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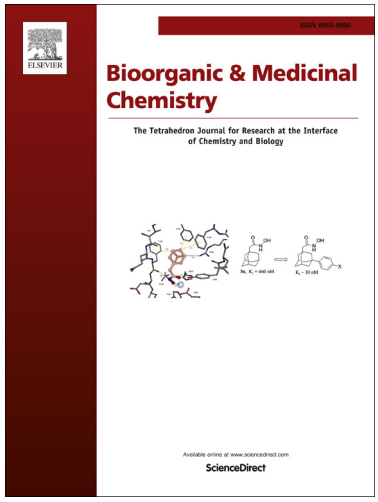

Catechol-rhodanine derivatives: specific and promiscuous inhibitors of *Escherichia coli* deoxyxylulose phosphate reductoisomerase (DXR)

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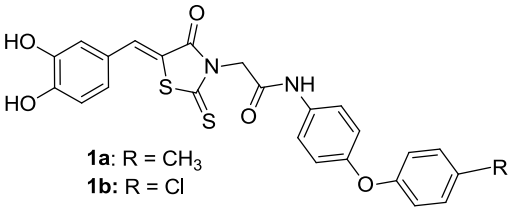
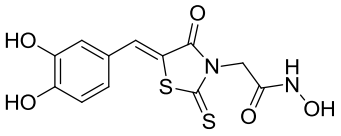
Promiscuous inhibitors of *E. coli* DXR



1a: R = CH_3
1b: R = Cl

Specific inhibitors of *E. coli* DXR



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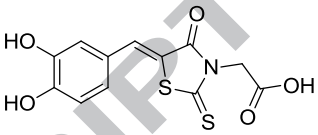

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Catechol-rhodanine derivatives: specific and promiscuous inhibitors of *Escherichia coli* deoxyxylulose phosphate reductoisomerase (DXR)

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Abstract

To develop more effective inhibitors than fosmidomycin, a natural compound which inhibits the deoxyxylulose 5-phosphate reductoisomerase (DXR), the second enzyme of the MEP pathway, we designed molecules possessing on the one hand a catechol that is able to chelate the magnesium dication and on the other hand a group able to occupy the NADPH recognition site. Catechol-rhodanine derivatives (**1** - **6**) were synthesized and their potential inhibition was tested on the DXR of *Escherichia coli*. For the inhibitors **1**and **2,** the presence of detergent in the enzymatic assays led to a dramatic decrease of the inhibition suggesting, that these compounds are rather promiscuous inhibitors. The compounds **4** and **5** kept their inhibition capacity in the presence of Triton X100 and could be considered as specific inhibitors of DXR. Compound **4** showed antimicrobial activity against *Escherichia coli*. The only partial protection of NADPH against the inhibition suggested that the catechol-rhodanine derivatives did not settle in the coenzyme binding site. This paper points out the necessity to include a detergent in the DXR enzymatic assays to avoid false positive when putative hydrophobic inhibitors are tested and especially when the IC_{50}, are in the micromolar range.

1. Introduction

The emergence of multi-drug resistant bacterial pathogens is a growing public health concern. The development of inhibitory compounds against novel target sites is needed for overcoming antibiotic resistance. The 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, involved in the biosynthesis of isoprenoids, is present in most eubacteria among them the pathogenic *Mycobacterium tuberculosis,* the causative agent of tuberculosis and in *Plasmodium*

falciparum, the parasite responsible for malaria.[1,2] As this pathway is absent in humans, all enzymes concerned represent attractive targets for the conception of new antimicrobial drugs, which could overcome the emergence of antibiotic resistance.[3] Since fosmidomycin, a natural compound originally isolated from *Streptomyces lavendulae* culture broth,[4] was found to inhibit 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR),[5] the second enzyme of the pathway, extensive investigations were undertaken to find new DXR inhibitors that should be more effective and more available than fosmidomycin.[6] Indeed, fosmidomycin has the great drawback that bacteria become rapidly resistant to it.[7]

The structures of most synthetic inhibitors are based on the structure of fosmidomycin with the idea to keep the same binding mode. The antibiotic is linked to the enzyme active site through its two extremities: the hydroxamate group chelates an essential divalent metal cation, and the phosphonate group is settled in the phosphate recognition site of the substrate.[8-13] The replacement of the phosphonate group by a carboxylate or a sulfonate moiety[14] as bioisosters afforded poor inhibitors of the DXR as well as the substitution of the hydroxamate moiety with different cation chelating groups such as hydroxyurea, benzoxazolone, benzoxazolethione, oxaloylpyridone, hydrazide or dithiocarbamate.[15-17]

