Protein Chemical Synthesis

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Synthesis of a Biologically Active Triazole-Containing Analogue of Cystatin A Through Successive Peptidomimetic Alkyne–Azide Ligations**

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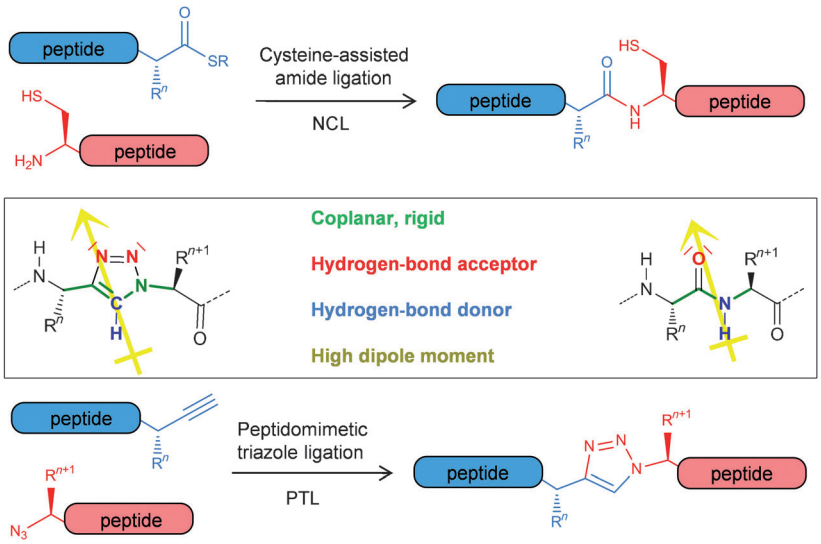
Amide surrogates are common in naturally occurring peptides and in synthetic peptides used in therapy. Whereas backbone-engineered proteins are, to date, extremely laborious to produce by genetic means,[1] the advent of chemoselective peptide chemical ligation reactions[2,3] paved the way to such complex molecular architectures of considerable potential for protein therapeutics. To date, the most popular strategy to introduce amide bond surrogates in proteins relies on an elaborate combination of 1) solution-phase synthesis to provide a suitably protected pseudo-dipeptide, 2) solid-phase peptide synthesis (SPPS) to incorporate the modification in a peptide fragment, and 3) native chemical ligation[2b] (NCL) to yield a full-length backbone-engineered protein.[4]

A valuable alternative for the introduction of amide-bond mimics in proteins would be a peptidomimetic ligation strategy combining in a single step the formation of the amide surrogate, its incorporation in a peptide backbone, and ligation of fragments. Besides the pioneering study on thioester backbone-engineered proteins,[2a] only few examples have been reported,[5] including a recent study concerning a ligation of thioacid- and aziridine-terminated model peptides, giving a reduced form ($\Psi[CH_2NH_2]$) of an amide bond.[6] To enlarge the palette of the synthetic protein chemist, we envisioned developing a new peptidomimetic ligation prototype that leads to bioactive backbone-modified proteins. Herein, we report for the first time the use of the Cu^I-mediated cycloaddition of azides and terminal alkynes (CuAAC)[7] for the assembly of unprotected peptide fragments into a bioactive triazole-containing protein.



Scheme 1. Protein synthesis through fragment condensation using NCL and PTL.[10]

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The rapid and regioselective method for the synthesis of 1,4-disubstituted 1,2,3-triazoles (Tz) through CuAAC, independently discovered by the Meldal[7a] and Sharpless[7b] groups, has incredibly accelerated the studies involving Tz-containing novel compounds. This heterocycle is well recognized as an amide bioisostere: it closely mimics the geometric, steric, and electronic features of a *trans*-amide bond and can likewise participate in hydrogen bonding and dipole–dipole interactions[8] (Scheme 1). The triazole moiety is considered as an universal peptidomimetic group that can accommodate any peptide secondary structure.[9] The introduction of triazole amide surrogates (Ψ[Tz]) by a peptidomimetic ligation strategy requires peptides modified at their C and N termini with α-amino alkynes and α-azido acids, respectively. The latter building blocks can be easily prepared[11] and introduced into peptide fragments using standard peptide chemistry, and the synthesis of chiral α-amino alkynes can be

