{24}N_6O_5$=380.39. Yield: 65 mg (68%); m.p.: 290°C (decomp.); m/z (TS-POS): 381 $(M+H)^+$; ^{1}H-NMR (300 MHz; D_2O): 1.32–1.60 (m, 2H, Lys–C$^{\gamma}$H$_2$), 1.64–1.78 (m, 2H, Lys–C$^{\delta}$H$_2$), 1.78–2.02 (m, 2H, Lys–C$^{\beta}$H$_2$), 3.02 (t, 2H, Lys–C$^{\varepsilon}$H$_2$), 3.13–3.37 (brm, 2H, His–C$^{\beta}$H), 4.30 (dd, 1H, Lys–C$^{\alpha}$H), 4.71 (t, 1H, His–C$^{\alpha}$H), 6.02 (d, 1H, HOOC–C*H*=), 6.35 (d, 1H =C*H*–CO–NH), 7.26 (s, 1H, >C=C*H*–NH–), 7.99 (s, 1H, –N=C*H*–NH–). Preparation of dihydrochloride was carried out by solvation of 10 mg of Mal–His-Lys–NH_2 in 0.2 ml of 0.4 N HCl and freeze-drying. ^{1}H-NMR (300 MHz; D_2O): 1.38–1.61 (m, 2H, Lys–C$^{\gamma}$H$_2$), 1.65–1.97 (m, 4H, Lys–C$^{\delta}$H$_2$ and Lys–C$^{\beta}$H$_2$), 3.05 (t, 2H, Lys–C$^{\varepsilon}$H$_2$), 3.10–3.42 (brm, 2H, His–C$^{\beta}$H), 4.34 (dd, 1H, Lys–C$^{\alpha}$H), 4.71 (His–C$^{\alpha}$H), 6.33 (d, 1H, HOOC–C*H*=), 6.41 (d, 1H, =C*H*–CO–NH), 7.36 (s, 1H, >C=C*H*–NH–), 8.68 (s, 1H, –N=C*H*–NH–).

4.15. Mal–His-Lys(Z)–NH_2

Preparation of Mal–His-Lys(Z)–NH_2 was carried out as described for Mal–His-Lys–NH_2 starting from 40.6 mg (0.1 mmol) of Mal–His–ONb and 116.6 mg (0.4 mmol) of H–Lys(Z)–NH_2 and 2.5 mg of α-chymotrypsin. The crude material was separated via RP-HPLC by gradient elution using 10–25% ACN in 0.1% trifluoroacetic acid as eluent. The product was obtained as the trifluoroacetate, $C_{24}H_{30}N_6O_7$=514.52. Yield: 56.7 mg (91%); m/z (TS-POS): 515 $(M+H)^+$; ^{1}H-NMR (300 MHz; D_2O): 1.30–1.47 (m, 2H, Lys–C$^{\gamma}$H$_2$), 1.47–1.60 (m, 2H, Lys–C$^{\delta}$H$_2$), 1.73–1.89 (m, 2H, Lys–C$^{\beta}$H$_2$), 3.12–3.21 (m, 2H, Lys–C$^{\varepsilon}$H$_2$), 3.21–3.40 (brm, 2H, His–C$^{\beta}$H), 4.26–4.38 (m, 1H, Lys–C$^{\alpha}$H), 4.71–4.75 (m, 1H, His–C$^{\alpha}$H), 5.14 (s, 2H, O–CH$_2$), 6.31 (d, 1H, HOOC–C*H*=), 6.35 (d, 1H, =C*H*–CO–NH), 7,32 (s, 1H, >C=C*H*–NH–), 7.39–7.53 (m, 5H, –C$_6$H$_5$), 8.65 (s, 1H, –N=C*H*–NH–).