We investigated the opportunity to replace the hydroxamate (or retro-hydroxamate) by another chelating group, such as a catechol moiety. Indeed the magnesium dication Mg^{2+}, described as a hard metal ion forms stable complexes with dioxygen based ligands such as hydroxamate[18] but also catecholates.[19] In previous studies, we synthesized analogs of fosmidomycin by replacing the hydroxamate group with a catechol group but such compounds, as confirmed by others, are weak inhibitors with only mM IC_{50} values.[17,20] These compounds do not fit well in the active site. Either the interactions between the catechol and the cation or those between the phosphonate and the enzyme do not take place. To overcome this problem, we designed molecules possessing simultaneously a chelating catechol group and a group able to occupy the NADPH recognition site or the hydrophobic site situated near the active site. Sem *et al.* reported the synthesis of molecules having a catechol-rhodanine scaffold targeting dehydrogenases requiring nicotinamide coenzymes.[21] As compound **1a** inhibits the *E. coli* DXR with a promising IC_{50} value (0.1 µM) we synthesized and tested derivatives of compound **1a** with the aim to discover inhibitors with a higher inhibition activity (Figure 1). These compounds have limited solubility in aqueous solutions and may give aggregates able to inhibit the DXR in a non-specific manner. Such inhibitors, also called promiscuous inhibitors, are considered as "false positive inhibitors" in high throughput screening. To strengthen our assumption, we compared on one hand, the inhibitory capacity

of these compounds in absence and presence of Triton X100 as suggested by Feng and Shoichet[22] and on the other hand, we tested these compounds on the malate dehydrogenase (MDH) of *Thermus flavus*, one of the model enzymes recommended to allow the identification of promiscuous inhibitors.[23] That dehydrogenase reduces oxaloacetate to L-malate in the presence of NADH and does not need a metal cation to be active.

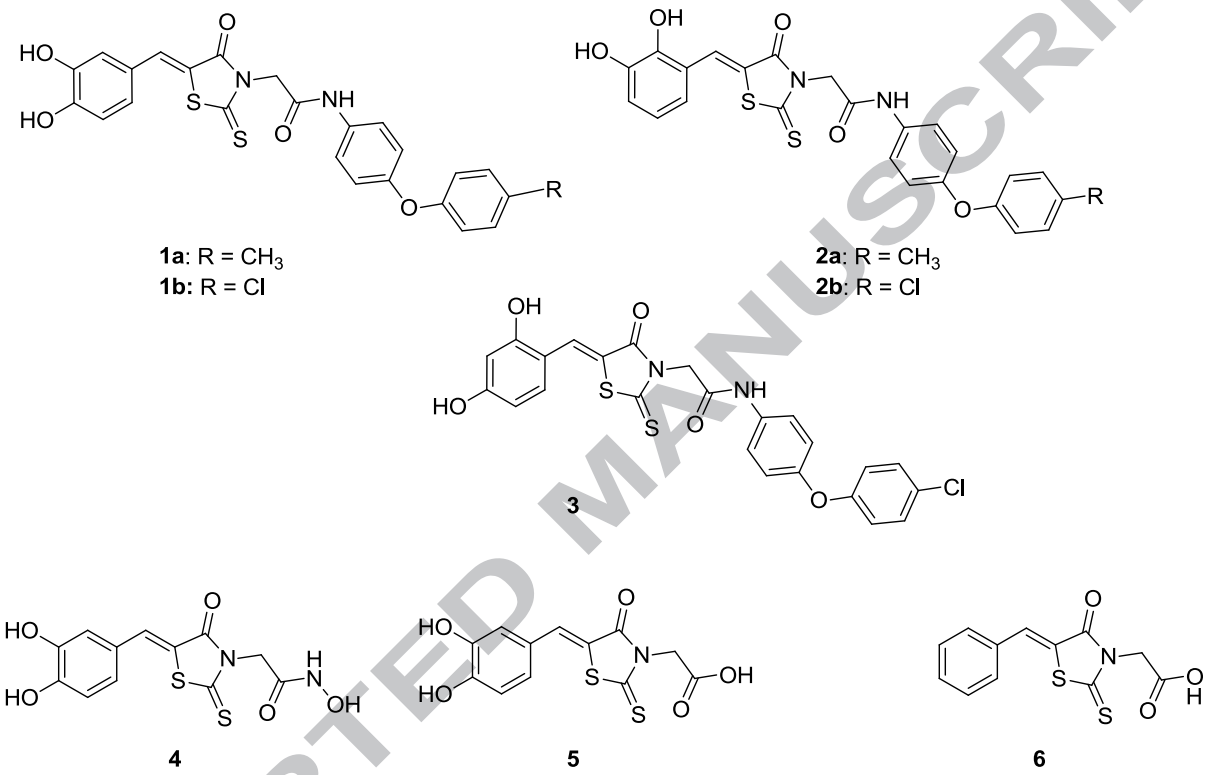

Figure 1: Catechol-rhodanine derivatives.

2. Results

2.1.Inhibition of His-tagged DXR (H-DXR) with rhodanine derivatives 1-6

For a given enzyme, the determination of half maximal inhibitory concentration (IC_{50}) is, in a first approach, often privileged to compare the effectiveness of the inhibition by several compounds. However, these values depend on assay conditions, for example on the concentration of the substrates relative to the Km. They are also influenced by the assay procedures: with or without preincubation of the enzyme with the inhibitor, order of addition of the reagents (addition of substrate or enzyme to initiate the enzymatic reaction), presence or not of BSA to stabilize the enzyme during the assay, nature of the di-cation (Mg^{2+} or Mn^{2+})

in the active site. Unfortunately, all these reasons make the comparison of the effectiveness of the tested potential inhibitors by IC_{50} values from different research groups difficult. We synthesized therefore **1a** and **5** as references to compare our new compounds with these two previously investigated rhodanines.[21]

Expecting that our compounds are most likely slow-binding inhibitors, as fosmidomycin, we tested the influence of preincubation of compound **1a**, considered as a compound of reference, on the inhibition activity of His-tagged DXR (H-DXR) from *E. coli*. Clearly, a significant increase of the inhibition was observed when it was preincubated with H-DXR. At a concentration of 10 µM, the inhibition of **1a** was very weak (< 5%) while it increased to about 53 % when the enzymatic reaction was initiated after a 2 min preincubation.

