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Enzyme Catalysis

A High-Throughput Assay for Arylamine Halogenation Based on a Peroxidase-Mediated Quinone–Amine Coupling with Applications in the Screening of Enzymatic Halogenations

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Abstract: Arylhalides are important building blocks in many fine chemicals, pharmaceuticals and agrochemicals, and there has been increasing interest in the development of more "green" halogenation methods based on enzyme catalysis. However, the screening and development of new enzymes for biohalogenation has been hampered by a lack of high-throughput screening methods. Described herein is the development of a colorimetric assay for detecting both chemical and enzymatic arylamine halogenation reactions in an aqueous environment. The assay is based on the unique UV/Vis spectrum created by the formation of an *ortho*-benzoquinone-amine adduct, which is produced by the peroxidase-catalysed benzoquinone generation, followed by Michael addition of either a halogenated or non-halogenated arylamine. This assay is sensitive, rapid and amenable to high-throughput screening platforms. We have also shown this assay to be easily coupled to a flavin-dependent halogenase, which currently lacks any convenient colorimetric assay, in a "one-pot" workflow.

Introduction

Arylhalides are key building blocks in many fine chemicals including pharmaceuticals, polymers and agrochemicals (Figure 1).[1] Halogenated arenes are also widely utilised in transition-metal-catalysed cross-coupling reactions.[2] Common routes for the production of halogenated aromatics primarily rely on electrophilic aromatic substitution, but these approaches lack regioselectivity[3] and often require Lewis acid catalysis. As a result they generate large amounts of waste acid that is environmentally unfriendly[4] and lacking in atom efficiency.[5] Due to the inherent usefulness of halogenated arylamine compounds, there has been significant interest in more efficient and selective routes to their production. Transition-metal-catalysed halogenations have been reported,[6] which have been shown to provide regioselectivity by ligand-chelate control.[7] An alternative approach that avoids the use of transition metals is to harness enzymes to perform the halogenation of arylamines. Biocatalysis is attractive because it potentially offers highly efficient synthesis in terms of yields and chemical selectivity, together with an inherent environmental sustainability stemming from an ability to promote reactions under mild conditions and a minimal reliance on toxic or expensive feedstocks.[8]

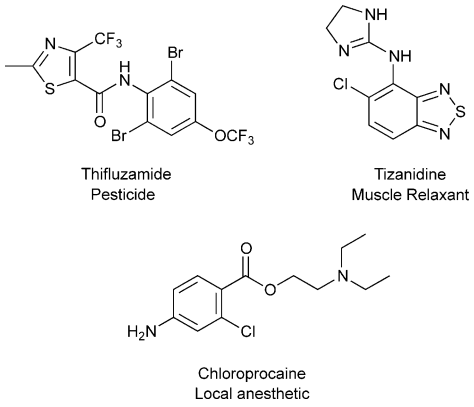

Figure 1. Examples of fine chemicals synthesised from halogenated arylamines.

To date, two discrete classes of enzymes are known to perform biological halogenation reactions, which are categorised by their oxidation partner.[9] Flavin-dependent and non-heme iron(II)-dependent enzymes require oxygen as an electron acceptor and have been termed oxygen-dependent halogenases, whereas heme- and vanadium-dependent enzymes use hydrogen peroxide (H_2O_2) and are termed haloperoxidases.[10] More recently, the use of biocatalysts to produce halogenated arylamines has been explored using regioselective flavin-dependent tryptophan halogenases.[11] These enzymes are particularly interesting because they have been shown to install a chlorine

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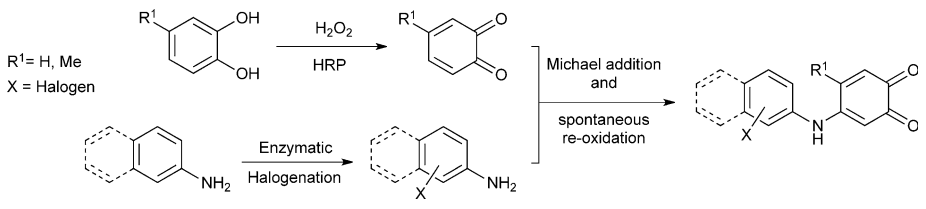
atom regioselectively on an array of aromatic substrates.[12] The proposed mechanism involves the in situ production of hypohalous acid, which binds to an active-site lysine residue; this residue is then thought to guide the regioselective attack of the substrate.[13] In contrast, heme-dependent enzymes release free hypohalous acid, which reacts with a substrate in solution and thus lacks good regiocontrol.[10] Early reports of vanadium-dependent enzymes suggested that they are also unselective due to the escape of hypohalous acid[10] but, more recently, a number of reports have now emerged indicating that good regiocontrol is possible.[14]

