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Identification and characterization of an anthrol reductase from *Talaromyces islandicus* (*Penicillium islandicum*) WF-38-12†

Shailesh Kumar Singh, ‡ Amit Mondal, ‡ Nirmal Saha and Syed Masood Husain *

An NADPH-dependent oxidoreductase from *Talaromyces islandicus* WF-38-12 has been identified through genome analysis. It has been shown to catalyze a regio- and stereoselective reduction of anthrols (formed *in situ* by the reduction of anthraquinones in the presence of $Na_2S_2O_4$) to (*R*)-dihydroanthracenones, with high enantiomeric excess (>99%). The implications of results on the biosynthesis of deoxygenated (bis)anthraquinones and modified (bis) anthraquinones are discussed.

The asymmetric reduction of tricyclic aromatic polyketides by fungal short-chain dehydrogenase/reductase (SDR) has gained significant attention in recent years, due to their role in the biosynthesis of monomeric and dimeric deoxyanthraquinones.[1–3] For example, emodin hydroquinone **2b** (formed *in situ* by the reduction of emodin (**1**) using $Na_2S_2O_4$) is reduced by MdpC from *Aspergillus nidulans* using NADPH to give a putative biosynthetic intermediate, (*R*)-3,8,9,10-tetrahydroxy-6-methyl-3,4-dihydroanthracene-1(2*H*)-one (**3**) (Fig. 1A).[1] This transformation is being recognized as a crucial step for the formation of a commonly isolated deoxyanthraquinone, chrysophanol (**4**), formed by dehydration and oxidation of **3**, during the biosynthesis of monodictyphenone (**5**),[1] agnestin A (**6**)[4] and cladofulvin (**7**)[5] (Fig. 1). As a result, several SDRs have been identified to possess anthrol reductase activity. They include 17β-HSD from *Cochliobolus lunatus*,[6] AflM from *Aspergillus paraticus*,[3] ClaC from *Cladosporium fulvum*,[5] and AgnL6 from *Paecilomyces variotii*,[4] which reduce emodin hydroquinone **2** to **3** in the same manner as that of MdpC (Fig. 1A). Being a key biosynthetic intermediate, **3** has been utilized in the biomimetic synthesis of a dimeric bisanthraquinone, (−)-flavoskyrin (**8**), which further supports the involvement of reduced anthrols like **3** in the biosynthesis of modified bisanthraquinones.[2] In yet another example, versicolorin A is being reduced, chemoenzymatically using AflM and NADPH in the presence of $Na_2S_2O_4$ to reduced versicolorin A hydroquinone **9**, which has been proposed as a putative biosynthetic intermediate for aflatoxin biosynthesis.[3]

Moreover, the isolation of related deoxyanthraquinones such as islandicin (**10**), aloe-emodin (**11**), and rhein (**12**); xanthone like ravenelin (**13**); and modified bisanthraquinones such as (+)-rugulosin (**14**), (+)-2,2′-*epi*-cytoskyrin A (**15**), (+)-rugulosin C (**16**), (−)-rubroskyrin (**17**), (−)-rugulosin (**18**),

