Posttranslational Modifications

International Edition: DOI: 10.1002/anie.201605366
German Edition: DOI: 10.1002/ange.201605366

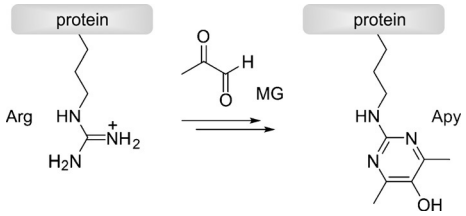
Impaired Chaperone Activity of Human Heat Shock Protein Hsp27 Site-Specifically Modified with Argpyrimidine

*Maria Matveenko, Elena Cichero, Paola Fossa, and Christian F. W. Becker**

Abstract: *Non-enzymatic posttranslational modifications (nPTMs) affect at least ~30% of human proteins, but our understanding of their impact on protein structure and function is limited. Studies of nPTMs are difficult because many modifications are not included in common chemical libraries or protein expression systems and should be introduced site-specifically. Herein, we probed the effect of the nPTM argpyrimidine on the structure and function of human protein Hsp27, which acquires argpyrimidine at residue 188 in vivo. We developed a synthetic approach to an argpyrimidine building block, which we then incorporated at position 188 of Hsp27 through protein semisynthesis. This modification did not affect the protein secondary structure, but perturbed the oligomeric assembly and impaired chaperone activity. Our work demonstrates that protein function can be altered by a single nPTM and opens up a new area of investigation only accessible by methods that allow site-selective protein modification.*

Protein structure and function are often modulated by enzyme-controlled chemical reactions with small molecules that result in covalent posttranslational modifications (PTMs).[1] In addition, protein modification can proceed in an enzyme-independent fashion through the binding of electrophilic metabolites to nucleophilic groups in protein side chains.[2] The ensuing non-enzymatic posttranslational modifications (nPTMs) comprise a large and expanding group of chemical moieties that include the so-called AGEs (advanced glycation end products)[3] and ALEs (advanced lipoxidation end products),[4] caused by metabolites originating from carbohydrate or lipid sources.[5] These modifications have become prominently linked to aging, diabetes, uremia, vascular disease, inflammatory disorders, neurodegenerative diseases, cancer, and cataract.[3,4,5b,6] They have been recognized not only as markers of pathology, but also as promoting disease initiation and progression.[5b] nPTM formation is subject to the general determinants of nucleophilic additions, such as reactant concentration, steric accessibility, acid-base catalysis, and reaction time, and thus the resulting distribution pattern of nPTMs under native conditions is not random[7] but determined by protein structure, microenvironment, and half-life. On the other hand, the majority of biochemical studies of nPTM-derivatized proteins have been conducted by incubating proteins with high concentrations of electrophiles, often precluding selectivity observed in vivo and favoring crosslink formation. Major differences may exist in the nature and location of nPTMs formed in this way compared to those found in vivo.[8] In addition, there are no effective mutagenesis-based strategies for introducing or mimicking nPTMs, and thus very little is known about the structural and functional consequences of selective, biologically-relevant nPTM formation. Therefore, new methods are required that can introduce nPTMs in a site-directed manner and provide homogenous modified proteins to pinpoint the biological effect of particular nPTMs. Promising steps toward this goal have recently been made by establishing chemical access to several nPTMs in the context of small peptides.[9]

Here, we focused on the effect of the formation of argpyrimidine (Apy), a nPTM that has been detected, to varying extents, in healthy and cataractous lenses,[10] in amyloid fibers from patients with familial amyloidotic polyneuropathy,[11] and in tissues from diabetic and cancer patients.[12] Apy is a fluorescent adduct formed between arginine and methylglyoxal (MG, Scheme 1),[13] a highly reactive electrophile generated as an unavoidable by-product of glycolysis and responsible for the modification of at least ~30% of human proteins.[14]



Scheme 1. Modification of arginine with methylglyoxal (MG) can produce argpyrimidine (Apy), a non-enzymatic posttranslational modification (nPTM).

Apy has been co-immunoprecipitated with human small heat shock protein Hsp27 (HspB1) in a number of diseased human samples including diabetic heart,[15] brunescent cataractous lenses,[16] hyperglycemic endothelial cells,[17] and several cancer cells and tissues.[12b,18] Hsp27 is an ATP-independent chaperone that is upregulated in response to a variety of stress conditions.[19] In cancer cells, expression of

[*] Dr. M. Matveenko, Prof. Dr. C. F. W. Becker
University of Vienna, Faculty of Chemistry
Institute of Biological Chemistry
Währinger Str. 38, 1090 Vienna (Austria)
E-mail: christian.becker@univie.ac.at
Dr. E. Cichero, Prof. Dr. P. Fossa
University of Genoa, Department of Pharmacy
Viale Benedetto XV n.3, 16132 Genoa (Italy)
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/anie.201605366.