In all cases, any efforts aimed at discovering new halogenases[10] or genetically reengineering existing enzymes[15] require the capacity to detect and quantify halogenation activity. One possible approach to this end is the application of assays for the detection of free hypohalous acid in solution,[16,17] but this is an indirect method of detecting enzyme turnover and would not be applicable to halogenating enzymes that bind hypohalous acid in their active site, such as the tryptophan halogenases. As a result, most analyses of these substrates has been conducted with serial and inherently low-throughput methods such as HPLC, LC–MS and NMR.[11,18] Therefore, the further development of halogenating enzymes would greatly benefit from a high-throughput screen, which would aid in the screening of enzyme libraries.

Previously, it has been shown that the oxidation of catechols by horseradish peroxidase (HRP) to their corresponding 1,2-benzoquinone (*ortho*-quinone) can be combined with a subsequent Michael addition of an aniline to the quinone in a "one-pot" reaction.[19] Because the resulting quinone–aniline adduct was highly coloured, this reaction sequence has been applied in an assay for peroxidase activity.[20] We have observed that different anilines, even those closely related in structure, result in the formation of adducts that have significantly different UV/Vis spectra. In comparing halogenated anilines to their unhalogenated parent compounds, these spectral differences were sufficiently large to be visually distinguishable.

Based on these observations, we now report the development of this reaction sequence into a colorimetric assay for the detection and quantification of halogenated arylamines. The assay described is generally applicable to a wide range of arylamines, rapid and high-throughput. Furthermore, since the oxidation of the catechol is also enzymatically driven, it can be performed sequentially after an enzymatic halogenation reaction in a "one-pot" procedure (Scheme 1).



Scheme 1. General scheme illustrating the HRP-catalysed oxidation of catechols to their corresponding *ortho*-quinone (upper pathway) and the formation of the arylamine–catechol adduct. This reaction can be coupled to a biocatalytic halogenation reaction (lower pathway).

Results and Discussion

Development of halogenation assay

The basis for the assay is the distinct UV/Vis spectrum of quinone–aniline adduct that is formed by the stoichiometric Michael addition of the amino component to the *ortho*-benzoquinone, formed by the oxidation of catechol by HRP.[20] Previously, this adduct formation had been used to quantify the concentration of peroxidases in solution, but by using an excess of HRP and H_2O_2 it is instead possible to use the adduct produced by the reaction for quantification of the amino component. If the reaction mixture contains multiple amino components, each resulting adduct can be differentiated by their respective λ_{max} peaks in the overall UV/Vis spectra. In the case of arylamines and halogenated arylamines it was found that the λ_{max} values were typically in the range of ≈520 and ≈425 nm, respectively (Figure 2).

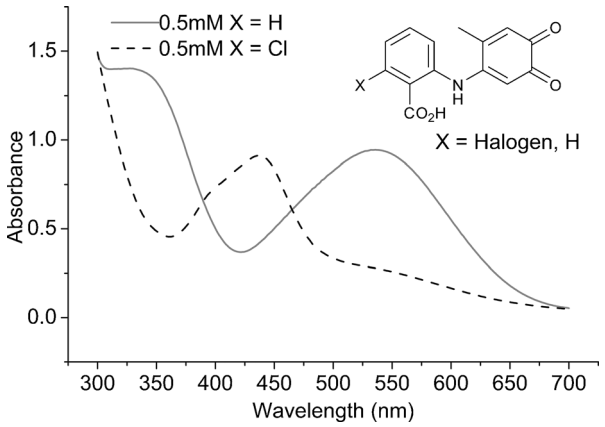


Figure 2. UV/Vis spectra of adduct formed from 4-methyl-catechol (4-MC) with either 2-aminobenzoic acid (0.5 mM, X = H) or 2-amino-6-chlorobenzoic acid (0.5 mM, X = Cl) in K_2HPO_4 (50 mM) at pH 7.4.

