

Published in final edited form as:

Bioorg Med Chem Lett. 2013 February 15; 23(4): 1001–1003. doi:10.1016/j.bmcl.2012.12.038.

Specific chlorination of isoquinolines by a fungal flavin-dependent halogenase

Jia Zeng^a, Anna K. Lytle^b, David Gage^a, Sean J. Johnson^b, and Jixun Zhan^{a,*}

^aDepartment of Biological Engineering, Utah State University, 4105 Old Main Hill, Logan, UT 84322, United States

^bDepartment of Chemistry and Biochemistry, Utah State University, 0300 Old Main Hill, Logan, UT 84322, United States

Abstract

Rdc2 is the first flavin-dependent halogenase identified from fungi. Based on the reported structure of the bacterial halogenase CmlS, we have built a homology model for Rdc2. The model suggests an open substrate binding site that is capable of binding the natural substrate, monocillin II, and possibly other molecules such as 4-hydroxyisoquinoline (**1**) and 6-hydroxyisoquinoline (**2**). *In vitro* and *in vivo* halogenation experiments confirmed that **1** and **2** can be halogenated at the position *ortho* to the hydroxyl group, leading to the synthesis of the chlorinated isoquinolines **1a** and **2a**, respectively, which further expands the spectrum of identified substrates of Rdc2. This work revealed that Rdc2 is a useful biocatalyst for the synthesis of various halogenated compounds.

Keywords

Fungal halogenase; Flavin-dependent; Homology model; Isoquinolines; Substrate specificity

Halogenated molecules are an important group of natural products because of their significant biological activities. Up to now, more than 4,500 halogenated compounds have been isolated from nature,^{1, 2} such as rebeccamycin, chloramphenicol and vancomycin. Recently, biological halogenation has drawn more and more attention as a highly selective and efficient substitute for classical organic chemistry approaches.^{3, 4} Consequently, discovery of novel halogenases with broad substrate specificity from natural product biosynthetic pathways is critical. Our group has recently discovered a promising halogenase Rdc2 that is involved in radicicol biosynthesis in *Pochonia chlamydosporia*.⁵ This enzyme specifically chlorinates at C-6 of monocillins such as monocillin II, a biosynthetic precursor of radicicol, as shown in Fig. 1. We have found that this enzyme can also work on other similar macrolactones such as dihydroresorcylide. This enzyme can catalyze both mono- and dichlorination. Additionally, it also accepts bromide as a halogen donor to conduct both mono- and dibromination.⁵ Taken these unique promising properties together, this enzyme has shown great potential as a powerful halogenating biocatalyst. Thus, we decided to further study this enzyme to understand its structure and test its applicability to other types

© 2012 Elsevier Ltd. All rights reserved.

*Corresponding author. Tel.: +1-435-797-8774; fax: +1-435-797-1248; jixun.zhan@usu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of substrates. In this work, we report a homology model of Rdc2 for substrate docking and chlorination of two isoquinolines by this enzyme.

Several structures of bacterial flavin-dependent halogenases have been reported, such as PrnA (AAB97504),⁶ RebH (CAC93722)⁷ and CmlS (AAK08979).⁸ Both PrnA and RebH are tryptophan 7-halogenase, involved in the biosynthesis of pyrrolnitrin and rebeccamycin, respectively, while CmlS is characterized from the biosynthetic pathway of chloramphenicol.

We constructed a homology model of Rdc2 using the LOMETS server.⁹ The highest scored solution was based on the crystal structure of CmlS (pdb ID 3i3l), using MUSTER¹⁰ to perform the threading alignment and MODELLER¹¹ to build the final structure. The model contains 505 residues, which corresponds to 94.7% of the Rdc2 sequence. The final model contains an N-terminal flavin monooxygenase domain and a C-terminal “winged-helix” domain (Fig. 2A). Additional models produced by the server vary in the C-terminal domain but show strong agreement in the N-terminal domain.