Hsp27 is abnormally high, and the protein may participate in oncogenesis and resistance to chemotherapy.[20] The 205-amino acid protein has 16 arginines (Figure 1 a), but while all of these are in principle susceptible to Apy formation, only mutation of Arg188 (to Gly) eliminated the recognition of the protein by an *anti*-argpyrimidine antibody and the take-up of ^{14}C-labeled MG.[18d]

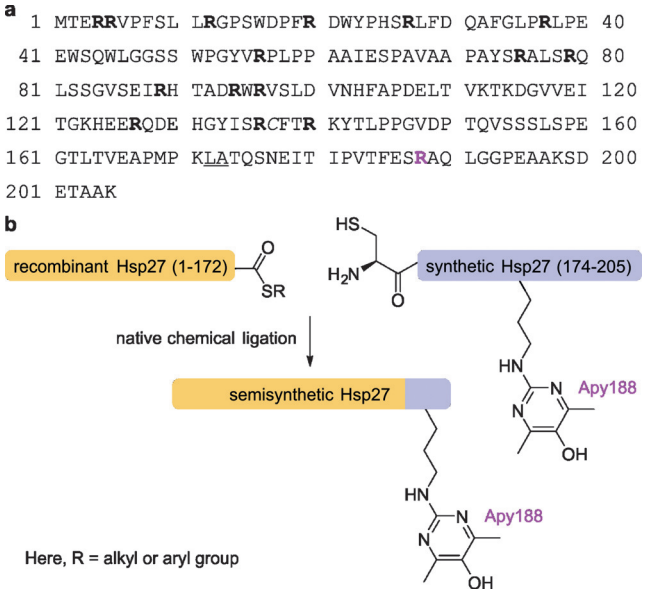
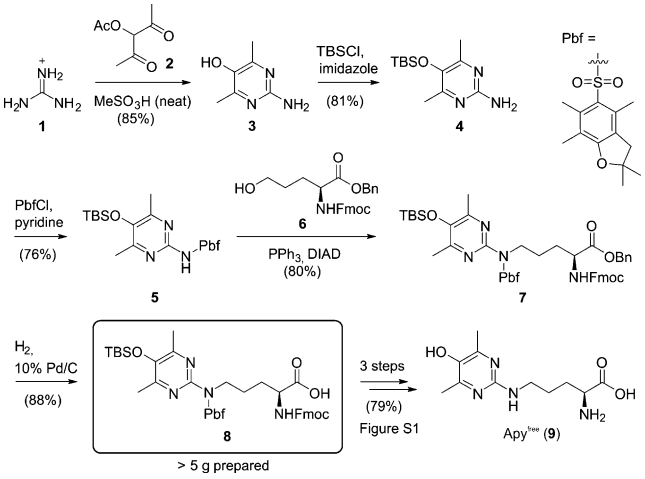


Figure 1. a) Amino acid sequence of Hsp27, indicating the position that has been observed to undergo the Apy modification in human cancer cells (R188, magenta); the only cysteine residue (C137) is shown in italic; the ligation site (see below) is underlined. b) Overview of the strategy for the semisynthesis of Hsp27 bearing the Apy188 modification.

Despite the biological significance of Apy formation at position 188 of Hsp27, no functional consequences of this modification at the molecular level have been reported to date. The required homogeneously Apy-modified protein is not attainable by incubation with MG, as it gives rise to mixtures of differentially modified and cross-linked proteins. Likewise, there are no amino acids that can mimic the unique electronic and structural features of Apy for use in mutagenesis. To address this limitation, we devised a robust semisynthetic strategy to introduce Apy site-specifically that requires the synthesis of a suitably protected Apy building block, its incorporation into a peptide, and subsequent ligation to a recombinantly produced Hsp27 segment (expressed protein ligation, EPL[21]; Figure 1 b). EPL is a powerful combination of recombinant protein expression and solid phase peptide synthesis (SPPS), which, along with chemical protein synthesis,[22] has enabled deciphering the impact of enzymatic PTMs on a large variety of proteins.[23] Herein, the selectively modified C-terminal peptide could be appended to the longer segment produced recombinantly as a protein α-thioester covering amino acids 1–172. In the absence of a strategically useful cysteine residue for the pivotal native chemical ligation (NCL),[24] peptide Hsp27 (174–205) was extended with an N-terminal cysteine (Cys173), which can be reverted, after the ligation, to the native Ala173 by chemical desulfurization.[25] While the desulfurization step is not selective and thus also reduces the only native cysteine 137 to alanine, this Cys137Ala conversion is functionally neutral in the context of our characterization.[26]

