

Supramolecular Assembly of Artificial Metalloenzymes Based on the Dimeric Protein LmrR as Promiscuous Scaffold

Jeffrey Bos,[†] Wesley R. Browne,[†] Arnold J. M. Driessen,[‡] and Gerard Roelfes^{*,†}

[†]Stratingh Institute for Chemistry, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands

[‡]Groningen Biomolecular Sciences and Biotechnology Institute and the Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands

S Supporting Information

ABSTRACT: Supramolecular anchoring of transition metal complexes to a protein scaffold is an attractive approach to the construction of artificial metalloenzymes since this is conveniently achieved by self-assembly. Here, we report a novel design for supramolecular artificial metalloenzymes that exploits the promiscuity of the central hydrophobic cavity of the transcription factor Lactococcal multidrug resistance Regulator (LmrR) as a generic binding site for planar coordination complexes that do not provide specific protein binding interactions. The success of this approach is manifested in the excellent enantioselectivities that are achieved in the Cu(II) catalyzed enantioselective Friedel–Crafts alkylation of indoles.

Artificial metalloenzymes open the way to engage the molecular recognition properties of biomolecules, such as proteins or DNA, in achieving enzyme-like activities and selectivities in transition metal catalysis.¹ While tremendous advances have been made already, the rational design of an artificial metalloenzyme for a given reaction requires knowledge of the relation between structure and activity that is still beyond the state of the art.² Indeed, the design of artificial metalloenzymes at present is focused first and foremost on the (re)design of the metal binding site.³ A popular approach involves the judicious positioning of a catalytically active transition metal complex within an existing protein scaffold.¹ This is often realized through supramolecular anchoring of the catalyst at a specific site in the protein, using metal complexes tethered via their ligand to high affinity binding motifs. This approach has been applied successfully in the design of a variety of artificial metalloenzymes, most notably the streptavidin/biotin system.^{4,5} Yet, the nature of the tether and its position on the ligand place restrictions as to the structural flexibility and dynamics of the metal complex. As a consequence, the conformational space and second coordination sphere interactions that can be accessed during catalysis are also restricted.

Here, we report a novel design for supramolecular artificial metalloenzymes that exploits the extensive promiscuity of the central hydrophobic cavity of the protein Lactococcal multidrug resistance Regulator (LmrR) as a generic, moderate affinity binding site for planar coordination complexes that do not provide specific protein binding interactions. The success of this approach is manifested in the excellent enantioselectivities

that are achieved in the Cu(II) catalyzed enantioselective Friedel–Crafts alkylation of indoles.

LmrR is a transcriptional repressor that is involved in the antibiotic resistance response of *Lactococcus lactis*.⁶ It is a homodimeric protein with a size of 13.5 kDa per monomer and contains an unusual large hydrophobic pore at the dimer interface. Here, flat aromatic organic molecules can bind as is shown in X-ray and NMR structures of LmrR with various planar drugs bound.⁷ Two tryptophan residues, one from each subunit, i.e., W96 and W96', play a key role in binding by sandwiching the organic molecules using π -stacking interactions (Figure 1). The LmrR structure has been reported to exhibit a broad and shallow conformation energy surface in which the conformation can shift readily to accommodate structurally unrelated compounds without prerequisite specific binding interactions.^{7b}

Recently, we have reported two new classes of artificial metalloenzymes containing a covalently linked Cu(II) complex at the dimer interface of LmrR.⁸ These artificial metalloenzymes were shown to catalyze Diels–Alder, hydration, and Friedel–Crafts alkylation reactions, with excellent enantiomeric excess (ee) in the first and moderate ee in the latter two reactions. These results demonstrated that the hydrophobic cavity of LmrR can accommodate many diverse substrates and reaction types. Encouraged by these results, it was decided to explore the supramolecular assembly of an LmrR-based artificial metalloenzyme, taking advantage of the promiscuity of the hydrophobic pocket at the dimer interface. We envisioned that Cu(II) complexes of planar aromatic ligands would be capable of binding partly or completely within the LmrR pocket, with the result that the catalyzed reaction takes place inside the chiral microenvironment provided by the LmrR hydrophobic pocket.

