doi:10.2533/chimia.2013.333 Chimia 67 (2013) 333–336

Thiohistidine Biosynthesis

Florian P. Seebeck*

Abstract: Ergothioneine and ovothiol A are sulfur-containing histidine derivatives produced by microorganisms including *Mycobacterium tuberculosis*, *Trypanosoma cruzi* or *Erwinia amylovora* and may also play important roles in human physiology. Based on our recent identification of thiohistidine biosynthetic enzymes from *Mycobacterium smegmatis* and *Erwinia tasmaniensis* we investigate several aspects of sulfur-based redox biochemistry. For example, we are characterizing the catalytic mechanism of two thiohistidine biosynthetic enzymes which afford O_2-dependent sulfur insertion into the C(5)–H and C(2)–H bonds of the imidazolyl side chain of histidine.

Keywords: Biosynthesis · Ergothioneine · Ovothiol · Oxidative stress

Florian P. Seebeck received his Diploma in Chemistry from the University of Bern, and a PhD from the ETH in Zürich. He worked as a postdoc at the Massachusetts General Hospital in Boston between 2005 and 2007 and in 2008 he became a group leader at the Max Planck Institute of Molecular Physiology in Dortmund. In 2011 he moved to Basel to assume the 'Stiftungsprofessur für Molecular Bionics'.

Cellular Redox Chemistry as a Research Focus

Molecular oxygen (O_2) is the common terminal electron acceptor in aerobic life. In its ground state O_2 is unreactive towards most organic molecules. Consequently, biological O_2 dependent oxidations require catalysis by transition metal or flavin dependent enzymes. In contrast, partially reduced oxygen species such as the hydroperoxyl radical (O_2 + 1 electron + 1 proton), hydrogen peroxide (O_2 + 2 electrons + 2 protons) or the hydroxyl radical (O_2 + 3 electrons + 2 protons) attack cellular components without the need for catalysis causing unspecific oxidation of vital biomolecules such as proteins and nucleic acids. The presence of 20% O_2 in the atmosphere and the many cellular processes which produce partially reduced O_2 species sets the cell under a constant pressure to repair and prevent oxidative damage. This oxidative stress is an important factor in causing cancer, cardiovascular, inflammatory, neurodegenerative, infective disease and – although this is not a disease – aging. As a result cellular redox biochemistry has emerged as a major research focus.

Sulfur is a Mayor Player in Cellular Redox Biochemistry

Much of oxidative stress is mediated, communicated, mitigated or amplified by a complex intracellular system of sulfur-containing proteins and small molecules. Reversible oxidations of specific cysteine and methionine residues on signaling proteins or enzymes allow the cell to detect reactive oxygen species[1–3] and to catalyze their reductive destruction. Small sulfur-containing metabolites on the other hand are found as messenger,[4] as pathogenicity factors,[5] as antibacterials[6] or as redox buffers.[7,8] For example, human cells contain millimolar concentrations of glutathione (**1**, Fig. 1), a tripeptide consisting of glutamic acid, cysteine and glycine. Many microorganisms depend on similar cysteine derivatives such as bacillithiol (**2**),[9] mycothiol (**3**)[10] or trypoanothione (**4**).[8]

The purpose of high cellular concentration of such thiols is to keep protein-based cysteine residues in reduced form, to trap electrophilic toxins, and to assist the trafficking of transition metals across the cell. The role of glutathione in neutralizing reactive oxygen species and O-, C- or N-based radicals is less clear: at physiological pH glutathione is predominantly protonated and does not react efficiently with peroxides. Reduction of oxygen superoxide and other radicals generate the glutathionyl radical which is almost as reactive as the parent radical and still poses a threat to proteins and nucleic acids. These shortcomings point to functional niches for alternative antioxidants with distinct chemical properties from the cysteine derivatives **1–4**. The research in our group is based on the hypothesis that ergothioneine (**5**, Fig. 1) and ovothiol A (**6**) represent such complementary thiols. To test this hypothesis we study the biosynthesis of thiohistidines and investigate which organisms depend on thiohistidines and under which circumstances.

