Bioinorganic Chemistry

DOI: 10.1002/anie.201203255

A Sulfur Tripod Glycoconjugate that Releases a High-Affinity Copper Chelator in Hepatocytes**

Anaïs M. Pujol, Martine Cuillel, Anne-Solène Jullien, Colette Lebrun, Doris Cassio, Elisabeth Mintz, Christelle Gateau, and Pascale Delangle**

The copper ion is essential for life as a cofactor in a number of vital processes. Its main role is to exchange electrons in cuproenzymes needed for oxidative metabolism, neurotransmitter and hormone biosynthesis, free-radical detoxification, or iron absorption. However, high concentrations of copper can be deleterious as they promote Fenton-like reactions, thereby leading to oxidative damage of all the cell constituents, proteins, lipids, and nucleic acids. Therefore, the intracellular copper concentration is strictly controlled so that copper is provided to the enzymes that need it and does not reach toxic levels.[1] Detoxification of the whole body occurs in the liver, where copper is pumped towards the bile for further elimination. The Wilson's disease patients whose *ATP7B* gene is mutated lack the protein that is responsible for pumping the copper ions out of the hepatocytes and suffer from copper overload.[2] They need lifelong treatments by chelators that help to lower dietary copper absorption and to detoxify their body; unfortunately, these treatments have harmful side effects and are not always efficient.[3] Since the pool of intracellular copper is in the +I oxidation state, we figured that a chelator that would enter the hepatic cells and be specific for Cu^{I} could potentially represent an improving alternative. Therefore, we are designing bifunctional molecules that are able to both efficiently complex Cu^{I} and specifically target hepatocytes.

An efficient strategy to target hepatocytes is to use ligands of the asialoglycoprotein receptor (ASGP-R), a hepatic lectin that is chiefly expressed at the surface of these cells[4] and a promising candidate for drug delivery into hepatocytes.[5] ASGP-R recognizes terminal galactose (Gal) and *N*-acetylgalactosamine (GalNAc) with a better affinity for GalNAc than for Gal.[6] Besides, sugar–protein interactions are greatly enhanced by multivalent events, commonly known as the "cluster glycoside effect", which leads to a 10^2–10^3-fold increase of the affinity for ASGP-R with each additional carbohydrate from mono to triantennary structures.[7]

On the other hand, proteins involved in copper homeostasis are outstanding sources of inspiration for the design of efficient Cu^{I} chelators. In metallochaperones, Cu^{I} is mainly bound by soft donors like cysteine thiolates in CXXC sequences. This binding motif has led us to design peptides including two cysteine residues;[8] these peptides show a similar affinity for Cu^{I} as the metallochaperones (K_d ca. 10^{-16})[9] and a high selectivity for Cu^{I} over Zn^{II}, another essential metal ion found in cells. Recently, one of these peptides was functionalized with a cluster of carbohydrates to target hepatocytes and shown to enter these cells and to chelate intracellular copper.[10] Even more efficient Cu^{I} chelators were obtained by favoring a CuS_3 coordination environment in mononuclear and cluster-type complexes, as found in metallothioneins. Indeed, the tripodal ligand $\mathbf{L^2}$ (Scheme 1), which is based on a nitrilotriacetic acid (NTA) moiety extended by three converging cysteines for metal binding, shows a very high affinity for Cu^{I}, similar to that of metallothioneins (K_d ca. 10^{-19}),[11] and a large selectivity over Zn^{II} (10^8–10^9-fold).[12]

