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Promiscuity of an unrelated anthrol reductase of *Talaromyces islandicus* WF-38-12†

Shailesh Kumar Singh, ‡[ab] Anshul Rajput, ‡[a] Arijit De,[a] Tapati Chakraborti[b] and Syed Masood Husain *[a]

An anthrol reductase of *Talaromyces islandicus* WF-38-12 (ARti-2) from an unrelated biosynthetic gene cluster (BGC) has been identified and characterized. It catalyses the NADPH-dependent reduction of anthrols (hydroanthraquinones), estrone and a naphthol with high stereo- and regioselectivity. The role of ARti-2, the *CRG89872.1* gene of the same BGC and non-enzymatic oxidation in the biosynthesis of (−)-flavoskyrin has been proposed.

The superfamily of short-chain dehydrogenases/reductases (SDRs) consists of enzymes with diverse functions and uses NAD(P)(H) as a cofactor to catalyse oxidation/reduction reactions in a highly stereo- and regioselective manner.[1] Among these, naphthol and anthrol reductases (NRs & ARs) can catalyse the NADPH-dependent reduction of polyhydroxy aromatics in general.[2] Phylogenetic studies reveal that both have emerged from keto reductases such as glucose dehydrogenase (GDH) & alcohol dehydrogenase (ADH), and catalyse the reduction of ketones (**1**) to secondary alcohols (**2**)[3] (Fig. 1). As a physiological function, NRs such as the 1,3,6,8-tetrahydroxy naphthalene reductase (T_4HNR) & 1,6,8-trihydroxy naphthalene reductase (T_3HNR) of *Magnaporthe grisea* catalyse the NADPH-dependent reduction of naphthols such as T_3HN (**3**) & T_4HN (**4**) to (*R*)-vermelone (**5**) and (*R*)-scytalone (**6**), respectively (Fig. 1).[4] Likewise, ARs reduce an unknown tautomer of an anthrol (**7**) formed *in situ* by the reduction of emodin (**9**) in the presence of $Na_2S_2O_4$ to give a putative biosynthetic intermediate, (*R*)-3,8,9,10-tetrahydroxy-6-methyl-3,4-dihydroanthracen-1(2*H*)-one (**8**) (Fig. 1).[2,5,6] ARs, which have been identified only recently, include MdpC of *Aspergillus nidulans*,[5] AflM of *A. parasiticus*,[7] 17β-HSD of *Curvularia lunata*,[6] and ARti of *Talaromyces islandicus* (previously *Penicillium islandicum*).[2] In a short span of time, the ARs have been identified for their role in the biosynthesis of chrysophanol (**11**), cladofulvin, monodictyphenones and xanthone derivatives.[8–10]

Furthermore, ARs might have a crucial role in the biosynthesis of dimeric bisanthraquinoids[11] such as (−)-flavoskyrin (**30**), (−)-rugulosin (**31**) and related metabolites isolated from *P. islandicum*.[12] Hence, apart from the recently identified ARti, we expect the presence of more such enzyme(s) which might be involved in the biosynthesis of **30**, **31** & related natural products.

The pairwise sequence alignment of 17β-HSDcl (GenBank *AAD12052.1*) against the *T. islandicus* (taxid: 28573) genome yielded two significant hits: *CRG89873.1* (83.71% identity, 6×10^{-167} *e*-value) and *CRG86682.1* (79.55% identity, 4×10^{-156} *e*-value) but only the latter one is characterized as an anthrol

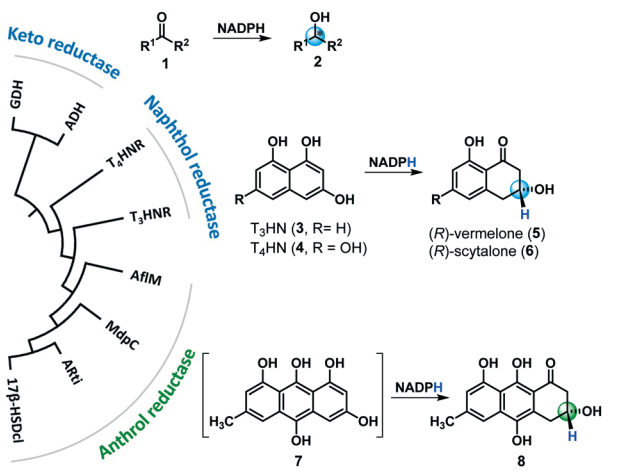

Fig. 1 Phylogenetic tree depicting the functional diversity of NAD(P)-dependent oxidoreductases.