The putative substrate binding site is located in a hydrophobic region of the N-terminal domain adjacent to the strictly conserved K74. The Rdc2 substrate monocillin II was modeled into the structure using a superimposed PrnA structure bound to 7-chlorotryptophan (PDB ID 2AR8) as a guide (Fig. 2B). The model suggests an open binding site that may accommodate a variety of compounds, raising the possibility that Rdc2 may be able to utilize various substrates.

To further expand the substrate spectrum and the application of Rdc2, we tested the ability of Rdc2 to chlorinate isoquinoline substrates. Isoquinoline is a common structural component of many bioactive natural products, such as renieramycin M from *Xestospongia* sp. and jorunnamycin C from *Jorunnafunebris*, both having promising anticancer activity with IC₅₀ values in the nanomolar range.¹² In this paper, we chose two typical isoquinolines, 4-hydroxyisoquinoline (**1**) and 6-hydroxyisoquinoline (**2**), to test whether Rdc2 could chlorinate this important type of molecule. As shown in Fig. 2C and 2D, both compounds can fit into the active site of Rdc2 and thus represent potential substrates for this enzyme.

We then conducted *in vitro* enzymatic assays to examine whether they can really be chlorinated by Rdc2. Rdc2 and the partner flavin reductase Fre from *Escherichia coli* were expressed and purified as previously reported.⁵ As shown in Fig. 3, LC-MS analysis revealed that both reactions¹³ yielded a product peak with lower polarity. ESI-MS revealed that both **1a** and **2a** had two [M+H]⁺ ion peaks with a 3:1 ratio in the positive mode (data not shown), which is a characteristic of monochlorinated molecules. **1** and **2** are isomers with a molecular weight of 145, while the molecular weight of both **1a** and **2a** is 179, which is 34 Da greater than the substrates, indicating that they are the chlorinated products.

In order to obtain sufficient amounts of **1a** and **2a** for structure elucidation to characterize the chlorination positions, we used an *in vivo* biocatalytic approach. A total of 30 mg of **1** and **2** were separately fed into the isopropyl β-D-1-thiogalactopyranoside (IPTG) induced fermentation broth of *E. coli* BL21-CodonPlus (DE3)-RIL/pJZ54 to yield 8.5 mg of **1a** and 11.4 mg of **2a**, respectively.¹⁴

The purified products were dissolved in methanol-*d*₄ and the NMR spectra were acquired on a JEOL instrument (300 MHz). The ¹H NMR spectrum of **1a** showed two doublet and two triplet signals that belong to H-5, H-6, H-7 and H-8, indicating that the halogenation has not occurred on the benzene ring. In contrast, only one singlet was observed for the pyridine ring, suggesting that either C-1 or C-3 is halogenated. The chemical shift of this signal is δ 8.64, which belongs to H-1. Thus, we can deduce that the halogenation occurred at C-3

position, which is *ortho* to the hydroxyl group. Thus, **1a** was identified as 3-chloro-4-hydroxyisoquinoline.

To determine the chlorination position in **2a**, both ^1H and ^1H - ^1H COSY spectra were recorded. As expected, there were only five proton signals in the ^1H NMR spectrum, confirming that one proton has been substituted by Cl. The ^1H - ^1H COSY correlations of H-3 to H-4 as well as H-7 to H-8 clearly revealed that these protons are not chlorinated. Meanwhile, the signal of H-1 can be easily located in the low field at δ 9.53, indicating that C-5 of **2** has been chlorinated, which is also *ortho* to the hydroxyl group. Therefore, **2a** was characterized as 5-chloro-6-hydroxyisoquinoline. The ^1H NMR signals of both products are shown in Table 1.