Herein, we report on the glycoconjugate **1**, which is derived from ligand $\mathbf{L^2}$ and has the properties of $\mathbf{L^2}$ once it is in the hepatocytes. Indeed, our synthetic strategy consists in tethering to each chelating thiolate of $\mathbf{L^2}$ one carbohydrate through a disulfide bond, which will be cleaved in the reducing intracellular medium. Therefore, glycoconjugate **1** will acquire its specificity for Cu^{I} chelation by entering the cells. Interestingly, among the structures proposed for optimal ASGP-R recognition are tripods bearing on each arm a β-linked GalNAc moiety that is kept 20 Å away from the branching point of the tripod by an ethylene glycol spacer.[13] The tripodal architecture of ligand $\mathbf{L^2}$ is thus perfectly suitable for the design of a triantennary glycoside cluster (**1** in Scheme 1). To ensure an optimal orientation of the three terminal β-linked GalNAc moieties for recognition by ASGP-R, an ethylene glycol spacer consisting of nine atoms has been chosen to establish the 20 Å connections between the GalNAc and the branching point. The analogue **1-TAMRA**, bearing a carboxytetramethylrhodamine fluorescent unit, was also synthesized to visualize the uptake into hepatocytes.

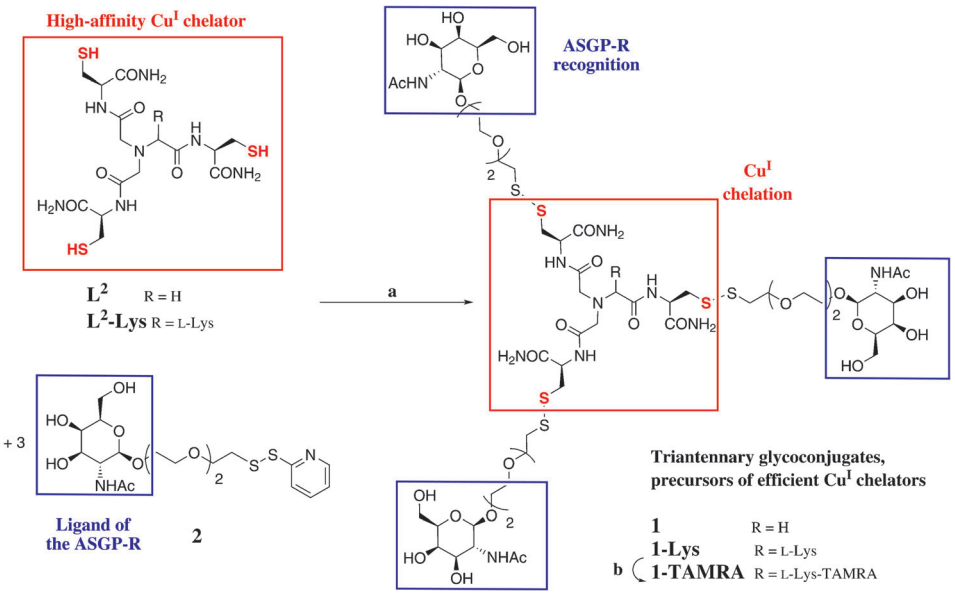
[*] Dr. A. M. Pujol, A.-S. Jullien, C. Lebrun, Dr. C. Gateau, Dr. P. Delangle
INAC, Service de Chimie Inorganique et Biologique (UMR_E 3 CEA UJF), Commissariat à l'Energie Atomique
17 rue des martyrs, 38054 Grenoble Cedex (France)
E-mail: pascale.delangle@cea.fr

Dr. M. Cuillel, Dr. E. Mintz
iRTSV, Laboratoire de Chimie et Biologie des Métaux (UMR 5249 CEA CNRS UJF), Commissariat à l'Energie Atomique
17 rue des martyrs, 38054 Grenoble Cedex (France)
E-mail: elisabeth.mintz@cea.fr

Dr. D. Cassio
INSERM UMR S757, Université Paris-Sud
91405 Orsay (France)

[**] The financial support from the "Cluster de recherche Chimie de la Région Rhône-Alpes" is duly acknowledged. We are grateful to Ignacio Sandoval (CIBEREHD, Madrid, Spain) for his generous gift of a polyclonal antibody anti-ATP7B and to Valérie Nicolas (Plateforme d'Imagerie Cellulaire, Chatenay Malabry, France) for help with confocal analysis.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201203255.



