Receptor Targeting

DOI: 10.1002/ange.201406842

A Multivalent Ligand for the Mannose-6-Phosphate Receptor for Endolysosomal Targeting of an Activity-Based Probe**

*Sascha Hoogendoorn, Gijs H. M. van Puijvelde, Johan Kuiper, Gijs A. van der Marel, and Herman S. Overkleeft**

Abstract: *The ubiquitously expressed mannose-6-phosphate receptors (MPRs) are a promising class of receptors for targeted compound delivery into the endolysosomal compartments of a variety of cell types. The development of a synthetic, multivalent, mannose-6-phosphate (M6P) glycopeptide-based MPR ligand is described. The conjugation of this ligand to fluorescent DCG-04, an activity-based probe for cysteine cathepsins, enabled fluorescent readout of its receptor-targeting properties. The resulting M6P-cluster–BODIPY–DCG-04 probe was shown to efficiently label cathepsins in cell lysates as well as in live cells. Furthermore, the introduction of the 6-O-phosphates leads to a completely altered uptake profile in COS and dendritic cells compared to a mannose-containing ligand. Competition with mannose-6-phosphate abolished all uptake of the probe in COS cells, and we conclude that the mannose-6-phosphate cluster targets the MPR and ensures the targeted delivery of cargo bound to the cluster into the endolysosomal pathway.*

The 300 kDa cation-independent mannose-6-phosphate receptor (CI-MPR/IGFII-R) and its smaller 46 kDa homologue, the cation-dependent mannose 6-phosphate receptor (CD-MPR), are essential for the correct trafficking of newly synthesized lysosomal enzymes from the trans-Golgi network to the lysosomes. The receptor population is largely localized intracellularly but a small (ca. 10%) fraction of the receptors shuttles continuously between the cell membrane and the endocytic pathway.[1] The endocytic nature of the receptor, combined with its ubiquitous expression on fibroblast cells of various tissues,[2] makes it a valid candidate for drug targeting. Indeed, current enzyme replacement therapies for the lysosomal storage disorders Fabry disease and Pompe disease make use of the binding interaction between mannose-6-phosphate (M6P) residues on the complex N-glycan chains of enzymes recombinantly expressed in CHO cells and membrane-bound CI-MPRs.[3] The internalization of recombinant enzyme bound to the CI-MPR ensures delivery into the endolysosomal compartments of cells deficient in the lysosomal enzyme. Structural and biochemical studies have revealed multiple M6P-binding domains in the CI-MPR[4] and the formation of receptor dimers for both types of MPR.[5] Furthermore, the CI-MPR has a relatively low affinity for M6P ($K_d = 7$ μM) compared to glycoproteins that contain multiple phosphorylated mannose residues ($K_d = 2$–20 nM).[6] The notion that multivalency increases the affinity of M6P-containing ligands towards the receptor has prompted several groups to investigate synthetic ligands since these enable the creation of defined structures for both the glycan chain and the spacing between the phosphate residues.[7] We previously reported a synthetic mannose cluster[8] that contains six flexibly spaced mannose units, and which functions as a ligand for mannose-binding lectins on dendritic cells. Herein, we describe the synthesis and biological evaluation of an analogous mannose-6-phosphate cluster (M6PC, **10**; Figure 1) with the aim of targeting the mannose-6-phosphate receptor. The flexible nature of the cluster may enable multivalent binding interactions, thereby increasing potency and possibly receptor internalization.[9] To investigate the MPR-targeting properties of this ligand, we conjugated the cluster to fluorescent BODIPY–DCG-04, an activity-based probe for cysteine cathepsins, a family of proteases that mostly reside in the endolysosomal compartments.[10] Cathepsins play important roles in both health and disease[11] and activity-based probes are powerful tools to visualize and modulate their activity.[12] We demonstrate the ability of the M6PC–BODIPY–DCG-04 probe (**11**; Figure 1) to label cathepsins in cell and tissue lysates. We further report an in situ analysis of the uptake and trafficking of **11** and its inhibition of cathepsins in both COS cells, a fibroblast-like cell line, and dendritic cells. We finally show that M6PC–BODIPY–DCG-04 (**11**) differs in its targeting ability from its nonphosphorylated counterpart.