A number of experimental considerations were taken into account to simplify the analysis and enable a one-pot procedure. Firstly, 4-methylcatechol (4-MC) was employed as the catechol component, because it was known to be a good substrate for HRP. The resulting *ortho*-quinone gives only a single-conjugate product, because the 4-methyl substituent blocks further addition at this position.[21] To prevent any oxidation of the arylamine by HRP at low pH,[22] all assays were carried out in pH 7.4 buffered conditions. To confirm that only the desired product was formed under these conditions, test reactions were carried out with 4-MC and either 2-aminobenzoic acid or 2-amino-6-chlorobenzoic acid; HPLC and MS analysis of the reaction mixtures confirmed single-product formation and that the reaction was complete within 5 min.

Substrate range

To confirm the trend that the λ_{max} values of adducts formed from the Michael addition were lower than the corresponding non-halogenated arylamines, a further series of analogues was tested (Figure 3). From the data collated, it can be observed that halogenated substrates have a lower λ_{max} than their non-halogenated counterparts throughout the series (Table 1). The results show that the trend is also seen with the more-substituted arylamines as well as with the aminopyridine tested.

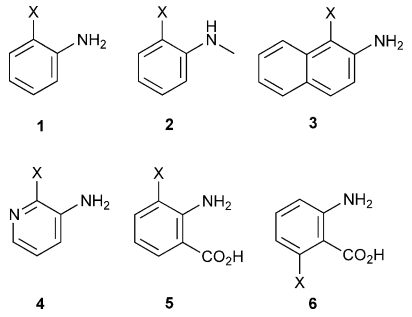


Figure 3. Structures of substrates tested with the coupled assay. X = H, Cl, Br or I.

Table 1. UV/Vis λ_{max} and ε values of the aniline–catechol adducts formed by the assay. "–" = not determined.

Arylamine[a]	X = H		X = Cl		X = Br		X = I	
	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$]	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$]	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$]	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$]
1	508	4141	441	2386	432	1964	431	2029
2	537	5050	403	1027	400	1108	–	–
3	516	1966	438	1489	437	1086	–	–
4	500	1919	398	1710	413	1244	–	–
5	535	4706	436	3789	436	2770	–	–
6	535	4706	437	4548	–	–	–	–

[a] See Figure 3 for structure.

Quantitative analysis

The corresponding molar absorption coefficients (ε) for the λ_{max} values that were determined in Table 1 were then used to allow quantification of binary mixtures of the halogenated arylamines and their unhalogenated analogues. For this purpose, calibration plots were constructed for each of the arylamines by reacting different ratios of known concentrations of the halogenated and non-halogenated arylamine. Here, the halogenated and non-halogenated arylamine were added in ratios from 10:0 to 0:10 to a total concentration 0.5 mM.

As an illustrative example, the UV/Vis spectra were recorded for mixtures of 2-aminobenzoic acid and 2-amino-6-chlorobenzoic acid (**6**) that were subjected to the assay. It was found that, as the ratio of 2-amino-6-chlorobenzoic acid increased, the absorbance at 437 nm increased with a concomitant decrease at 535 nm, with a corresponding visually observable colour change from red to yellow (Figure 4a and b, full-colour version available in the Supporting Information). A calibration graph was then constructed by plotting the absorbance of the peak in the 500–550 nm region, corresponding to λ_{max} of the arylamine–catechol adduct produced (which, in the case of 2-amino-6-chlorobenzoic acid, was at 535 nm), against the concentration of the halogenated component (Figure 4c). This methodology was then extended to a range of other halogenated and unhalogenated mixture-pairs and, in all cases, the resulting calibration plot fitted a linear relationship with a high statistical significance ($R^2 > 0.99$, Table 2). The detection limit for the halogenated product was dependent on the sensitivity of the UV/Vis assay. In these cases, it was possible to detect conversion rates as low as 5% (0.025 mM in 0.5 mM of total adduct).