[a] *Molecular Synthesis and Drug Discovery Unit, Centre of Biomedical Research, SGPGIMS Campus, Raebareli Road, Lucknow-226014, India. E-mail: smhusain@cbmr.res.in, smhusain.cbmr@gmail.com*

[b] *Department of Biochemistry and Biophysics, University of Kalyani, Nadia-741235, West Bengal, India*

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‡ These authors contributed equally to this work.

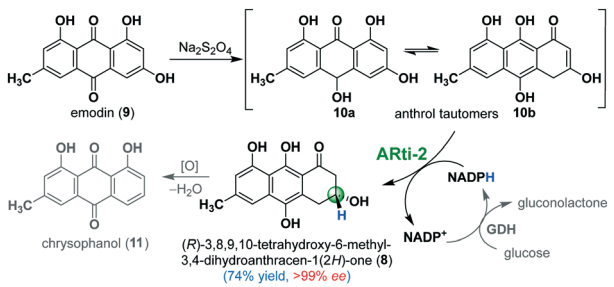
reductase (ARti) based on its conserved domains and biosynthetic gene cluster (BGC) analysis.[2] All other alignments were less significant due to low sequence identity (<51%). A conserved domain search (CDD v3.18) of *CRG89873.1* at NCBI predicts it as a classical SDR with a characteristic T_3HN/T_4HN reductase domain belonging to naphthol/anthrol reductases. More specifically *CRG89873.1* is proposed as an anthrol reductase (ARti-2) as inferred from the phylogenetic tree (see the ESI,† Fig. S2). Also, its multiple sequence alignment with known ARs (MdpC, AflM, 17β-HSDcl & ARti) shows a common NAD(P)-binding site as well as a conserved YXXXK active site motif (Fig. S1†). Serine (S-122) & asparagine (N-148) are also present upstream of tyrosine (Y-162) & lysine (K-166) residues forming a catalytic tetrad.[13] Further, fungiSmash analysis of the *T. islandicus* genome predicts the corresponding gene of ARti-2 as part of a putative cluster of unknown type (see the ESI,† Table S1).[14] However, ARti-2 doesn't belong to any known BGC, and in turn, we failed to deduce its physiological functions based on the location. In contrast, all the previously characterized ARs: ARti, MdpC, AflM and 17β-HSDcl from different fungi were predicted to be part of a BGC that might be involved in the synthesis and modifications of anthraquinones like emodin (**9**) to chrysophanol (**11**).[2,5–7] Hence, the existence of a second AR in *T. islandicus* at an unrelated locus raises questions about its catalytic function. Altogether bioinformatic analysis suggested us to screen the catalytic activity of ARti-2 *in vitro* starting with the substrates accepted by 17β-HSDcl, T_4HNR and/or T_3HNR.

The codon-optimized gene of ARti-2 cloned into the pET-19b vector was overexpressed in *E. coli* BL21(DE3) and the protein was purified using Ni–NTA affinity chromatography (see the ESI,† page S6). The catalytic activity of the purified ARti-2 was tested using emodin (**9**) as a substrate. For this, **9** was incubated with ARti-2 in the presence of $Na_2S_2O_4$ & NADPH [generated using $NADP^+$/glucose/glucose dehydrogenase (GDH)] in 50 mM KPi buffer (pH 7.0, 1 mM EDTA, 1 mM DTT) under anoxic conditions for 14 h (Scheme 1). $Na_2S_2O_4$ was used for the reduction of **9** to a tautomeric mixture of emodin hydroquinone **10a**/**10b** *in situ*, one of which acts as a substrate for the anthrol reductases.[2] This resulted in the formation of (*R*)-3,8,9,10-tetrahydroxy-6-methyl-3,4-dihydroanthracen-1(2*H*)-one (**8**) obtained in 74% yield. Herein, the absolute configuration was assigned using CD spectroscopy. Chiral HPLC confirms the enantiopurity of (*R*)-**8** to be >99% ee (see the ESI,† pages S10 and S21).[15]

Along with **8**, we also obtained chrysophanol (**11**) as a side product, which is known to be formed by non-enzymatic oxidation, followed by dehydration.[5] We also performed the above transformation by taking NADH as a cofactor. However, no conversion to the product was observed indicating NADPH to be the accepted cofactor for ARti-2. Also, no product was obtained when the transformation was performed in the absence of $Na_2S_2O_4$, indicating that ARti-2 does not accept emodin (**9**) as a direct substrate.