Peptide scaffold **7** was used as a starting point for the solid-phase peptide synthesis (SPPS) approach to mannose-6-phosphate cluster **10** (Figure 1).[8] We envisaged that a triazole moiety could be used to connect the sugar to the peptide backbone since it has been shown that the aglycone part of the mannoside does not interfere with receptor binding.[7c] For this purpose, a propargyl mannose-6-phosphate building block was designed and synthesized that contains acid-labile *tert*-butyl protective groups on the phosphate group and base-labile benzoyl groups on the hydroxy groups (compound **6**, Figure 1, and Scheme S1 in the Supporting Information),

[*] Dr. S. Hoogendoorn, Prof. Dr. G. A. van der Marel, Prof. Dr. H. S. Overkleeft
Bio-organic Synthesis, Leiden Institute of Chemistry
Leiden University
Einsteinweg 55, 2300 RA, Leiden (The Netherlands)
E-mail: h.s.overkleeft@chem.leidenuniv.nl
Dr. G. H. M. van Puijvelde, Prof. Dr. J. Kuiper
Dept of Biopharmaceutics, LACDR, Leiden (The Netherlands)

[**] We thank H. van den Elst for assisting with HPLC–MS purifications and C. Erkelens, F. Lefeber, and K. B. S. S. Gupta for NMR measurements. This research was funded by the Netherlands Organization for Scientific Research (NWO-CW) and the European Research Council.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201406842.

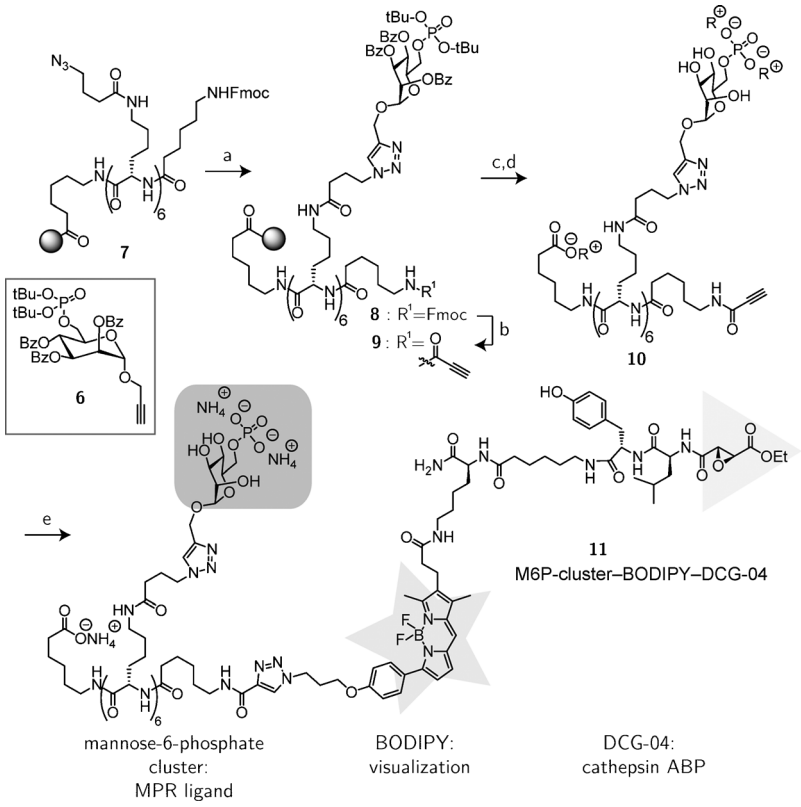

Figure 1. Design and synthesis of the fluorescent MPR-targeted activity-based cathepsin probe **11**. Reagents and conditions: a) Mannose-6-phosphate (**6**), sodium ascorbate, $CuSO_4$, CH_2Cl_2/H_2O, RT, 72 h; b) 1. 20% piperidine/NMP, RT, 15 min; 2. propiolic acid, EEDQ, CH_2Cl_2, RT, 1.5 h; c) TFA/TIS (98/2, v/v), 3×15 min; d) 1. K_2CO_3, MeOH, RT, 24 h; 2. Citric acid (aq); 3. RP-HPLC. R = NH_4^+ (used in the next step) or R = Na^+ (after Chelex-Na^+ ion exchange); e) BODIPY(TMR)–DCG-04 (**12**), sodium ascorbate, $CuSO_4$, DMF/H_2O (26%). NMP = *N*-methyl-2-pyrrolidinone, EEDQ = 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroxyquinoline, DMF = dimethylformamide.