achieved in a few steps[4b,9] from the protected parent amino acids. Propargylamine, a precursor for GlyΨ[Tz]Xaa, is even commercially available (Gly = G = glycine, Xaa = any α-amino acid). Because Gly is one of the most common and well-distributed residues in proteins, retrosynthetic disconnections at Gly-Xaa sites are particularly attractive. The incorporation of 1,4-disubstituted triazoles into the backbone of long peptides has been reported and was based on a classical SPPS approach involving triazole-containing pseudodipeptides as building blocks.[8c] To our knowledge, the few examples of post-SPPS backbone engineering by triazole formation involve protected peptide fragments.[12–14] Most examples concern small cyclic peptides. Given that CuAAC is extremely robust, orthogonal to most other reactions, and tolerant of an extensive variety of functional groups, including unprotected peptide side chains, we decided to explore the potential of CuAAC to assemble unprotected peptides into backbone-engineered protein analogues.

An iterative version of peptidomimetic triazole ligation (PTL) is crucial for the synthesis of medium-sized (> 80 amino acids) bioactive proteins: the temporary masking of a terminal alkyne with a silyl protecting group[15] enables multiple successive CuAAC steps under the mildest conditions compatible with complex biomacromolecules. We and others have shown that triisopropylsilyl (TIPS) is a group of choice for multiple linear CuAAC steps,[16] and we have demonstrated its full compatibility with the conditions of fluorenylmethoxycarbonyl (Fmoc) SPPS.[16a]

For the proof of concept of a multiple-PTL approach, we focused on human cystatin A (also known as stefin A), a natural cysteine-protease inhibitor about 100 amino acids long and without any cysteine[17] (see Figure 1 a for its amino acid sequence).

Cystatin A potently inhibits papain-like cysteine proteases of the family 1, including cysteine cathepsins. Besides their primary role in end-stage breakdown of endocytosed proteins in lysosomal compartments, these proteases are also associated with numerous major pathological disorders;[18,19] this association makes the proteases emerging therapeutic targets.[20] The inhibitory site of cystatin A involves three noncontiguous regions comprising an N-terminal part with a conserved glycyl residue, a central β-hairpin loop containing a