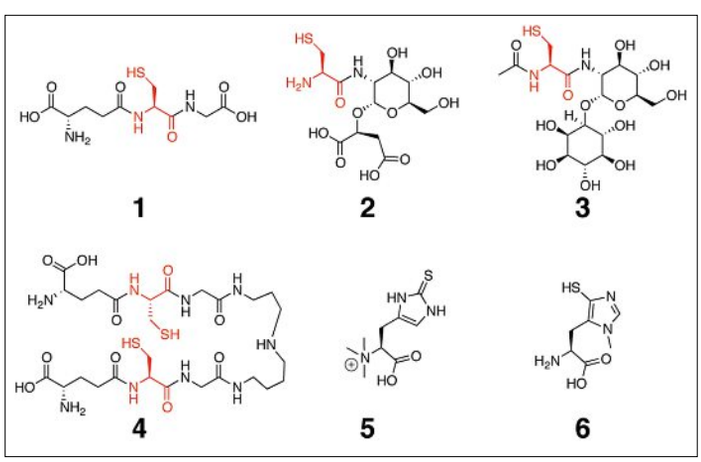


Fig. 1. Structures of the cysteine derivatives glutathione (**1**), bacillithiol isolated from *Bacillus* species (**2**),[9] mycothiol from mycobacteria (**3**),[10] trypanothione from trypanosoma (**4**).[8] The cysteine portions are indicated in red. The thiohistidine derivatives ergothioneine (**5**)[11] and ovothiol A (**6**).[12]

*Correspondence: Prof. Dr. F. P. Seebeck
University of Basel
Department of Chemistry
St. Johanns-Ring 19
CH-4056 Basel
Tel.: +41 61 267 1143
E-mail: florian.seebeck@unibas.ch

Ergothioneine Biology

In addition to their respective main intracellular thiols **1** and **3**, fungi and mycobacteria have long been known to produce the 2-thiohistidine derivative ergothioneine (**5**, Fig. 1).[13] Although ergothioneine is formally a thiol, physiological conditions stabilize its thione form.[14] Other than glutathione, ergothioneine does not engage in disulfide bond formation or reduction, is not prone to autooxidation and is characterized by a significantly higher p*K*a (>10) of the thioimidazole group than most alkylthiols.[14] Despite a long series of reports on the *in vitro* properties of ergothioneine,[14] its physiological roles remain a matter of speculation. The most exciting development in this field came from the recent discovery of a human ergothioneine transporter protein (OCTN1).[15] Specific human tissues such as liver, kidney, central nervous system, bone marrow, and red blood cells assimilate ergothioneine from dietary sources up to millimolar concentrations.[14] Its seems that tissue-specific expression of the onct1 gene is responsible for this non-uniform distribution of ergothioneine. Gain-of-function mutations of onct1 are associated with Crohn's disease,[16] while elimination of ergothioneine transport activity in cultured HeLa cells reduce resilience towards oxidative stress.[17] Evidently, the relation between cellular health and ergothioneine is complex. Although ergothioneine research is still in its infancy, the California-based company OXIS (*http://www.oxis.com/*) already markets ergothioneine as additive in food products and cosmetics. Several *in vitro* studies attest ergothioneine excellent affinity for heavy metals such as copper which loses redox activity upon ergothioneine complexation.[14,18] Ergothioneine is not particularly reactive towards peroxides or oxygen superoxide, but does react with hydroxyl radicals, hypochlorous acid, peroxynitrite or singlet oxygen.[14] However, it is not clear whether this reactivity profile is relevant in living cells. For example, ergothioneine seems to protect *Neurospora crassa* from peroxides despite the lack of *in vitro* reactivity towards peroxides.[19]