Scheme 1. Design and synthetic approach for the glycoconjugates **1**, **1-Lys**, and **1-TAMRA**. Reagents and yields: a) DMF; 36% (**1**) and 20% (**1-Lys**); b) TAMRA-NHS, diisopropylethylamine (DIEA), DMF; 16% (**1-TAMRA**).

The synthetic strategy that leads to the glycoconjugates (Scheme 1), consists in assembling the two building blocks $\mathbf{L^2}$ and **2** through three disulfide bonds in the last step. The synthesis of the tripodal building block $\mathbf{L^2}$ was reported recently[12b] and a similar procedure was used to obtain $\mathbf{L^2}$**-Lys** from *N*α,*N*α-bis(carboxymethyl)-L-lysine, which affords a primary amine for the introduction of the fluorophore (Scheme S1 in the Supporting Information). The GalNAc-containing intermediate **2** was prepared from peracetylated galactosamine, which was O-glycosylated through an oxazoline intermediate as the glycosyl donor, to favor the stereoselective formation of the β anomer (Scheme S2 in the Supporting Information).[13c,14] The two tripods $\mathbf{L^2}$ and $\mathbf{L^2}$**-Lys** were then coupled to three equivalents of intermediate **2** to afford glycoconjugates **1** and **1-Lys** in 36% and 20% yield, respectively (Scheme 1). The reaction of **1-Lys** with the *N*-succinimidyl ester of carboxytetramethylrhodamine (TAMRA-NHS) gave the fluorescent analogue **1-TAMRA** in 16% yield after reverse-phase HPLC using a C18 column.

The Cu-chelating properties of **1** were evaluated in the test tube, by competition with sodium bathocuproine disulfonate (Na_2(bcs)). The $[Cu^I(bcs)_2]^{3-}$ complex is formed as shown in Equation (1) and is easily detected by its visible absorption band (483 nm, 13300 $M^{-1}cm^{-1}$).[9a,10,12b] Thus, when bcs ligand is added to any Cu^IL complex, as shown in Equation (1), the more intense is the band at 483 nm, the lower is the stability of the Cu^IL complex.

$$Cu^{+} + 2\,bcs^{2-} \rightarrow [Cu^{I}(bcs)_2]^{3-} \quad (1)$$

As expected, **1** did not bind Cu^I as it contains three disulfide bridges and no free thiolate functions: 100% Cu is bound in a complex with bcs (Figure 1). Glutathione (GSH, 1 mM) was used to mimic the reducing intracellular medium: in agreement with the low affinity of GSH for Cu^I, it induced a 10% decrease of the amount of Cu complexed in $[Cu^I(bcs)_2]^{3-}$. However, when GSH was added together with **1**, the amount of Cu bound to the bcs ligand decreased dramatically to 8% of the total copper, thereby evidencing the release of the high-affinity chelator $\mathbf{L^2}$ after reduction of the disulfide bridges of **1** into free thiolates. For comparison, in the presence of $\mathbf{L^2}$ only 2% of the total copper is bound in the $[Cu^I(bcs)_2]^{3-}$ complex.[12b] These experiments demonstrate that the glycoconjugate **1** is not a Cu^I chelator. However, the reduction of **1** by GSH converts it into an efficient Cu^I chelator, thus confirming its potential ability to bind Cu^I once entered in the reducing medium of the hepatic cells.

