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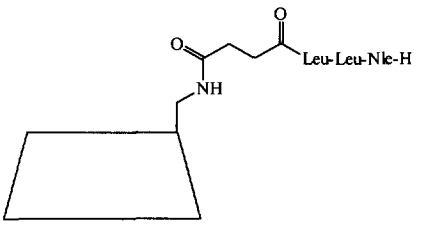
OLIGOPRESENTATION OF PROTEASE INHIBITORS WITH β-CYCLODEXTRIN AS TEMPLATE

Norbert Schaschke,[1] Hans-Jürgen Musiol,[1] Irmgard Assfalg-Machleidt,[2] Werner Machleidt,[2] and Luis Moroder[1*]

[1]*Max-Planck-Institut für Biochemie, 82152 Martinsried, Germany*
[2]*Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der LMU München, 80336 München, Germany*

Abstract: Perfacial functionalization of β-cyclodextrin as heptakis-(6-mercapto-6-deoxy)- and heptakis-(6-succinylamido-6-deoxy)-derivative was used to graft the tripeptide aldehyde Leu-Leu-Nle-H to the strongly solubilizing carbohydrate template. Oligopresentation of this inhibitor and thus *in loco* increase of the concentration was not found to enhance inhibition of cathepsin B and μ-calpain on molar basis if compared to the mono-conjugated form. © 1997 Elsevier Science Ltd.

Since the early studies on multiple conjugation of several peptide hormones to tobacco mosaic virus[1] much effort was devoted to enhance biological responses via oligopresentation of bioactive molecules. While such approach proved to be very efficient for induction of immunological responses,[2] in the case of receptor ligands multivalent constructs and particularly bivalent peptides led to controversial effects.[3-8] Cyclodextrins have been extensively used for solubilization and transport of hydrophobic drugs because of their ability to trap in their hydrophobic cavity via host-guest complexation a variety of compounds.[9,10] We have recently investigated the effect of such carbohydrate templates on the accessibility of peptide aldehydes as inhibitors of



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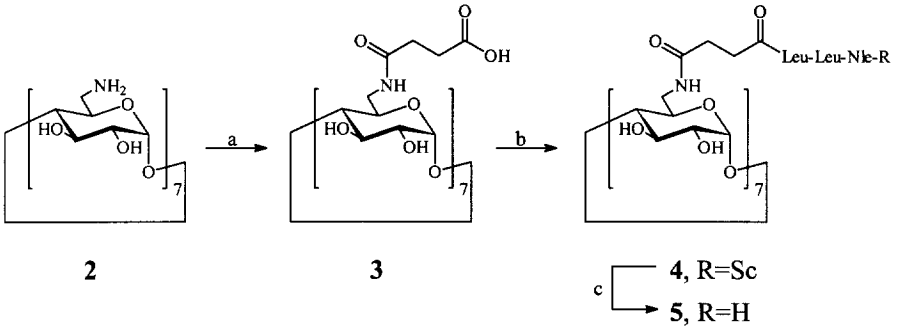
cysteine proteases when covalently linked to β-cyclodextrin.[11] Using the peptide aldehyde Ac-Leu-Leu-Nle-H[12] as model compound and a C4 spacer for grafting the tripeptide aldehyde to the template (compound **1**) only a slighty increased K_i value was observed for cathepsin B whereas a significantly stronger reduction of the

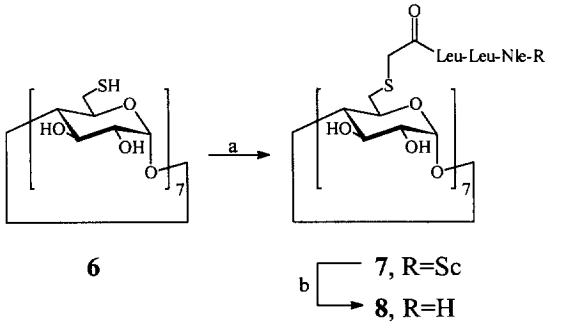
* E-mail: moroder@biochem.mpg.de Fax: +49 89 8578 3905

inhibitory potency was induced in the case of μ-calpain (Table 1). These results stimuleted us to analyse the effect of a multiple presentation of the peptide aldehyde inhibitor on β-cyclodextrin.