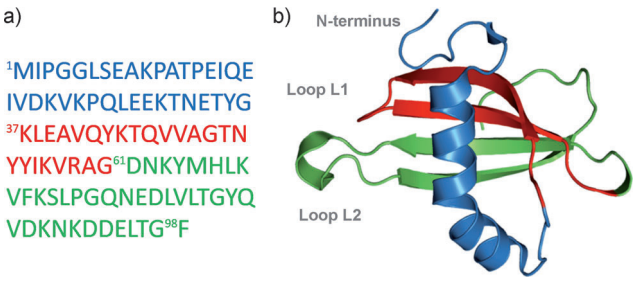


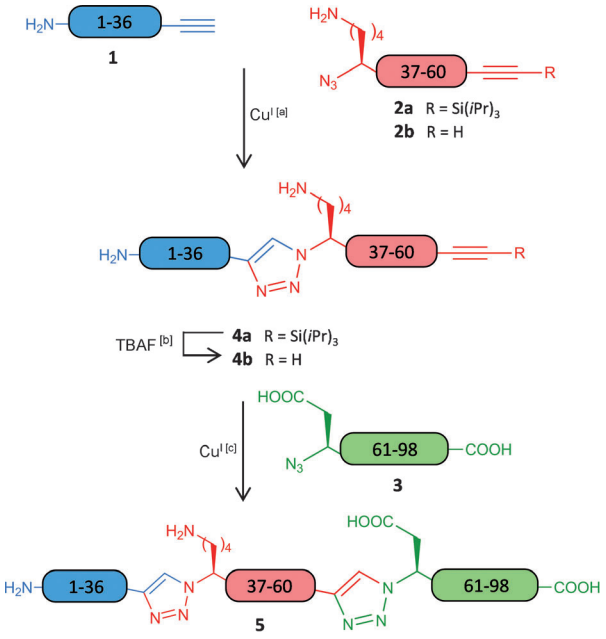
Figure 1. a) Amino acid sequence of human cystatin A. M = Met = methionine, I = Ile = isoleucine, P = Pro = proline, L = Leu = leucine, S = Ser = serine, E = Glu = glutamic acid, A = Ala = alanine, K = Lys = lysine, T = Thr = threonine, Q = Gln = glutamine, V = Val = valine, D = Asp = aspartic acid, Y = Tyr = tyrosine, N = Asn = asparagine, R = Arg = arginine, H = His = histidine, F = Phe = phenylalanine. b) Schematic three-dimensional structure of human cystatin A.

consensus QXVXG motif (Figure 1 b, loop L1), and a C-terminal β-hairpin loop (Figure 1 b, loop L2).[21] Together they form a large, wedge-shaped binding surface that inserts into the cathepsin active-site cleft.[22]

From a retrosynthetic point of view, the 97 amino acid sequence of cystatin A has been dissected into three sections,[23] to ensure an optimal length of the fragments for their synthesis by SPPS (Figure 1, green, red, and blue parts). The C-terminal propargylamide peptides **1** and **2a** were obtained using a general approach based on a backbone-amide-linker[24,25] (BAL) strategy (Scheme 1 in the Supporting Information). Propargylamine and 3-(triisopropylsilyl)prop-2-yn-1-amine[16a] were subjected to reductive amination with 4-(4-formyl-3,5-dimethoxyphenoxy)butanoic acid, and the resulting secondary amine was subsequently Fmoc-protected and loaded onto aminomethyl ChemMatrix resin through standard amide coupling. Fmoc deprotection furnished the solid-supported secondary amine, which was efficiently coupled to the next amino acid using HATU (*N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide) as the coupling reagent. The SPPS elongations of the three peptide segments **1**, **2a**, and **3** were achieved using the Fmoc/*t*Bu strategy. The main challenge at this stage was to obtain crude peptides with a high standard purity and yield. Residue ^{1}Met, which only minimally participates in the inhibitory properties of the protein,[26,27] was substituted by an isosteric norleucyl residue in peptide segment **1**.

Although the synthesis of **3** was straightforward, the low yields of the first elongations of peptide **1** and **2a** carried out with HBTU (*N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide) as the coupling reagent were very disappointing. We exploited recent advancements in SPPS by combining the use of pseudoproline dipeptide derivatives,[28] a polar resin,[29] and HATU as a powerful coupling reagent, and we conducted a meticulous step-by-step optimization of the synthesis of **1** and **2a** (see the Supporting Information for details). The synthesis of **2a** was particularly demanding,[30] and its purification by conventional reversed-phase (RP)-HPLC resulted in very low yields. A careful analysis of the crude peptide revealed the main coproducts to be N-truncated peptides bearing terminal acetamides, which would not interfere with the PTL (see the Supporting Information). We thus decided to directly engage crude, lyophilized **2a** in the CuAAC step, whereas peptide segments **1** and **3** were purified by RP-HPLC before ligation.

We then determined appropriate reaction conditions to carry out the first ligation (Scheme 2) between **1** and **2a**. We chose the most standard $CuSO_4$/sodium ascorbate protocol for the generation of the active Cu^{I} species, and all the triazole ligations were conducted in an argon atmosphere in a carefully degassed phosphate buffer at pH 8. A slight excess of **2a** was engaged in a CuAAC with **1** (ca. 1 mM) in a 1:1 HFIP (hexafluoroisopropyl alcohol)/buffer mixture.[31] Gratifyingly, under these conditions, peptide **1** was totally consumed in less than 15 min to cleanly yield the triazolopeptide **4a** (Figure 2 a, trace 2 and the Supporting Information). Further treatment with an excess of tetra-*n*-butylammonium



Scheme 2. Chemoselective assembly of **5**. [a,b] 47% yield over two steps. [c] 57% yield.