The LmrR variant used in this study, referred to as LmrR_LM, contains a C-terminal Strep tag to facilitate purification and two mutations in the DNA binding domain, i.e., K55D and K59Q, which facilitated expression and purification.^{8b} A second mutant was prepared in which the tryptophan residues at the dimer interface were replaced by alanine, i.e., LmrR_LM_W96A. Both mutants were expressed in *E. coli* BL21(DE3)C43. Typical yields of purified protein were 20–30 mg L⁻¹ culture.

Received: June 4, 2015

Published: July 27, 2015

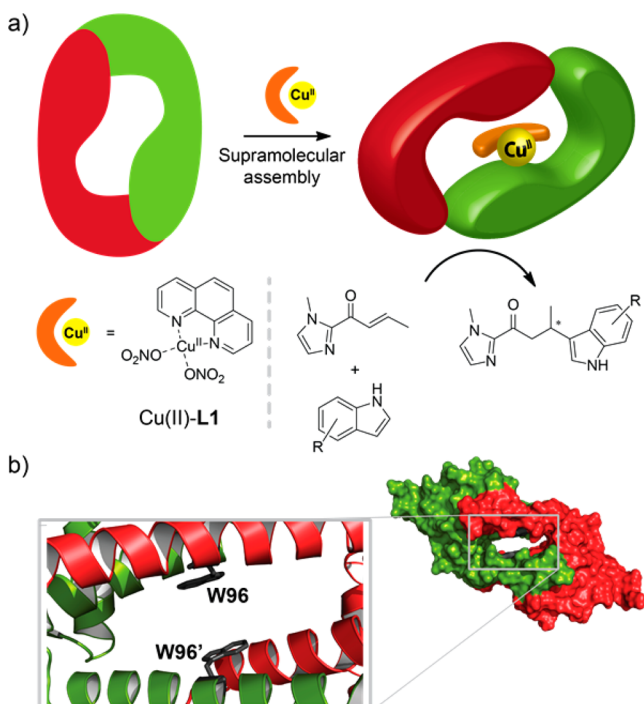
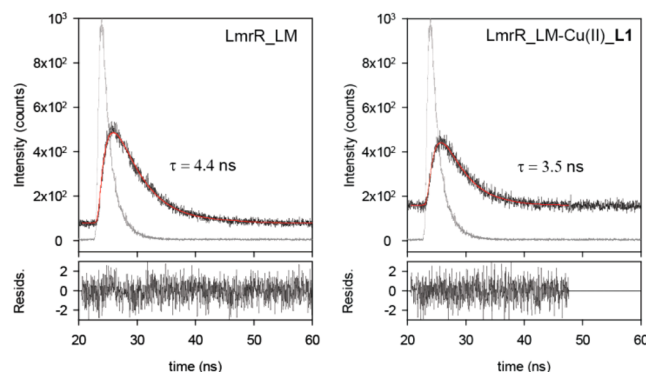


Figure 1. (a) Schematic representation of the design of the supramolecular artificial metalloenzyme in which a Cu(II) complex is bound at the dimer interface of LmrR, followed by application in a catalyzed Friedel–Crafts alkylation. (b) Space filled representation of dimeric LmrR (pdb 3F8B) and ribbon representation of the ligand binding pocket (inset). Tryptophan residues W96 and W96' are shown in gray. Pictures were generated with Pymol.

The artificial metalloenzymes were prepared through self-assembly, by combining LmrR_LM with a Cu(II) complex of a planar aromatic ligand L1–7 (Figure S1) in a buffered solution (20 mM MOPS, 150 mM NaCl, pH 7.0). The binding affinity of the Cu(II) complex to LmrR_LM was determined by titration through monitoring of the quenching of tryptophan fluorescence, taking into account the quenching of fluorescence of the tryptophan residues at the exterior of the protein, i.e., W67 and W119 (Figure S2), by using LmrR_LM_W96A as control (Figure S6). A dissociation constant (K_d) of $2.6 \pm 2 \mu\text{M}$ was determined for binding of Cu(II)-L1 to LmrR_LM, which is significantly higher than the K_d reported for the tight-binding drugs H33342 and daunomycin (21 ± 8 and 236 ± 53 nM, respectively).⁶ The binding of Cu(II)-L1 to LmrR_LM_W96A mutant, which does not contain tryptophan in the hydrophobic pocket, was significantly weaker: the K_d was determined to be one order of magnitude lower, i.e., $45 \mu\text{M}$. Analytical size exclusion chromatography of the LmrR_LM \subset Cu(II)-L1 and LmrR_LM_W96A \subset Cu(II)-L1 hybrids was performed. Both eluted as a single band with an apparent molecular weight of 29 kDa, showing that the addition of Cu(II)-L1 does not disrupt the homodimeric structure (Figure S7).⁸

