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Unprecedented Role of Hydronaphthoquinone Tautomers in Biosynthesis**

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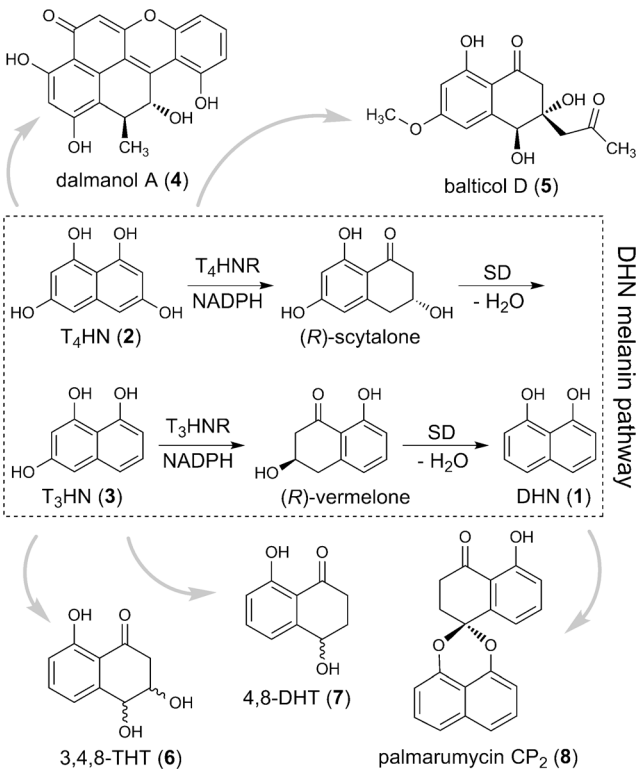
Abstract: *Quinones and hydroquinones are among the most common cellular cofactors, redox mediators, and natural products. Here, we report on the reduction of 2-hydroxynaphthoquinones to the stable 1,4-diketo tautomeric form of hydronaphthoquinones and their further reduction by fungal tetrahydroxynaphthalene reductase. The very high diastereomeric and enantiomeric excess, together with the high yield of cis-3,4-dihydroxy-1-tetralone, exclude an intermediary hydronaphthoquinone. Labeling experiments with NADPH and NADPD corroborated the formation of an unexpected 1,4-diketo tautomeric form of 2-hydroxyhydronaphthoquinone as a stable intermediate. Similar 1,4-diketo tautomers of hydronaphthoquinones were established as products of the NADPH-dependent enzymatic reduction of other 1,4-naphthoquinones, and as substrates for different members of the superfamily of short-chain dehydrogenases. We propose an essential role of hydroquinone diketo tautomers in biosynthesis and detoxification processes.*

Quinones and hydroquinones, together with their non-oxidized precursors such as polyhydroxynaphthalenes, play a central role as biosynthetic precursors for primary and secondary metabolites and for polymers such as melanin. Most fungal melanins and related metabolites are formed by a polyketide route and are derived from the common precursor molecule 1,8-dihydroxynaphthalene (DHN, **1**). The first known intermediate of the pathway is 1,3,6,8-tetrahydroxynaphthalene (T_4HN, **2**). This metabolite is reduced by tetrahydroxynaphthalene reductase (T_4HNR); the product (*R*)-scytalone is then dehydrated to 1,3,8-trihydroxynaphthalene (T_3HN, **3**), which undergoes another reduction to (*R*)-vermelone and dehydration to give DHN (**1**) (Scheme 1). In 1985 when Wheeler and Stipanovic provided a comprehensive summary of DHN melanin biosynthesis in the fungus *Wangiella dermatitidis*, it became clear that fungal



Scheme 1. Fungal DHN melanin biosynthesis (boxed) represents a branching point towards several secondary metabolites (**4**–**8**).[1–6] THT: trihydroxytetralone, DHT: dihydroxytetralone, T_4HN: tetrahydroxynaphthalene, T_4HNR: T_4HN reductase, T_3HN: trihydroxynaphthalene, T_3HNR: T_3HN reductase, SD: scytalone dehydratase.