For our approach to Apy-modified Hsp27, we required an efficient, multigram-scale synthetic route that affords Apy as a building block for standard Fmoc-based SPPS. By contrast, the syntheses of Apy published so far are limited to providing protecting-group-free compounds on analytical scale.[9b,13,27] Our synthesis commenced with the construction of the aminopyrimidine ring of Apy by subjecting readily available guanidine sulfate to an excess of the known 1,3-diketone **2**[28] in methanesulfonic acid (Scheme 2). TBS-protection of the



Scheme 2. Chemical synthesis of multi-gram quantities of Apy as a building block for Fmoc-based SPPS and access to the unprotected amino acid, Apy^{free}.

ensuing 2-aminopyrimidinol **3**, followed by the activation of the amine function as the corresponding 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) sulfonamide, allowed the resulting intermediate **5** to engage in a smooth Mitsunobu reaction[29] with the glutamate derivative **6**.[30] Gentle hydrogenolysis of the resulting benzyl ester **7** then furnished the desired Apy building block **8**. Our synthesis allowed us to prepare over 5 g of this valuable material in five steps with an overall yield of 37 %. We then went on to obtain pure samples of the free amino acid Apy (Apy^{free}, **9**) for analytical purposes through global deprotection of **8** (see Figure S1 and the Supporting Information). The spectral and physical data derived from this amino acid were in excellent agreement with the literature values.[9b,13,27] In particular, the UV/Vis spectrum of this material displays the expected absorption maxima at 230 and 320 nm (Figure 2 a), consistent with the values reported by Nagaraj et al.[13] Likewise, the characteristic Apy excitation and emission maxima at 320 and 385 nm, respectively,[13] are observed in the fluorescence spectrum of our synthetic Apy^{free} (Figure S2).

With the Apy building block in hand, we proceeded to synthesize the 33-mer peptide that comprises the C-terminus