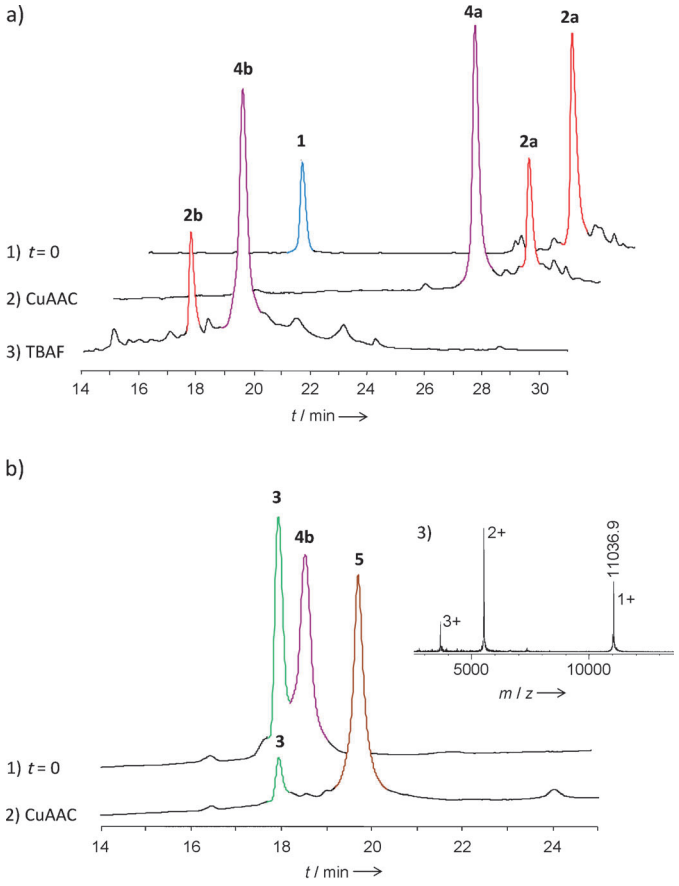


Figure 2. a) HPLC profiles (detection at 275 nm) of a mixture of **1** and **2a** before (trace 1) and after 15 min CuAAC (trace 2), and after subsequent fluoride treatment for one hour (trace 3). b) HPLC profiles (275 nm) of a mixture of **3** and purified **4b** before (trace 1) and after 15 min CuAAC (trace 2). Inset 3) MALDI-TOF spectrum of purified **5**. Full details on synthesis and characterization can be found in the Supporting Information.

fluoride (TBAF) in dimethylformamide (DMF) for one hour afforded desilylated **4b** (Figure 2a, trace 3), which was purified by RP-HPLC and recovered in a good 47% yield over two steps. Nonligated, truncated acetamidopeptides that had been contaminating crude **2a** were easily removed at this step, owing to the advantages associated with an N-to-C protein assembly.

Peptide alkyne **4b** and a slight excess of azidopeptide **3** were then treated under purely aqueous CuAAC conditions. A complete conversion was once again observed within 15 min (Figure 2b). Although peptide **3** contains Fenton-oxidation-sensitive His and Met residues, the MS data did not show any significant [+16] adducts typically associated with deleterious oxidative side reactions. Chromatographic purification afforded pure triazole-modified cystatin A **5** in a good overall 27% yield over three steps based on starting peptide **1**.