DHN melanin is not formed by a straightforward pathway, but rather a complex metabolic network.[1,2] Subsequently, hundreds of metabolites, such as the dalmanols (e.g., **4**),[3] balticols (e.g., **5**),[4] 3,4-dihydroxy-1-tetralones (e.g., **6**),[1] 4-hydroxy-1-tetralones (e.g., **7**),[1] and spirodioxynaphthalenes (e.g., **8**),[5] have been shown to be derived from the intermediate polyhydroxynaphthalenes **1**–**3**. It has been proposed that 4-hydroxy-1-tetralones and 3,4-dihydroxy-1-tetralones are the products of the oxidation of polyhydroxynaphthalenes to naphthoquinones, followed by a double reduction via hydronaphthoquinones.[1,2,6] Accordingly, it has been suggested that the formation of 4-hydroxy-1-tetralone derivatives represents a detoxification process.[1,2]

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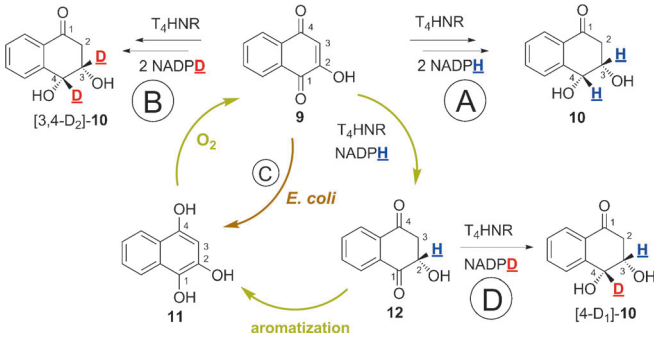
Moreover, quinones are among the most common cellular cofactors and natural products. From a traditional point of view, "primary" quinones are widely distributed as electron acceptors, while "secondary" quinoid metabolites play a defense role by generating reactive oxygen species (ROS) through redox cycling, which results in (nonspecific) superoxide toxicity. Redox cycling arises from the one-electron reduction of the quinone to the activated semiquinone form (toxification), or from the two-electron reduction to the hydroquinone form, back-oxidation of which generates ROS. Under the prevailing reduced intracellular redox state, two-electron reduction normally leads to detoxification.[7,8] In contrast, the producing cell in the "redox state", which constitutes all redox-active molecules, has to maintain cellular homeostasis and the ability to deal with redox changes in a highly regulated manner.[9] In this context, it has remained unclear how organisms producing quinoid metabolites handle potentially toxic intermediates and byproducts.[10,11]

We show herein that NADPH-dependent enzymatic reduction of 2-hydroxynaphthoquinones, resulting in 3,4-dihydroxy-1-tetralones, proceeds via the stable 1,4-diketo tautomer of the hydronaphthoquinones. This is in contrast to the well-known two-electron reduction of quinones resulting in the respective hydroquinones. We propose that hydronaphthoquinone tautomers play an unprecedented and essential role in the biosynthesis of many natural products and are involved in breaking the redox cycle of quinones–hydroquinones.

We recently reported the reduction of 2-hydroxy-1,4-naphthoquinones to the corresponding *cis*-3,4-dihydroxy-1-tetralones by the NADPH-dependent enzyme T_4HNR from the rice blast fungus *Magnaporthe grisea*.[12] This enzyme plays an integral part in the biosynthesis of DHN melanin, a virulence factor of many filamentous fungi.[13] To elucidate the mechanism of these reactions, we employed lawsone (**9**) as a model substrate (Scheme 2). Lawsone was reduced with 2 equivalents of NADPH to (3*S*,4*R*)-3,4-dihydroxy-1-tetralone (**10**) by T_4HNR with high diastereoselectivity (d.r.$_{cis/trans}$ $\geq$ 99:1), in high enantiomeric excess (> 99 % *ee*), and in 90 % yield (see Scheme 2 A and the Supporting Information).