protecting groups that are compatible with SPPS conditions. Protected propargyl mannose-6-phosphate **6** was attached to the six azide groups in peptide **7** on solid support by Cu^I-catalyzed Huisgen 1,3-cycloaddition.[13] Next, the Fmoc group was removed and propiolic acid was coupled to the primary amine with the use of EEDQ. The peptide was cleaved from the resin by using acidic conditions, which also ensured complete deprotection of the phosphate groups. We consider this protecting-group strategy to be a considerable improvement compared to previously reported methods towards M6P derivatives. These rely on either 2,2,2-trichloroethyl protection of the phosphate group, which requires harsh solution-phase conditions for cleavage,[7a] or the use of unprotected phosphate, which results in electronic repulsion and coupling difficulties.[7b] Global deprotection was accomplished by using a saturated solution of K_2CO_3 in methanol, followed by quenching with citric acid. The glycopeptide was purified under neutral NH_4OAc conditions and lyophilized to give the ammonium salt of compound **10**. ^{31}P NMR analysis showed a very broad peak, which is indicative of multiple salt forms of the peptide. The peptide was ion-exchanged to the sodium form by using Chelex-Na^+ resin, thereby resulting in a homogeneous salt form of **10** as seen by a sharp phosphate peak in the NMR spectrum. However, the final compound M6PC–BODIPY–DCG-04 (**11**) was only successfully synthesized by using the heterogeneous NH_4^+ phosphate cluster because of solubility issues with either the starting material or the product. The use of the more reactive propiolic acid handle instead of the previously employed pentynoic acid[8,14] resulted in a drastic improvement of the click reaction (1 h, room temperature) between the mannose-6-phosphate cluster **10** and BODIPY(TMR)–DCG-04 (**12**; Figure S1 in the Supporting Information). Subsequent purification by HPLC–MS under NH_4OAc conditions provided the (mostly) ammonium salt form of probe **11**, which was used without further cation-exchange procedures.

As shown in Figure 2, we first determined the ability of compound **11** to function as an activity-based probe for cathepsins by performing lysate labeling experiments. For this, increasing concentrations of the probe were added to lysates of different origins (mouse liver, mouse dendritic cells, or monkey COS cells) at pH 5.5, the optimal pH for most of the cathepsin activities.[10] Concentration-dependent labeling of active cathepsins was observed in each lysate, with a shift in molecular weight corresponding to the mass of the probe (Figure 2, compare lane 5 (1 μM **11**) to lane 11 (1 μM **14,** BODIPY(FL)-DCG-04; Figure S1). Inactivation of the cathepsins by preincubation with 1 or 10 μM of the known cathepsin inhibitors AS44[8] or various DCG-04 deriva-

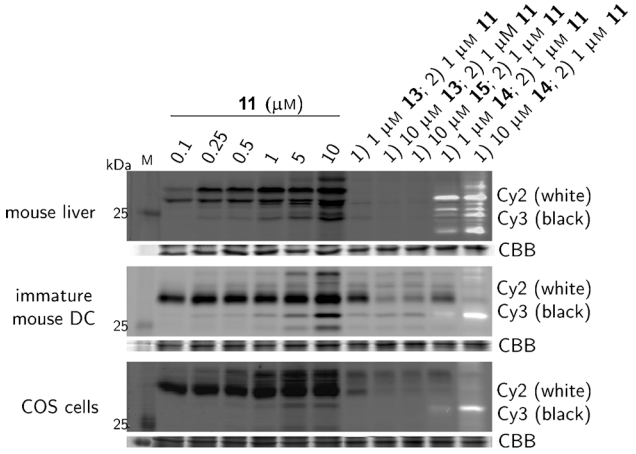

Figure 2. Lysate cathepsin labeling. Various cell lysates (10–20 μg total protein) were incubated (1 h, 37°C) with increasing concentrations of probe **11** at pH 5.5. Alternatively, lysates were incubated (1 h, 37°C) with azido-DCG-04 (**13**, 1 or 10 μM), AS44 (**15**, 10 μM) or BODIPY(FL)–DCG-04 (**14**, 1 or 10 μM), before treatment with **11** (1 μM, 1 h, 37°C). Proteins were resolved by 12.5% SDS-PAGE, followed by fluorescence scanning [Cy2: BODIPY(FL), Cy3: BODIPY(TMR)] and total protein staining with coomassie brilliant blue (CBB).

tives[8,15] (for structures, see Figure S1) resulted in loss of labeling with probe **11**, thus showing that it competes for the same set of cathepsins.