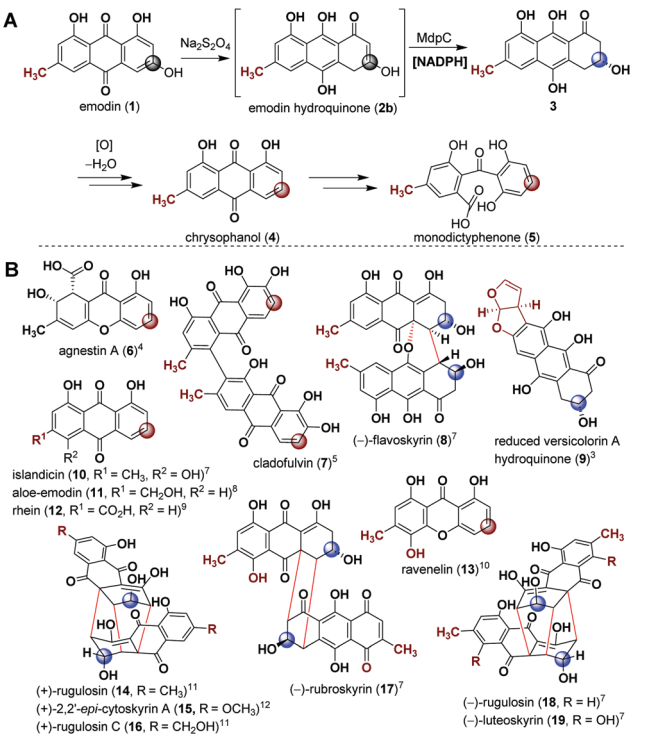

Fig. 1 (A) Chemoenzymatic reduction of emodin (**1**) to reduced emodin hydroquinone **3** by MdpC of *Aspergillus nidulans*.[1] (B) Secondary metabolites (**1**, **4**, **8**, **10**, **17–19**)[7] and (**6**, **7**, **11–19**)[4,5,8–12] have been isolated from *P. islandicum* and other fungi, respectively.

Centre of Biomedical Research, Sanjay Gandhi Postgraduate Institute of Medical Sciences Campus, Raebareli Road, Lucknow 226014, India.
E-mail: smhusain@cbmr.res.in, smhusain.cbmr@gmail.com

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‡These authors contributed equally.

and (−)-luteoskyrin (**19**) from different fungal species, implies for a broader substrate scope for anthrol reductases (Fig. 1B). Therefore, in this work, we aim to identify a new fungal anthrol reductase, which can catalyze the reduction of natural anthraquinones or their hydroquinones to give access to unknown, not yet isolated putative biosynthetic intermediates. This would have implications on the understanding of the biosynthesis of modified anthraquinones and bisanthraquinones.

Considering the isolation of secondary metabolites **1**, **4**, **8**, **10**, and **17–19**[7] from *Talaromyces islandicus* (formerly *Penicillium islandicum* Sopp), which appears to involve a regio- and stereoselective reduction of anthraquinones or their hydroquinones, we propose the existence of anthrol reductase (s) in *T. islandicus*. Such an enzyme might be directly involved in the biosynthesis of these natural products (Fig. 1B). To identify such an anthrol reductase(s) and its biosynthetic gene cluster (BGC), we analyzed the genomic sequence of the *T. islandicus* WF-38-12 genome, which has been published recently.[13] We searched for a BGC containing a putative oxidoreductase with high sequence homology to the known anthrol reductases MdpC, AflM, and 17β-HSDcl, all belonging to the short-chain dehydrogenase/reductase (SDR) superfamily. The fungiSMASH (fungal version of antiSMASH)[14] analysis of the *T. islandicus* genome predicted nineteen type 1 polyketide synthase gene clusters, out of which only one contains all the essential genes coding for proteins involved in the biosynthesis and biotransformation of anthraquinones (Fig. S2†). In Table 1, this cluster shows high similarity with other known BGCs such as monodictyphenone (*mdp*)[15] of *A. nidulans*, cladofulvin (*cla*)[5] of *C. fulvum*, and agnestin (*agn*)[4] of *P. divaricatus* (for more details see Table S1†).

CRG86674.1 (polyketide synthase) and CRG86680.1 (β-lactamase) show strong similarities to *mdpG*/*claG* and *mdpF*/*claF*, respectively which can synthesize atrochrysone. CRG86683.1 (decarboxylase or anthrone oxygenase) shows strong similarity with *mdpH*/*claH* that catalyzes the decarboxylation of atrochrysone to produce emodin (**1**). CRG86682.1 (SDR) and CRG86684.1 (dehydratase) show strong similarities with *mdpC*/*claC* and *mdpB*/*claB*, respectively. The former catalyzes the reduction of emodin (**1**) while the latter might facilitate the dehydration of the reduced form of emodin that is **2b** leading to the formation of chrysophanol (**3**).[5,15] The putative anthrol reductase from *T. islandicus* (named herein "ARti", accession CRG86682.1) shows 80% sequence identity with 17β-HSD from *C. lunatus*, 77% with MdpC from *A. nidulans*, and 66% with AflM from *A. parasiticus*. In addition, the alignment of protein sequences of the well-known naphthol reductases, 1,3,6,8-tetrahydroxynaphthalene reductase (T_4HNR) and 1,6,8-trihydroxynaphthalene reductase (T_3HNR) of *Magnaporthe grisea* involved in the dearomatization of naphthols during melanin biosynthesis[16,17] with ARti shows 53% and 65% sequence identity, respectively. The conserved domain analysis of the ARti with T_4HNR, T_3HNR, MdpC, AflM, and 17β-HSDcl shows a consensus of the NADP-binding site for all the enzymes (Fig. S1†). The comparison of ARti with 17β-HSDcl in Table 2, shows common amino acid residues for the substrate binding and active site.[18] The high sequence homology and putative domain architecture hint for a similar physiological function for ARti.