Scheme 2. Overview of the reactions performed for elucidation of the mechanism of vicinal *cis*-ketodiol formation by T_4HNR. Reduction of lawsone (**9**) with 2 equivalents of NADPH results in the vicinal *cis*-ketodiol **10** (A), while reduction with 2 equivalents of NADPD leads to double deuteration (B). Redox cycling of lawsone (**9**) is catalyzed by crude extracts from *E. coli* (C), and by aromatization of intermediate **12**. The reduction of **12** with NADPD yields [4-D_1]-**10** (D).

In order to verify the involvement of hydronaphthoquinones in such transformations, as proposed by Wheeler et al.,[1,2] we synthesized the hydroquinoid 1,2,4-trihydroxynaphthalene (**11**) by reduction of lawsone (**9**) using sodium dithionite, and applied it as a potential substrate of T_4HNR. The reaction was performed under anoxic conditions to prevent back-oxidation of **11** to **9**. Surprisingly, the expected *cis*-ketodiol **10** was not formed, in contrast to the previous proposal for analogous transformations.[1,2,6] Hence, the putative two-step enzymatic formation of **10** from 2-hydroxyquinone **9** does not involve the hydroquinone **11**.

To clarify the mechanism of the reduction of **9**, we performed deuterium-labeling experiments. The use of in situ generated NADPD [[1-D]-D-glucose/glucose dehydrogenase (GDH) cofactor regeneration system] resulted in double incorporation of deuterium at positions 3 and 4 ([3,4-D_2]-**10**, Scheme 2 B). In the inverse experiment, using NADPH (D-glucose/GDH) in D_2O buffer, no incorporation of deuterium at these positions occurred. This clearly demonstrates that both hydrogen atoms are transferred in the enzymatic reduction process using either NADPH or NADPD, rather than being taken up from the solvent through keto–enol tautomerism.

When the reaction was terminated before completion by fast extraction of the reaction mixture, we observed hydronaphthoquinone **11** (by NMR spectroscopy) and, unexpectedly, the correspondding 1,4-diketo tautomer **12**. Hydronaphthoquinone **11** is formed by aromatization of **12** and, as expected, it was shown to be the product of redox cycling of **9**. Such redox cycling is generally considered to be the cause of quinone toxicity;[14] here, the cycle is the result of catalytic activity of crude extracts from *E. coli*, the host organism used for heterologous expression (Scheme 2 C).[15] In contrast, the diketo tautomer **12** was formed only in the presence of T_4HNR and its surprising stability suggested that aromatization proceeds sufficiently slow, allowing for draining the cycle through subsequent reduction to ketodiol **10**.

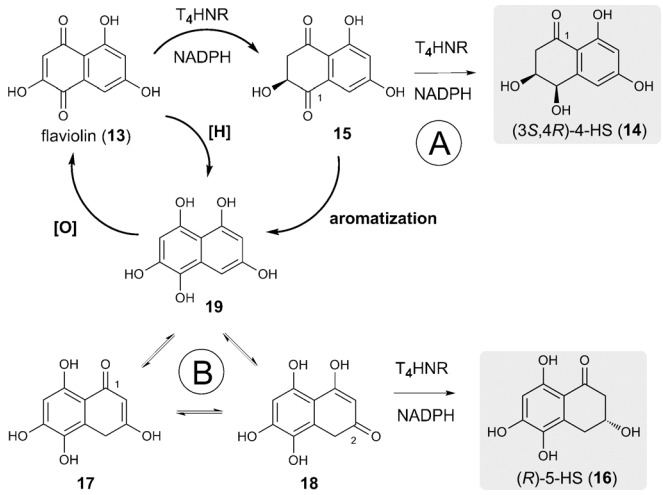
To prove formation of the supposedly reactive 1,4-diketo tautomers of hydroquinones, we optimized the reaction conditions in order to isolate sufficient amounts of the putative intermediate **12** to be used as a substrate. Conversion of **12** (synthesized by monoreduction of **9** with NADPH and T_4HNR), using NADPD and T_4HNR under anoxic conditions, resulted in the formation of the monodeuterated *cis*-ketodiol [4-D_1]-**10** (Scheme 2 D). Similarly, conversion of [2-D_1]-**12** (synthesized by monoreduction of **9** with NADPD and T_4HNR), using NADPH and T_4HNR under anoxic conditions, resulted in the formation of the monodeuterated *cis*-ketodiol [3-D_1]-**10** (see the Supporting Information).