Having established that attachment of the mannose-6-phosphate cluster does not interfere with cathepsin profiling, we compared the new probe head-to-head with the previously reported mannose-cluster–BODIPY–DCG-04 probe (**16**;[8] Figure S1). To elucidate whether our probe is taken up by MPR-mediated endocytosis, we used COS cells, an African green monkey fibroblast-like cell line.

Cells were incubated for 16 h with 0.5 μM of the MPR-targeted probe **11**, nontargeted fluorescent BODIPY(TMR)-DCG-04 (**12**), or the mannose receptor (MR)-targeted probe **16** and subsequently analyzed by flow cytometry. As shown in Figure 3 A, only cells that were treated with probes **11** or **12** showed a shift in the population fluorescence indicative of probe binding/uptake. Cellular fluorescence was time-dependent in case of probe **11** (50 % positive after 6 h compared to 74 % after 16 h of incubation) and was almost completely inhibited in the presence of 1 mM M6P. COS cells treated for 6 h with 0.5 μM of probe **11** and imaged by confocal fluorescence microscopy showed bright intracellular fluorescent vesicles, thus establishing that the compound was taken up into the endocytic pathway (Figure 3 B). Next, as additional proof for MPR-dependent uptake and trafficking of the probe into the lysosomes, we determined the cathepsin labeling. Cells were treated under various conditions, washed, lysed, and analyzed by 12.5 % SDS-PAGE followed by in-gel fluorescence scanning. In agreement with the flow cytometry data, no labeling was observed for mannose-cluster–BODIPY–DCG-04 (**16**), a result in accordance with the selectivity of this probe for mannose-binding lectins. The M6P probe **11**, on the other hand, showed both concentration- and time-dependent labeling of cathepsins (Figure 3 C, D). As shown in Figure 3 E, cathepsin labeling was inhibited when using increasing concentrations of M6P, with almost complete inhibition at 1 mM M6P. Even when taking into account that the probe contains six M6P residues, a large molar excess of monovalent M6P is needed to inhibit its uptake. Somewhat surprisingly, pretreatment of the cells with nonfluorescent azido-DCG-04 (**13**) did not compete with cathepsin labeling by the targeted probe. To clarify this, we used green fluorescent nontargeted BODIPY(FL)-DCG-04 (**14**) in COS cathepsin labeling studies and indeed observed only background bands and not the expected labeling as seen in lysates (Figure 2 and Figure S2). Presumably, diffusion of the nontargeted probes into the endolysosomal compartments of COS cells is ineffective. Together, these findings highlight the efficiency with which the targeted probe **11** is taken up by MPR-mediated endocytosis into COS cells and trafficked to the endolysosomal compartments, thereby leading to the inactivation of cathepsins.

In a next set of experiments, we treated live immature mouse dendritic cells (DCs) with varying concentrations of either probe. This resulted in similar concentration-dependent labeling profiles, which are completely absent after preincubation with unlabeled azido-DCG-04 (**13**; Figure S3).[16] Confocal fluorescence microscopy and SDS-PAGE analysis confirmed that blockage of the mannose-binding lectins present on DCs with the yeast oligomannoside mannan (3 mg mL^{-1}) results in inhibition of the uptake of the mannose-cluster probe **16**.[8,14] By contrast, the uptake and