The binding of Cu(II)-L1 to LmrR_LM was studied further by fluorescence decay lifetime experiments. The tryptophan residues can be divided into two groups: those within the hydrophobic pocket (W96/W96') and those distant from the hydrophobic pocket (W67/W67' and W119/W119'). The observed fluorescence lifetime is a weighted sum of the individual lifetimes of both groups (note that these are too similar to be resolved reliably by a bi- or multiexponential decay

fit, given the much greater emission intensity of the tryptophans within the pocket, see Supporting Information for further details) and was 4.4 ns in the case of LmrR_LM (Figures 2 and



Protein	[Cu(II)-L1] (μM)	Lifetime (ns)
LmrR_LM	0	4.4
LmrR_LM	44	3.5
LmrR_LM_W96A	0	3.7
LmrR_LM_W96A	44	3.7

Figure 2. Representative fluorescence decays (in black), IRF corrected exponential fits (in red), and residuals for LmrR_LM and LmrR_LM \subset Cu(II)-L1. In both cases the χ^2 values for the fits are ca. 1.01. Further addition of Cu(II) reduces emission intensity but has no additional effect on lifetime as expected for static quenching. The instrumental response function (IRF) was recorded using BaSO₄ (in gray).

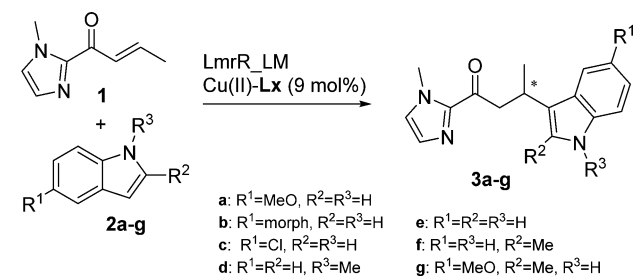
S8). Upon saturation of LmrR_LM with Cu(II)-L1, i.e., with the latter at $44 \mu\text{M}$, a decrease beyond experimental uncertainty in the fluorescence lifetime to 3.5 ns was observed. The final lifetime is close to the lifetime observed for LmrR_LM_W96A, i.e., the lifetimes for W67/W119, which is 3.7 ns (Figure 2). Combined, these data indicate that Cu(II)-L1 binds predominantly in proximity to W96/W96', which strongly suggests it is bound at or near to the hydrophobic pore of LmrR (for a detailed discussion, see Supporting Information).

The binding of a series of copper complexes of nitrogen based bidentate ligands (Cu(II)-(L2-L7)) (Table S1) to LmrR was studied also. The K_d for binding of these Cu(II) complexes to LmrR_LM was found to be within the same order of magnitude as for Cu(II)-L1, suggesting that indeed the hydrophobic pore of LmrR acts as a generic binding pocket and that significant specific interactions to the metal complex are not required.

The potential of these new artificial metalloenzymes in catalysis was evaluated in the enantioselective vinylogous Friedel–Crafts (FC) alkylation of 5-methoxy-1H-indole (2a) with 1-(1-methyl-1H-imidazol-2-yl)but-2-en-1-one (1) to generate 3a.⁹ This is a reaction that we previously showed to be compatible with dynamic catalytic assemblies.^{9a} Artificial metalloenzymes were prepared *in situ* by self-assembly from 9 mol % of Cu(II)-L1 ($90 \mu\text{M}$) with a slight excess (1.3 equiv) of LmrR_LM in 3-(N-morpholine)propanesulfonic acid buffer (MOPS) (20 mM, pH 7.0), containing 150 mM NaCl. Based on the K_d more than 90% of Cu(II)-L1 is bound to LmrR_LM under these conditions. The catalytic reaction was started by addition of 1 mM 1 and 5 mM indole 2a. After 16 h at 4 °C, a

conversion of 57% was observed, and **3a** was obtained with an ee of 61% in the (+) enantiomer (Table 1, entry 1).