Coupling with flavin-dependent halogenase system

In order to demonstrate the applicability of this assay to biocatalytic halogenation reactions, efforts were then directed to the development of an end-point assay that could be conducted sequentially in the same reaction vessel.

As a model tryptophan halogenase enzyme, the tryptophan-7-halogenase RebH from *Leche-*

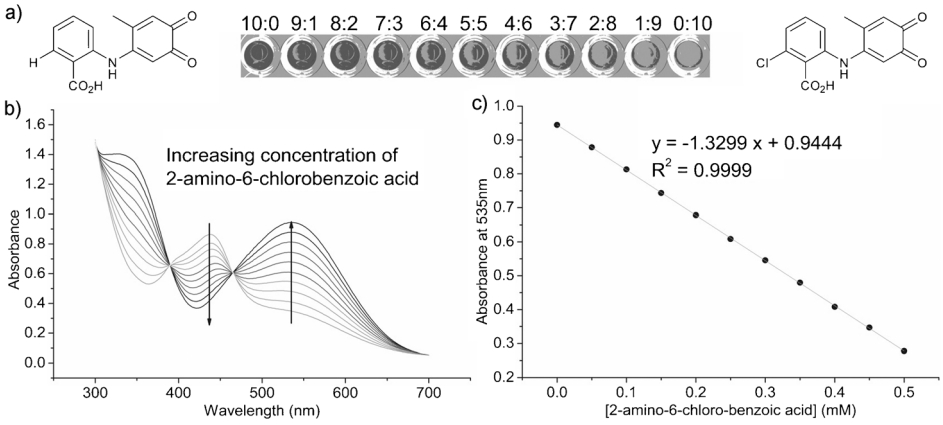


Figure 4. Illustrative data for mixtures of 2-aminobenzoic acid and 2-amino-6-chlorobenzoic acid solutions. a) Photograph of one row of wells in a microtitre plate with adducts produced from mixtures of 2-aminobenzoic acid and 2-amino-6-chlorobenzoic acid (total concentration 0.5 mM). b) UV/Vis spectra for the mixtures in a range of ratios to a total concentration of 5 mM. c) The calibration graph produced by plotting the absorbance of the peak at 535 nm against the concentration of 2-amino-6-chlorobenzoic acid.

Table 2. Parameters calculated from the best fit of calibration plots for the binary mixtures of the halogenated and unhalogenated arylamine–catechol adducts.

Arylamine[a]	Ring substituent X	Gradient	y-intercept	Coefficient of determination (R^2)
1	H vs. Cl	−0.9916	0.8139	0.9977
	H vs. Br	−1.2304	0.8099	0.9951
	H vs. I	−0.9916	0.8139	0.9977
2	H vs. Cl	−1.706	1.0078	0.9997
	H vs. Br	−1.7988	1.0479	0.9992
3	H vs. Cl	−0.4588	0.4082	0.9982
	H vs. Br	−0.6210	0.4132	0.9910
4	H vs. Cl	−0.3803	0.3973	0.9906
	H vs. Br	−0.7111	0.4744	0.9933
5	H vs. Cl	−1.4900	0.9705	0.9921
	H vs. Br	−1.5853	0.9781	0.9910
6	H vs. Cl	−1.3299	0.9444	0.9999

[a] See Figure 3 for structure.

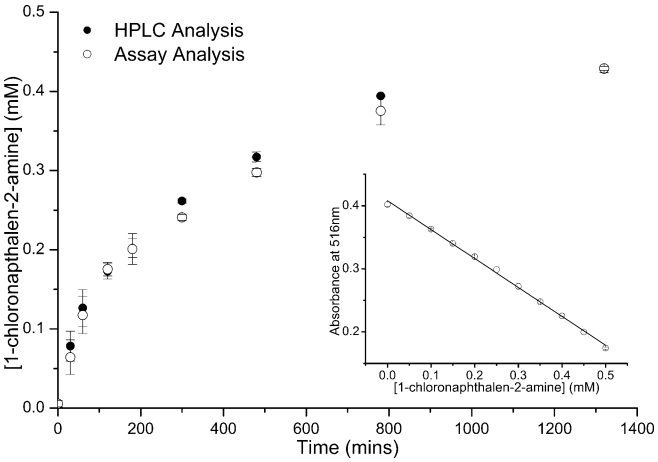
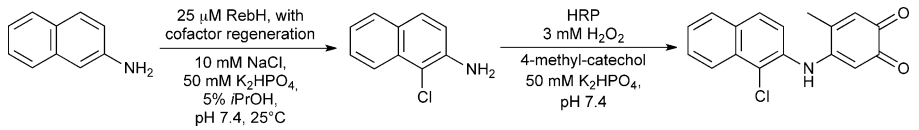