Ovothiol A Biology

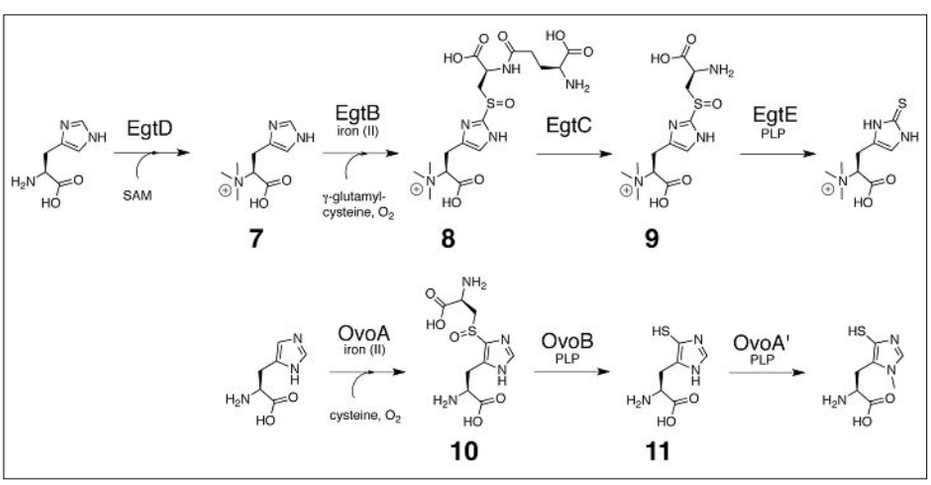
Ovothiol A (**6**, Fig. 1) has been discovered in unfertilized eggs of sea urchin.[12,20,21] The thiol group in ovothiol A is characterized by a very low pK_a (1.4)[22] and by a much more positive redox potential (–0.09 V vs. SHE) relative to that of glutathione (–0.26V).[22] The 2- and 5-thioimidazole groups in ergothioneine and ovothiol A have remarkably different properties. It seems therefore possible that the two thiohistidine isomers serve completely different biological roles. For example, ovothiol A is a much better reducing agent for peroxides than ergothioneine and glutathione. This reactivity is believed to protect the DNA content of the sea urchin eggs from the oxidative burst that concludes the fertilization process.[7] Sporadic reports over the last thirty years have described ovothiol A as a redox regulator in *Dunaliella salina* (a micro-algae),[23] as a sex-pheromone in *Platynereis dumerilii* (a marine worm)[24] and as building block for various secondary metabolites from marine invertebrates.[25,26] *Trypanosoma cruzi, Trypanosoma brucei* and *Leishmania major* are the human pathogens which cause tropical diseases such as sleeping disease, Chagas disease and leishmaniasis also produce ovothiol A.[27–30] Elucidation of the corresponding biosynthetic pathway may reveal novel strategies to treat these conditions, for which no efficient therapy is known. Furthermore, identification of the ovothiol A biosynthetic genes (see below) revealed that several plant pathogens such as *Erwinia amylophora,* the causative agent of fire blight and *Phytophthora infestans* which causes potato blight, are also ovothiol A producers. At the present time, however, it is not known whether ovothiol A is essential for viability or pathogenicity of any of these organisms.[31]

Thiohistidine Biosynthesis

Early biosynthetic studies in cell-free extracts from *Neurospora crassa* (an ascomycete fungus) revealed that ergothioneine is assembled from histidine, cysteine, and methionine with Nα-trimethylhistidine (**7**, Scheme 1) as a first, and 2-Nα-trimethylhistidyl cysteine sulfoxide (**9**) as a third intermediate.[34] These observations also implicated the involvement of an S-adenosyl methionine (SAM)-dependent methyltransferase, an iron(II)-dependent oxidase, and a pyridoxal 5-phosphate (PLP)-dependent β-lyase as possible catalysts. An analogous study with cell-free extracts from *Crithidia fasciculata* (a kinetoplastid), suggested that ovothiol A biosynthesis proceeds in a similar manner *via* a 5-histidinyl cysteine sulfoxide intermediate (**10**, Scheme 1).[30] When we started our own research in this field, none of the proposed thiohistidine biosynthetic enzymes were known.

Gene Identification

We were intrigued by the mysterious reaction which affords sulfurization of histidine either at the imidazole C(2) or C(5) position. Because this reaction appeared to be iron(II) and O_2 dependent it was clear that thiohistidine biosynthesis entailed unprecedented enzymatic activity. The wealth of sequenced microbial genomes and the availability of user friendly bioinformatics tools (*www.ncbi.nlm.nih.gov/pubmed/*) enabled our non-expert fishing expedition to identify the genes involved. Briefly, we searched for a methyltransferase (EgtD, Scheme 1) gene which occurs in *bona fide* ergothioneine producers such as *Mycobacterium avium* and *N. crassa*,[35] but not in bacteria which do not produce ergothioneine (Fig. 2).[36] These two criteria reduced a list of 78 annotated mycobacterial methyltransferases to ten candidate genes (red segments, Fig. 2) one of which is encoded next to a pyridoxal 5-phosphate (PLP)-binding protein in the context of a five-gene cluster. We cloned these genes from *Mycobacterium smegmatis* and found that the encoded enzymes indeed synthesized ergothioneine in a test tube (Scheme 1).[32] The key reactions are catalyzed by a histidine-specific methyltransferase