Next, we sought to determine the thermal stability & optimum pH of ARti-2. For this, the melting temperature (T_m) was determined by recording the ellipticity of ARti-2 at 222 nm as a function of temperature (20–85 °C) and was found to be 43 °C (Fig. 2a). Furthermore, the ellipticity *versus* temperature plot gave us the idea to choose the optimum range of temperature for enzymatic transformations, *i.e.*, 20–30 °C at which ARti-2 remains in native conformation. Also, the optimal pH of ARti-2 was determined by performing the chemoenzymatic reduction of **9** at pH ranging from 5.5 to 8.0. Emodin was incubated with ARti-2 and NADPH (generated using $NADP^+$/glucose/GDH) in buffer of different pH values for 5 h under anoxic conditions and the conversion to product **8** was determined by analysing ^1H NMR in acetone-d_6 of the crude reaction mixture. It showed maximum conversions of 93% at pH 6.5 and 90% at pH 7.0 (Fig. 2b). However, considering the little difference in the conversions, we performed most of the transformation using potassium phosphate buffer of pH 7.0.

We planned to investigate the kinetic profile of ARti-2 using **10a**/**10b** as a substrate which is formed *in situ* by the reduction of emodin (**9**) in the presence of $Na_2S_2O_4$ under anoxic conditions. However, maintaining such anoxic conditions to prevent oxidation of **10a**/**10b** back to emodin (**9**) during kinetic measurements remained a challenge. In addition, the spectral overlap of the substrate, cofactor and the product using the UV-vis spectrophotometric method prevented us from determining the kinetic parameters for ARti & other related enzymes. That is why thus far no report has been published that investigates the kinetic profile of enzymes catalysing the reduction of anthrols. Therefore, we



Scheme 1 Chemoenzymatic asymmetric reductive dearomatization of emodin (**9**). ARti-2 catalyses the NADPH-dependent reduction of the tautomer of emodin hydroquinone **10a**/**10b** to **8**.

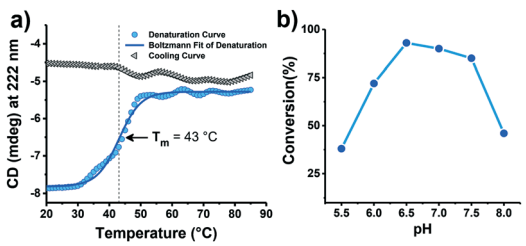


Fig. 2 a) Denaturation curve of ARti-2. b) pH profile of ARti-2 determined from chemoenzymatic reduction of emodin (**9**) in buffer of different pH values.

used the reverse-phase HPLC method to analyse the ARti & ARti-2-catalysed NADPH-dependent reduction of anthrols **10a**/**10b** to (*R*)-**8**. At first, we varied the substrate concentration and analysed the amount of (*R*)-**8** formed to measure the velocity. K_M and V_{max} (= k_{cat}·[E_T]) were determined by plotting the reaction velocity *versus* substrate concentration that is fitted to the Michaelis–Menten equation. The catalytic efficiency ($\eta_{cat} = k_{cat}/K_M$) of ARti-2 was found to be 4.01 × 10^3 s^{-1} M^{-1} which is higher than that of ARti (η_{cat} = 3.56 × 10^3 s^{-1} M^{-1}) when **9** was employed as a substrate in the reaction (Fig. 3). This is the first time that the impression of the kinetics with any of the ARs for anthrol reduction has been obtained, which provides significant catalytic insights into such biotransformation.

To further validate the anthrol reductase activity of ARti-2, we also used lunatin (**12**) and citreorosein (**15**) as substrates. For this, anthraquinones **12** or **15** were incubated with ARti-2 in the presence of $Na_2S_2O_4$ and NADPH, which resulted in the formation of the corresponding dihydroanthracenones **14** and **17** in 62% and 72% yields, respectively (Fig. 4a).

Again, no product was obtained when the transformations were performed without $Na_2S_2O_4$, ruling out anthraquinones **12** and **15** to be direct substrates for ARti-2 (Fig. 4a). To determine the kinetic profile of ARti-2-catalysed reduction of hydroquinones lunatin (**13a**/**13b**) and citreorosein (**16a**/**16b**) as substrates, reactions with varying anthraquinone (**12** or **15**) concentrations were performed in a closed system and analysed using HPLC to determine the reaction velocity. Kinetic parameters were calculated from the Michaelis–Menten plot of substrate concentration *versus* reaction velocity (see the ESI,† page S15). The catalytic efficiencies (K_{cat}/K_M) of ARti-2-catalysed reduction with **13** and **16** were found to be 6.81 × 10^2 s^{-1} M^{-1} and 3.95 × 10^3 s^{-1} M^{-1}, respectively (Fig. 4b and c).