Figure 5. Graph showing the increase in the concentration 1-chloronaphthalen-2-amine against length of time of the halogenation reaction. Inset: assay graph calibration plot for assay analysis produced using RebH halogenation reagents and conditions.

valieria aerocolonigenes was chosen because it is known to catalyse the chlorination of 2-naphthylamine to 1-chloronaphthalen-2-amine (**3**).[11] Because RebH is a flavin-dependent enzyme, the biocatalytic halogenation system required the inclusion in the reaction mixture of a co-factor-regeneration system composed of flavin reductase[23] (Fre, from *Escherichia coli*) and glucose dehydrogenase[24] (GDH2, from *Bacillus megaterium*).

The overall sequential biocatalytic halogenation and assay procedure thus involved the execution of the halogenation, which was terminated at pre-determined time points by heating to 95 °C. After a brief cooling period, HRP, H_2O_2 and 4-MC were added and incubated for a further 5 min (Scheme 2). Each mixture was then analysed to quantify the amount of the respective halogenated and unhalogenated 4-MC adducts by using the UV/Vis method described above, and the time course of the biotransformation was plotted (Figure 5). The quantifications were also conducted by HPLC as a comparator and a good correlation between both analytical methods was observed, thus validating the spectrometric method (see the Supporting Information for detailed UV/Vis absorbance data and HPLC chromatograms). Due to the rapidity of the quinone generation and adduct formation, it was also possible to perform the second step of the assay without the need to stop the halogenation reaction beforehand.



Scheme 2. Reaction sequence for RebH halogenation followed by in situ HRP oxidation of 4-MC and coupling of the naphthylamine(s).

Conclusion

In summary, a UV/Vis spectrometric assay for the halogenation of arylamines based on in situ oxidation of 4-methylcatechol by HRP and conjugate addition has been developed. The presented assay is rapid (5 min), sensitive (0.025 mM detection limit) and provides both qualitative and quantitative analysis. In all the examples described, the reaction results in a sufficiently large λ_{max} shift that it can even be visually observed, making it a genuine colorimetric assay. It has also been shown to be amenable to coupling with reactions carried out by flavin-dependent halogenase enzymes in a one-pot procedure and can be applied in a high-throughput multi-well format. Because the assay functions under mild physiological conditions, this assay can in principle be used in a similar manner to halogenation reactions catalysed by other classes of enzymes.

It is thus envisaged that a key application of this assay will be in efforts to discover and reengineer new halogenase enzymes for synthetic applications, and is an improvement over previously described methods that only identify enzyme activity indirectly by the detection of hypohalous acid production. Indeed, previous programmes in the development of new biocatalysts have shown that the availability of a suitable high-throughput screening methodology has been crucial to their success.[25]

Ideally, an authenticated sample of the desired product is required in order to provide accurate quantification, but, in most cases, standards can be obtained through conventional (non-enzymatic) chemistry. Also, it is possible to derive semi-quantitative data by applying computationally simulated UV/Vis spectra.[26] Even in cases for which information on the product is limited, a simple absorbance measurement of any wavelength in the 500–550 nm range can serve as a qualitative test, which is usually sufficient as a first-pass library screening tool.

Though the original intention was to develop a colorimetric assay for enzymatic arylamine halogenations, it could also be equally applied to any halogenation reaction of aryl systems that possess an exocyclic nucleophile (e.g., arylmercaptans, arylphosphines).[19,27] Thus, this general procedure of in situ HRP-

mediated quinone oxidation, followed by conjugate addition, has substantial scope for further development into assays for a wide range of substrates.