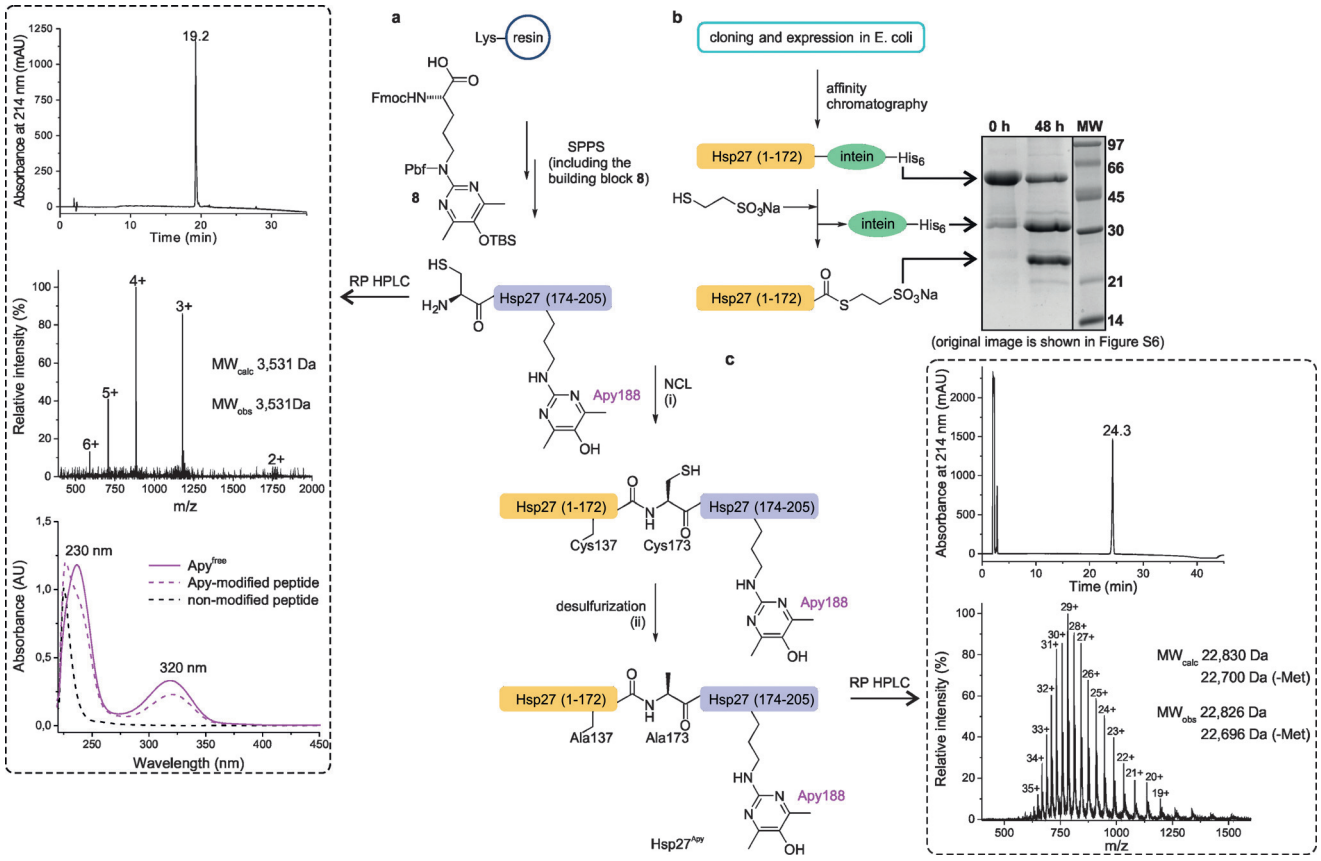


Figure 2. Semisynthesis of Hsp27^{Apy}. a) Fmoc-based SPPS afforded Apy-modified peptide that was characterized by analytical HPLC, ESI-MS, and UV/Vis spectroscopy. b) Thiolysis of the expressed fusion protein Hsp27 (1–172)-Mxe GyrA intein-His$_6$ afforded Hsp27 (1–172) α-thioester. c) NCL of the two segments followed by radical desulfurization provided Hsp27^{Apy} that was characterized by analytical HPLC and ESI-MS: i) 6 M Gdn·HCl, 0.2 M NaPi, 250 mM MESNA, 50 mM TCEP, pH 7.8; ii) 6 M Gdn·HCl, 0.2 M NaPi, 200 mM TCEP, 16 mM V-50, pH 6.7.

of Hsp27 including Apy188 and an N-terminal cysteine residue (Apy-modified peptide; Figure 2a). As anticipated, automated Fmoc-SPPS using the building block **8** proceeded readily to afford sufficient quantities (~20 mg) of the required peptide in a purity of >95% and in 6% yield (based on the crude material) after HPLC. Notably, global deprotection and cleavage of the peptide from the resin went smoothly and did not require additional steps or unconventional reagents. The corresponding peptide without the Apy modification (non-modified peptide) was prepared as well in a similar purity and yield (>95% and 10%, respectively; Figure S4). Fluorescence properties of Apy were retained after its incorporation into the peptide with an absorption and emission maxima at 320 and 385 nm, respectively (Figure S5), along with the expected absorptions in the UV region around 230 and 320 nm (Figure 2a).

In parallel experiments, protein α-thioester encompassing the bulk of Hsp27 was generated (Figure 2b). Hsp27 (1–172) segment was expressed in *E. coli* as an N-terminal fusion with Mxe GyrA intein and a polyhistidine purification tag, and this protein was then harvested within inclusion bodies. Solubilized proteins were subjected to nickel affinity chromatography to obtain the required fusion protein accompanied by traces of prematurely excised intein-His$_6$-containing segments (Figure 2b, SDS-PAGE analysis, lane 1). Thiolysis of this material using an excess of sodium 2-mercaptoethanesulfonate (MESNA; Figure 2b, SDS-PAGE analysis, lane 2), followed by HPLC purification, afforded sufficient amounts of the protein α-thioester (3.3 mg liter^{-1} of *E. coli* culture) in high purity. The only inhomogeneity here was due to the inconsequential partial removal of the N-terminal methionine (ΔMW 131 Da), as often observed for proteins overexpressed in bacteria[31] (Figure S7).