Hence, our results suggest an unprecedented mechanism for the conversion of 2-hydroxy-1,4-naphthoquinone into the vicinal ketodiol. This pathway proceeds as follows: 1) initial reduction of the 2-hydroxynaphthoquinone at the enolic position generates the nonaromatic 1,4-diketo tautomer of the hydronaphthoquinone, either by Michael addition of hydride or by tautomerism to the 2-ketone before keto reduction; 2) a second reduction at the benzylic position of the 1,4-diketo tautomer results in the vicinal diol; and 3) partial aromatization of the 1,4-diketone forms the hydro-

naphthoquinone, whose back-oxidation to the quinone substrate enables complete conversion. In contrast to the well-established two-electron reduction of quinones resulting in hydroquinones, themselves prone to (nonenzymatic) reoxidation to the quinones, this new mechanism proceeds via a two-step transformation with decisive consequences for this redox cycle: although the first enzymatic reduction product, the 1,4-diketo tautomer, can aromatize to the hydronaphthoquinone, alternatively it can be reduced in a second NADPH-dependent enzymatic reduction, eventually resulting in a drain of the quinone–hydroquinone compounds. If the mechanism established for the T_4HNR-catalyzed reduction of 2-hydroxynaphthoquinone **9** proves applicable to other quinone–enzyme combinations, it would challenge the general notion of quinone–hydroquinone transformations as the only underlying equilibrium reactions.

The T_4HNR-catalyzed reduction was next tested with other related metabolites as substrates. The reduction of flaviolin (**13**), a putative intermediate in the biosynthesis of several monomeric and oligomeric polyketide metabolites, to the naturally occurring *cis*-(3*S*,4*R*)-4-hydroxyscytalone (**14**)[12] and isolation of the nonaromatic, monoreduced 1,4-diketo tautomer **15** strongly suggests the general applicability of these results for DHN melanin biosynthesis (Scheme 3A). In addition, when lower enzyme concentrations were used, naturally occurring (*R*)-5-hydroxyscytalone (**16**)[1,16] was formed as a side product (Scheme 3B).

Compound **16** represents the product of the reduction of the tautomeric forms **17** and **18** of flaviolin hydroquinone (**19**), either by Michael addition of hydride to the 1-ketone **17** or by hydride addition to the 2-ketone **18**. Again, the hydroquinone **19** is formed by aromatization of **15** and/or quinone reduction by crude extracts from *E. coli*. Moreover, the phenolic and ketonic forms are in equilibrium, as demonstrated by the reduction to **16**, and back-oxidation to **13** competes with enzymatic reduction. Therefore, to obtain



Scheme 3. Enzymatic reduction of flaviolin (**13**) by T_4HNR. A) Reduction of **13** by T_4HNR results in the vicinal *cis*-ketodiol *cis*-(3*S*,4*R*)-**14**.[12] B) As a side product, (*R*)-5-HS (**16**) was observed, the product of reduction of the tautomeric forms of hydroquinone **19**. The final products are highlighted with gray boxes. HS: hydroxyscytalone, PHN: pentahydroxynaphthalene.

16 as the main product, flaviolin (**13**) was reduced in situ with sodium dithionite under a nitrogen atmosphere. Subsequent conversion with T_4HNR gave **16** in 24% yield.

Tautomers are different isomers of "one" compound in the same oxidation state: due to the mostly rapid interconversion, they are commonly considered to be the same chemical compound. The formation of tetralones **14** and **16** nicely demonstrates that different tautomers can be regio- and stereoselectively reduced by one and the same enzyme. This not only corroborates our proposed new mechanism, but also sheds light on the diversity-oriented biosynthesis of melanins: here, starting from one substrate, namely flaviolin (**13**), the three distinctly different products **14**, **15**, and **16** are regio- and enantioselectively formed by the action of one enzyme.