Scheme 1. Top: Reaction sequence of ergothioneine *in vitro* biosynthesis catalyzed by recombinant enzymes from *Mycobacterium smegmatis*.[32] Bottom: Reaction sequence of ovothiol A biosynthesis catalyzed by recombinant enzymes from *Erwinia tasmaniensis*.[33]

(EgtD) and an iron(II)-dependent sulfoxide synthase (EgtB). The latter protein is the first representative of an entirely new enzyme family. When we searched the some 2000 genome sequences in the public domain for EgtD and EgtB homologs it became clear that ergothioneine biosynthesis is a frequent trait found in actinobacteria, cyanobacteria, pezizomycotina and basidiomycota, and also in numerous bacteroidetes, proteobacteria and firmicutes.[32] This finding certainly graduates ergothioneine from a rare curiosity to an important player in microbial redox biochemistry.

The search for EgtB homologs also led to the identification of a second class of sulfoxide synthases. We termed these enzymes OvoA because *in vitro* reconstitution of homologs from *Erwinia tasmaniensis*, *Trypanosoma cruzi* and *Leishmania mayor* revealed that these catalysts mediate ovothiol A biosynthesis (Scheme 1).[33] Again, it appears that ovothiol A biosynthesis is far more widespread than previously thought.[33]

Sulfoxide Synthases and their Catalytic Mechanism

EgtB and OvoA are the central enzymes in the biosynthetic pathway of ergothioneine and ovothiol A (Scheme 1).[32,33] The unique ability of sulfoxide synthases to insert sulfur into non-electrophilic carbon scaffolds raises the question as to whether such enzymes could be used for biotechnological production of low molecular thiols. However, application of OvoA and EgtB as preparative thiolation catalysts may be limited by a narrow substrate scope owing to strict active site – substrate shape complementarity. Secondly the catalytic mechanism might be highly specific for imidazole rings as sulfur acceptors and might not be permissible for other hydrocarbons. To address this question we are currently working on four mechanistic proposals for the OvoA- and EgtB-catalyzed reactions.

OvoA and EgtB bind iron(II) through the side chains of two histidine and one glutamate residue within a seven amino acid motif.[33] Both enzymes require O_2 as four electron acceptor to mediate C–S bond formation and concomitant sulfoxidation of the thioether bond. The first three mechanisms (**1**–**3**, Scheme 2) predict that sulfoxidation of the iron-coordinated substrate cysteine allows the enzyme to form a highly reactive oxo iron (IV) species (**a**, Scheme 2) which then mediates C–S bond formation. This second step may proceed *via* hydrogen atom abstraction from the imidazolyl ring at position C(5) in the active site of OvoA, or at position C(2) in the active site of EgtB (**b**, mechanism **1**); However, formation of an imidazolyl sp2 radical is energetically very difficult[37] and would therefore be rate-limiting. The absence of a measurable kinetic substrate isotope effect when we assayed OvoA with deuterated histidine therefore suggests that this mechanism does not apply.[33,37] A second mechanism proposes homolytic cleavage of an N–H bond of the imidazolyl ring to generate an imidazolyl π radical (**c**, mechanism **2**). This radical would then attack the iron-coordinated cysteine sulfoxide. In the third mechanism the cysteine sulfoxide is attacked by the imidazole ring acting as a nucleophile (**d**, mechanism **3**). A fourth mechanism assumes that the enzyme-based iron(III)-superoxide complex oxidizes the substrate cysteine to a thiyl radical which then attacks the imidazole ring (**e**), followed by rearomatization of the imidazole ring (**f**). Subsequent oxidation of the sulfur atom restores the ferrous state of the enzyme and concludes the catalytic cycle. This mechanism appears particularly convincing because even aqua complexes of iron(III) generate cysteine thiyl radicals during iron-catalyzed oxidation of cysteine to cystine. The second step, attack of a thiyl radical onto a carbon sp2 center, is also observed in uncatalyzed systems, namely in the thiol-ene reaction where photogenerated thiyl radicals have been shown to attack a broad range of unsaturated hydrocarbons.[38] We are looking forward to experimentally testing these and other mechanistic proposals to decipher the intriguing reactivity of the new class of sulfoxide synthases.