Keywords: halogenation · enzyme catalysis · Michael addition · high-throughput screening

Experimental Section

Reagents and materials

All reagents and enzyme substrates were purchased from commercial suppliers and used without additional purification. Peroxidase from horseradish (Type VI-A), β-nicotinamide adenine dinucleotide dipotassium salt (NAD), flavin adenine dinucleotide disodium salt (FAD) and sequencing primers were purchased from Sigma Aldrich (UK). The RebH gene was amplified from the genomic DNA of *Lechevalieria aerocolonigenes* using primers 5′–GTACGT*CATATG*TCCGGCAAGATTGACAAG–3′ and 5′–GTCAGC*AAGCTT*TCAGCGGCCGTGCTGTGCC–3′with NdeI and HindIII restriction sites. The gene was cloned into the corresponding restriction sites of pET28a.[28] The flavin reductase (Fre) gene was amplified from the genomic DNA of *E. coli* BL21 (DE3) using primers 5′–AAAAAA*GGTACC*ATGACAACCTTAAGCTGTAAA–3′ and 3′–AAAAAA*CTCGAG*TCAGATAAATGCAAACGCATC–5′, and digested using KpnI and XhoI before ligating into the pET45b expression vector. The pET21b/GDH2 plasmid[24] was a kind gift from Prof. N. S. Scrutton's laboratory. All vectors were confirmed by DNA sequencing. *E. coli* ArcticExpress (DE3) cells were purchased from Agilent and BL21(DE3) cells were purchased from Invitrogen. Details of halogenase enzyme production, enzymatic halogenation and assay analysis can be found in the Supporting Information.

General assay procedure

All UV/Vis data was collected on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments). Calibration curves were constructed from experiments performed at a 150 μL per well scale by combining, 4-methyl-catachol (0.5 mM), hydrogen peroxide (3 mM) and the mixture of arylamine substrates to be analysed (0.5 mM total concentration) in potassium phosphate buffer (20 mM) at pH 7.4 with isopropanol (5% v/v) to aid the dissolution of the organic amine. HRP (1 μL, 0.1 mg mL^{-1} in potassium phosphate buffer (20 mM) at pH 7.4) was then added to commence the reaction. The plate was then agitated for 5 min and the UV/Vis spectra recorded. In all cases, the reactions were performed in triplicate. OriginPro 8 was used for all data analysis, using linear least-squares fitting to calculate the calibration curve.