For this purpose two hepta-conjugates (**5** and **8**) were synthesized using different strategies to graft the peptide aldehyde portion to the cyclomaltoheptaose. Heptakis-(6-amino-6-deoxy)-β-cyclodextrin (**2**), obtained by known procedures,[13] was acylated with succinic anhydride to yield the spacer-functionalized β-cyclodextrin derivative **3**[14] upon saponification of esters as byproducts of the acylation step (Scheme 1). Then the tripeptide aldehyde protected as semicarbazone, i.e. H-Leu-Leu-Nle-Sc, and synthesized as described previously[11] was linked to the heptakis-(6-succinylamido-6-deoxy)-β-cyclodextrin derivative by the PyBOP method.[15] Weak acid hydrolysis of the semicarbazone in presence of excesses of formaldehyde led in good overall yield to the desired conjugate **5** as homogeneous and well characterized compound.[16]



Scheme 1. Synthesis of the peptide aldehyde/β-cyclodextrin hepta-conjugate with a C4 spacer: a) succinic anhydride/pyridine (39%), b) TFA×H-Leu-Leu-Nle-Sc/PyBOP/NEt_3/DMF (85%), c) 37% formaldehyde/AcOH/MeOH (77%).



Scheme 2. Synthesis of the peptide aldehyde/β-cyclodextrin hepta-conjugate with a C2 spacer: a) Br-CH_2-CO-Leu-Leu-Nle-Sc/diisopropylethylamine/DMF (77%), b) 37% formaldehyde/AcOH/MeOH (70%).

Conversely, the heptakis-(6-mercapto-6-deoxy)-β-cyclodextrin (**6**), also obtained by known procedures,[17] was reacted with 3-bromo-acetyl-Leu-Leu-Nle-Sc to yield the heptakis-peptide conjugate **8**[18], upon weak acid hydrolysis of the semicarbazone protcting group (Scheme 2).

For the chromatographic analysis of the heptakis-conjugates in the protected and deprotected form HPLC served only to demonstrate the absence of low-mass contaminants, e.g. educts and reagents, since broad peaks were obtained both at 25 °C and 50 °C as well as changing from acid to neutral eluents. Using micellar

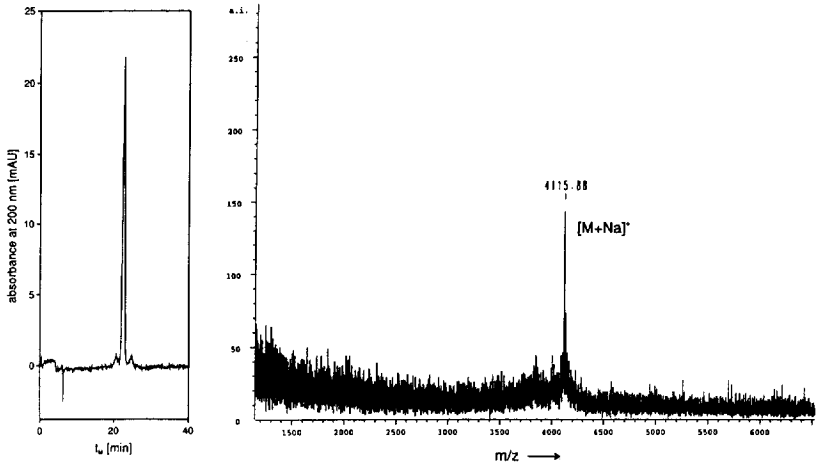


Figure 1. Capillary electropherogramm and MALDI-TOF mass spectrum of the heptakis-(6-succinylamido-6-deoxy)-β-cyclodextrin/Leu-Leu-Nle-H conjugate (**5**).