The efficiency of **1** was tested in two hepatic cell lines. In vivo, hepatocytes are polarized cells, displaying apical poles in contact with the outer medium, basal poles in contact with the inner medium, and lateral poles in contact with adjacent cells. In the liver, the outer medium is the bile that is collected by a network of canaliculi formed by the union of apical poles of adjacent hepatocytes, sealed by tight junctions (Figure 2e). ASGP-R is embedded in the basal membrane in

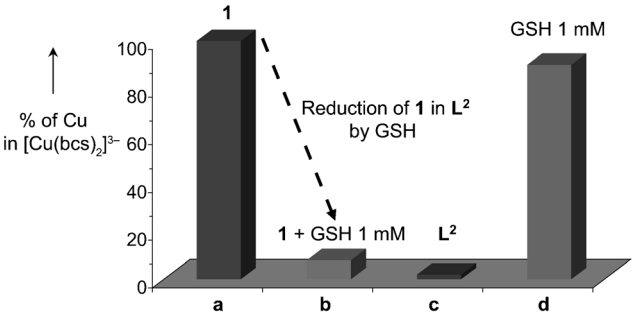


Figure 1. Percentage of Cu in the complex $[Cu(bcs)_2]^{3-}$ for samples containing $[Cu(CH_3CN)_4]PF_6$ (45 μM), bcs (100 μM) in phosphate buffer (20 mM), pH 7.4 at 298 K, and a) 50 μM **1**; b) 50 μM **1** and 1 mM GSH; c) 50 μM $\mathbf{L^2}$; d) 1 mM GSH.

contact with the blood. We checked that **1-TAMRA** enters HepG2 cells, the most commonly used human hepatic cell line, and WIF-B9 cells, which are able to reconstitute remarkably stable and polarized epithelia with functional bile canaliculi within seven to ten days.[15] After two hours, the dye was in all the cells and concentrated in a cell compartment facing the canalicular spaces reconstituted by WIF-B9 cells. Importantly, we also checked whether **1-TAMRA** could enter HeLa cells, a cell line deprived from ASGP-R.[16] This

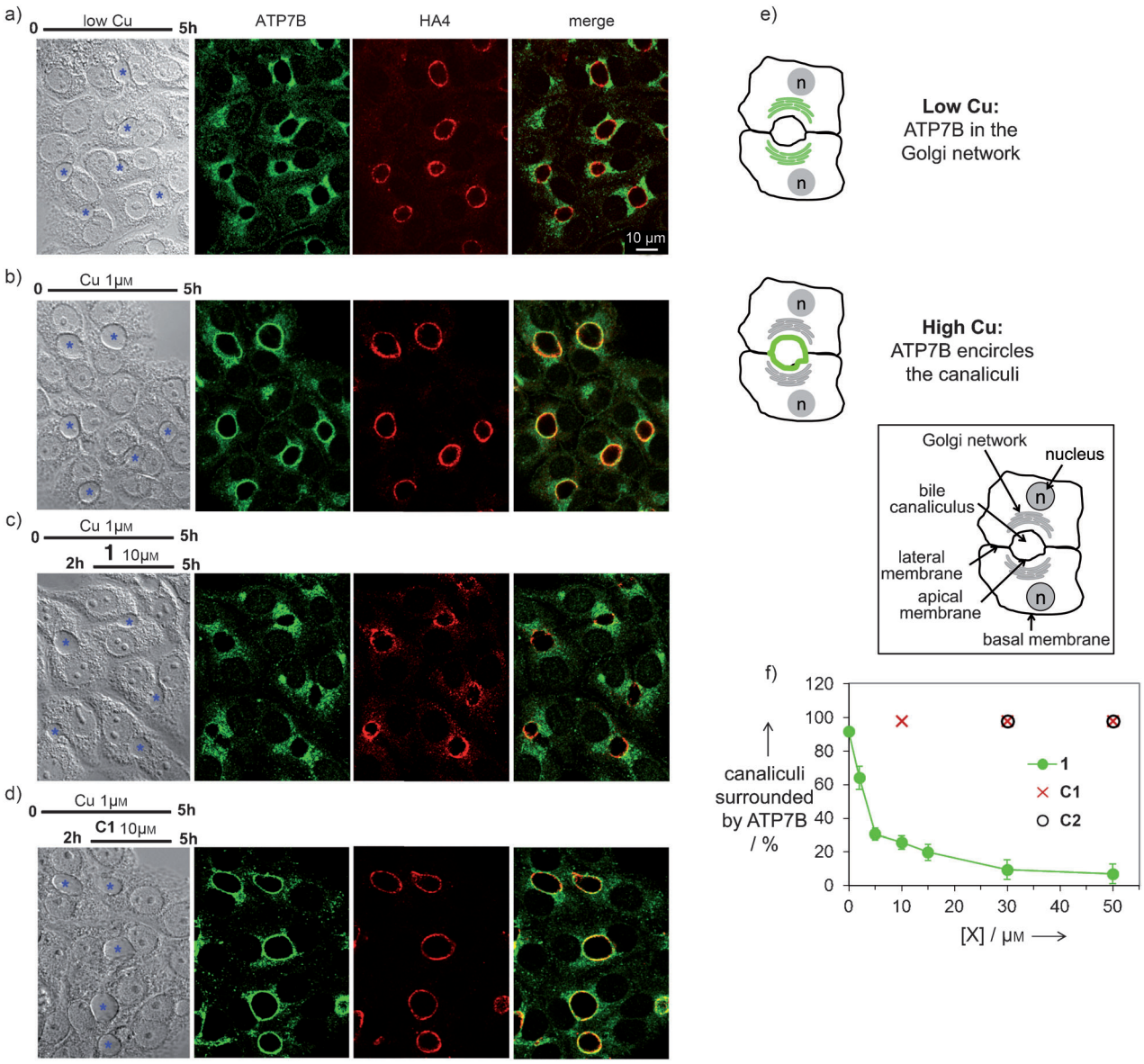
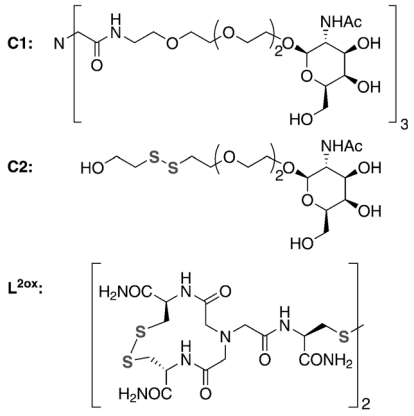