In addition, we tested compounds **4**, **18**, and **20**–**23** as substrates with ARti-2, which were reduced by either T_4HNR or 17β-HSDcl using NADPH (Fig. 5). HPLC analysis shows that ARti-2 could reduce 1,3,6,8-tetrahydroxynaphthalene (**4**) to (*R*)-scytalone (**6**) & estrone (**18**) to 17β-estradiol (**19**) in a highly stereo- and regioselective manner (Fig. 5a). This shows the ability of ARti-2 to reduce naphthol and a keto group of estrone (**18**).

However, unlike T_4HNR, ARti-2 could not catalyse the reduction of lawsone (**20**), 2-hydroxyjuglone (**21**), 3-hydroxyjuglone (**22**) & 2-tetralone (**23**).[16,17] ARti-2 also failed

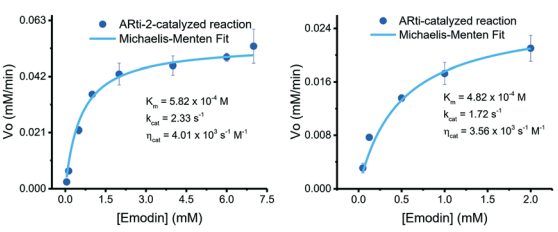


Fig. 3 Kinetic profiles of ARti-2 & ARti-catalysed reduction of emodin (**9**) in the presence of $Na_2S_2O_4$.

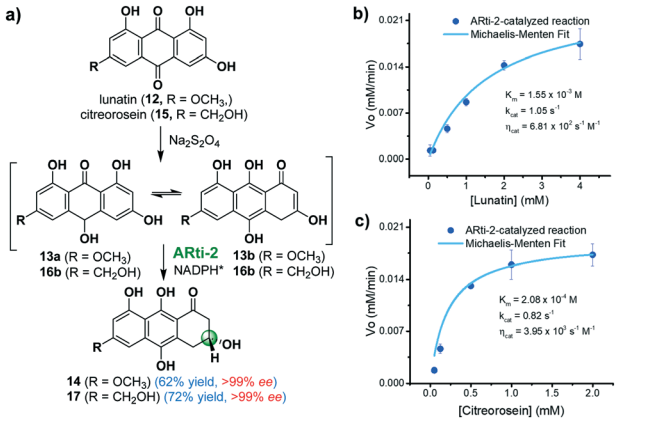


Fig. 4 a) Chemoenzymatic reduction of lunatin (**12**) and citreorosein (**15**) in the presence of sodium dithionite, ARti-2 & NADPH. b and c) Kinetic profiles of ARti-2-catalysed reduction of **13** & **16**.

to reduce simple cyclic ketone **24** or 2-methyl-2-cyclohexen-1-one (**25**). Likewise, emodin anthrone **26** was also not reduced by ARti-2. Moreover, plausible reduction of anthrol tautomer **10b** by ARti-2 hints towards its ene reductase activity (Scheme 1). However, neither **25** nor menadione (**27**), both of which contain an alkene bond, was reduced by ARti-2. Altogether, substrate screening suggests that ARti-2 specifically reduces anthrols but shows little promiscuity towards other substrates such as estrone & T_4HN.

Finally, characterization of ARti-2 as an AR & its presence in an unrelated BGC prompted us to explore its relevance to the biogenesis of dimeric (−)-flavoskyrin (**30**) and (−)-rugulosin (**31**), which have been isolated from fungus *P. islandicum* in particular.[18] In support, we found that *T. islandicus* WF-38-12 also contains an atypical SDR (GenBank *CRG89872.1*) in the BGC containing ARti-2 (see the ESI,† Table S1), which shows 57% sequence identity (1 × 10^{-107} *e*-value) with AgnL4 (GenBank *A0A411PQN7.1*) of the agnestin BGC. The knockout experiments by Simpson and co-workers have revealed that the deletion of the AgnL4 gene accumulated **9** in *Paecilomyces variotii*.[9] Based on that, AgnL4 has been proposed to catalyse the reduction of **9** to **7** during

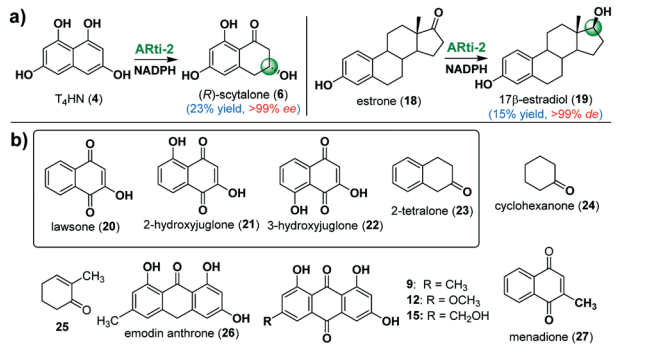


Fig. 5 a) Reduction of T_4HN (**4**) and estrone (**18**) by ARti-2 in the presence of NADPH showcases the catalytic promiscuity of ARti-2. b) Substrates not accepted by ARti-2.