4.16. Mal–His-Lys–OMe

H–Lys–OMe·2HCl (233 mg, 1 mmol) and 9.5 mg of α-chymotrypsin were dissolved in 3.58 ml water. A solution of 101.5 mg (0.25 mmol) of Mal–His–ONb in 1 ml DMSO was added and the reaction was started by addition of 0.42 ml of 5 N KOH. The mixture was quickly shaken and placed in liquid nitrogen to achieve shock-freezing. Then it was incubated in a constant temperature thermostat at −25°C for three days. After complete ester consumption, 5 ml of 3 N acetic acid was added and the enzyme was removed by filtering through a polyester membrane (pore size 0.4 μm, Reichelt Chemietechnik, Germany). The filtrate was freeze-dryed and seperated via isocratic preparative RP-HPLC using 2.5% acetonitrile in 0.1% aqueous trifluoroacetic acid as eluent. The dipeptide was obtained as the ditrifluoroacetate, $C_{17}H_{25}N_5O_6$=365.40. Yield: 43 mg (28%); m/z (TS-POS): 396 $(M+H)^+$; ^{1}H-NMR (300 MHz; D_2O): 1.28–1.51 (m, 2H, Lys–$C^{\gamma}H_2$), 1.55–1.77 (m, 2H, Lys–$C^{\delta}H_2$), 1.77–1.89 (m, 2H, Lys–$C^{\beta}H_2$), 2.89–3.02 (m, 2H, Lys–$C^{\varepsilon}H_2$), 3.02–3.44 (brm, 2H, His–$C^{\beta}H$), 3.71 (s, 3H, –OCH_3), 4.28–4.48 (m, 1H, Lys–$C^{\alpha}H$), 4.63–4.71 (m, 1H, His–$C^{\alpha}H$), 6.30 (d, 1H, HOOC–C*H*=), 6.44 (d, 1H, =C*H*–CO–NH), 7.30 (s, 1H, >C=C*H*–NH–), 8.62 (s, 1H, –N=C*H*–NH–).

4.17. Mal–His-Lys–OH (Mal–His-$^{\alpha}$Lys–OH) and H–Lys(Mal–His)–OH (Mal–His-$^{\varepsilon}$Lys–OH)

The preparation of Mal–His-Lys–OH and H–Lys(Mal–His)–OH was carried out as described for Mal–His-Lys–OMe starting from 365 mg (2 mmol) of H–Lys–OH·HCl, 203 mg (0.5 mmol) of Mal–His–ONb and 19 mg of α-chymotrypsin at pH 11. The freeze-dried residue was eluted from silica gel 60 column (70–230 mesh ASTM; E. Merck, Germany) using methanol:$CHCl_3$:8% aqueous NH_3 (5.1:3.4:1.5) as eluent. The first fraction contained a mixure of Mal–His–OH and H–Lys(Mal–His)–OH, which was further purified by isocratic elution via RP-HPLC using 2% methanol in 0.1% aqueous trifluoroacetic acid as eluent to give H–Lys(Mal–His)–OH·2TFA, $C_{16}H_{23}N_5O_6$=381.37. Yield: 34 mg (12%); m/z (TS-POS): 382 $(M+H)^+$; ^{1}H-NMR (300 MHz; D_2O): 1.36–1.53 (m, 2H, Lys–$C^{\gamma}H_2$), 1.53–1.66 (m, 2H, Lys–$C^{\delta}H_2$), 1.89–2.04 (m, 2H, Lys–$C^{\beta}H_2$), 3.20–3.32 (m, 2H, Lys–$C^{\varepsilon}H_2$), 3.32–3.42 (brm, 2H, His–$C^{\beta}H$), 4.00 (t, 1H, Lys–$C^{\alpha}H$), 4.68–4.78 (m, 1H, His–$C^{\alpha}H$), 6.37 (d, 1H, HOOC–C*H*=), 6.53 (d, 1H, =C*H*–CO–NH), 7.36 (s, 1H, >C=C*H*–NH–), 8.68 (s, 1H, –N=C*H*–NH–). The second fraction of column chromatography contained Mal–His-Lys–OH. Yield: 72 mg (38%); m.p.: 134–145°C; m/z (TS-POS): 382 $(M+H)^+$; ^{1}H-NMR (300 MHz; D_2O): 1.36–1.55 (m, 2H, Lys–$C^{\gamma}H_2$), 1.64–1.84 (m, 2H, Lys–$C^{\delta}H_2$), 1.84–1.95 (m, 2H, Lys–$C^{\beta}H_2$), 3.03 (t, 2H Lys–$C^{\varepsilon}H_2$), 3.12–3.40 (brm, 2H, His–$C^{\beta}H$), 4.13–4.22 (m, 1H, Lys–$C^{\alpha}H$), 4.71 (t, 1H, His–$C^{\alpha}H$), 5.97 (d, 1H, HOOC–C*H*=), 6.40 (d, 1H, =C*H*–CO–NH), 7.20 (s, 1H, >C=C*H*–NH–), 7.26 (s, 1H, –N=C*H*–NH–). Preparation of the dihydrochloride of Mal–His-Lys–OH was carried out as described for Mal–His-Lys–NH_2. ^{1}H-NMR (300 MHz; D_2O): 1.38–1.58 (m, 2H, Lys–$C^{\gamma}H_2$), 1.68–1.78 (m, 2H, Lys–$C^{\delta}H_2$), 1.78–2.04 (m, 2H, Lys–$C^{\beta}H_2$), 3.04 t, 2H, Lys–$C^{\varepsilon}H_2$), 3.19–3.51 (brm, 2H, His–$C^{\beta}H$), 4.36–4.49 (m, 1H, Lys–$C^{\alpha}H$), 6.38 (d, 1H, HOOC–C*H*=), 6.54 (d, 1H, =C*H*–CO–NH), 7.37 (s, 1H, >C=C*H*–NH–), 8.67 (s, 1H, –N=C*H*–NH–).