Figure 2. Effect of Cu on ATP7B localization in WIF-B9 cells and efficiency of the glycoconjugate **1**. a–d) ATP7B and HA4 (a canalicular membrane marker) were detected by indirect immunofluorescence and imaged by confocal microscopy. In each panel are presented from left to right, the Nomarski image, the green and red images of the sum of two confocal sections taken in the middle of the cell layer, and the merge image. On the Nomarski images, bile canaliculi are indicated by blue asterisks. a) Cells kept in the basal culture medium for 5 h. b) Cells kept in the basal culture medium plus $CuCl_2$ (1 µM) for 5 h. c) Cells kept with $CuCl_2$ (1 µM) for 2 h and further treated with **1** (10 µM) for 3 h. d) Cells kept with $CuCl_2$ (1 µM) for 2 h and further treated with **C1** (10 µM) for 3 h. e) Scheme of polarized hepatic cells showing the localization of ATP7B (green) in presence of high and low Cu concentrations. f) Dose-dependence effect of **1**, **C1**, **C2**; the % of bile canaliculi surrounded by ATP7B is presented as a function of the compound concentrations; all the experiments used in this curve were performed as in c) or d).

experiment showed that **1-TAMRA** does not enter HeLa cells under the conditions where it enters HepG2 and WIF-B9 cells, thereby illustrating the role of the hepatic receptor in the cellular uptake of the glycoconjugate (Figure S2 in the Supporting Information).