A complete biological characterization of the synthetic cystatin A **5** was then carried out. The kinetics of cathepsin B, H, and L inhibition were determined under standard conditions. We used the commercially available recombinant His-tagged version of human cystatin A as a control (six His residues inserted between residues ^{1}Met and ^{2}Ile). The obtained inhibition constants K_i (nanomolar to picomolar range) were similar and fully consistent with the values published in the literature (Table 1), as were the rate constants for dissociation (k_{diss}) and for association (k_{ass}) with cathepsin B and L. The concentration-independent circular dichroism spectra with minima at 217 nm suggested that **5** folds as a monomer with a predominant β-sheet structure, as observed for the native protein[35] (Figure 1 in the Supporting Information). This observation demonstrated that the triazole-containing protein analogue was able to correctly display the three noncontiguous interacting regions, thereby adopting a three-dimensional structure similar to the native proteins (see Figure 5 in the Supporting Information for a homology-based model).

Similar to native cystatins, synthetic cystatin A **5** is insensitive to thermal inactivation, because incubation for

Table 1: Cathepsin inhibition assays.

Enzyme	cystatin A K_i [nM][a]	rec. cystatin A K_i [nM][b]	synthetic cystatin A **5** K_i [nM]	k_{ass} [$M^{-1}s^{-1}$]	k_{diss} [s^{-1}]
cathepsin B	0.9[32]/1.8[33]/8.2[34]	4.0 (3)	3.0 (3)	4.4×10^{4} (4)	1.3×10^{-4}
cathepsin H	0.9[32]/0.3[34]	0.4 (2)	0.2 (3)	n.d.[c]	n.d.[c]
cathepsin L	<0.01[32]/0.0018[33]/1.3[34]	0.005 (3)	0.001 (4)	2.9×10^{7} (3)	2.9×10^{-5}

[a] Published results. [b] Kinetics data were determined by using Enzfitter software (Biosoft, Cambridge, UK). Z-Phe-Arg-AMC was the fluorogenic substrate for cathepsins B and L and H-Arg-AMC for cathepsin H. Measured values are given as means without errors, and test numbers are given in parentheses. AMC = 7-amino-4-methylcoumarin, Z = CBz = benzyloxycarbonyl. [c] Not determined.

one hour at 60, 80, or 95 °C did not impact its inhibitory potential towards cysteine protease papain (Figure 3 in the Supporting Information). Similarly, the activity of **5** was unchanged after both treatment at acidic and basic pH values (Figure 2 in the Supporting Information).[36]

After we established that the presence of the triazoles did not impair the stability of the folded protein, we next investigated the ability of **5** to inhibit the cathepsin-dependent invasiveness of highly metastatic MDA-MB-231 breast cancer cells. This cell line expresses functional cysteine cathepsins that, as we found, play a predominant role in cancer invasiveness through degradation of the extracellular matrix.[37] First, a qualitative assay was performed using cells cultured in Matrigel[38] that contained quenched fluorescein linked to gelatin (DQ-Gelatin), which shows green fluorescence upon proteolysis. The recombinant cystatin A and E-64, a broad-spectrum cysteine-protease inhibitor, were included in the trial as controls. Synthetic cystatin A **5** impaired the extracellular gelatinolytic activity at a concentration of 100 nM (Figure 3), comparable to the recombinant protein, and inhibtor E-64 impaired the gelatinolytic activity at a concentration of 100 μM. This inhibitory activity was further confirmed by the quantification (cell count) of the inhibition of the MDA-MB-231 cell invasiveness using Boyden chambers separated by Matrigel, (ca. 50% inhibition[39] for both proteins and E-64, see Figure 4 in the Supporting Information). These data demonstrate that the introduction of the two peptidomimetic bonds is not detrimental to the functionality of the synthetic cystatin A **5**, which retains the biological potency of its natural counterpart in a living environment.