Almost all the previously characterized flavin-dependent halogenases are highly substrate specific, such as those reported tryptophan halogenases.^{15–18} In order to develop a useful halogenating biocatalyst, an enzyme with broad substrate specificity is needed. Rdc2 is a halogenase different from those previously reported. It is a late tailoring enzyme in radicicol biosynthesis and can halogenate other macrolactones besides its natural substrates monocillins.⁵ In this work, we report a homology model of the first identified fungal flavin-dependent halogenase Rdc2, which predicts that this enzyme can accommodate various substrates. We demonstrated that Rdc2 can function on another important group of molecule, isoquinolines. This is the first report of enzymatic synthesis of chlorinated isoquinolines. Structural characterization of **1a** and **2a** indicated that the chlorine atom has been introduced at the position *ortho* to the hydroxyl group. A hydroxyl group is a strongly activating substituent. The resonance effect of the hydroxyl group directs the electron toward the ring and may contribute to the chlorination of the *ortho* position. Further investigation of the Xray structure of Rdc2 will reveal more information about the catalytic mechanism of this enzyme, which will allow us to use the structure model to find more potential substrates. Moreover, this research also demonstrated a convenient and effective way to prepare halogenated molecules with Rdc2. *In vitro* enzymatic reaction allows a quick screening of the substrates and initial analysis of the products, while *in vivo* biocatalysis through engineered *E. coli* provides an economic way to obtain sufficient amounts of halogenated products on a large scale for structure elucidation and bioactivity studies.

Acknowledgments

This work was partly supported by a Utah Science Technology and Research (USTAR) Veterinary Diagnostics and Infectious Disease Seed Project and a National Institutes of Health grant (AI065357). We thank Dr. Chad Testa at Frontier Scientific, Inc. for providing the substrates.

References and notes

1. Gribble GW. J. Chem. Educ. 2004; 81:1441.
2. Vaillancourt FH, Yeh E, Vosburg DA, Garneau-Tsodikova S, Walsh CT. Chem. Rev. 2006; 106:3364. [PubMed: 16895332]
3. Wagner C, El Omari M, König GM. J. Nat. Prod. 2009; 72:540. [PubMed: 19245259]
4. Zeng J, Valiente J, Zhan J. Nat. Prod. Commun. 2011; 6:223. [PubMed: 21425679]
5. Zeng J, Zhan J. ChemBioChem. 2011; 11:2119. [PubMed: 20827793]
6. Dong C, Flecks S, Unversucht S, Haupt C, van Pée K-H, Naismith JH. Science. 2005; 309:2216. [PubMed: 16195462]
7. Bitto E, Huang Y, Bingman CA, Singh S, Thorson JS, Phillips GN Jr. Proteins: Struct. Funct. Bioinf. 2007; 70:289.
8. Podzelinska K, Latimer R, Bhattacharya A, Vining LC, Zechel DL, Jia Z. J. Mol. Biol. 2010; 397:316. [PubMed: 20080101]

9. Wu S, Zhang Y. *Nucleic Acids Res.* 2007; 35:3375. [PubMed: 17478507]
10. Wu S, Zhang Y. *Proteins: Struct. Funct. Bioinf.* 2008; 72:547.
11. Sali A, Blundell TL. *J. Mol. Biol.* 1993; 234:779. [PubMed: 8254673]
12. Charupant K, Suwanborirux K, Daikuhara N, Yokoya M, Ushijima-Sugano R, Kawai T, Owa T, Saito N. *Mar. Drugs.* 2009; 7:483. [PubMed: 20098592]
13. A 100- μ L halogenation assay consisted of 100 μ M FAD, 10 mM NADH, 0.1 mM **1** or **2**, 10 mM NaCl, 16 μ M Fre, and 16 μ M Rdc2 in 100 mM phosphate buffer (pH 7.0). The reaction mixtures were placed at 30°C for 5 h and then quenched by addition of 50 μ L of methanol. The mixtures were then subjected to analysis on an Agilent 6130 LC-MS instrument using a Zorbax SB-C18 (5 μ m, 4.6 \times 150 mm). A gradient of acetonitrile/H₂O system (10–90% over 25 min) containing 0.1% trifluoroacetic acid (TFA) was programmed for the analysis.
14. 30 mg of **1** was fed into the IPTG induced fermentation broth of *E. coli* BL21-CodonPlus (DE3)-RIL/pJZ54 that expresses Rdc2. The culture was maintained at 28°C with shaking at 250 rpm for 36 h. The ethyl acetate extract of the broth was fractionated on a Sephadex LH-20 (20 g) column eluted with methanol to give 14 fractions, 5 mL each. Fractions 3–6 were combined and further separated by reverse-phase HPLC (Eclipse XDB-C18 column, 5 μ m, 4.6 \times 150 mm) with isocratic elution of 25% acetonitrile in H₂O (each containing 0.1% TFA) for 20 min at a flow rate of 1 mL/min to yield 8.5 mg of **1a**. Similarly, 30 mg of **2** was also incubated with *E. coli* BL21-CodonPlus (DE3)-RIL/pJZ54 under the same conditions. The ethyl acetate extract was fractionated on a Diaion HP-20 (30 g) column eluted with a stepwise gradient of isopropanol-water (0:100, 20:80, 40:60, 60:40, 80:20, 100:0, each 250 mL) to give 6 fractions. Further separation of fraction 3 by reverse-phase HPLC with isocratic elution of 10% acetonitrile in H₂O (each containing 0.1% TFA) over 20 min at a flow rate of 1 mL/min afforded 11.4 mg of **2a** in pure form.
15. Glenn WS, Nims E, O'Connor SE. *J. Am. Chem. Soc.* 2011; 133:19346. [PubMed: 22050348]
16. Jiang W, Heemstra JR Jr, Forseth RR, Neumann CS, Manaviazar S, Schroeder FC, Hale KJ, Walsh CT. *Biochemistry.* 2011; 50:6063. [PubMed: 21648411]
17. van Pée K-H. *ChemBioChem.* 2011; 12:681. [PubMed: 21344584]
18. Zeng J, Zhan J. *Biotechnol. Lett.* 2011; 33:1607. [PubMed: 21424165]