Our results also provide insight into the role of 4-hydroxy-1-tetralones, another group of metabolites observed in the DHN melanin pathway. It has been suggested that such compounds are the products of hydronaphthoquinone reduction by polyhydroxynaphthalene reductases.[1,2,6] However, the 1,4-diketo tautomers, and not the hydronaphthoquinones, might just as well represent the basic substrates for keto reduction, even though the course of their generation in biosynthesis remains unknown. Accordingly, we tested chemically synthesized 1,4-diketo tautomers of the hydronaphthoquinones as putative substrates of T_4HNR. 2,3-Dihydronaphthalene-1,4-dione (**20a**) was indeed reduced by T_4HNR (21% yield) to (*S*)-4-hydroxy-1-tetralone (**21**) (Figure 1). (*S*)-4,8-

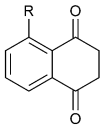
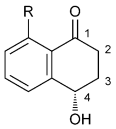
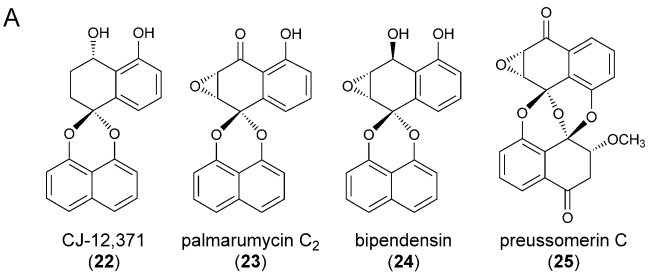
Substrate	Product	Enzyme		Yield [%]	*ee* [%]	Abs. config.
		T_4HNR	**21**	21	98	(*S*)
			7	9	95	(*S*)
		GDH	**21**	14	11	(*S*)
			7	26	<5	n.d.
20a: R = H **20b**: R = OH	**21**: R = H (*S*)-**7**: R = OH					

Figure 1. Reduction of the 1,4-diketo tautomers of hydronaphthoquinones. 2,3-Dihydronaphthalene-1,4-diones (**20**) were reduced by T_4HNR and GDH leading to 4-hydroxy-1-tetralones **7** and **21**. n.d.: not determined.

Dihydroxy-1-tetralone [(*S*)-**7**] was obtained by T_4HNR-catalyzed reduction (9% yield) of 5-hydroxy-2,3-dihydronaphthalene-1,4-dione (**20b**). Again, in both cases, the respective hydronaphthoquinone showed no conversion, but was observed as a side product. Thus, it can be assumed that the installation of a 1,4-diketo-tautomer-forming process results in complete conversion, as observed for the vicinal ketodiols **10** and **14**.

The results demonstrate that DHN melanin biosynthesis constitutes a diversity-oriented metabolic network, comprising vicinal ketodiol and 4-hydroxy-1-tetralone biosyntheses as branching points. Presumably, polyhydroxynaphthalene reductases are involved in promiscuous reduction reactions, reflecting the idea of a matrix biosynthetic pathway.[17,18]

These results directed us towards a group of fungal secondary metabolites with a great variety of biological activities: namely, the spirodioxynaphthalenes, in which naphthalene and dihydronaphthalene units are bridged by a spiroketal linkage (Figure 2A). The monomers putatively



Enzyme		Yield [%]	*ee* [%] (d.r.)	Abs./rel. config.[a]
T_4HNR-his	**27a**	74	>99 (86:14)	(2*S*,3*S*,4*R*) *trans*
	27b	<10% conversion		
GDH	**27a**	64	53 (87:13)	(2*S*,3*S*,4*S*) *cis*
	27b	40	50 (88:12)	(2*S*,3*S*,4*S*) *cis*
	and **27c**	9	95 (>97:3)	(2*S*,3*S*,4*S*) *cis*