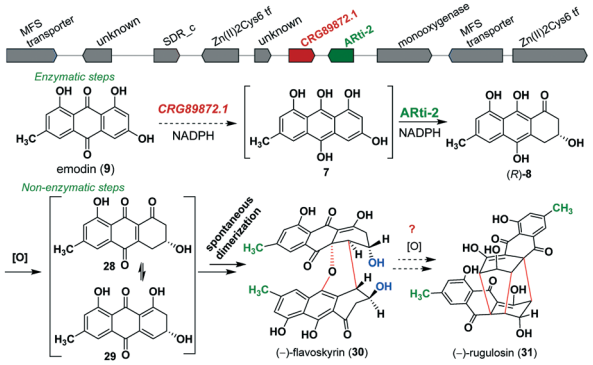


Fig. 6 Proposed role of ARti-2 in the biosynthesis of bisanthraquinoid metabolites isolated from *T. islandicus* (previously *P. islandicum*).

agnestin biosynthesis.[9] Although the catalytic functions of either AgnL4 or other related enzymes are not yet verified through *in vitro* experiments, we aim to identify the function of *CRG89872.1*. For this purpose, we tried to express *CRG89872.1* in *E. coli* BL21(DE3) from a codon-optimised gene cloned into the pET-19b vector. However, despite several attempts and applying different conditions, the *CRG89872.1* gene could not be expressed. Then, we tried to express its analogous proteins: AgnL4,[9] AflX (GenBank *Q6UEF2.1*),[19] and MdpK (GenBank *C8VQ62.1*)[20] in *E. coli*. However, we could not obtain the desired protein in any case & hence couldn't verify the *in vitro* role of *CRG89872.1*.

Nevertheless, we considered the role of *CRG89872.1* in the NAD(P)H-dependent reduction of **9** to its hydroquinone (**7**) based on earlier studies.[9] This is followed by the stereoselective reduction of **7** to a biosynthetic intermediate (*R*)-**8** catalysed by ARti-2 during the biosynthesis of bisanthraquinones **30** & **31** (Fig. 6). The absence of an oxidative coupling enzyme in the BGC containing ARti-2 & *CRG89872.1* hints at the role of non-enzymatic transformation in the formation of **30**. This is supported by the view that non-enzymatic, spontaneous transformations do play a significant role in natural product biosynthesis.[21] The same has been shown by us recently where (*R*)-**8** undergoes autoxidation in KPi buffer (pH 6.0) to form a putative biosynthetic intermediate, (*R*)-3,4-dihydroemodin (**28**).[22,23] **28** can also exist as a dienol tautomer (**29**) which spontaneously undergoes a cycloaddition reaction following an *exo*-anti arrangement to form (−)-flavoskyrin (**30**),[11,23] being in concurrence with its proposed biosynthesis.[12] Thus, the biosynthetic transformation of (*R*)-**8** into **30** might take place non-enzymatically (Fig. 6). Also, **30** has been identified as a biosynthetic intermediate for **31** based on feeding experiments.[12,24] This is supported by the cascade conversion of **30** to **31** reported by Shibata and co-workers in the presence of a base.[18] Therefore, we speculate that this step might also take place spontaneously under appropriate conditions (Fig. 6).

In summary, we have identified a new anthrol reductase (ARti-2) of *T. islandicus* WF-38-12 which catalyses the NADPH-dependent asymmetric reduction of anthrols, estrone (**18**) and a naphthol (**4**). For the first time, we could determine the kinetic parameters of anthrol reductases (ARti & ARti-2) using HPLC. Although the role of another associated gene, *CRG89872.1*, could not be verified through *in vitro* experiments, it might be crucial for the biosynthesis of (*R*)-**8**. Therefore, considering the isolation of bisanthraquinones, (−)-flavoskyrin (**30**) & (−)-rugulosin (**31**), we have speculated the role of *CRG89872.1*, ARti-2 and the non-enzymatic transformations in their biosynthesis. As no BGC is yet identified for these complex products, the current study may provide useful insight into the biogenesis of this class of dimeric natural products.

Conflicts of interest

There are no conflicts to declare.

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