This work established the proof-of-concept of a new peptidomimetic ligation strategy through successive CuAAC steps. The azide and alkyne peptides were readily synthesized by SPPS, and the use of the TIPS group as a temporary alkyne-masking group for the construction of proteins containing multiple triazole groups was validated. Peptide ligations were quantitative after 15 min at millimolar peptide concentrations, thus emphasizing the advantages associated with a metal-catalyzed reaction, the kinetics of which can be fully controlled by varying the catalyst loading. Synthetic cystatin A **5** containing two triazole groups was prepared in high yields from three fragments, and no deleterious oxidized coproducts were observed. Triazole-containing protein **5** folded properly and exhibited the expected enzymatic and functional properties, thereby suggesting a high potential for triazole-modified proteins. Our approach enables sequential ligations in the N-to-C direction, compatible with the presence of unreacted truncated SPPS coproducts, thus giving us the opportunity to use crude peptide segments. We expect that the combination of PTL and NCL, which is mostly carried out in the opposite C-to-N direction, may enable a fully convergent synthesis of large triazole-containing proteins. Collectively, our results provide the first example of a bioactive protein obtained by PTL, and we believe that PTL will become a general and useful tool for the synthetic protein chemist.

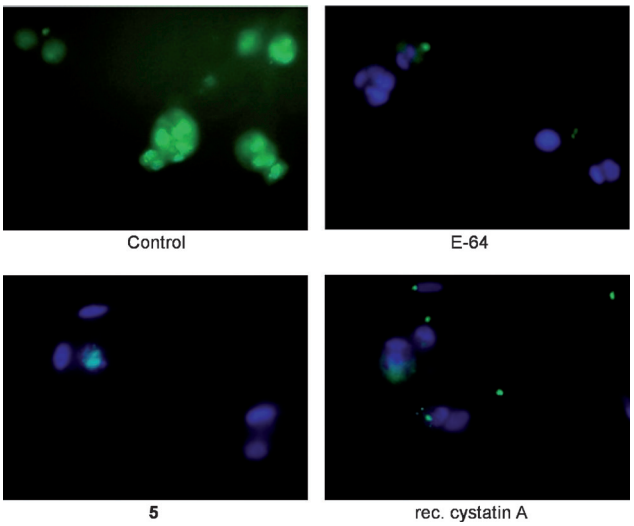


Figure 3. Imaging of the inhibition of the gelatinolytic activity of MDA-MB-231 breast cancer cells. Glass coverslips were coated with 25 $\mu g\,mL^{-1}$ DQ-gelatin in Matrigel. Green fluorescence indicates gelatinolytic activity. Cell nuclei are stained in blue by 4′,6-diamidino-2-phenylindole (DAPI). E-64 was used at a concentration of 100 μM, whereas both cystatins were used at a concentration of 0.1 μM. The control corresponds to untreated cells. Representative picture of triplicate assays. See the Supporting Information for details.

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[1] C. C. Liu, P. G. Schultz, *Annu. Rev. Biochem.* **2010**, *79*, 413 – 444.
[2] a) M. Schnölzer, S. B. Kent, *Science* **1992**, *256*, 221 – 225; b) P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776 – 779.
[3] Such chemical ligation approaches do not necessarily require the protection of any side chain, which is a revolutionary improvement compared to earlier peptide coupling methods.
[4] a) For an example of introduction of a depsipeptidic bond: D. W. Low, M. G. Hill, *J. Am. Chem. Soc.* **2000**, *122*, 11039 – 11040; b) for an example of introduction of a *cis*-peptide bond mimic: A. Tam, U. Arnold, M. B. Soellner, R. T. Raines, *J. Am. Chem. Soc.* **2007**, *129*, 12670 – 12671.
[5] a) M. Baca, S. B. Kent, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11638 – 11642; b) H. F. Gaertner, R. E. Offord, R. Cotton, D. Timms, R. Camble, K. Rose, *J. Biol. Chem.* **1994**, *269*, 7224 – 7230; c) D. R. Englebretsen, B. Garnham, P. F. Alewood, *J. Org. Chem.* **2002**, *67*, 5883 – 5890.
[6] N. Assem, A. Natarajan, A. K. Yudin, *J. Am. Chem. Soc.* **2010**, *132*, 10986 – 10987.
[7] a) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057 – 3064; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708 – 2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596 – 2599.
[8] See for example: a) A. Brik, J. Alexandratos, Y. Lin, J. H. Elder, A. J. Olson, A. Wlodawer, D. S. Goodsell, C.-H. Wong, *ChemBioChem* **2005**, *6*, 1167 – 1169; b) Y. Hua, A. H. Flood, *Chem. Soc. Rev.* **2010**, *39*, 1262 – 1271; c) W. S. Horne, M. K. Yadav, C. D. Stout, M. R. Ghadiri, *J. Am. Chem. Soc.* **2004**, *126*, 15366 – 15367.