Thus, based on sequence identity, conserved domains, and position in the genome, we propose that the physiological function of ARti is to catalyze the NAD(P)H-dependent regio- and stereoselective reduction of naphthols and/or anthrols.[6,19] Therefore, we tested compounds that are known to be reduced by T_4HNR of *M. grisea*, 17β-HSD of *C. lunatus* and also those which might be reduced by related enzymes to identify the physiological function of ARti (Fig. 2). For enzyme preparation, the codon-optimized gene of ARti was synthesized and cloned into the pET-19b vector between 5′-NdeI and 3′-XhoI encoding N-terminal his_{10}-tag. The recombinant plasmid was then, transformed into *E. coli* BL21 (DE3) and the protein was expressed and purified using Ni-NTA affinity chromatography. The purity and size of the protein were confirmed by SDS-PAGE (Fig. S3†). To test the substrate scope, at first, we synthesized 1,3,6,8-tetrahydroxynaphthalene (T_4HN, **20**)[20] and incubated it with NADPH (regenerated through the glucose/GDH system) and ARti_his in potassium phosphate buffer

Table 2 Comparison of amino acid residues at binding and active sites between 17β-HSDcl and ARti

Binding site		Active site	
17β-HSDcl	ARti	17β-HSDcl	ARti
Ser153, Asn154, Tyr167, Gly199, Met204, Phe205, Ser209 and Tyr212	Ser148, Asn149, Tyr162, Gly194, Met199, Phe200, Ala204, Tyr207	Asn127, Ser153, Tyr167, Lys171	Asn122, Ser148, Tyr162, Lys166

Table 1 Alignment of the BGC containing ARti of *T. islandicus* with other known BGCs involved in the biosynthesis of monodictyphenone (*mdp*),[15] cladofulvin (*cla*),[5] and agnestin (*agn*)[4]

	Sequence identity		
Proteins with putative function	*mdp*	*cla*	*agn*
CRG86674.1 (polyketide synthase)	*mdpG* (45%)	*claG* (43%)	*agnpks1* (44%)
CRG86680.1 (β-lactamase)	*mdpF* (48%)	*claF* (47%)	*agnL7* (50%)
CRG86682.1 (SDR, "ARti")	*mdpC* (77%)	*claC* (77%)	*agnL6* (72%)
CRG86683.1 (decarboxylase)	*mdpH* (57%)	*claH* (68%)	—
CRG86684.1 (dehydratase)	*mdpB* (56%)	*claB* (60%)	*agnL8* (57%)

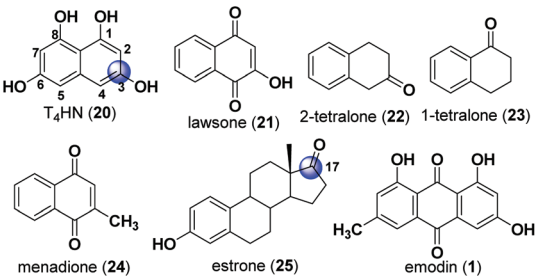
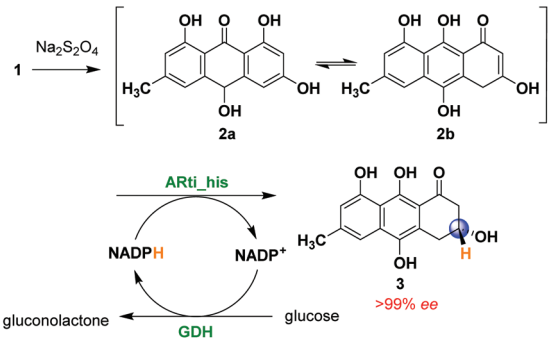


Fig. 2 Substrates **1**, **20–25** tested for reduction with ARti_his using NADPH, regenerated through the glucose/GDH system.