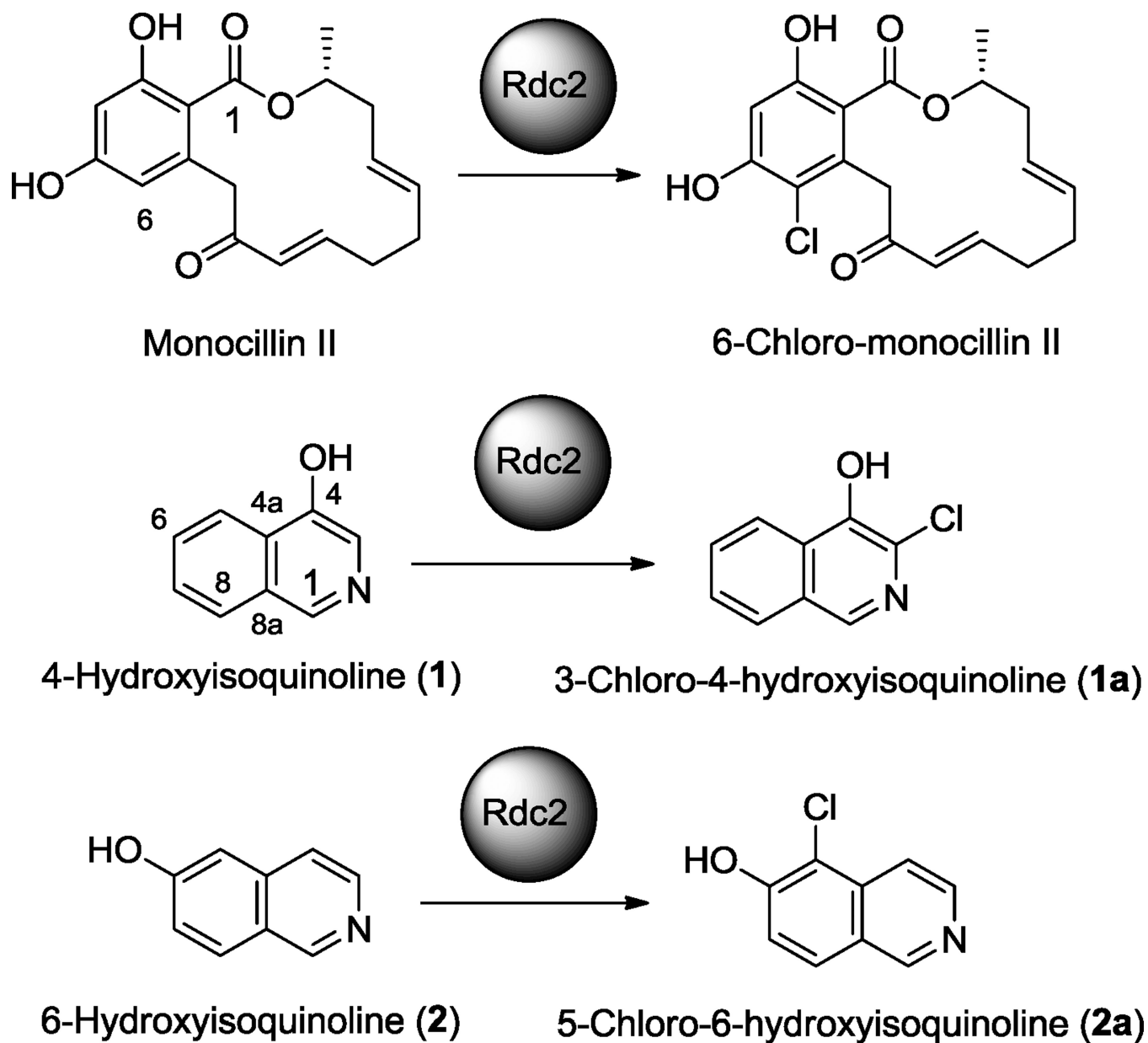


Figure 1.
Chlorination of **1** and **2** by Rdc2.