With the ligation partners in hand, we focused on the assembly of full-length Hsp27 by the key NCL reaction (Figure 2c). Submitting Hsp27 (1–172) α-thioester to a 2-fold excess of the Apy-modified or non-modified synthetic peptide under denaturing and reducing conditions (6 M Gdn·HCl, 50 mM *tris*(2-carboxyethyl)phosphine (TCEP)) in the presence of MESNA as a mediator afforded the desired ligation products after 48 h in high conversion (Figures 2c, S8). The subsequent in situ radical desulfurization,[32] mediated by additional TCEP as the thiyl radical acceptor, MESNA as the hydride source and the water-soluble radical initiator V-50,[25b,33] proceeded smoothly in 16 h (Figure S9) to afford, after HPLC purification including separation of traces of hydrolyzed α-thioester, the required semisynthetic Hsp27 variants in milligram amounts. These two proteins featured the native Ala173 at the ligation site as well as the Cys137Ala conversion, and differed only in residue 188 being Apy

(Hsp27^{Apy}) or Arg (Hsp27^{NM}). The compounds were obtained in high purity (Figures 2c, S10, S11) and with isolated yields of ~45% over two steps. Successful folding by dilution with appropriate buffer was indicated by circular dichroism (CD) (Figure 3a and see below). The fluorescent properties of Apy allowed us to directly observe its incorporation by the characteristic absorption and emission maxima at 320 nm and 380 nm, respectively (Figure 3b).

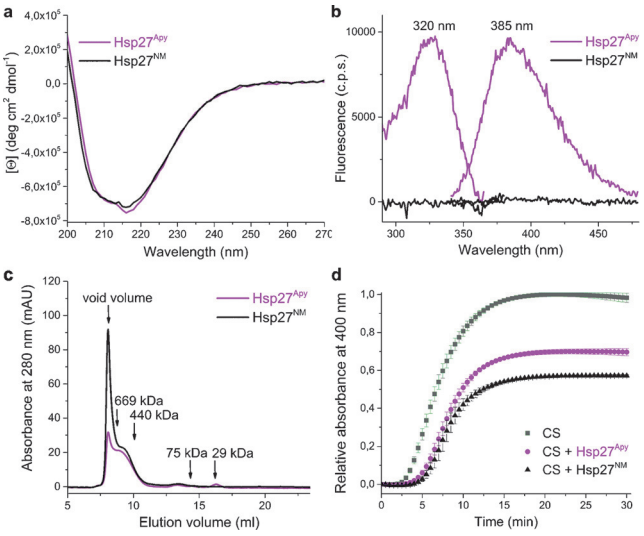


Figure 3. Structural and functional characterization of Hsp27^{Apy}. a) Far-UV CD spectra of folded semisynthetic proteins, Hsp27^{Apy} and Hsp27^{NM}. b) Fluorescence spectrum derived from the above proteins. c) Size exclusion chromatogram obtained for Hsp27^{Apy} and Hsp27^{NM}. d) Functional assay of Hsp27^{Apy} and Hsp27^{NM} using citrate synthase (CS) as a client protein.

To investigate the effect of Apy188 on the structural properties and stability of Hsp27, we analyzed the folded semisynthetic proteins by CD spectroscopy. The far-UV CD spectra of Hsp27^{Apy} and the non-modified variant are characterized by a broad negative maximum between 207 and 220 nm (Figure 3a), and the position and the amplitude of this maximum are consistent with those of the recombinant wild-type Hsp27 (hereafter, Hsp27^{WT}; Figure S12b), indicating the correct folding of the semisynthetic proteins at the secondary structure level. The spectrum of Hsp27^{NM} was indistinguishable from that of its analogue that had been obtained by subjecting Hsp27^{WT} to the same procedure of desulfurization, purification, and refolding as for the semisynthetic proteins (Hsp27^{D}; Figures S12b, S13). Notably, Figure 3a shows that the control Hsp27^{NM} exhibits a practically identical CD spectrum to that of Hsp27^{Apy}, dominated by the β-sheet structural element as for Hsp27^{WT} (Table S1). Variable temperature CD analysis (Figure S14) indicated similar transition temperatures for both variants (59 and 60 °C for Hsp27^{Apy} and Hsp27^{NM}, respectively) that are only slightly lower than the previously measured values of 62–64 °C for recombinant Hsp27.[34] Together, these results suggest that the Arg188Apy modification did not cause significant changes in the secondary structure and the overall stability of Hsp27.

Next, we examined the degree of oligomerization of the semisynthetic proteins using size exclusion chromatography (SEC). Hsp27^{WT} can exist as a multimer of 12 to 40 subunits, and the quaternary structure of the protein greatly influences its activity.[19] The elution profile of semisynthetic Hsp27^{NM} is dominated by a sharp peak at the void volume of the column (~8 mL, Figure 3c), indicating the formation of very large oligomers with an apparent molecular weight of more than 1,000 kDa. As expected, the Hsp27^{D} analogue behaved identically (Figure S15b). On the other hand, Hsp27^{Apy} eluted predominantly as oligomers of ~600 kDa (peak at ~9 min, Figure 3c) corresponding to a complex of ~26 monomers, accompanied by a decreased peak at 8 mL at the same protein concentration. In contrast to the spectrum of the non-modified counterpart, the monomer peak is clearly visible at ~16 mL elution volume of Hsp27^{Apy}. These results indicate that the Apy modification destabilizes the formation of very large oligomers or aggregates of the protein, and induces the dissociation to monomers.