4.18. Z–His-Lys–NH_2

The preparation of Z–His-Lys–NH_2 was carried out as described for Mal–His-Lys–NH_2 starting from 218 mg (1 mmol) of H–Lys–NH_2, 76 mg (0.25 mmol) of Z–His–OMe and 9.5 mg of α-chymotrypsin. The product was separated by isocratic RP-HPLC using 10% acetonitrile in 0.1% aqueous trifluoroacetic acid as eluent to give Z–His-Lys–NH_2·2TFA, $C_{20}H_{28}N_6O_4$=416.46. Yield: 98 mg (61%); MS (TS-POS):

417 $(M+H)^+$; ^{1}H-NMR (300 MHz; D_2O): 1.28–1.52 (m, 2H, Lys–$C^{\gamma}H_2$), 1.54–1.72 (m, 2H, Lys–$C^{\delta}H_2$), 1.72–1.86 (m, 2H, Lys–$C^{\beta}H_2$), 2.99 (t, 2H, Lys–$C^{\varepsilon}H_2$), 3.09–3.36 (brm, 2H, His–$C^{\beta}H$), 4.30–4.40 (m, 1H, Lys–$C^{\alpha}H$), 4.50–4.58 (dd, 1H, His–$C^{\alpha}H$), 5.15 (s, 2H, O–CH_2–), 7.28 (s, 1H, >C=C*H*–NH–), 7.40 (d, 2H, =C*H*–C(CH_2)–C*H*–), 7.45–7.54 (m, 3H, =C*H*–C*H*=C*H*–), 8.57 (s, 1H, –N=C*H*–NH–).

4.19. Z-Gly-His-Lys-NH_2

The synthesis of Z–Gly-His-Lys–NH_2 was carried out as described for Mal–His-Lys–NH_2 starting from 350 mg (0.25 mmol) of Z–Gly-His–ONb, 218 mg (1 mmol) of H–Lys–NH_2 and 0.47 mg of α-chymotrypsin. The product was isolated from a silica gel 60 column (70–230 mesh ASTM; E. Merck, Germany) using methanol:$CHCl_3$:8% aqueous NH_3 (5.1:3.4:1.5) as eluent, $C_{22}H_{31}N_7O_5$=473.51. Yield: 93 mg (79%); MS (TS-POS): 474 $(M+H)^+$; ^{1}H-NMR (300 MHz; D_2O): 1.36–1.54 (m, 2H, Lys–$C^{\gamma}H_2$), 1.65–1.78 (m, 2H, Lys–$C^{\delta}H_2$), 1.78–1.93 (m, 2H, Lys–$C^{\beta}H_2$), 3.02 (t, 2H, Lys–$C^{\varepsilon}H_2$), 3.10–3.35 (brm, 2H, His–$C^{\beta}H$), 3.89 (s, 2H, Gly–CH_2), 4.23–4.35 (m, 1H, Lys–$C^{\alpha}H$), 4.67–4.75 (m, 1H, His–$C^{\alpha}H$), 5.20 (s, 2H, O–CH_2–), 7.19 (s, 1H, >C=C*H*–NH–), 7.48 (brs, 5H, C_6H_5), 8.29 (s, 1H, –N=C*H*–NH–). The dihydrochloride was obtained as described for Mal–His-Lys–NH_2. MS (TS-POS): 474 $(M+H)^+$; ^{1}H-NMR (300 MHz; D_2O): 1.38–1.58 (m, 2H, Lys–$C^{\gamma}H_2$), 1.63–1.78 (m, 2H, Lys–$C^{\delta}H_2$), 1.78–1.94 (m, 2H, Lys–$C^{\beta}H_2$), 3.02 (t, 2H, Lys–$C^{\varepsilon}H_2$), 3.16–3.40 (brm, 2H, His–$C^{\beta}H_2$), 3.89 (s, 2H, Gly–$C^{\alpha}H_2$), 4.29–4.38 (m, 1H, Lys–$C^{\alpha}H$), 4.70–4.75 (m, 1H, His–$C^{\alpha}H$), 5.21 (s, 2H, O–C*H*$_2$–CO–NH–), 7.30 (s, 1H, >C=C*H*–NH), 7.49 (brs, 5H, C_6H_5), 8.63 (s, 1H, –N=C*H*–NH–).

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