By following the work of Hubbard and co-workers,[17] we have recently used the localization of ATP7B, the protein responsible for copper detoxification, as an intracellular copper sensor in WIF-B9 cells.[10] WIF-B9 cells were incubated under basal conditions or with added copper ions (1 µM) and the localizations of ATP7B protein (in green) and HA4 protein (a canalicular membrane protein[18] in red) were monitored by immunofluorescence. A complementary experiment with the detection of a Golgi protein,[19] instead of the HA4 protein is shown in Figure S3 in the Supporting Information. In agreement with previous work,[17] under basal conditions where the copper concentration is low, ATP7B was in the Golgi network between the nucleus and the apical membrane (Figure 2a), whereas with copper ions (1 µM) in the culture medium, ATP7B was mainly localized in a continuous crown around the canaliculus to excrete the excess of copper into the bile (Figure 2b). The latter localization of ATP7B bears all the hallmarks of the physiological needs for copper detoxification and can therefore be used as an

evidence for the ability of our compounds to chelate excess of intracellular Cu. Hence, ATP7B and HA4 localizations were monitored in the presence of copper ions (1 μM) and either **1**, or **C1**, **C2**, $\mathbf{L^{2ox}}$ as control compounds (Scheme 2 and the Supporting Information). **C1** is a molecule similar to **1** with no sulfur atoms at all, unable to release the sulfur tripod. **C2** mimics one arm of **1** and is able to release in the intracellular medium low-affinity monothiol compounds that resemble glutathione. $\mathbf{L^{2ox}}$ is the oxidation product of $\mathbf{L^2}$, a dimer containing three disulfide bridges but lacking the sugar targeting units; $\mathbf{L^{2ox}}$ could become a chelator if it entered the cells.



Scheme 2. Structures of control compounds.

After incubation for three hours with **1** (10 μM), the pericanalicular distribution of ATP7B changed dramatically as the green fluorescence localized at a large compartment reminiscent of the Golgi network (Figure 2c), close to the nucleus; this distribution resembles that observed in basal conditions (Figure 2a). In similar experiments using **C1** (10–50 μM) or **C2** (30–90 μM) or $\mathbf{L^{2ox}}$ (50 μM), ATP7B distribution did not change, thus indicating that the cells still needed detoxification (Figure 2d and Figure S4 in the Supporting Information). The proportion of bile canaliculi surrounded by ATP7B increases with excess Cu concentration and is therefore related to the ability of the compounds to chelate toxic excess copper and to cancel the detoxification process by ATP7B. This proportion clearly decreased as the concentration of the sulfur tripod glycoconjugate **1** increased, whereas the pericanalicular distribution of ATP7B is not sensitive to the other compounds (Figure 2f). These experiments demonstrate that the two functions present in **1**, the glycoside cluster for ASPG-R targeting and the sulfur tripod for efficient Cu^I chelation, are essential to promote the release of a high-affinity intracellular Cu^I chelator once it is in hepatic cells.

In conclusion, we demonstrated that the tripodal Cu^I chelator $\mathbf{L^2}$ derived from NTA by an extension with three cysteines, which exhibits an affinity for Cu^I as high as that of metallothioneins, could be derived to enter hepatic cells and complex intracellular copper. The glycoconjugate **1** can thus be considered as a vector delivering $\mathbf{L^2}$, the active compound, at the right place to chelate excess Cu^I. For the Wilson's disease patients, **1** could represent an interesting concept of a prodrug to be further developed.

Received: April 27, 2012
Published online: June 14, 2012

Keywords: bioinorganic chemistry · cell recognition · copper · drug delivery · glycoconjugates