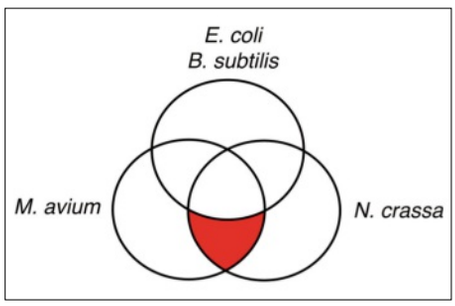


Fig. 2. Qualitative Venn diagram representing all methyltransferase genes encoded by the genomes of *Mycobacterium avium, Neurospora crassa, Escherichia coli* and *Bacillus subtilis* (black circles). Some methyltransferases occur in all four genomes (central section), some are specific for each species (peripheral sections). An ergothioneine biosynthetic methyltransferase (EgtD, Scheme 1) has to occur in the genomes of *M. avium* and *N. crassa*, but has to be absent in *E. coli* and *B. subtilis* (red segment). *M. avium* and *N. crassa* share only ten homologous methyltransferases, one of which proved to be EgtD.

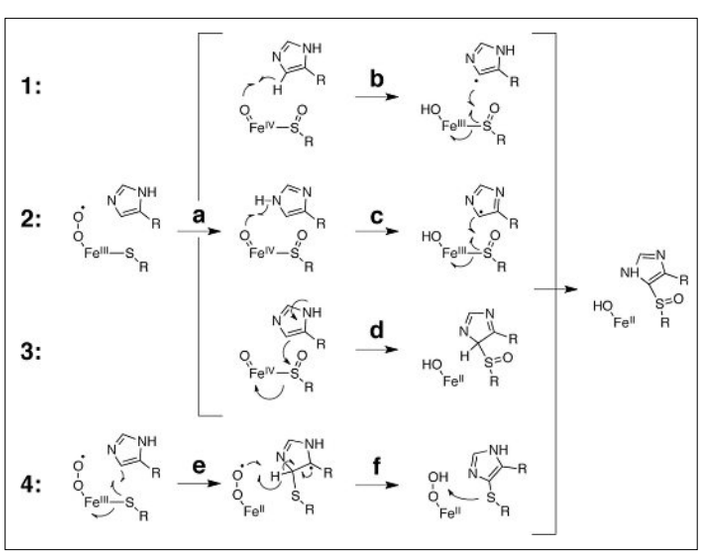
In conclusion, we propose that ergothioneine and ovothiol A play a fundamental but previously underappreciated role in the physiology of humans, human pathogens, plant pathogens and biomass degrading fungi and bacteria. Identification of thiohistidine biosynthetic enzymes established the basis to test this hypothesis, and also revealed a novel class of iron(II)-dependent enzymes which afford unprecedented oxidative sulfur transfers onto non-electrophilic carbon scaffolds. I am convinced that this research field will keep us busy and excited for many years to come.

Acknowledgments

This project is funded by the Swiss National Science Foundation. F.P.S. is supported by the 'Professur für Molecular Bionics'.

Received: April 4, 2013

[1] C. E. Paulsen, T. H. Truong, F. J. Garcia, A. Homann, V. Gupta, S. E. Leonard, K. S. Carroll, *Nat. Chem. Biol.* **2012**, *8*, 57.
[2] D.-H. Cho, T. Nakamura, J. Fang, P. Cieplak, A. Godzik, Z. Gu, S. A. Lipton, *Science* **2009**, *324*, 102.
[3] R. Vanacore, A.-J. L. Ham, M. Voehler, C. R. Sanders, T. P. Conrads, T. D. Veenstra, K. B. Sharpless, P. E. Dawson, B. G. Hudson, *Science* **2009**, *325*, 1230.
[4] G. Yang, L. Wu, B. Jiang, W. Yang, J. Qi, K.



Scheme 2. Proposed catalytic mechanisms **1–4** for OvoA catalyzed oxidative sulfur insertion into the C(5)–H bond of the imidazolyl side chain of histidine.[33]