To determine the potential inhibition of the catechol rhodanine derivatives, we privileged to preincubate the DXR with the potential inhibitors and to initiate the enzymatic reaction by addition of the coenzyme. Indeed, as these compound should settle in the NADPH binding site, the presence of the coenzyme might prevent their binding to the DXR.[21] However, as the enzyme follows an Ordered Bi Bi mechanism in which NADPH binds first before the DXP, the latter was added in the preincubation medium for practical convenience. [24]

The catechol rhodanine derivatives **1-5** inhibited the H-DXR at a micromolar range whereas the phenyl rhodanine acetate **6** was inefficient to inhibit the enzyme. The resorcinol derivative **3** was about 15 – 20 times less efficient that the catechol derivatives (Table 1). These results showed the importance of the presence of the hydroxyl groups and their positioning on the aromatic ring for the recognition by the H-DXR. The catechol rhodanine **1a** and **5** have been reported by Ge *et al.* to inhibit the DXR with a better inhibition in favor of **1a**.[21] This discrepancy may be due to different experimental conditions.

All compounds have limited solubility in aqueous solution and may form aggregates, not visible with the naked eye, capable of inhibiting enzymes as promiscuous inhibitors.[23, 25-27] Such inhibitors, also called aggregating inhibitors, act rather non-specifically. Indeed, the inhibition seems to occur *via* a partial unfolding of the enzymes when they are bound to such aggregates.[28] To discriminate between specific and non-specific inhibition, we compared the inhibition capacity of the compounds in the presence and absence of Triton X100 acting as dispersing agent. The assays were performed at a concentration of 0.01%, which did not modify the activity of H-DXR.[22] For compounds **1** and **2**, no inhibition was observed in the presence of Triton X100 (Table 1). These compounds are probably promiscuous inhibitors, i.e. they self-associate into aggregates that inhibit the enzymes non-specifically. In contrast, with compounds **4** and **5**, the inhibition remained almost unchanged, suggesting that these

compounds were specific inhibitors of the H-DXR. Light scattering analyses of compounds **1a**, **4** and **5** revealed that, at the concentrations used for the inhibition studies (< 20 µM), compounds **4** and **5** did not show detectable aggregates. On the other hand, catechol derivative **1a** seems to form aggregates already at 10 µM (Supporting information Figure S1).

	IC_{50} (µM) or *% of inhibition*	
Compounds	No Triton	Triton
1a	**4.1**	*10* [a]
1b	**3.2**	*< 5* [a]
2a	**3.6**	*< 5* [a]
2b	**3.3**	*< 5* [a]
3	**58.0**	ND
4	**3.8**	**6.1**
5	**3.1**	**4.4**
6	*< 5* [a]	ND
Fosmidomycine	**0.032**[b]	-

Table 1: Influence of 0.01% Triton X100 on the inhibition of H-DXR with rhodanine derivatives

H-DXR was pre-incubated during 2 min in the presence of the different compounds at variable concentration and 480 µM DXP. NADPH (160 µM final concentration) was then added to measure the residual activity. The inhibition studies were conducted with or without 0.01% Triton X100. When feasible, **IC_{50}** were determined, otherwise the *percentages of inhibition* at an inhibitor concentration of 20 µM (indicated by [a]). ND: not determined. (b) See reference 50

2.2. Determination of the inhibition mechanism of compounds 4 and 5

As the compounds may target the coenzyme binding site, we determined the influence of the coenzyme (NADPH) on the inhibition level (Table 2). For H-DXR, even at saturating concentration of NADPH, the protection was only partial. The compounds did not bind into the NADPH binding site.

Compounds	Inhibition (%) No NADPH	Inhibition (%) NADPH 160 µM
1a	38	30
1b	64	41
2a	60	38
2b	63	43
4	70	29
5	61	41

Table 2: Influence of NADPH on the inhibition of H-DXR by rhodanine derivatives

H-DXR was pre-incubated during 2 min with the inhibitors at a 6 µM concentration in the presence or in the absence of NADPH (160 µM).

The equilibrium between the enzyme, its substrate, and its inhibitor that might occur in solution may be expressed in a general way represented in Figure 2.