Higher order oligomers formed by interaction of monomeric or dimeric subunits, as well as binding to client proteins (see below), have been previously attributed to the C-terminal domain of Hsp27.[35] Here, homology modeling revealed a change in the surface charges within this region upon Apy188 modification (see Table S2, Figure S16, and the Supporting Information), providing a possible mechanism for the observed changes.

To study the potential effect of the Apy modification on the function of Hsp27, we analyzed the in vitro activity of the folded semisynthetic variants as molecular chaperones using the standard non-native client protein citrate synthase (CS).[36] We examined the ability of the proteins to suppress the aggregation of CS that was denatured at 45 °C by monitoring UV absorbance at 400 nm as a measure of amorphous precipitation of this client protein. In the presence of 0.2 molar equivalents of Hsp27^{NM}, the expected suppression of aggregation was observed (Figure 3d), identical to the activity of the analogue Hsp27^{D} (Figure S17b), indicating that the semisynthetic proteins were functional. On the other hand, Hsp27^{Apy} was less effective in preventing CS aggregation, observed by both the faster onset and higher level of precipitation of the client protein (Figure 3d). At maximum aggregation (~21 min), there was a 22% decrease in chaperone activity for Hsp27^{Apy} relative to the control protein. Our results suggest that a single Apy modification that occurs in the C-terminal domain of Hsp27 had a significant detrimental effect on the chaperone function of the protein. Our site-specifically modified Hsp27 behaved very differently to the MG-modified mixtures reported by Nagaraj et al. that showed a strong enhancement of chaperone activity, which the authors themselves found counterintuitive.[16,37]

In summary, we were able to obtain molecular level details of the impact of the formation of the nPTM Apy on human Hsp27. Combining organic chemistry and protein semisynthesis, we produced Hsp27 bearing the pathologically relevant Apy188 modification that showed impaired chaperone activity. To our knowledge, this work represents the first instance of the generation of a site-specifically nPTM-modified, functional protein, and may serve as a paradigm

for obtaining molecular insights into the influence of nPTMs on proteins.

Acknowledgements

We thank M. Felkl, A. Körner, and G. Niederacher for technical assistance; Dr. A. Kravchuk for expert advice and assistance; Dr. M. Haslbeck (Technische Universität München) for the Hsp27 expression plasmid; A. Roller and Dr. V. Arion for X-ray crystallographic analysis; and Dr. E. Mymrikov (Technische Universität München) for helpful discussions. This work was supported by the Austrian Academy of Sciences. M.M. is a recipient of an APART fellowship of the Austrian Academy of Sciences.

Keywords: argpyrimidine · biological activity · chaperone proteins · protein modifications · protein semisynthesis

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 11397–11402
Angew. Chem. **2016**, *128*, 11569–11574