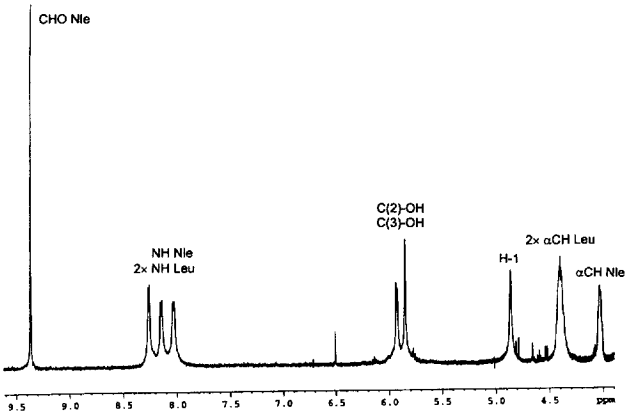


Figure 2. Portion of the ^{1}H-NMR spectrum of the heptakis-(6-mercapto-6-deoxy)-β-cyclodextrin/CH_2-CO-Leu-Leu-Nle-H conjugate (**8**) in DMSO-d_6.

concentrations of SDS, capillary zone electrophoresis was found to be the most efficient method as shown exemplarily for the conjugate **5** in Fig. 1. By mass spectrometry (Fig. 1) and ^{1}H-NMR (Fig. 2) structures and homogeneity of the compounds were confirmed. Samples varied in the degree of hydration of the aldehyde group as well as of racemization of the Nle-H residue according to the NMR spectra in DMSO-d_6.

As shown in Table 1, the K_i values of these cyclodextrin conjugates remain in the nanomolar range of the parent unconjugated inhibitor Ac-Leu-Leu-Nle-H. A significant effect is exerted by the spacer size as well evidenced by comparing the inhibitory potencies of the C2- and C4-spaced inhibitors.

Inhibitors	K_i (nM)	
	Cathepsin B	μ-Calpain
Ac-Leu-Leu-Nle-H	1.0	3.4
1	2.0	91
5	11.3	41.5
8	46.3	203

Table 1. Equilibrium dissociation constants (K_i) for the inhibition of human cathepsin B and human μ-calpain; values are averages of 6-15 measurements at 12 °C and were determined as described previously.[11] Since autolysis of the Ca^{2+}-activated calpain is known to occur rapidly measurements with both enzymes were performed at 12 °C for comparative analysis.

Further elongation of the spacer to C6 and C8 was without significant effect on the inhibitory potency of the mono-conjugate (unpublished results). These findings agree with our docking experiments of the mono-conjugate **1** to the X-ray structure of cathepsin B.[19] In fact, with a C4 spacer sterical clashes for binding of the tripeptide aldehyde to the active-site cleft are avoided. Since the X-ray structure of μ-calpain is not yet available, the different effect of the template on the K_i values observed for the two enzymes cannot be fully rationalized. Regarding mono- *vs.* oligopresentation opposite effects were obtained with the two enzymes, i.e. a lowered inhibition of cathepsin B, but a 2-fold improved inhibition of μ-calpain.

Sterical hindrance or hydrophobic collapses of the peptide moieties in the β-cyclodextrin conjugates seem not to be the main cause of the observed lack of enhanced inhibition as suggested by comparing the K_i values for μ-calpain. Thus the multivalent presentation to the recognition process is preserved. Multivalency can theoretically increase apparent binding affinities by orders of magnitude due to the entropic effects on avidity.[20,21] But usually the observed affinity gain is modest and in the present case where high excesses of inhibitors over the enzyme concentration are required for inhibition, multivalent interaction of single β-cyclodextrin conjugates with the enzyme molecules is statistically unfavored. Nevertheless such constructs could represent interesting tools for inhibiting membrane-bound enzymes, since upon the initial recognition

(three-dimensional collision) the second recognition process should be favored by the two-dimensional pathway. In this context it is worthy to note that various tumor cells were found to contain populations of membrane-bound cathepsin B.[22]