Cao, Q. Meng, A. K. Mustafa, W. Mu, S. Zhang, S. H. Snyder, R. Wang, *Science* **2008**, *322*, 587.
[5] D. M. Gardiner, P. Waring, B. Howlett, *J. Microbiology* **2005**, *151*, 1021.
[6] E. Block, *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1135.
[7] E. Turner, L. J. Hager, B. M. Shapiro, *Science* **1988**, *242*, 939.
[8] A. H. Fairlamb, P. Blackburn, P. Ulrich, B. T. Chait, A. Cerami, *Science* **1985**, *227*, 1485.
[9] G. L. Newton, M. Rawat, J. J. La Clair, V. K. Jothivasan, T. Budiarto, C. J. Hamilton, A. Claiborne, J. D. Helmann, R. C. Fahey, *Nat. Chem. Biol.* **2009**, *5*, 625.
[10] H. S. C. Spies, D. J. Steenkamp, *Eur. J. Biochem.* **1994**, *224*, 203.
[11] C. Tanret, *Ann. Chim. Phys.* **1909**, *18*, 114.
[12] A. Palumbo, M. Dischia, G. Misuraca, G. Prota, *Tetrahedron Lett.* **1982**, *23*, 3207.
[13] D. B. Melville, W. H. Horner, C. C. Otken, M. L. Ludwig, *J. Biol. Chem.* **1955**, *213*, 61.
[14] P. E. Hartman, *Methods Enzymol.* **1990**, *186*, 310.
[15] D. Gruendemann, S. Harlfinger, S. Golz, A. Geerts, A. Lazar, R. Berkels, N. Jung, A. Rubbert, E. Schoemig, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 5256.
[16] V. D. Peltekova, R. F. Wintle, L. A. Rubin, C. I. Amos, Q. Q. Huang, X. J. Gu, B. Newman, M. Van Oene, D. Cescon, G. Greenberg, A. M. Griffiths, P. H. St George-Hyslop, K. A. Siminovitch, *Nat. Genet.* **2004**, *36*, 471.
[17] B. D. Paul, S. H. Snyder, *Cell. Death Differ.* **2009**, *47*, S149.
[18] I. K. Cheah, B. Halliwell, *Biochim. Biophys. Acta* **2012**, *1822*, 784.
[19] M. H. Bello, V. Barrera-Perez, D. Morin, L. Epstein, *Fungal Genet. Biol.* **2012**, *49*, 160.
[20] E. Turner, R. Klevit, P. B. Hopkins, B. M. Shapiro, *J. Biol. Chem.* **1986**, *261*, 3056.
[21] E. Turner, R. Klevit, L. J. Hager, B. M. Shapiro, *Biochem.* **1987**, *26*, 4028.
[22] K. H. Weaver, D. L. Rabenstein, *J. Org. Chem.* **1995**, *60*, 1904.
[23] S. Selmanreimer, R. J. Duhe, B. J. Stockman, B. R. Selman, *J. Biol. Chem.* **1991**, *266*, 182.
[24] I. Röhl, B. Schneider, B. Schmidt, E. Zeek, *Zeitschrift Naturforsch. C* **1999**, *54*, 1145.
[25] S. Ito, G. Nardi, G. Prota, *J. Chem. Soc. Chem. Comm.* **1976**, 1042.
[26] C. Pathirana, R. J. Andersen, *J. Am. Chem. Soc.* **1986**, *108*, 8288.
[27] R. L. Krauth-Siegel, H. Bauer, H. Schirmer, *Angew. Chem. Int. Ed*. **2005**, *44*, 690.
[28] D. J. Steenkamp, H. S. C. Spies, *Eur. J. Biochem.* **1994**, *223*, 43.
[29] D. J. Steenkamp, D. Weldrick, H. S. C. Spies, *Eur. J. Biochem.* **1996**, *242*, 557.
[30] R. N. Vogt, H. S. C. Spies, D. J. Steenkamp, *Eur. J. Biochem.* **2001**, *268*, 5229.
[31] M. R. Ariyanayagam, A. H. Fairlamb, *Mol. Biochem. Parasitol.* **2001**, *115*, 189.
[32] F. P. Seebeck, *J. Am. Chem. Soc*. **2010**, *132*, 6632.
[33] A. Braunshausen, F. P. Seebeck, *J. Am. Chem. Soc.* **2011**, *133*, 1757.
[34] Y. Ishikawa, S. E. Israel, D. B. Melville, *J. Biol. Chem.* **1974**, *249*, 4420.
[35] D. S. Genghof, *J. Bacteriol.* **1970**, *103*, 475.
[36] D. B. Melville, D. S. Genghof, E. Inamine, V. Kovalenko, *J. Biol. Chem.* **1956**, *223*, 9.
[37] E. A. Bushnell, G. B. Fortowsky, J. W. Gauld, *Inorg. Chem.* **2013**, *51*, 13351.
[38] C. E. Hoyle, C. N. Bowman, *Angew. Chem. Int. Ed.* **2010**, *49*, 1540.