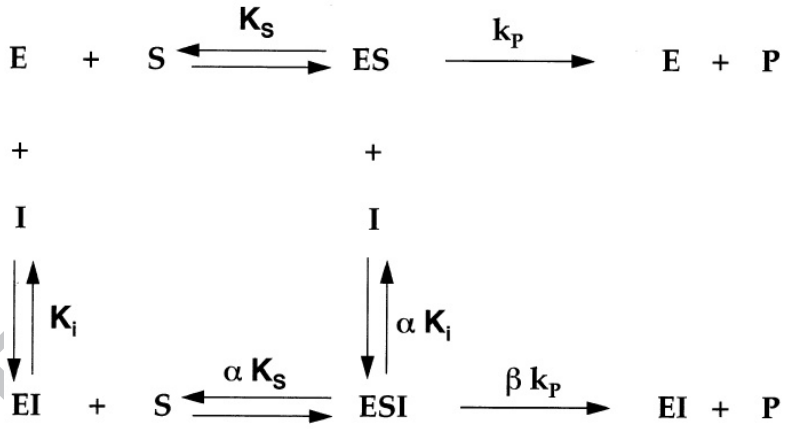

Figure 2: General inhibition mechanism of enzymes

Kinetic studies analyzing the influence of the concentration of the substrate S and of the inhibitor I on the enzymatic activity would give some information on the mechanism of inhibition (competitive, non-competitive, uncompetitive or mixed type). The DXR has two substrates: DXP and NADPH. We investigated only the influence DXP on the inhibition as a study with NADPH is experimentally not feasible owing to its low Km (0.5 µM)[29] and the need of non-saturating concentrations in the assays. These constraints were incompatible with enzymatic rate measurements based on the oxidation of the coenzyme.

With compound **4**, we obtained, in the graphical representation of Lineweaver-Burck, a series of parallel lines with increasing concentration of inhibitor, characteristic for uncompetitive inhibition (Figure 3). This observation was surprising as it supported that DXP is able to bind to DXR in the absence of NADPH. Indeed DXR is described to catalyse the transformation of DXP to MEP according to an ordered Bi Bi mechanism with the coenzyme binding first.[24] Probably in the presence of the two compounds, NADPH binds faster to the enzyme than DXP. According to our results, it is not possible to distinguish between a pure uncompetitive mechanism and a partial mixed-type inhibition, both have a similar inhibition pattern, namely a series of parallel lines in the double reciprocal Lineweaver Burk representation. To discriminate between the two mechanisms, we plotted 1/slope or 1/v-axis intercept *versus* inhibitor concentration.[30] In the first case, pure uncompetitive mechanism, the inhibitor bound only to the binary complex ES with $\beta = 0$ and replots are linear. When partial mixed-type mechanism takes place, the ternary complex ESI is active and the decrease of the enzymatic rate is exactly compensated by a decrease of the K_m ($\alpha = \beta$) and replots are hyperbolic. With **4**, as replots (1/v –axis intercept and slope *versus*) seem to be not linear, a rather mixed-type inhibition mechanism, probably hyperbolic uncompetitive should take place (Supporting information Figure S2). The ternary complex (ESI) is still productive, with $\alpha = \beta$.

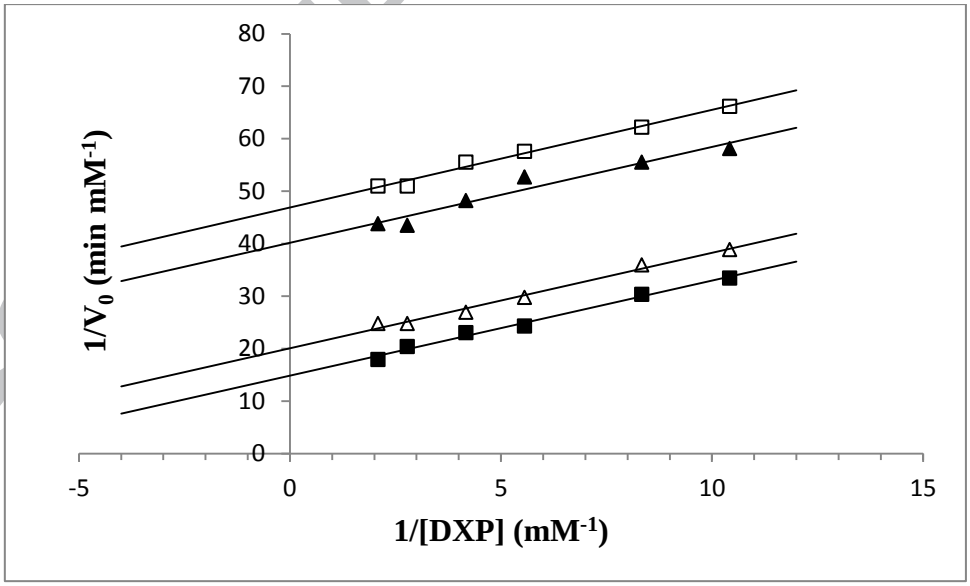

Figure 3 **Inhibition of H-DXR by rhodanine derivative** 4**: double reciprocal plot of initial velocities versus DXP concentrations at fixed concentrations of compound** 4

Reactions were conducted at 37 °C in a 50 mM Tris/HCl buffer pH 7.5 containing 3 mM $MgCl_2$ and 2 mM DTT. H-DXR was pre-incubated during 2 min in the presence of compound (**4**) and DXP. NADPH (160 µM final concentration) was then added to

measure the residual activity. The inhibitor concentrations were 0 (■), 1 (△), 4 (▲), 8 (□) µM. The concentration of DXP was varied between 96 and 480 µM. The initial rates are expressed as mM produced $NADP^+$ per min.