Scheme 1 ARti_his catalyzed reduction of emodin (**1**) hydroquinones (**2a**/**2b**) formed *in situ* using $Na_2S_2O_4$ to (*R*)-3,8,9,10-tetrahydroxy-6-methyl-3,4-dihydroanthracene-1(2*H*)-one (**3**) in the presence of NADPH.

(50 mM, 1 mM EDTA, 1 mM DTT, pH 7.0) under anoxic conditions. The transformation resulted in the reduction of **20** to scytalone with >99% ee and 36% yield. (*R*)-Configuration was assigned to scytalone based on the CD spectra (Fig. S14†).[21] However, when we tested other substrates such as lawsone (**21**) and 2-tetralone (**22**) which are known to be reduced by T_4HNR[21,22] in the presence of NADPH, none of them were reduced by ARti (Fig. 2). This indicates that the two enzymes have different physiological functions, which motivated us to test substrates such as 1-tetralone (**23**) and menadione (**24**), which are not reduced by T_4HNR or even T_3HNR of *M. grisea* (Fig. 2).[22] However, none of these substrates resulted in any reduced product on incubation with ARti under the same conditions.

Considering, the high sequence identity (80%) of ARti with that of 17β-HSDcl, which is known to reduce estrone (**25**) using NADPH, we tested **25** as a probable substrate for ARti (Fig. 2).[23] We incubated **25** with ARti_his in the presence of NADPH (regenerated using the glucose/GDH system) in the same buffer for 12 h using DMF as a co-solvent. ARti_his catalyzed the reduction of carbonyl of estrone (**25**) in the 17-position to 17β-estradiol (>99% de) with 26% conversion (determined with ^{1}H NMR spectroscopy). The product was purified in 22% yield by silica gel chromatography and characterized by NMR spectroscopy and mass spectrometry (ESI). The above transformation indicated that ARti might have a similar physiological function as that of 17β-HSD from *C. lunatus*. Similar to MdpC, 17β-HSDcl has also been identified recently to catalyze the regio- and stereoselective reduction of emodin anthrols (**2a**/**2b**) formed *in situ* by the reduction of **1** using $Na_2S_2O_4$, to **3** in the presence of NADPH (Scheme 1).[6]

This motivated us to test the reduction of emodin (**1**) with ARti in the presence of $Na_2S_2O_4$ which might also result in the formation of **3**. We incubated emodin (**1**) with ARti_his in the same buffer using DMSO as a co-solvent in the presence of $Na_2S_2O_4$ and NADPH (regenerated using glucose/GDH) for 12 h under anoxic conditions (Scheme 1). This resulted in 93% conversion of emodin (**1**) to (*R*)-3,8,9,10-tetrahydroxy-6-methyl-3,4-dihydroanthracene-1(2*H*)-one (**3**) with >99% ee. Due to the overlap of absorbance bands between the substrate and cofactor, UV based assay was not feasible; therefore to analyze the activity of ARti, we determined the conversion of **1** to **3** using ^{1}H NMR spectra. (*R*)-Configuration was assigned to **3** in comparison with the CD spectra of (*R*)-**3** synthesized chemoenzymatically using 17β-HSDcl as reported earlier (Fig. S6†).[6] We also incubated emodin (**1**) with ARti_his in potassium phosphate buffer (50 mM, 1 mM EDTA, 1 mM DTT, pH 7.0) using DMSO as a co-solvent for 12 h in the absence of $Na_2S_2O_4$, under anoxic conditions in the presence of NADPH, regenerated through glucose/GDH. However, no reduced product was formed in such a case. To further characterize the catalytic function of ARti under different conditions, such as various co-solvents, pH and temperature, we used the reduction of emodin (**1**) by ARti_his in the presence of $Na_2S_2O_4$ and NADPH to **3**, as a model reaction (Scheme 1). At first, we tested different co-solvents. For this, we incubated **1** dissolved in different co-solvents (8% v/v) such as 2-propanol, ethanol, *t*-butanol, acetone, acetonitrile, DMSO, DMF, or 1,4-dioxane, with ARti_his in the presence of $Na_2S_2O_4$ (20 equiv.) and NADPH, regenerated through the glucose/GDH system under anoxic conditions for 6 h. The conversions (%) under different co-solvents were determined from ^{1}H NMR spectra of the crude reaction mixture in acetonitrile-d_3. The result shows DMSO as the best co-solvent with 87% conversion of **1** into **3** (Fig. 3a). The use of 2-propanol as a co-solvent resulted in only 34% conversion. This could be due to the low solubility of the substrate in 2-propanol. All other solvents gave reasonable conversion between 73–84% (Fig. 3a). Therefore, DMSO is chosen as a co-solvent for further experiments. To determine the cofactor preference, we performed the transformation (Scheme 1) using NADPH or NADH, generated using the glucose/GDH system keeping everything else the same. We observed that only the NADPH containing reaction resulted in the formation of **3**, which shows ARti as an NADPH-dependent enzyme. To investigate the effect of pH on the conversion of emodin (**1**) to **3**, we performed the same chemoenzymatic transformation at pH 5.5–8.0 and determined the conversion using the ^{1}H NMR spectra of the crude reaction mixture. The results show a low conversion (~63%) at pH 5.5 and 8.0, higher conversion (87–94%) at pH in between, and the highest (94%) at pH 7.0 (Fig. 3b).