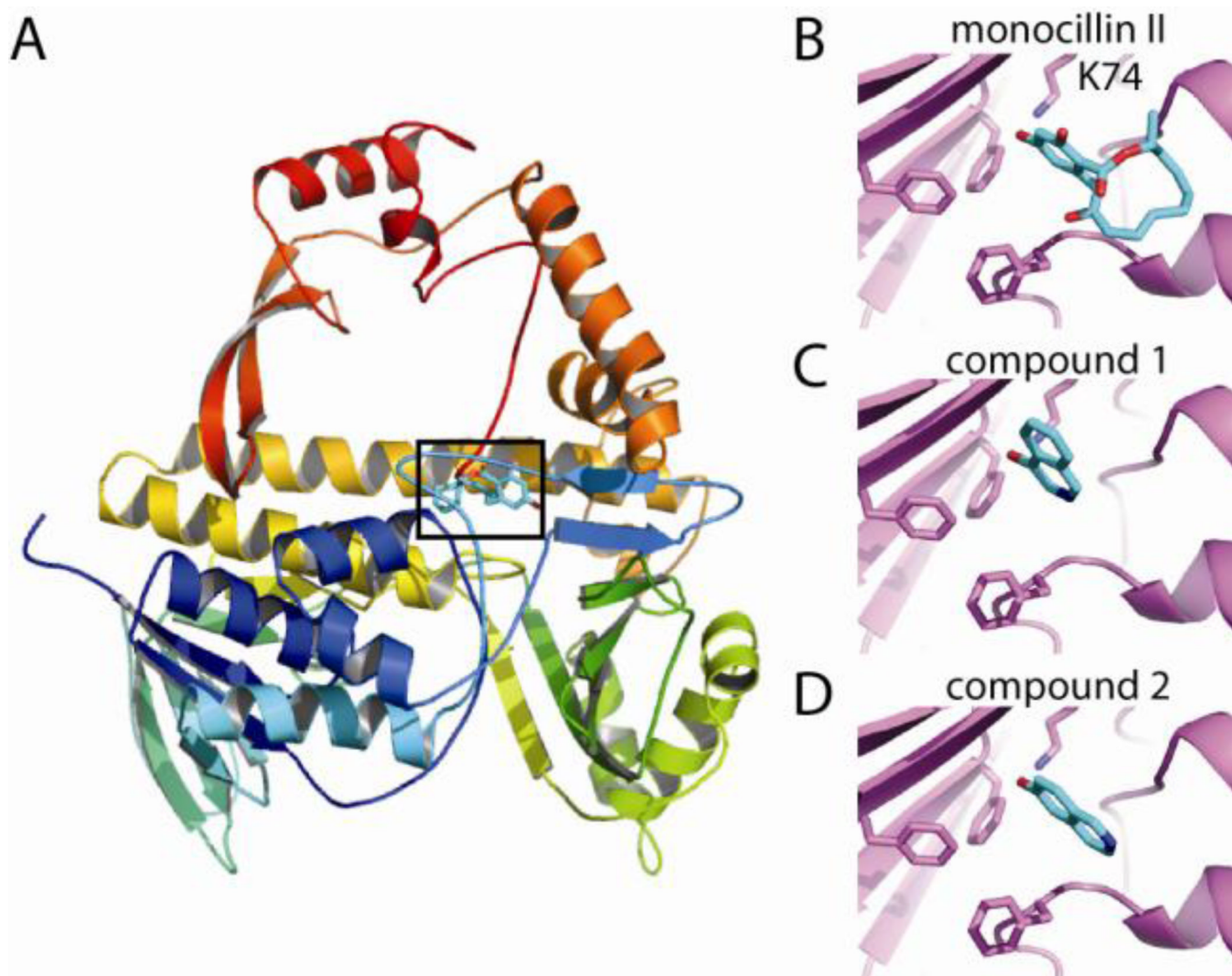


Figure 2. Homology model of Rdc2. (A) Overview of model colored from N (blue) to C terminus (red). The box indicates the putative active site. Modeling of monocillin II and two unnatural substrates **1** and **2** into the active site region are indicated in B–D, respectively.

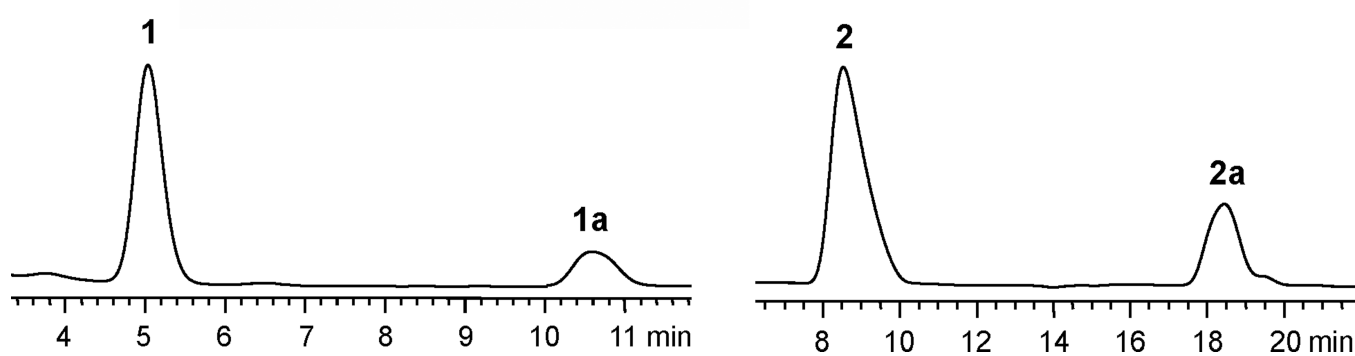


Figure 3.
HPLC analysis of the *in vitro* chlorination of **1** (left) and **2** (right) by Rdc2.

Table 1¹H NMR data for **1a** and **2a** (CD₃OD, 300 MHz, *J* in Hz)

| Position | 1a | 2a |
|----------|------------------------------------|------------------------------|
| 1 | 8.64 (1H, s) | 9.53 (1H, s) |
| 3 | | 8.51 (1H, d, <i>J</i> = 6.9) |
| 4 | | 8.47 (1H, d, <i>J</i> = 6.9) |
| 5 | 8.26 (1H, d, <i>J</i> = 8.3) | |
| 6 | 7.79 (1H, dd, <i>J</i> = 8.3, 6.9) | |
| 7 | 7.68 (1H, dd, <i>J</i> = 8.3, 6.9) | 7.68 (1H, d, <i>J</i> = 9.0) |
| 8 | 8.06 (1H, d, <i>J</i> = 8.3) | 8.34 (1H, d, <i>J</i> = 9.0) |