Table 1. Results of Friedel–Crafts Alkylation Reactions Catalyzed by LmrR-Based Artificial Metalloenzymes^a



entry	indole	equiv of indole	product	conversion (%) ^b	ee (%) ^c
LmrR_LM ⊂ Cu(II)-L1					
1	2a	5	3a	57 ± 7	61 ± 8(+)
2	2a	1	3a	84 ± 5	75 ± 13(+)
3	2a	0.5	3a	90 ± 2 ^d	84 ± 3(+)
4	2b	1	3b	94 ± 4	82 ± 1
5	2c	1	3c	19 ± 8	64 ± 7(+)
6	2d	1	3d	79 ± 2	18 ± 2(-)-R
7	2e	1	3e	full	94 ± 0(+)-R
8	2f	1	3f	full	93 ± 1
9	2f	0.5	3f	full ^d	93 ± 1
10	2g	1	3g	56 ± 25 ^e	81 ± 1
LmrR_LM_W96A ⊂ Cu(II)-L1					
11	2a	1	3a	53 ± 28	<5

^aTypical conditions: 75% Cu(II)-L1 (9 mol %; 90 μM) loading with respect to LmrR_LM or LmrR_LM_W96A, 1 mM **1**, 0.5–5 mM **2**, in 20 mM MOPS buffer (pH 7.0), 150 mM NaCl, at 4 °C for 16 h (**2a**) or 64 h (other indoles). Conversions and ee values are an average of at least two independent experiments, both carried out in duplicate. ^bConversions are determined by HPLC and are based on substrate **1**. ^cSign of rotation and absolute configuration based on elution order in chiral HPLC. ^dConversion is based on substrate **2**. ^eAdditional unidentified products were detected (Figure S4).

Interestingly, a strong dependence of the results of catalysis on the indole concentration was observed. When the concentration of **2a** was decreased 5-fold, i.e., to 1 mM, the conversion and ee increased to 84% and 75%, respectively (Table 1, entry 2). Further decreasing the concentration of indole to 0.5 mM, i.e., using a ratio of **1/2a** of 1:0.5, resulted in an ee of 84% (Table 1, entry 3) This suggests that the indole competes with Cu(II)-L1 for binding to LmrR_LM,¹⁰ i.e., at higher indole concentrations Cu(II)-L1 is displaced from the hydrophobic pore of LmrR_LM. This results in an increased fraction of unbound Cu(II)-L1 that catalyzes the reaction in a racemic fashion, causing a decrease in ee of **3a**.

Artificial metalloenzymes based on Cu(II)-(L2-L7) provided similar enantioselectivities in the catalyzed reaction. The presence of a ligand is required since ee was not obtained when using LmrR_LM + Cu(NO₃)₂ (Table S2).

Using LmrR_LM_W96A ⊂ Cu(II)-L1 as catalyst in the reaction of **1** and **2a** resulted in moderate conversion and <5% ee of **3a**. (entry 11). This is in agreement with the weaker binding affinity of Cu(II)-L1 in the absence of W96/W96', i.e., under the conditions of catalysis only a small fraction of the Cu(II) complex is actually bound to the LmrR_LM_W96A.

The substrate scope of LmrR_LM ⊂ Cu(II)-L1 was evaluated by variation of the 2-acyl indazole and of the indole. The artificial metalloenzyme did not tolerate larger substituents on the β position of the enone (Table S3). In contrast, a broad scope of indoles **2** was accepted. Replacing the methoxy group at the 5 position of the indole with an *N*-morpholine moiety (**2b**) resulted in a similar ee, whereas a chloride at this position (**2c**) resulted in a lower ee (Table 1, entries 4–5). With *N*-methylindole (**2d**) the ee decreased to 18% (Table 1, entry 6). The best results were obtained with nonsubstituted indole (**2e**) and 2-methylindole (**2f**), that is, full conversion of **1** and an excellent ee of 94% (*R*-enantiomer) and 93% for the corresponding products **3e** and **3f**, respectively (Table 1, entries 7–9). The double substituted indole **2g** gave results comparable to **2a**, albeit that additional unidentified products were detected (Table 1, entry 10; Figure S4). The ee's achieved are significantly higher than those obtained with our previous artificial metalloenzyme design containing a covalently linked bipyridine ligand.^{8a}