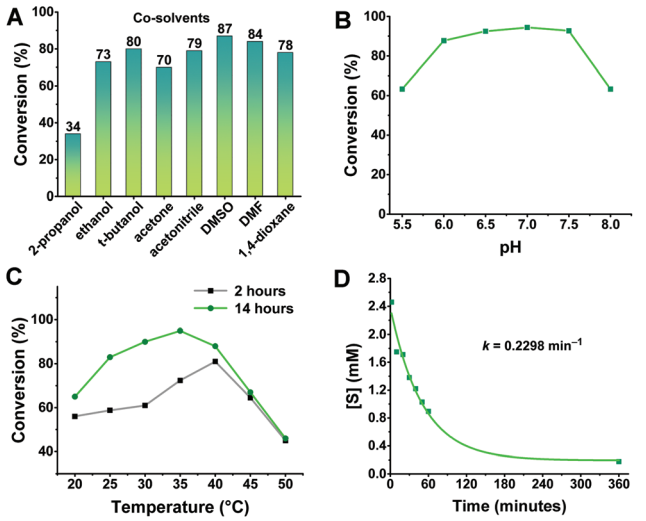


Fig. 3 (A) Effect of the co-solvent on the reduction of emodin hydroquinones (**2a**/**2b**) by ARti_his. (B) pH profile of ARti_his with **2a**/**2b**. (C) Temperature profile of ARti_his with **2a**/**2b**. (D) Plot of substrate concentration ([S]) *versus* time fitted exponentially.

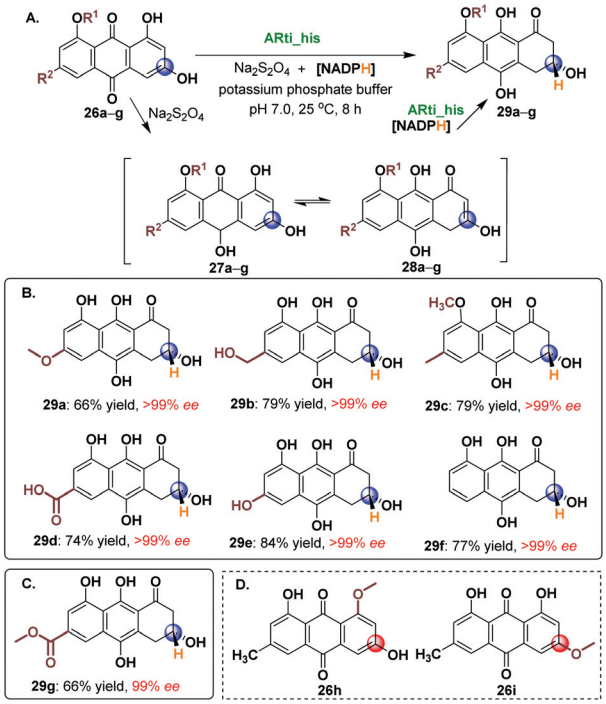


Fig. 4 (A) Regio- and stereoselective, NADPH-dependent reduction of anthrols formed *in situ* using $Na_2S_2O_4$ by ARti_his. NADPH regenerated using the glucose/GDH system. (B) Reduced compounds **29a–f** obtained by the reduction of natural anthraquinones **26a–f**. (C) Reduced compounds **29g** obtained by the chemoenzymatic reduction of non-natural anthraquinone **26g**. (D) Substrates **26h–i** were not reduced by ARti_his chemoenzymatically.