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14. Compound **3** was isolated by preparative HPLC; yield: 39%; TLC (acetonitrile/water 7:3): R_f 0.35; ^{1}H NMR (D_2O): δ = 2.50–2.78 (br. m, 4H, C<u>H</u>$_2$-C<u>H</u>$_2$-COOH), 3.50 (t, 1H, *J* = 9.3 Hz, H-4), 3.57 (dd, 1H, *J* = 14.1 Hz, *J* = 7.2 Hz, H-6a), 3.72 (dd, 1H, *J* = 9.9 Hz, *J* = 3.6 Hz, H-2), 3.90 (br. d, 1H, *J* = 14.1 Hz, H-6b), 4.01 (t, 1H, *J* = 9.3 Hz, H-3), 3.97–4.07 (m, 1H, overlap with triplet at 4.01 ppm, H-5), 5.09 (d, 1H, *J* = 3.6 Hz, H-1); ^{13}C NMR (D_2O): δ = 32.08, 33.01 (<u>C</u>H$_2$-<u>C</u>H$_2$-COOH), 42.59 (C-6), 73.18, 74.85, 75.60, 85.84 (C-2, C-3, C-4, C-5), 104.53 (C-1), 177.39, 179.32 (2×CO); FAB-MS: *m/z* = 1828.7 $[M+H]^+$; calcd. for $C_{70}H_{105}N_7O_{49}$: 1827.6.
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16. Compound **4** was isolated by precipitation of the crude product from DMF/water; yield: 85%; TLC (chloroform/methanol/water 14:6:1): R_f 0.40; ^{1}H NMR (DMSO-d_6): δ = 0.70–1.00 (m, 15H, εCH$_3$ Nle, 2×δ$_1$CH$_3$ Leu, 2×δ$_2$CH$_3$ Leu), 1.10–1.70 (br. m, 12H, βCH$_2$ Nle, γCH$_2$ Nle, δCH$_2$ Nle, 2×βCH$_2$ Leu, 2×γCH Leu), 2.40 (br. s, 4H, NHCO-C<u>H</u>$_2$-C<u>H</u>$_2$-CO), 3.20–3.80 (br. m, 6H, H-2, H-3, H-4, H-5, H-6a, H-6b, overlap with water signal in DMSO), 4.25–4.45 (m, 3H, 2×αCH Leu, αCH Nle), 4.80 (s, 1H, H-1), 5.85, 5.90 (2s, 2H, C(2)-OH, C(3)-OH), 6.22 (s, 2H, NH-CO-N<u>H</u>$_2$), 7.09 (s, 1H, N<u>H</u>-CO-NH$_2$), 7.80–8.20 (m, 4H, 2×NH Leu, NH Nle, N<u>H</u>CO-CH$_2$-CH$_2$-CO), 9.87 (s, 1H, C<u>H</u>=N); MALDI-TOF-MS: *m/z* = 4514.62 $[M+Na]^+$, 4530.59 $[M+K]^+$; calcd. for: $C_{203}H_{357}N_{49}O_{63}$: 4489.6.

   Compound **5** was isolated by precipitation of the crude product from water as mixture of diasteriomers due to partial racemisation of the Nle-H residue; yield: 77%; TLC (chloroform/methanol/water 14:6:1): R_f 0.55; CE (underivatized fused silica capillary (67 cm × 75 μm; lenght × diameter), 25 kV, 50 mM sodium borate