The combined data of the binding studies, fluorescence lifetime experiments, and catalysis demonstrate that the catalysis takes place in the hydrophobic pore of LmrR-LM and that tryptophan residues W96 and W96' in the newly created active site are of key importance to the catalysis. Possibly, they are involved in intercalation-like binding of the Cu(II) complex.¹² Alternatively, instead of binding the metal complex, it is also possible that either the enone or the indole substrates are bound between the tryptophan residues via π stacking, analogous to the observed binding mode of H33342 and daunomycin,⁶ and are thus positioned for the reaction, resulting in enantioselectivity. This model is in agreement with the observed preference in catalysis for planar conjugated molecules such as **1a** and unsubstituted indole **2e**. The observed competition between **2a** and Cu(II)-L1 in binding LmrR implies that both substrates and Cu(II) complex prefer to bind, at least partly, in the same location, which suggests significant dynamics during catalysis.

In conclusion, we have introduced a novel design of an artificial metalloenzyme, created by supramolecular binding of a catalytically active Cu(II) complex in the hydrophobic cavity of the dimer interface of LmrR. A key element of the present design is the promiscuity of the hydrophobic cavity of LmrR that can accept many compounds, including substrates and catalytically active transition metal complexes, without requiring specific ligand–protein interactions, as is the case in most supramolecular artificial metalloenzyme designs. The success of this approach was manifested in the catalyzed asymmetric vinylogous Friedel–Crafts alkylation of indoles in water, giving rise to excellent ee's. The results of this study suggest that precise design of second coordination sphere interactions may not always be the most appropriate approach *a priori* to achieving high selectivity in artificial metalloenzyme catalysis, provided that substrates and metal complexes have enough freedom to find themselves the optimum orientation and interactions in a promiscuous chiral space. The present design is highly flexible and is envisioned to be adapted readily for binding other metal complexes and catalysis of other reactions. It is notable also that LmrR is the first scaffold that has been used successfully in multiple anchoring strategies, thus underlining the versatility of LmrR as a scaffold for artificial metalloenzymes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05790.

Detailed experimental procedures, characterization data, and additional results of catalysis experiments (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*j.g.roelfes@rug.nl

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We wish to thank P. Dijkstra, I. Drienovská, and L. Villarino Palmaz for assistance with protein expression and the fluorescence decay lifetime experiments. Support from the NRSC-Catalysis, the European Research Council (starting grant no. 280010), and the Ministry of Education, Culture, and Science (Gravitation program no. 024.001.035) is gratefully acknowledged. A.J.M.D. acknowledges the research program on biobased ecologically balanced sustainable industrial chemistry (BE-BASIC) for support.