Next, we study the effect of heat on the above transformation by performing the enzymatic reduction of emodin (**1**) using ARti_his in the presence of $Na_2S_2O_4$ and NADPH for 2 h and 14 h at temperatures from 15 °C to 50 °C at the interval of 5 °C. ARti shows the highest activity at 40 °C and 35 °C as observed by conversions of **1** to **3** when incubated for 2 h and 14 h, respectively (Fig. 3c). This shows that ARti remained active for a longer period of time at a lower temperature of 35 °C. However, considering the formation of a deoxygenated side product, chrysophanol (**4**) at a higher temperature, we performed all further transformations at room temperature. In addition, we determined the melting temperature (T_m) for ARti_his using CD spectroscopy, to be 51 °C (Fig. S4, ESI, p. S7†). We also calculated the rate constant for the above transformation from the plot of substrate concentration *versus* time (Fig. 3d). For this, we carried out emodin (**1**) reduction in the presence of $Na_2S_2O_4$ with ARti_his using NADPH, regenerated through the glucose/GDH system for different time intervals. The substrate concentration was calculated from the conversion of **1** into **3**, determined through ^{1}H NMR spectra in acetonitrile-d_3. The rate constant was found to be 0.2298 min^{-1} (Fig. 3d) (ESI, pp. S8 and S9†).

To support further the reduction of anthrols by the putative oxidoreductase of *T. islandicus*, ARti, as its physiological function, we aimed to test the reduction of anthrols of several natural and non-natural anthraquinones **26a–i** as substrates for the newly characterized enzyme (Fig. 4A). For this purpose, naturally occurring anthraquinones, lunatin (**26a**, R^1 = H, R^2 = OCH_3), 1-methylemodin (**26c**, R^1 = CH_3, R^2 = CH_3), 1,3,6,8-tetrahydroxyanthraquinone (**26e**, R^1 = H, R^2 = OH), questin (**26h**), and physicon (**26i**) were synthesized using 2,6-dichlorobenzoquinone and various mixed vinyl ketene acetals *via* two consecutive Diels–Alder reactions (ESI†). Other anthraquinones such as citreorosein (**26b**, R^1 = H, R^2 = CH_2OH), emodic acid (**26d**, R^1 = H, R^2 = CO_2H) and its methyl ester (**26g**, R^1 = H, R^2 = CO_2CH_3) were synthesized starting from emodin (**1**) in 3–4 steps (ESI†). Anthraquinones **26a–e** and **26h–i** have been reportedly isolated from different fungi, so they could be appropriate biosynthetic precursors for other modified anthraquinones and bisanthraquinones involving the reduction of anthrols.[24] Therefore, anthraquinones **26a–e** and **26h–i** were incubated one by one with ARti_his in potassium phosphate buffer (50 mM, 1 mM EDTA, 1 mM DTT, pH 7.0) in the presence of $Na_2S_2O_4$ (20 equiv.) and NADPH, regenerated through the glucose/GDH system, using DMSO as a co-solvent under anoxic conditions (ESI, pp. S22–S30†). After 14 h, the reaction mixture was extracted in ethyl acetate and conversion to the reduced product formed, if any, was determined using ^{1}H NMR spectra in acetone-d_6. This resulted in the formation of reduced hydroanthraquinones **29a–e** (Fig. 4B), expectedly formed by the reduction of tautomers of anthrols **27a–e**/**28a–e** catalyzed by ARti_his using NADPH (Fig. 4A). Except for lunatin (**26a**) which shows 82% conversion, we observed the quantitative conversion of anthraquinones **26b–e** to their respective products **29b–e** formed by the reduction of the respective anthrols (**27a–e**/**28a–e**) catalyzed by ARti. No reduced product was obtained in the case of questin (**26h**), and physicon (**26i**) (Fig. 4D).