Figure 2. Reduction of 2,3-epoxy-1,4-naphthoquinones. Spirodioxynaphthalenes often exhibit a 2,3-epoxy-4-hydroxy-1-tetralone subunit[5] (A) which can be obtained by reduction of the corresponding 2,3-epoxy-1,4-naphthoquinone (B). [a] The absolute configuration was determined by VCD spectroscopy.

originate from fungal DHN melanin biosynthesis and often resemble a highly variable 1,4-naphthoquinone-derived backbone, such as in palmarumycin CP_2 (**8**) (see Scheme 1), CJ-12,371 (**22**), palmarumycin C_2 (**23**), bipendensin (**24**), and preussomerin C (**25**). The generation of different oxidation states has been proposed to occur after formation of the spiroketal linkage of two DHN units by oxidative phenol coupling.[5] However, our findings from T_4HNR-catalyzed reductions show that this heterogeneity may well be introduced at the monomer stage, prior to formation of the spiroketal linkage by acetalization (see, for example, the 1,4-diketonic substructures in **8** and **25**, and the 4-hydroxy-1-tetralone substructure in **22**). In addition, a variety of spirodioxynaphthalenes show a highly variable 2,3-epoxy-4-hydroxy-1-tetralone-derived backbone (e.g., **24**). Accordingly, we assumed that polyhydroxynaphthalene reductases are also involved in the formation of these derivatives and we tested 2,3-epoxy-1,4-naphthoquinones as substrates of T_4HNR.

Due to partial epoxide opening by crude extracts from *E. coli*, His-tagged and purified T_4HNR-his was used in these experiments. 2,3-Epoxy-1,4-naphthoquinone (**26a**) was reduced by T_4HNR-his to *trans*-(2*S*,3*S*,4*R*)-**27a** in 74% yield, whereas menadione epoxide (**26b**) showed only a poor conversion (< 10%, mainly *cis*-**27b**, Figure 2B). Hence, the present work suggests also the involvement of polyhydroxynaphthalene reductases in spirodioxynaphthalene biosynthesis; the details of which metabolic steps occur before and after formation of the spiroketal linkage are yet to be elaborated.

T_4HNR is a member of the short-chain dehydrogenases/reductases (SDR) enzyme family, which constitutes one of the largest enzyme superfamilies.[19] We hypothesized that other SDR should behave in a similar manner to T_4HNR, thus being able to reduce carbonyls, aromatic hydroxyls (e.g., polyhydroxynaphthalenes), 2-hydroxyquinones, and/or 1,4-diketo tautomers of hydroquinones. This view was strongly supported by the serendipitous observation that glucose dehydrogenase (GDH) indeed showed activity for (2-hydroxy)-1,4-diketones: when glucose/GDH was used as a regeneration system for NADPH in the T_4HNR-catalyzed reduction of **12** (see Scheme 2), the ketodiol **10** was obtained as a mixture of diastereomers ($d.r._{cis/trans} \leq 92:8$). This lower diastereoselectivity is due to a background reaction caused by GDH. When the cofactor regeneration system was changed to L-malic acid/malic enzyme, the d.r. was restored to $d.r._{cis/trans} > 99:1$ (see above), clearly showing that **12** is a substrate for GDH.

In addition, reduction of **20a** by GDH gave **21** in 14% yield, while **20b** as substrate gave virtually racemic **7** in 26% yield (Figure 1).[20] GDH also reduced epoxyquinone **26a**, leading to *cis*-(2*S*,3*S*,4*S*)-**27a** in 64% yield. Menadione epoxide (**26b**) was reduced by GDH to *cis*-(2*S*,3*S*,4*S*)-**27b** in 40% yield, along with *cis*-(2*S*,3*S*,4*S*)-**27c** in 9% yield (Figure 2B).

Previously, it was shown that 17β-hydroxysteroid dehydrogenase from *Cochliobolus lunatus* (a homologue of T_4HNR) and T_3HNR catalyze reductions of polyhydroxynaphthalenes with a high degree of regio- and stereocontrol.[20] Due to their close relationship to T_4HNR we propose that these and other SDR, such as GDH, will behave in a diversity-oriented manner similar to T_4HNR, resulting, for instance, in *cis*- and *trans*-3,4-dihydroxy-1-tetralones.