With compounds **5** (Figure 4), the lines intersect below the 1/[S] axis. We are in the presence of a mixed-type inhibition, with a ternary (ESI) complex also productive but, in this case, α and β have different values below 1 (Supporting information Figure S3). The inhibition constants of rhodanine derivative **5** on H-DXR are given in Supplementary information (Table S3).

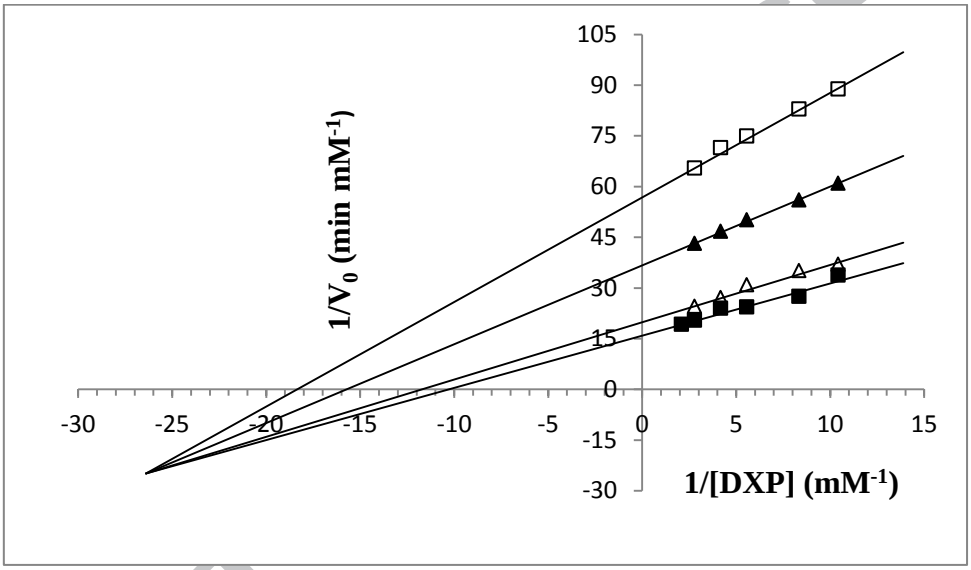

Figure 4 **Inhibition of H-DXR by rhodanine derivative** 5**: double reciprocal plot of initial velocities versus DXP concentrations at fixed concentrations of compound** 5

Reactions were conducted as described in **Figure 3**. The concentrations of **5** were 0 (■), 1 (△), 4 (▲), 8 (□) µM.

Comparison of the kinetics properties of the inhibition of H-DXR with compounds **4** and **5** seem to indicate that, while structurally very close, they did not bind completely in the same manner to the enzyme. As it seems that the complex (DXR, DXP, inhibitor) remained functional, NADPH is able to bind to this complex and to catalyse the production of MEP. This observation suggests that the inhibitors probably did not settle in the NADPH binding site, in agreement with the lack of whole protection by the coenzyme.

2.3. Inhibition of MDH with rhodanine derivatives 1-6

Except compound **6**, all the tested rhodanine derivatives inhibited the MDH nearly in the same extent (Table 3). Even the resorcinol derivative **3** was as potent as the catechol derivatives in the case of MDH. This is the most important difference between the two enzymes as it was much less efficient with H-DXR (IC_{50} = *ca* 3.5 µM *vs* 58 µM). These results showed the importance of the presence of the hydroxyl groups and their positioning on the aromatic ring to be recognized by the enzymes. In the presence of Triton X100, compounds **1** – **3** were inefficient to inhibit MDH. In contrast with H-DXR, compounds **4** and **5** seemed to be less specific inhibitors of MDH since their IC_{50} values, especially for derivative **4**, increased significantly when the surfactant is present in the reaction medium.

Contrary to H-DXR, the coenzyme NADH protected the MDH from the inhibition with the tested rhodanine-derivatives. The compounds could effectively settle into the coenzyme recognition site but the coenzyme could also stabilize the enzyme preventing a possible unfolding by the aggregates.

	IC_{50} (µM) or *% of inhibition*	
Compounds	No Triton	Triton
1a	**0.4**	*< 5* [b]
1b	**0.5**	*< 5* [b]
2a	**1.0**	*< 5* [a]
2b	**0.7**	*< 5* [a]
3	**1.3**	*< 5* [a]
4	**3.4**	*53* [b]
5	**4.7**	**15.0**
6	*< 5* [b]	ND

Table 3: Influence of 0.01% Triton X100 on the inhibition MDH with rhodanine derivatives

MDH was pre-incubated during 4 min in the presence of the different compounds at variable concentration and 480 µM DXP. Oxaloacetate and NADPH, at final concentration of 250 and 160 µM respectively, were then added to measure the residual activity. The inhibition studies were conducted with or without 0.01% Triton X100. When feasible, **IC_{50}**

were determined, otherwise the *percentages of inhibition* at an inhibitor concentration of 10 µM (indicated by [a]) or 50 µM (indicated by [b]) are given. ND: not determined.

2.4. Antimicrobial activity of rhodanine derivatives

Among the tested rhodanine derivatives, only the compound **4** was able to inhibit the growth of *E. coli*. The inhibition power is weak as the deposit of 400 and 800 nanomoles was necessary to observe clear inhibition zones, 14 and 20 mm respectively (Supporting information Figure S4).