The deoxygenation of **29e** by heating under an oxygen atmosphere gave access to another natural anthraquinone **26f** (R^1 = R^2 = H), which was also subjected to the chemoenzymatic reduction with ARti_his under the same condition to obtain **29f** with quantitative conversion (Fig. 4B). A non-natural anthraquinone **26g** also resulted in the formation of **29g** in 75% conversion (Fig. 4C), when incubated with ARti_his in the presence of $Na_2S_2O_4$ and NADPH (regenerated using glucose/GDH system). The *in situ* formation of anthrols **27a–g**/**28a–g** by the reduction of respective anthraquinones **26a–g** through $Na_2S_2O_4$, was confirmed by the ^{1}H NMR spectra, supported by their respective HRMS data. The reduced compounds **29a–g** were purified using silica gel column chromatography in 66–84% yield and characterized by NMR spectroscopy and mass spectrometry. The configuration (*R*) has been assigned to all the reduced compounds **29a–g** by comparison of their CD spectra with that of (*R*)-3,8,9,10-tetrahydroxy-6-methyl-3,4-dihydroanthracene-1(2*H*)-one (**3**) synthesized by the chemoenzymatic reduction of emodin (**1**) using 17β-HSDcl as reported previously (Fig. S4–S12†).[6] We could also determine the enantiomeric excess for all the reduced compounds **29a–g** using HPLC by comparison with the racemic **29a–g** made by the reduction of anthraquinones **26a–g** using $NaBH_4$ in the presence of $Na_2S_2O_4$ in water. For all the reduced anthraquinones **29a–g** obtained through chemoenzymatic reduction, ee was found to be >99%. Overall, the result shows tolerance of various substituents on the ring A of the anthraquinones as observed for the reduction of **26a–g** while any changes to the ring C of anthraquinones, as in the case of **26h–i**, were not tolerated. Therefore, no reduced products were obtained in the case of questin (**26h**) and physicon (**26i**). The asymmetric reduction of hydroanthraquinones **27a–g**/**28a–g** shown above by ARti, supports anthrol reduction as a physiological function for the putative oxidoreductase (accession no. CRG86682.1) from *T. islandicus*. As shown here, the use of ARti provides easy access to many of the putative biosynthetic intermediates **29a–f** in optically pure form for the first time and had also broadened the scope of anthrol reductases which remained limited to the reduction of emodin anthrols (**2a**/**2b**). In comparison with the only non-enzymatic procedure that has been reported for the synthesis of (*S*)-**29a** and involves 14 steps with an overall yield of 5% (Scheme S7†),[25] our biocatalytic procedure is simple, atom economical, green and results in high yields.

In conclusion, an SDR from *Talaromyces islandicus* WF-38-12 has been identified through genome analysis and proposed to possess anthrol reductase activity. The enzyme has been shown to catalyze an asymmetric reduction of anthrols **27a–g**/**28a–g**, formed *in situ* by the reduction of variously substituted natural anthraquinones **26a–f** and a non-natural analog **26g** in the presence of $Na_2S_2O_4$, using NADPH to **29a–g** (Fig. 4). This chemoenzymatic transformation supports anthrol reduction as the physiological function of ARti. Based on the earlier work of Shibata[7,26] and our recent biomimetic approach towards the synthesis of bisanthraquinones,[2,27] we propose that **29a–f** may act as precursors for the biosynthesis of deoxy (bis)anthraquinones like **7**, **10–12**, xanthones like **6**, **13** and modified (bis) anthraquinones **8**, **14–18** (Fig. 1). In addition, the use of enantiomerically pure, chemoenzymatically reduced anthrols **29a–f** presented here, will facilitate method development to synthesize various natural products and their analogs and will provide clues for the elucidation of the (bio)synthesis of such natural products. We also believe that many more secondary metabolites with dimeric structures related to bisanthraquinone such as (−)-flavoskyrin (**8**) and **14–18** (Fig. 1) with a diverse array of biological activities will be isolated in future.

Conflicts of interest

There are no conflicts to declare.

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