Acknowledgements

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[1] a) S. A. Lawrence, *Amines: Synthesis Properties and Applications*, Cambridge University Press, Cambridge, **2006**; b) M. J. O'Neil, *The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals, Vol. 15*, Royal Society of Chemistry, Cambridge, **2013**.
[2] N. Miyaura, A. Suzuki, *Chem. Rev.* **1995**, *95*, 2457 – 2483.
[3] P. Kovacic, N. O. Brace, *J. Am. Chem. Soc.* **1954**, *76*, 5491 – 5494.
[4] M. Noè, A. Perosa, M. Selva, L. Zambelli, *Green Chem.* **2010**, *12*, 1654 – 1660.
[5] B. M. Trost, *Science* **1991**, *254*, 1471 – 1477.
[6] D. Kalyani, A. R. Dick, W. Q. Anani, M. S. Sanford, *Org. Lett.* **2006**, *8*, 2523 – 2526.
[7] K. M. Engle, J. Q. Yu, *J. Org. Chem.* **2013**, *78*, 8927 – 8955.
[8] a) M. T. Reetz, *J. Am. Chem. Soc.* **2013**, *135*, 12480 – 12496; b) M. A. Fisher, D. Tullman-Ercek, *Curr. Opin. Biotechnol.* **2013**, *24*, 1010 – 1016; c) G. W. Huisman, S. J. Collier, *Curr. Opin. Chem. Biol.* **2013**, *17*, 284 – 292; d) U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185 – 194.
[9] C. D. Murphy, *J. Appl. Microbiol.* **2003**, *94*, 539 – 548.
[10] a) F. H. Vaillancourt, E. Yeh, D. A. Vosburg, S. Garneau-Tsodikova, C. T. Walsh, *Chem. Rev.* **2006**, *106*, 3364 – 3378; b) A. Butler, M. Sandy, *Nature* **2009**, *460*, 848 – 854.
[11] J. T. Payne, M. C. Andorfer, J. C. Lewis, *Angew. Chem.* **2013**, *125*, 5379 – 5382; *Angew. Chem. Int. Ed.* **2013**, *52*, 5271 – 5274.
[12] K. H. van Pée, E. Patallo, *Appl. Microbiol. Biotechnol.* **2006**, *70*, 631 – 641.
[13] a) C. Dong, S. Flecks, S. Unversucht, C. Haupt, K. H. Van Pée, J. H. Naismith, *Science* **2005**, *309*, 2216 – 2219; b) E. Yeh, L. C. Blasiak, A. Koglin, C. L. Drennan, C. T. Walsh, *Biochemistry* **2007**, *46*, 1284 – 1292.
[14] a) J. M. Winter, B. S. Moore, *J. Biol. Chem.* **2009**, *284*, 18577 – 18581; b) L. Kaysser, P. Bernhardt, S.-J. Nam, S. Loesgen, J. G. Ruby, P. Skewes-Cox, P. R. Jensen, W. Fenical, B. S. Moore, *J. Am. Chem. Soc.* **2012**, *134*, 11988 – 11991.
[15] W. S. Glenn, E. Nims, S. E. O'Connor, *J. Am. Chem. Soc.* **2011**, *133*, 19346 – 19349.
[16] a) E. Verhaeghe, D. Buisson, E. Zekri, C. Leblanc, P. Potin, Y. Ambroise, *Anal. Biochem.* **2008**, *379*, 60 – 65; b) L. P. Hager, D. R. Morris, F. S. Brown, H. Eberwein, *J. Biol. Chem.* **1966**, *241*, 1769 – 1777.
[17] C. Wagner, I. M. Molitor, G. M. König, *Phytochemistry* **2008**, *69*, 323 – 332.
[18] M. Frese, P. H. Guzowska, H. Voß, N. Sewald, *ChemCatChem* **2014**, *6*, 1270 – 1276.
[19] V. K. Tandon, H. K. Maurya, *Tetrahedron Lett.* **2009**, *50*, 5896 – 5902.
[20] A. Molaei Rad, H. Ghourchian, A. A. Moosavi-Movahedi, J. Hong, K. Nazari, *Anal. Biochem.* **2007**, *362*, 38 – 43.
[21] a) S. Uchiyama, Y. Hasebe, J. Nishimoto, H. Hamana, Y. Maeda, Y. Yoshida, *Electroanalysis* **1998**, *10*, 647 – 650; b) S. Witayakran, A. J. Ragauskas, *Eur. J. Org. Chem.* **2009**, *2009*, 358 – 363.
[22] W. Liu, J. Kumar, S. Tripathy, K. J. Senecal, L. Samuelson, *J. Am. Chem. Soc.* **1999**, *121*, 71 – 78.
[23] G. Spyrou, E. Haggard-Ljungquist, M. Krook, H. Jornvall, E. Nilsson, P. Reichard, *J. Bacteriol.* **1991**, *173*, 3673 – 3679.
[24] T. Nagao, T. Mitamura, X. H. Wang, S. Negoro, T. Yomo, I. Urabe, H. Okada, *J. Bacteriol.* **1992**, *174*, 5013 – 5020.
[25] V. Köhler, Y. M. Wilson, M. Dürrenberger, D. Ghislieri, E. Churakova, T. Quinto, L. Knörr, D. Häussinger, F. Hollmann, N. J. Turner, T. R. Ward, *Nat. Chem.* **2012**, *5*, 93 – 99.
[26] V. Barone, *Computational Strategies for Spectroscopy*, John Wiley & Sons, Hoboken, NJ, **2011**.
[27] D. V. Moiseev, B. O. Patrick, B. R. James, T. Q. Hu, *Inorg. Chem.* **2007**, *46*, 9389 – 9399.
[28] E. Yeh, S. Garneau, C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 3960 – 3965.

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