3. Discussion

Rhodanine derivatives and especially ylidene rhodanines are privileged scaffold in drug discovery in so far as the heterocyclic nucleus is able to build diverse interactions (hydrogen bonding, hydrophobic and π-stacking interactions) with amino acids located in the active site. The possibility of various chemical derivatizations on the rhodanine ring made such compounds very attractive in the development of enzyme inhibitors.[31-42] Multiple biological activities as anticonvulsants, antibacterials, antivirals, and antidiabetics were ascribed to rhodanine derivatives.[43-45] The most representative example is Epalrestat, an inhibitor of aldose reductase used to prevent or slow down the progression of diabetic neuropathy.[46] Ge *et al.* reported the synthesis of molecules having a catechol-rhodanine scaffold targeting dehydrogenases requiring nicotinamide coenzymes.[21] The two most striking features are the (methylene)-2-thioxothiazolidin-4-one ring in the inhibitors participating in π-stacking interactions with the nicotinamide ring of the NAD(P)H cofactor and the catechol core chelating the metal cation in the DXR active site. Among them, rhodanine **1a** inhibited DXR significantly (IC_{50} = 0.1 µM). Based on these data, we synthesized several rhodanines bearing on the one side a metal cation chelating group and on the other side a group able to settle in the NADPH recognition site and explore their inhibition activity on H-DXR.

Among the studied compounds, only compounds **4** and **5** seem acting as specific inhibitors of H-DXR. Unlike compounds **1**, **2** and **3**, they do not contain a hydrophobic moiety and are accordingly more polar and probably more water-soluble than the former compounds. The partition coefficients (logP) for compounds **4** and **5** in the non-ionized form are 1.8 and 1.0 respectively, compared to *ca*. 5.3 for the other compounds. In the enzymatic assays, at the used concentrations, the rhodanine derivatives **4** and **5**, contrary to compounds **1**-**2**, did not self-assemble into aggregates. As the resorcinol derivative **3** was less efficient that the

catechol derivatives **1**, **2**, **4** and **5** we assumed that the catechol group might play a role in the inhibition of H-DXR. However, no significant difference in the inhibition level between the four catechol-rhodanine derivatives **1** and **2** was observed whatever are the respective positions of the hydroxyl groups (3,4 in compounds **1a** and **1b** or 2,3 in compounds **2a** and **2b**) while a difference between them would be expected when the catechol group is implied in a chelation in the inhibition mechanism. A definitive answer about the ability of a catechol group to coordinate to the di-cation present in the active site of DXR cannot be given. The presence of a hydroxamate in compound **4**, able to interact with the di-cation, while modifying slightly the binding mode, did not result in a better inhibition. Nevertheless, it's the sole compound showing an antimicrobial activity.

We aimed with these compounds a binding in the NADPH recognition site with simultaneous interactions of the catechol group with the cation but this apparently did not take place. The absence of the hydrophobic tail, preventing the formation of aggregates, would not be in favor of a binding in the NADPH recognition site. Compounds **4** and **5** could settle in the hydrophobic site highlighted by Deng *et al.* and situated near the active site.[29]

Our study confirms the necessity to include a detergent in the enzymatic assays when putative hydrophobic inhibitors are tested and especially when the IC_{50}, in the absence of detergent, are in the micromolar range. The recently introduced notion of Pan Assay Interference Compounds or PAINS has to be considered in high throughput screening to avoid false positive inhibitors.[47] Among the substructures to be excluded, rhodanine derivatives and catechols are proposed, but these compounds, owing to their valuable binding properties, are not to be neglected. As recommended by Mendgen *et al.*, particular conditions of assay have to be investigated to be sure that these compounds could be considered as new hits as, for instance, affinity in the nanomolar range and high selectivity.[48] Moreover, one synthetized catechol-rhodanine compound **4** inhibits specifically the DXR and has antimicrobial activity suggesting that further development of such compounds may be of interest.

4. Experimental Section

4.1. General methods

All non-aqueous reactions were run in dry solvents under an argon atmosphere. All reagents and solvents were reagent grade. After extraction, extracts were dried over anhydrous sodium sulfate. Flash chromatography was performed on silica gel 60 230-400 mesh with the solvent system as indicated. TLC plates were revealed by spraying with an ethanolic solution of *p*-

anisaldehyde (2.5 %), sulfuric acid (3.5 %) and acetic acid (1.6 %) or with an ethanolic solution of phosphomolybdic acid (20 %) followed by heating. NMR experiments were recorded on a Bruker AV300 spectrometer. NMR experiments were performed in ($^2H_6$)acetone or ($^2H_6$)DMSO using as an internal standard $CH^2H_2COC^2H_3$ (δ = 2.05 ppm) or $CH^2H_2SOC^2H_3$ (δ = 2.50 ppm) for ^{1}H-NMR, $C^2H_3COC^2H_3$ (δ = 29.9 ppm) or $C^2H_3COC^2H_3$ (δ = 39.5 ppm) for ^{13}C-NMR. Negative or positive-mode electrospray MS were performed on a Bruker Daltonics microTOF spectrometer (Bruker Daltonik GmgH, Bremen, Germany) equipped with an orthogonal electrospray (ESI) interface. Calibration was performed using a solution of 10 mM sodium formate. Sample solutions were introduced into the spectrometer source with a syringe pump (Harvard type 55 1111: Harvard Apparatus Inc., South Natick, MA, USA) with a flow rate of 5 µL.min^{-1}.