[1] S. Lutsenko, *Curr. Opin. Chem. Biol.* **2010**, *14*, 211–217.
[2] a) D. Huster, *Best Pract. Res. Clin. Gastroenterol.* **2010**, *24*, 531–539; b) P. Delangle, E. Mintz, *Dalton Trans.* **2012**, *41*, 6359–6370.
[3] a) B. Sarkar, *Chem. Rev.* **1999**, *99*, 2535–2544; b) O. Andersen, *Chem. Rev.* **1999**, *99*, 2683–2710.
[4] a) M. Meier, M. D. Bider, V. N. Malashkevich, M. Spiess, P. Burkhard, *J. Mol. Biol.* **2000**, *300*, 857–865; b) M. Spiess, *Biochemistry* **1990**, *29*, 10009–10018.
[5] J. Wu, M. H. Nantz, M. A. Zern, *Front. Biosci.* **2002**, *7*, d717–725.
[6] J. U. Baenziger, Y. Maynard, *J. Biol. Chem.* **1980**, *255*, 4607–4613.
[7] a) Y. C. Lee, R. R. Townsend, M. R. Hardy, J. Lonngren, J. Arnarp, M. Haraldsson, H. Lonn, *J. Biol. Chem.* **1983**, *258*, 199–202; b) Y. C. Lee, R. T. Lee, *Acc. Chem. Res.* **1995**, *28*, 321–327.
[8] a) P. Rousselot-Pailley, O. Seneque, C. Lebrun, S. Crouzy, D. Boturyn, P. Dumy, M. Ferrand, P. Delangle, *Inorg. Chem.* **2006**, *45*, 5510–5520; b) O. Sénèque, S. Crouzy, D. Boturyn, P. Dumy, M. Ferrand, P. Delangle, *Chem. Commun.* **2004**, 770–771.
[9] a) Z. Xiao, F. Loughlin, G. N. George, G. J. Howlett, A. G. Wedd, *J. Am. Chem. Soc.* **2004**, *126*, 3081–3090; b) R. Miras, I. Morin, O. Jacquin, M. Cuillel, F. Guillain, E. Mintz, *J. Biol. Inorg. Chem.* **2008**, *13*, 195–205.
[10] A. M. Pujol, M. Cuillel, O. Renaudet, C. Lebrun, P. Charbonnier, D. Cassio, C. Gateau, P. Dumy, E. Mintz, P. Delangle, *J. Am. Chem. Soc.* **2011**, *133*, 286–296.
[11] P. Faller, *FEBS J.* **2010**, *277*, 2921–2930.
[12] a) A. M. Pujol, C. Gateau, C. Lebrun, P. Delangle, *J. Am. Chem. Soc.* **2009**, *131*, 6928–6929; b) A. M. Pujol, C. Gateau, C. Lebrun, P. Delangle, *Chem. Eur. J.* **2011**, *17*, 4418–4428.
[13] a) P. C. N. Rensen, S. H. van Leeuwen, L. A. J. M. Sliedregt, T. J. C. van Berkel, E. A. L. Biessen, *J. Med. Chem.* **2004**, *47*, 5798–5808; b) O. Khorev, D. Stokmaier, O. Schwardt, B. Cutting, B. Ernst, *Bioorg. Med. Chem.* **2008**, *16*, 5216–5231; c) U. Westerlind, J. Westman, E. Tornquist, C. I. E. Smith, S. Oscarson, M. Lahmann, T. Norberg, *Glycoconjugate J.* **2004**, *21*, 227–241.
[14] S. Nakabayashi, C. D. Warren, R. W. Jeanloz, *Carbohydr. Res.* **1986**, *150*, C7–C10.
[15] C. Decaens, M. Durand, B. Grosse, D. Cassio, *Biol. Cell* **2008**, *100*, 387–398.
[16] J. H. Park, E. W. Cho, S. Y. Shin, Y. J. Lee, K. L. Kim, *Biochem. Biophys. Res. Commun.* **1998**, *244*, 304–311.
[17] Y. Guo, L. Nyasae, L. T. Braiterman, A. L. Hubbard, *Am. J. Physiol. Gastrointest. Liver Physiol.* **2005**, *289*, G904–G916.
[18] A. L. Hubbard, J. R. Bartles, L. T. Braiterman, *J. Cell Biol.* **1985**, *100*, 1115–1125.
[19] J. Saraste, G. E. Palade, M. G. Farquhar, *J. Cell Biol.* **1987**, *105*, 2021–2029.