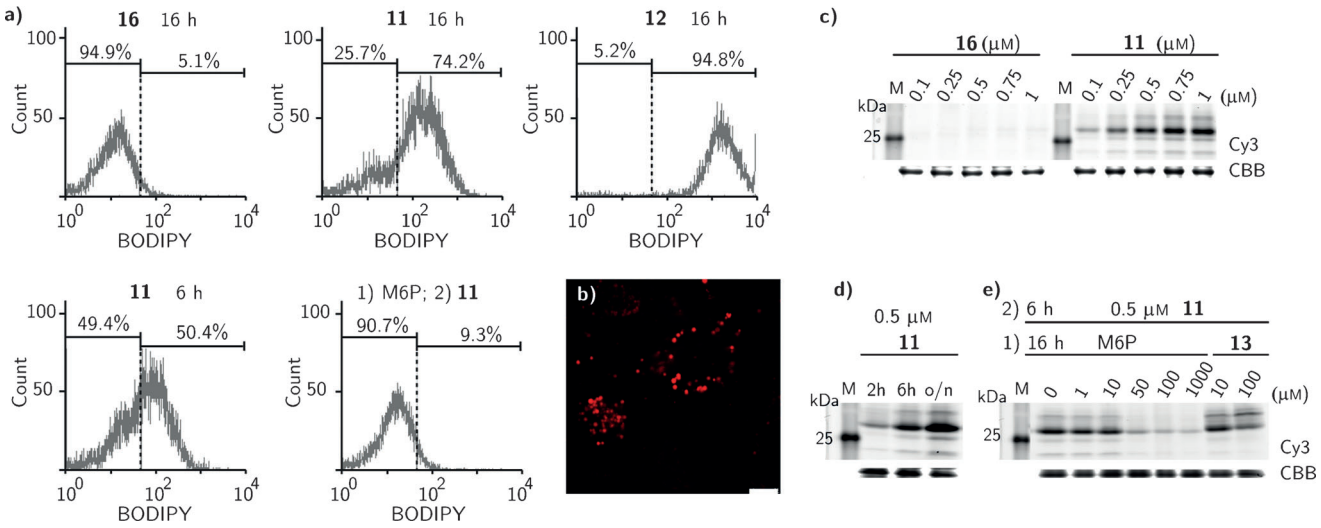

Figure 3. M6PC–BDP–DCG-04 (**11**) uptake and cathepsin labeling in live COS cells. a) Flow cytometry analysis of COS cells that were treated with 0.5 μM M6PC–BDP–DCG-04 (**11**, 6 h or 16 h), MC–BDP–DCG-04 (**16**, 16 h), the nontargeted probe BODIPY(TMR)–DCG-04 (**12**, 16 h), or first with 1 mM M6P (16 h) followed by treatment with probe **11** for 6 h. Mean percentages of cells in the negative (left) and positive (right) populations from three independent experiments are given. b) Representative confocal microscopy image of live COS cells treated with probe **11** (0.5 μM) for 6 h, showing fluorescently labeled intracellular compartments. Scale bar: 7.5 μm. c,d) COS cells were treated with increasing concentrations of probe **11** or **16** for 16 h (c) or with a 0.5 μM concentration of **11** for different periods of time (d) before being washed, lysed, and analyzed by 12.5 % SDS-PAGE. Representative fluorescence scans (Cy3) and total protein stains (CBB) are depicted. e) Competition experiments between different concentrations of M6P or azido-DCG-04 (**13**; 16 h, 37 °C) and **11** (0.5 μM, 6 h, 37 °C). After treatment, cells were washed with PBS, lysed, and resolved on a 12.5 % SDS-PAGE gel. BDP = BODIPY, M6P(C) = mannose-6-phosphate (cluster), MC = mannose cluster, PBS = phosphate-buffered saline.

cathepsin binding of phosphorylated probe **11** is not inhibited by mannan, thus indicating that the phosphorylated mannose residues are unable to bind to the MR and that the uptake of this probe occurs through other means. An attempt at uptake inhibition through the addition of 1 mM of M6P to the medium did not succeed. Since DCs are highly endocytotically active, other processes besides receptor-mediated endocytosis (such as macropinocytosis) may be responsible for probe uptake in these cells.[17]

In conclusion, we have shown that peptide-based targeting devices bearing 6-*O*-phosphorylated mannose units have completely altered targeting properties compared to non-phosphorylated mannose-bearing peptide conjugates. Since the MPR is widely expressed on a variety of cells and tissues, we envisage that our artificial M6P cluster may provide an effective means of broad targeted delivery. Cathepsins are efficiently inhibited by the targeted probe, a result that has wider implications given that these enzymes are associated with various diseases.[11b] The ability to monitor and perturb cathepsin activities in a variety of cell types or tissues, depending on the choice of ligand, could thus be greatly advantageous. The conjugation of our M6P cluster to other biological entities, such as recombinant lysosomal enzymes, may thus be a suitable alternative for their targeted delivery into cells affected by lysosomal storage disorders such as Pompe or Fabry disease.