buffer (pH 8.5) containing 42 mM SDS): t_M 22.7 min; ^{1}H NMR (DMSO-d_6): δ = 0.70–0.95 (m, 15H, εCH$_3$ Nle, 2×δ$_1$CH$_3$ Leu, 2×δ$_2$CH$_3$ Leu), 1.05–1.80 (br. m, 12H, βCH$_2$ Nle, γCH$_2$ Nle, δCH$_2$ Nle, 2×βCH$_2$ Leu, 2×γCH Leu), 2.40 (br. s, 4H, NHCO-CH$_2$-CH$_2$-CO), 3.10–3.80 (br. m, 6H, H-2, H-3, H-4, H-5, H-6a, H-6b, overlap with water signal in DMSO), 4.00 (m, 1H, αCH Nle) 4.35 (m, 2H, 2×αCH Leu,), 4.80 (m, 1H, H-1), 5.88, 5.92 (2s, 2H, C(2)-OH, C(3)-OH), 7.82–8.25 (m, 4H, 2×NH Leu, NH Nle, NHCO-CH$_2$-CH$_2$-CO), 9.36, 9.58 (2s, 1H, CH=O, ratio 2:1); MALDI-TOF-MS: *m/z* = 4115.88 [M+Na]$^+$, 4131.84 [M+K]$^+$; calcd for: $C_{196}H_{336}N_{28}O_{63}$: 4090.4.

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18. Compound **7** was isolated by precipitation of the crude product from methanol/ethyl acetate; yield: 77%; TLC (chloroform/methanol/acetic acid/water 80:40:5:10): R_f 0.60; ^{1}H NMR (DMSO-d_6): δ = 0.70–1.02 (m, 15H, εCH$_3$ Nle, 2×δ$_1$CH$_3$ Leu, 2×δ$_2$CH$_3$ Leu), 1.10–1.75 (br. m, 12H, βCH$_2$ Nle, γCH$_2$ Nle, δCH$_2$ Nle, 2×βCH$_2$ Leu, 2×γCH Leu), 2.70–3.90 (br. m, 8H, H-2, H-3, H-4, H-5, H-6a, H-6b, S-CH$_2$-CO, overlap with the water signal in DMSO), 4.30–4.50 (m, 3H, 2×αCH Leu, αCH Nle), 4.87 (s, 1H, H-1), 5.85 (s, 1H, OH), 5.93 (d, 1H, *J* = 5.6 Hz, OH), 6.25 (s, 2H, NH-CO-NH$_2$), 7.07 (s, 1H, *J* = 3.8 Hz, NH-CO-NH$_2$), 8.02 (d, 2H, J = 7.2 Hz, 2×NH), 8.12 (d, 1H, J = 7.2 Hz, NH), 9.88 (s, 1H, CH=N); MALDI-TOF-MS: m/z = 4339.27 [M+Na]$^+$, 4355.24 [M+K]$^+$; calcd. for: $C_{189}H_{336}N_{42}O_{56}S_7$: 4314.3.

    Compound **8** was purified by precipitation of the crude product from water; yield: 70%; TLC (chloroform/methanol/water 26:10:1): R_f 0.80; CE (underivatized fused silica capillary (67 cm × 75 μm; lenght × diameter), 25 kV, 50 mM sodium borate buffer (pH 8.5) containing 42 mM SDS): t_M 18.2 min; ^{1}H-NMR (DMSO-d_6): δ = 0.70–1.00 (m, 15H, εCH$_3$ Nle, 2×δ$_1$CH$_3$ Leu, 2×δ$_2$CH$_3$ Leu), 1.14–1.80 (br. m, 12H, βCH$_2$ Nle, γCH$_2$ Nle, δCH$_2$ Nle, 2×βCH$_2$ Leu, 2×γCH Leu), 2.20–3.90 (br. m, 8H, H-2, H-3, H-4, H-5, H-6a, H-6b, S-CH$_2$-CO, overlap with the water signal in DMSO), 4.03 (m, 1H, αCH Nle), 4.40 (m, 2H, 2×αCH Leu), 4.86 (s, 1H, H-1), 5.84 (s, 1H, OH), 5.93 (d, 1H, *J* = 6.5 Hz, OH), 8.03 (d, 1H, *J* = 6.7 Hz, NH), 8.14 (d, 1H, *J* = 7.7 Hz, NH), 8.26 (d, 1H, *J* = 6.7 Hz, NH) 9.36 (s, 1H, CH=O); MALDI-TOF-MS: m/z = 3936.32 [M+Na]$^+$, 3952.96 [M+K]$^+$; calcd. for: $C_{182}H_{315}N_{21}O_{56}S_7$: 3915.0.
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