The quinone–hydroquinone equilibrium is of utmost importance for many biological processes. The selective formation of *cis*-3,4-dihydroxy-1-tetralones in the T_4HNR-catalyzed reduction of 2-hydroxyquinones challenged the generally accepted mechanism of NADPH-dependent quinone reductions. The elucidation of the mechanism of the enzymatic reduction of the model substrate lawsone (**9**) resulted in the identification and isolation of the unexpectedly stable 1,4-diketo tautomer **12** of hydronaphthoquinone **11**. The involvement of such 1,4-diketo tautomers in enzymatic redox reactions of quinones was subsequently demonstrated for several compounds. The stability of the 1,4-diketo tautomers was sufficient for their isolation and application in asymmetric transformations. In addition to obvious bio-

catalytic and nonenzymatic[21–23] transformations,[24] these results may have major implications for the biosynthesis and the physiological role of primary and secondary quinone metabolites.

Our findings contribute to the understanding of melanin biosynthesis. In the "classical" DHN melanin pathway, the equilibrium of the phenolic and ketonic tautomers facilitates the chemoenzymatic reduction.[25] In contrast, we have demonstrated that 1,4-diketonic tautomers represent true intermediates of reduction and not of tautomerism of the hydronaphthoquinones. This represents a new unifying concept for the fungal metabolism of polyhydroxynaphthalenes, which is achieved simply by enzyme promiscuity.

The results presented herein might have a broader significance regarding quinoid pathways. Vicinal ketodiols and 4-hydroxy-1-tetralones, such as **7**,[1,6,26] **21**,[27] catalponol,[28] isoshinanolone,[29] and juglanoside A,[27] are common among fungi, but have also been isolated from plants. We postulate that diketo tautomers often do not represent the product of tautomerism, but rather emerge as true intermediates in biosynthesis. Accordingly, a 1,4-diketo tautomer has been described in actinorhodin biosynthesis,[30] and diketonic dihydrofusarubins have been suggested as representing the physiological metabolites.[31] Other diketones of reduced pyranonaphthoquinone antibiotics have been isolated[32] and the leuco forms of anthrahydroquinones have been described as products of enzymatic quinone reduction.[33,34] For menaquinone biosynthesis, two metabolic steps have even been proposed to proceed via diketo tautomeric forms.[35,36] We have shown that the keto tautomers of emodin hydroquinone represent the true substrates for enzymatic reduction by a T_4HNR homologous enzyme in the biosynthesis of monodictyphenone; the hydroquinone was not observed.[37]

Finally, the generation and reduction of 1,4-diketo tautomers of hydronaphthoquinones represents a strategy to bypass potentially toxic byproducts in biosynthesis. The redox cycling of (undirected) quinoid metabolites such as 1,4-naphthoquinones and 2-hydroxy-1,4-naphthoquinones as byproducts in biosyntheses poses a potential risk to the producing cell. It has been suggested previously that the formation of 4-hydroxytetralone derivatives represents a detoxification process.[1,2] Hence, our finding of the reduction of nonaromatic hydroquinone tautomers indicates a possibility to break the redox cycle. Moreover, increasing evidence suggests that these "secondary" metabolites also directly influence redox signaling; for example, stress hormesis of naphthoquinones has been described recently.[38] In all cases, upon accumulation of the metabolites, indirect effects occur by changes in the glutathione/oxidized glutathione and NAD(P)H/$NAD(P)^+$ ratios, as well as by a direct action of reactive oxygen species. However, redox cycling of lawsone (**9**) and of anticancer drugs, such as β-lapachone, cannot be arrested in the hydroquinone state by two-electron reduction via DT-diaphorase [NAD(P)H:quinone reductase].[7,8] Therefore, we conclude that diketo tautomers emerge as true intermediates in biosynthesis and that their formation breaks the (redox) cycle, thus protecting the cell from stress-related redox events.

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