Received: July 3, 2014
Published online: August 22, 2014

Keywords: activity-based protein profiling · drug delivery · endolysosomal pathway · fluorescent probes · mannose-6-phosphate

[1] P. Ghosh, N. M. Dahms, S. Kornfeld, *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 202 – 213.
[2] M. R. Natowicz, M. M.-Y. Chi, O. H. Lowry, W. S. Sly, *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 4322 – 4326.
[3] a) Y. A. Ioannou, D. F. Bishop, R. J. Desnick, *J. Cell Biol.* **1992**, *119*, 1137 – 1150; b) J. van Hove, H. W. Yang, J.-Y. Wu, R. O. Brady, Y.-T. Chen, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 65 – 70.
[4] M. K. Hancock, D. J. Haskins, G. Sun, N. M. Dahms, *J. Biol. Chem.* **2002**, *277*, 11255 – 11264.
[5] a) D. L. Roberts, D. J. Weix, N. M. Dahms, J.-J. P. Kim, *Cell* **1998**, *93*, 639 – 648; b) L. J. Olson, J. Zhang, N. M. Dahms, J.-J. P. Kim, *J. Biol. Chem.* **2002**, *277*, 10156 – 10161; c) J. C. Byrd, J. H. Park, B. S. Schaffer, F. Garmroudi, R. G. MacDonald, *J. Biol. Chem.* **2000**, *275*, 18647 – 18656.
[6] P. Y. Tong, W. Gregory, S. Kornfeld, *J. Biol. Chem.* **1989**, *264*, 7962 – 7969.
[7] a) M. K. Christensen, M. Meldal, K. Bock, H. Cordes, S. Mouritsen, H. Elsner, *J. Chem. Soc. Perkin Trans. 1* **1994**, 1299 – 1310; b) H. Franzyk, M. K. Christensen, R. M. Jorgensen, M. Meldal, H. Cordes, S. Mouritsen, K. Bock, *Bioorg. Med. Chem.* **1997**, *5*, 21 – 40; c) J. J. Distler, J. Guo, G. W. Jourdian, O. P. Srivastava, O. Hindsgaul, *J. Biol. Chem.* **1991**, *266*, 21687 – 21692.
[8] U. Hillaert, M. Verdoes, B. I. Florea, A. Saragliadis, K. L. L. Habets, J. Kuiper, S. Van Calenbergh, F. Ossendorp, G. A. van der Marel, C. Driessen, H. S. Overkleeft, *Angew. Chem. Int. Ed.* **2009**, *48*, 1629 – 1632; *Angew. Chem.* **2009**, *121*, 1657 – 1660.
[9] S. J. York, L. S. Arneson, W. T. Gregory, N. M. Dahms, S. Kornfeld, *J. Biol. Chem.* **1999**, *274*, 1164 – 1171.
[10] K. Brix, A. Dunkhorst, K. Mayer, S. Jordans, *Biochimie* **2008**, *90*, 194 – 207.
[11] a) M. M. Mohamed, B. F. Sloane, *Nat. Rev. Cancer* **2006**, *6*, 764 – 775; b) J. Reiser, B. Adair, T. Reinheckel, *J. Clin. Invest.* **2010**, *120*, 3421 – 3431.
[12] a) M. Verdoes, K. Oresic Bender, E. Segal, W. A. van der Linden, S. Syed, N. P. Withana, L. E. Sanman, M. Bogyo, *J. Am. Chem. Soc.* **2013**, *135*, 14726 – 14730; b) S. D. Wiedner, L. N. Anderson, N. C. Sadler, W. B. Chrisler, V. K. Kodali, R. D. Smith, A. T. Wright, *Angew. Chem. Int. Ed.* **2014**, *53*, 2919 – 2922; *Angew. Chem.* **2014**, *126*, 2963 – 2966; c) J. A. Joyce, A. Baruch, K. Chehade, N. Meyer-Morse, E. Giraudo, F.-Y. Tsai, D. C. Greenbaum, J. H. Hager, M. Bogyo, D. Hanahan, *Cancer Cell* **2004**, *5*, 443 – 453.
[13] 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. Int. Ed.* **2002**, *41*, 2596 – 2599; *Angew. Chem.* **2002**, *114*, 2708 – 2711.
[14] S. Hoogendoorn, K. L. Habets, S. Passemard, J. Kuiper, G. A. van der Marel, B. I. Florea, H. S. Overkleeft, *Chem. Commun.* **2011**, *47*, 9363 – 9365.
[15] D. Greenbaum, K. F. Medzihradszky, A. Burlingame, M. Bogyo, *Chem. Biol.* **2000**, *7*, 569 – 581.
[16] D. Greenbaum, A. Baruch, L. Hayrapetian, Z. Darula, A. Burlingame, K. F. Medzihradszky, M. Bogyo, *Mol. Cell. Proteomics* **2002**, *1*, 60 – 68.
[17] F. Sallusto, M. Cella, C. Danieli, A. Lanzavecchia, *J. Exp. Med.* **1995**, *182*, 389 – 400.