NADPH and Tris base were purchased from Sigma. DXP was synthesized according to Meyer *et al.*[49] The cloning and purification of His-tagged DXR (H-DXR) was previously described.[50]

4.2. Synthesis of catechol-rhodanine derivatives 1-6.

4.2.1. General procedure for coupling rhodanine and aromatic aldehyde.

To a solution of aldehyde in acetic acid (3 mL.mmol^{-1}) was added in one portion rhodanine acetic acid (1.1 eq.). Then the reaction mixture was stirred at 100°C overnight and was allowed to cool at room temperature, resulting in the formation of a precipitate. The suspension was filtered, and the solid was washed two fold with cold acetic acid. The crude product was used for the next step without purification.

4.2.1.1. (Z)-2-(5-(3,4-Dimethoxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid. Yellow solid (230 mg, 0.67 mmol, 56 %).^{1}H-NMR (300 MHz, ($^2H_6$)acetone): δ (ppm) = 3.92 (3H, s, O-C*H*$_3$), 3.93 (3H, s, O- C*H*$_3$), 4.88 (2H, s, C*H*$_2$), 7.17 (1H, d, *J* = 8.4 Hz, C*H*$_{Ph}$), 7.22 (1H, d, *J* = 2.4 Hz, C*H*$_{Ph}$), 7.28 (1H, dd, *J* = 2.4 Hz, 8.4 Hz, C*H*$_{Ph}$), 7.78 (1H, s, C*H*). ^{13}C-NMR (75.5 MHz, ($^2H_6$)acetone): δ (ppm) = 45.5, 56.3, 56.4, 113.0, 114.4, 120.2, 126.2, 127.0, 135.1, 150.8, 153.3, 167.5, 167.6, 194.5. MS (EI$^+$) *m/z* calculated for $C_{14}H_{13}NO_5S_2Na$ $[M+Na]^+$: 362.016, found 362.014.

4.2.1.2. (Z)-2-(5-(2,3-Dimethoxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid. Yellow solid (254 mg, 0.75 mmol, 63 %). ^{1}H-NMR (300 MHz, ($^{2}H_6$)acetone): δ (ppm) = 3.89 (3H, s, O- CH_3), 3.91 (3H, s, O- CH_3), 4.88 (2H, s, CH_2), 7.09 (1H, dd, *J* = 3.3 Hz, 6.3 Hz, CH_{Ph}), 7.21-7.27 (2H, m, CH_{Ph}), 8.04 (1H, s, C*H*). ^{13}C-NMR (75.5 MHz, ($^{2}H_6$)acetone): δ (ppm) = 45.5, 56.5, 61.8, 116.8, 121.9, 124.3, 125.7, 127.8, 129.8, 150.0, 154.2, 167.5, 167.6, 195.1. MS (EI$^-$) *m/z* calculated for $C_{14}H_{12}NO_5S_2$ [M-H]$^-$: 338.015, found 338.013.

4.2.1.3. (Z)-2-(5-(2,4-Dimethoxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid. Yellow solid (286 mg, 0.84 mmol, 70 %).^{1}H-NMR (300 MHz, ($^{2}H_6$)acetone): δ (ppm) = 3.91 (3H, s, O- CH_3), 3.98 (3H, s, O- CH_3), 4.87 (2H, s, CH_2), 6.69 (1H, d, *J* = 2.4 Hz, CH_{Ph}), 6.73 (1H, dd, *J* = 2.4 Hz, 8.7 Hz, CH_{Ph}), 7.43 (1H, d, *J* = 8.7 Hz, CH_{Ph}), 8.03 (1H, s, C*H*). ^{13}C-NMR (75.5 MHz, ($^{2}H_6$)acetone): δ (ppm) = 45.4, 56.3, 56.4, 99.4, 107.7, 115.9, 119.5, 130.3, 133.0, 161.6, 165.5, 167.6, 167.8, 195.1. MS (EI$^-$) *m/z* calculated for $C_{14}H_{12}NO_5S_2$ [M-H]$^-$: 338.015, found 338.015.

4.2.1.4. *(*Z)-2-(5-Benzylidene-4-oxo-2-thioxothiazolidin-3-yl)acetic acid 6. Yellow solid (151 mg, 0.54 mmol, 38 %).^{1}H-NMR (300 MHz, ($^{2}H_6$)DMSO): δ (ppm) = 4.74 (2H, s, CH_2), 7.54-7.61 (3H, m, CH_{Ph}), 7.68 (2H, dd, *J* = 2.1 Hz, 8.1 Hz, CH_{Ph}), 7.90 (1H, s, C*H*). ^{13}C-NMR (75.5 MHz, ($^{2}H_6$)DMSO): δ (ppm) = 45.1, 121.9, 129.6, 130.8, 131.2, 132.8, 133.6, 166.4, 167.3, 193.3. MS (EI$^+$) *m/z* calculated for $C_{12}H_9NO_3S_2Na$ [M+Na]$^+$: 301.99, found 301. 99.