[9] E. Ko, J. Liu, L. M. Perez, G. Lu, A. Schaefer, K. Burgess, *J. Am. Chem. Soc.* **2011**, *133*, 462 – 477.
[10] Dipole moments were estimated using DFT calculations (B3LYP/6-31++G**) on simple model compounds; see the Supporting Information.
[11] J. T. Lundquist IV, J. C. Pelletier, *Org. Lett.* **2001**, *3*, 781 – 783.
[12] Triazole formation with peptide fragments containing only nonfunctionalized side chains, which therefore do not have to be protected, is known.
[13] For a recent review about the use of triazoles in peptidomimetics, see: D. J. Pedersen, A. Abell, *Eur. J. Org. Chem.* **2011**, 2399 – 2411.
[14] See for example: a) W. S. Horne, C. A. Olsen, J. M. Beierle, A. Montero, M. R. Ghadiri, *Angew. Chem.* **2009**, *121*, 4812 – 4818; *Angew. Chem. Int. Ed.* **2009**, *48*, 4718 – 4724; b) J. M. Beierle, W. S. Horne, J. H. van Maarseveen, B. Waser, J. C. Reubi, M. R. Ghadiri, *Angew. Chem.* **2009**, *121*, 4819 – 4823; *Angew. Chem. Int. Ed.* **2009**, *48*, 4725 – 4729; c) V. D. Bock, D. Speijer, H. Hiemstra, J. H. van Maarseveen, *Org. Biomol. Chem.* **2007**, *5*, 971 – 975.
[15] a) V. Aucagne, D. A. Leigh, *Org. Lett.* **2006**, *8*, 4505 – 4507; b) O. D. Montagnat, G. Lessene, A. B. Hughes, *Tetrahedron Lett.* **2006**, *47*, 6971 – 6974.
[16] a) I. E. Valverde, A. F. Delmas, V. Aucagne, *Tetrahedron* **2009**, *65*, 7597 – 7602; b) P. M. Gramlich, S. Warncke, J. Gierlich, T. Carell, *Angew. Chem.* **2008**, *120*, 3491 – 3493; *Angew. Chem. Int. Ed.* **2008**, *47*, 3442 – 3444; c) B. C. Doak, M. J. Scanlon, J. S. Simpson, *Org. Lett.* **2011**, *13*, 537 – 539.
[17] We excluded Cys-containing proteins which are readily obtainable using the NCL approaches.
[18] F. Lecaille, J. Kaleta, D. Brömme, *Chem. Rev.* **2002**, *102*, 4459 – 4488.
[19] a) O. Vasiljeva, T. Reinheckel, C. Peters, D. Turk, V. Turk, B. Turk, *Curr. Pharm. Des.* **2007**, *13*, 387 – 403; b) J. Reiser, B. Adair, T. Reinheckel, *J. Clin. Invest.* **2010**, *120*, 3421 – 3431.
[20] a) C. Palermo, J. A. Joyce, *Trends Pharmacol. Sci.* **2008**, *29*, 22 – 28; b) M. Kasabova, A. Saidi, C. Naudin, J. Sage, F. Lecaille, G. Lalmanach, *Clin. Rev. Bone Miner. Metab.* **2011**, *9*, 148 – 161; c) B. Turk, *Nat. Rev. Drug Discovery* **2006**, *5*, 785 – 799.
[21] S. Jenko, I. Dolenc, G. Guncar, A. Dobersek, M. Podobnik, D. Turk, *J. Mol. Biol.* **2003**, *326*, 875 – 885.
[22] W. Bode, R. Huber, *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **2000**, *1477*, 241 – 252.
[23] We focused on Gly-Xaa disconnections for the present proof-of-concept study. Among the eight Gly residues, ^{36}Gly-Lys and ^{60}Gly-Asp amide bonds were the best suited to ensure an optimal length of the three fragments.
[24] J. Alsina, T. S. Yokum, F. Albericio, G. Barany, *J. Org. Chem.* **1999**, *64*, 8761 – 8769.