■ REFERENCES

- (1) (a) Bos, J.; Roelfes, G. *Curr. Opin. Chem. Biol.* **2014**, *19*, 135. (b) Lewis, J. C. *ACS Catal.* **2013**, *3*, 2954–2975. (c) Rosati, F.; Roelfes, G. *ChemCatChem* **2010**, *2*, 916. (d) Wilson, M. E.; Whitesides, G. M. *J. Am. Chem. Soc.* **1978**, *100*, 306.
- (2) Muñoz Robles, V.; Ortega-Carrasco, E.; Alonso-Cotchico, L.; Rodríguez-Guerra, J.; Lledós, A.; Maréchal, J.-D. *ACS Catal.* **2015**, *5*, 2469. (b) Dürrenberger, M.; Ward, T. R. *Curr. Opin. Chem. Biol.* **2014**, *19*, 99. (c) Kiss, G.; Çelibî-Ölçüm, N.; Moretti, R.; Baker, D.; Houk, K. N. *Angew. Chem., Int. Ed.* **2013**, *52*, 5700.
- (3) (a) Bordeaux, M.; Tyagi, V.; Fasan, R. *Angew. Chem., Int. Ed.* **2015**, *54*, 1744. (b) Yu, F.; Cangelosi, V. M.; Zastrow, M. L.; Tegoni, M.; Plegaria, J. S.; Tebo, A. G.; Mocny, C. S.; Ruckthong, L.; Qayyum, H.; Pecoraro, V. L. *Chem. Rev.* **2014**, *114*, 3495. (c) Song, W. J.; Tezcan, F. A. *Science* **2014**, *346*, 1525. (d) Coelho, P. S.; Brustad, E. M.; Kannan, A.; Arnold, F. H. *Science* **2013**, *339*, 307. (e) Mills, J. – H.; Khare, S. D.; Bolduc, J. M.; Forouhar, F.; Mulligan, V. K.; Lew, S.; Seetharaman, J.; Tong, L.; Stoddard, B. L.; Baker, D. *J. Am. Chem. Soc.* **2013**, *135*, 13393. (f) Zastrow, M. L.; Peacock, A. F. A.; Stuckey, J. A.; Pecoraro, V. L. *Nat. Chem.* **2012**, *4*, 118. (g) Lu, Y.; Yeung, N.; Sieracki, N.; Marshall, N. M. *Nature* **2009**, *460*, 855.
- (4) (a) Zimbron, J. M.; Heinisch, T.; Schmid, M.; Hamels, D.; Nogueira, E. S.; Schirmer, T.; Ward, T. R. *J. Am. Chem. Soc.* **2013**, *135*, 5384. (b) Hyster, T. K.; Knörr, L.; Ward, T. R.; Rovis, T. *Science* **2012**, *338*, 500. (c) Ward, T. R. *Acc. Chem. Res.* **2011**, *44*, 47.
- (5) For other examples see: (a) Chevalley, A.; Cherrier, M. V.; Fontecilla-Camps, J. C.; Ghasemi, M.; Salmain. *Dalton Trans.* **2014**, 43, 5482. (b) Esmieu, C.; Cherrier, M. V.; Amara, P.; Girgenti, E.; Marchi-Delapierre, C.; Odon, F.; Iannello, M.; Jorge-Robin, A.; Cavazza, C.; Ménage, S. *Angew. Chem., Int. Ed.* **2013**, *52*, 3922. (c) Monnard, F. W.; Nogueira, E. S.; Heinisch, T.; Schirmer, T.; Ward, T. R. *Chem. Sci.* **2013**, *4*, 3269. (d) Reetz, M. T.; Jiao, N. *Angew. Chem., Int. Ed.* **2006**, *45*, 2416. (e) Mahammed, A.; Gross, Z. *J. Am. Chem. Soc.* **2005**, *127*, 2883. (f) Ohashi, M.; Koshiyama, T.; Ueno, T.; Yanase, M.; Fujii, H.; Watanabe, Y. *Angew. Chem., Int. Ed.* **2003**, *42*, 1005.
- (6) Agustindari, H.; Lubelski, J.; Bart den Berg van Saparoea, H.; Kuipers, O. P.; Driessen, A. J. M. *J. Bacteriol.* **2008**, *190*, 759.
- (7) (a) Madoori, P. K.; Agustindari, H.; Driessen, A. J. M.; Thunnissen, A. -M. W. H. *EMBO J.* **2009**, *28*, 156. (b) Takeuchi, K.; Tokunaga, Y.; Imai, M.; Takahashi, H.; Shimada, I. *Sci. Rep.* **2014**, *4*, 6922.

(8) (a) Drienovská, I.; Rioz-Martínez, A.; Draksharapu, A.; Roelfes, G. *Chem. Sci.* **2015**, *6*, 770. (b) Bos, J.; García-Herraiz, A.; Roelfes, G. *Chem. Sci.* **2013**, *4*, 3578. (c) Bos, J.; Fusetti, F.; Driessen, A. J. M.; Roelfes, G. *Angew. Chem., Int. Ed.* **2012**, *51*, 7472.

(9) (a) Boersma, A. J.; Feringa, B. L.; Roelfes, G. *Angew. Chem., Int. Ed.* **2009**, *48*, 3346. (b) Evans, D. A.; Fandrick, K. R.; Song, H.; Scheidt, K. A.; Xu, R. *J. Am. Chem. Soc.* **2007**, *129*, 10029.

(10) The binding constant of indole **2a** to LmrR_{LM} could not be established due to spectral overlap.

(11) Due to the strong dependence on the indole (**2**) concentration, relatively large errors were sometimes observed in the conversions.

(12) Draksharapu, A.; Boersma, A. J.; Leising, M.; Meetsma, A.; Browne, W. R.; Roelfes, G. *Dalton Trans.* **2015**, *44*, 3647.