[1] C. T. Walsh, S. Garneau-Tsodikova, G. J. Gatto, *Angew. Chem. Int. Ed.* **2005**, *44*, 7342–7372; *Angew. Chem.* **2005**, *117*, 7508–7539.
[2] R. E. Moellering, B. F. Cravatt, *Science* **2013**, *341*, 549–553.
[3] a) R. Singh, A. Barden, T. Mori, L. Beilin, *Diabetologia* **2001**, *44*, 129–146; b) N. Nass, B. Bartling, A. Navarrete Santos, R. J. Scheubel, J. Borgermann, R. E. Silber, A. Simm, *Z. Gerontol. Geriatr.* **2007**, *40*, 349–356.
[4] A. Negre-Salvayre, C. Coatrieux, C. Ingueneau, R. Salvayre, *Br. J. Pharmacol.* **2008**, *153*, 6–20.
[5] a) T. Miyata, K. Kurokawa, C. V. De Strihou, *J. Am. Soc. Nephrol.* **2000**, *11*, 1744–1752; b) S. Jaisson, P. Gillery, *Clin. Chem.* **2010**, *56*, 1401–1412; c) G. Vistoli, D. De Maddis, A. Cipak, N. Zarkovic, M. Carini, G. Aldini, *Free Radical Res.* **2013**, *47*, 3–27.
[6] a) S. Y. Goh, M. E. Cooper, *J. Clin. Endocrinol. Metab.* **2008**, *93*, 1143–1152; b) N. Rabbani, P. J. Thornalley, *Biochem. Biophys. Res. Commun.* **2015**, *458*, 221–226; c) J. Li, D. Liu, L. Sun, Y. Lu, Z. Zhang, *J. Neurol. Sci.* **2012**, *317*, 1–5; d) Z. Hegab, S. Gibbons, L. Neyses, M. A. Mamas, *World J. Cardiol.* **2012**, *4*, 90–102.
[7] N. Ahmed, D. Dobler, M. Dean, P. J. Thornalley, *J. Biol. Chem.* **2005**, *280*, 5724–5732.
[8] R. A. Gomes, L. M. A. Oliveira, M. Silva, C. Ascenso, A. Quintas, G. Costa, A. V. Coelho, M. S. Silva, A. E. N. Ferreira, A. P. Freire, C. Cordeiro, *Biochem. J.* **2008**, *416*, 317–326.
[9] a) P. Gruber, T. Hofmann, *J. Pept. Res.* **2005**, *66*, 111–124; b) M. Hellwig, S. Geissler, R. Matthes, A. Peto, C. Silow, M. Brandsch, T. Henle, *ChemBioChem* **2011**, *12*, 1270–1279; c) T. N. Wang, R. Kartika, D. A. Spiegel, *J. Am. Chem. Soc.* **2012**, *134*, 8958–8967; d) T. M. Woods, M. Kamalov, P. W. R. Harris, G. J. S. Cooper, M. Brimble, *Org. Lett.* **2012**, *14*, 5740–5743.
[10] a) N. Ahmed, P. J. Thornalley, J. Dawczynski, S. Franke, J. Strobel, G. Stein, G. M. Haik, *Invest. Ophthalmol. Vis. Sci.* **2003**, *44*, 5287–5292; b) P. S. Padayatti, A. S. Ng, K. Uchida, M. A. Glomb, R. H. Nagaraj, *Invest. Ophthalmol. Vis. Sci.* **2001**, *42*, 1299–1304.
[11] R. Gomes, M. S. Silva, A. Quintas, C. Cordeiro, A. Freire, P. Pereira, A. Martins, E. Monteiro, E. Barroso, A. P. Freire, *Biochem. J.* **2005**, *385*, 339–345.
[12] a) T. Oya, N. Hattori, Y. Mizuno, S. Miyata, S. Maeda, T. Osawa, K. Uchida, *J. Biol. Chem.* **1999**, *274*, 18492–18502; b) J. W. J. van Heijst, H. W. M. Niessen, K. Hoekman, C. G. Schalkwijk, *Ann. N. Y. Acad. Sci.* **2005**, *1043*, 725–733.
[13] I. N. Shipanova, M. A. Glomb, R. H. Nagaraj, *Arch. Biochem. Biophys.* **1997**, *344*, 29–36.
[14] P. J. Thornalley, S. Battah, N. Ahmed, N. Karachalias, S. Agalou, R. Babaei-Jadidi, A. Dawney, *Biochem. J.* **2003**, *375*, 581–592.
[15] T. Gawlowski, B. Stratmann, I. Stork, B. Engelbrecht, A. Brodehl, K. Niehaus, R. Korfer, D. Tschoepe, H. Milting, *Horm. Metab. Res.* **2009**, *41*, 594–599.
[16] T. Oya-Ito, B. F. Liu, R. H. Nagaraj, *J. Cell. Biochem.* **2006**, *99*, 279–291.
[17] C. G. Schalkwijk, J. van Bezu, R. C. van der Schors, K. Uchida, C. D. A. Stehouwer, V. W. M. van Hinsbergh, *FEBS Lett.* **2006**, *580*, 1565–1570.