[25] a) D. Bonnet, S. Riché, S. Loison, R. Dagher, M. C. Frantz, L. Boudier, R. Rahmeh, B. Mouillac, J. Haiech, M. Hibert, *Chem. Eur. J.* **2008**, *14*, 6247 – 6254; b) J. Springer, K. R. de Cuba, S. Calvet-Vitale, J. A. J. Geenevasen, P. H. H. Hermkens, H. Hiemstra, J. H. van Maarseveen, *Eur. J. Org. Chem.* **2008**, 2592 – 2600.
[26] S. Estrada, A. Pavlova, I. Björk, *Biochemistry* **1999**, *38*, 7339 – 7345.
[27] An initial attempt for the synthesis of the N-terminal fragment **1** including the genuine ^{1}Met residue warned us about the propensity to oxidation of this N-terminal Met: the major peak in HPLC after final cleavage from the SPPS resin was accompanied by a significant peak with a corresponding measured $[M+H]^+$ mass that was shifted by 16 Da, which we attributed to ^{1}Met sulfoxide. We concluded that this oxidation was due to "sequence-dependent" oxidation susceptibility and therefore decided to avoid the ^{1}Met residue in the final triazole-containing construct.
[28] T. Wöhr, F. Wahl, A. Nefzi, B. Rohwedder, T. Sato, X. Sun, M. Mutter, *J. Am. Chem. Soc.* **1996**, *118*, 9218 – 9227.
[29] a) G.-A. Cremer, H. Tariq, A. F. Delmas, *J. Pept. Sci.* **2006**, *12*, 437 – 442; b) F. García-Martín, P. White, R. Steinauer, S. Côté, J. Tulla-Puche, F. Albericio, *Biopolymers* **2006**, *84*, 566 – 575.
[30] In the folded protein, this hydrophobic fragment adopts an extended antiparallel β-sheet substructure (blue portion, Figure 1b), usually linked to difficult synthesis and propensity for aggregation.
[31] Compound **2a** possesses a very limited aqueous solubility at neutral pH values, and a cosolvent or an additive was required. Although it was fully soluble in guanidine hydrochloride (6 M) or urea (8 M), the cycloaddition was extremely slow in our hands, and the target compound **4a** was accompanied with unidentified coproducts.
[32] E. Pol, S. L. Olsson, S. Estrada, T. W. Prasthofer, I. Björk, *Biochem. J.* **1995**, *311*, 275 – 282.
[33] E. A. Auerswald, D. K. Nägler, A. J. Schulze, R. A. Engh, G. Genenger, W. Machleidt, H. Fritz, *Eur. J. Biochem.* **1994**, *224*, 407 – 415.
[34] G. D. Green, A. A. Kembhavi, M. E. Davies, A. J. Barrett, *Biochem. J.* **1984**, *218*, 939 – 946.
[35] B. Japelj, J. P. Waltho, R. Jerala, *Proteins Struct. Funct. Genet.* **2004**, *54*, 500 – 512.
[36] A lower stability of recombinant cystatin A at pH 3.0 compared to the synthetic triazole-containing version could be attributed to the His-tag.
[37] L. Gillet, S. Roger, P. Besson, F. Lecaille, J. Gore, P. Bougnoux, G. Lalmanach, J. Y. Le Guennec, *J. Biol. Chem.* **2009**, *284*, 8680 – 8691.
[38] Matrigel is a marketed mixture that effectively mimics the extracellular matrix.
[39] As expected, the cell migration is strongly and reproducibly inhibited, but not completely abolished, because cysteine cathepsins are not the sole actors of this process.