[18] a) J. W. J. van Heijst, H. W. M. Niessen, R. J. Musters, V. W. M. van Hinsbergh, K. Hoekman, C. G. Schalkwijk, *Cancer Lett.* **2006**, *241*, 309–319; b) W. B. Bair, C. M. Cabello, K. Uchida, A. S. Bause, G. T. Wondrak, *Melanoma Res.* **2010**, *20*, 85–96; c) T. Oya-Ito, Y. Naito, T. Takagi, O. Handa, H. Matsui, M. Yamada, K. Shima, T. Yoshikawa, *Biochim. Biophys. Acta Mol. Basis Dis.* **2011**, *1812*, 769–781; d) H. Sakamoto, T. Mashima, K. Yamamoto, T. Tsuruo, *J. Biol. Chem.* **2002**, *277*, 45770–45775.
[19] M. Haslbeck, T. Franzmann, D. Weinfurtner, J. Buchner, *Nat. Struct. Mol. Biol.* **2005**, *12*, 842–846.
[20] C. G. Concannon, A. M. Gorman, A. Samali, *Apoptosis* **2003**, *8*, 61–70.
[21] T. W. Muir, D. Sondhi, P. A. Cole, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6705–6710.
[22] S. Bondalapati, M. Jbara, A. Brik, *Nat. Chem.* **2016**, *8*, 407–418.
[23] a) T. W. Muir, *Annu. Rev. Biochem.* **2003**, *72*, 249–289; b) T. Durek, C. F. W. Becker, *Biomol. Eng.* **2005**, *22*, 153–172; c) T. W. Muir, *Biopolymers* **2008**, *90*, 743–750; d) D. P. Gamblin, S. I. van Kasteren, J. M. Chalker, B. G. Davis, *FEBS J.* **2008**, *275*, 1949–1959; e) P. Siman, A. Brik, *Org. Biomol. Chem.* **2012**, *10*, 5684–5697; f) M. Holt, T. Muir, *Annu. Rev. Biochem.* **2015**, *84*, 265–290.
[24] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776–779.
[25] a) L. Z. Yan, P. E. Dawson, *J. Am. Chem. Soc.* **2001**, *123*, 526–533; b) Q. Wan, S. J. Danishefsky, *Angew. Chem. Int. Ed.* **2007**, *46*, 9248–9252; *Angew. Chem.* **2007**, *119*, 9408–9412.
[26] a) N. Pasupuleti, M. Gangadhariah, S. Padmanabha, P. Santhoshkumar, R. H. Nagaraj, *J. Cell. Biochem.* **2010**, *110*, 408–419; b) A. Zavialov, R. Benndorf, M. Ehrnsperger, V. Zav′yalov, I. Dudich, J. Buchner, M. Gaestel, *Int. J. Biol. Macromol.* **1998**, *22*, 163–173.
[27] a) N. Sreejayan, X. Yang, K. Palanichamy, K. Dolence, J. Ren, *Eur. J. Pharmacol.* **2008**, *593*, 30–35; b) B.-L. Renard, B. Boucherle, B. Maurin, M.-C. Molina, C. Norez, F. Becq, J.-L. Décout, *Eur. J. Med. Chem.* **2011**, *46*, 1935–1941.
[28] L. Valgimigli, G. Brigati, G. F. Pedulli, G. A. DiLabio, M. Mastragostino, C. Arbizzani, D. A. Pratt, *Chem. Eur. J.* **2003**, *9*, 4997–5010.
[29] K. C. K. Swamy, N. N. B. Kumar, E. Balaraman, K. V. P. P. Kumar, *Chem. Rev.* **2009**, *109*, 2551–2651.
[30] J. Broddefalk, K. E. Bergquist, J. Kihlberg, *Tetrahedron* **1998**, *54*, 12047–12070.
[31] P. H. Hirel, M. J. Schmitter, P. Dessen, G. Fayat, S. Blanquet, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 8247–8251.
[32] P. Siman, O. Blatt, T. Moyal, T. Danieli, M. Lebendiker, H. A. Lashuel, A. Friedler, A. Brik, *ChemBioChem* **2011**, *12*, 1097–1104.
[33] V-50, unlike the more commonly applied counterpart VA-044, is readily commercially available in Europe through Sigma–Aldrich.

[34] T. Rogalla, M. Ehrnsperger, X. Preville, A. Kotlyarov, G. Lutsch, C. Ducasse, C. Paul, M. Wieske, A. P. Arrigo, J. Buchner, M. Gaestel, *J. Biol. Chem.* **1999**, *274*, 18947 – 18956.
[35] M. V. Sudnitsyna, E. V. Mymrikov, A. S. Seit-Nebi, N. B. Gusev, *Curr. Protein Pept. Sci.* **2012**, *13*, 76 – 85.
[36] F. Narberhaus, M. Haslbeck in *Protein Folding Handbook* (Eds.: J. Buchner, T. Kiefhaber), Wiley-VCH, Weinheim, **2005**, pp. 830 – 857.
[37] R. H. Nagaraj, T. Oya-Ito, P. S. Padayatti, R. Kumar, S. Mehta, K. West, B. Levison, J. Sun, J. W. Crabb, A. K. Padival, *Biochemistry* **2003**, *42*, 10746 – 10755.

Received: June 1, 2016
Revised: June 28, 2016
Published online: July 21, 2016