4.2.2. General procedure for amide formation and deprotection of the dimethoxy group. A solution of the acid (1 eq.), the amine (1.5 eq.) and 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (2 eq.) in THF (1 mL.0.25 mmol^{-1}) was stirred at room temperature overnight. The reaction mixture was quenched by addition of water and extracted several times with EtOAc. The combined organic layers were dried with anhydrous Na_2SO_4, filtered and evaporated to dryness. The crude product was dissolved in CH_2Cl_2 (3 mL.0.05 mmol^{-1}) and boron tribromide (4 eq.) was added dropwise at room temperature. Then the reaction mixture was stirred for 3 h, water (0.5 mL.0.05 mmol^{-1}) was added, and the reaction mixture was stirred overnight. The two layers were separated. The organic layer was washed with a saturated aqueous NaCl solution, dried, filtered and evaporated to dryness. The orange solid was triturated with CH_2Cl_2 and filtered. The solid was washed several times with cold CH_2Cl_2 and dried to give the desired compound.

4.2.2.1. (Z)-2-(5-(3,4-Dihydroxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)-N-(4-(p-tolyloxy)phenyl)acetamide 1a*:* Greenish solid (16 mg, 32.5 µmol, 70 %). ^{1}H-NMR (300 MHz, ($^{2}H_6$)acetone): δ (ppm) = 2.29 (3H, s, C*H*$_3$), 4.98 (2H, s, C*H*$_2$), 6.85-6.94 (4H, m, C*H*$_{Ph}$), 7.01-7.04 (1H, m, C*H*$_{Ph}$), 7.11-7.17 (4H, m, C*H*$_{Ph}$), 7.60-7.68 (3H, m, C*H*$_{Ph}$), 8.65-8.90 (1H, m, C*H*), 9.65 (1H, br, N*H*). ^{13}C-NMR (75.5 MHz, ($^{2}H_6$)acetone): δ (ppm) = 20.7, 47.6, 117.2, 117.3, 117.9, 119.4, 119.6, 119.8, 121.8, 121.9, 126.2, 126.5, 131.2, 135.1, 149.8, 154.6, 156.3, 163.9, 164.0, 168.0, 194.9. MS (EI$^+$) *m/z* calculated for $C_{25}H_{20}N_2O_5S_2Na$ $[M+Na]^+$: 515.07, found 515.07.

4.2.2.2. (Z)-N-(4-(4-Chlorophenoxy)phenyl)-2-(5-(3,4-dihydroxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)acetamide 1b*:* Dark red solid (18 mg, 35.1 µmol, 64 %). mp: decomposition at 270°C. ^{1}H-NMR (300 MHz, ($^{2}H_6$)acetone 6): δ (ppm) = 4.98 (2H, s, C*H*$_2$), 6.97-7.02 (5H, m, C*H*$_{Ph}$), 7.11-7.16 (2H, m, C*H*$_{Ph}$), 7.34-7.40 (2H, m, C*H*$_{Ph}$), 7.65-7.69 (3H, m, C*H*$_{Ph}$ and C*H*), 9.65 (1H, br, N*H*). ^{13}C-NMR (75.5 MHz, ($^{2}H_6$)acetone): δ (ppm) = 47.6, 117.3, 117.8, 120.5, 120.7, 120.8, 122.0, 126.2, 126.3, 128.3, 130.7, 135.2, 135.9, 147.0, 150.5, 153.4, 157.8, 164.0, 167.9, 194.9. UV-vis (H_2O): λ_{max} = 410 nm. MS (EI$^+$) *m/z* calculated for $C_{24}H_{17}ClN_2O_5S_2Na$ $[M+Na]^+$: 535.02, found 535.01.

4.2.2.3. (Z)-2-(5-(2,3-Dihydroxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)-N-(4-(p-tolyloxy)phenyl)acetamide 2a: Brownish solid (11 mg, 22 µmol, 39 %). mp: decomposition at 270°C. ^{1}H-NMR (300 MHz, ($^{2}H_6$)acetone): δ (ppm) = 2.29 (3H, s, C*H*$_3$), 5.00 (2H, s, C*H*$_2$), 6.86-7.00 (6H, m, C*H*$_{Ph}$), 7.03-7.07 (1H, m, C*H*$_{Ph}$), 7.15-7.18 (2H, m, C*H*$_{Ph}$), 7.61-7.65 (2H, m, C*H*$_{Ph}$), 8.53 (1H, s, C*H*), 9.68 (1H, br, N*H*). ^{13}C-NMR (75.5 MHz, ($^{2}H_6$)acetone): δ (ppm) = 19.8, 46.7, 118.1, 118.4, 118.9, 120.0, 120.2, 120.1, 120.2, 120.9, 129.0, 130.2, 132.2, 134.1, 145.3, 146.2, 153.6, 155.4, 163.0, 167.0, 194.5. UV-vis (H_2O): λ_{max} = 397 nm. MS (EI$^+$) *m/z* calculated for $C_{25}H_{20}N_2O_5S_2Na$ $[M+Na]^+$: 515.07, found 515.07.

4.2.2.4. (Z)-N-(4-(4-Chlorophenoxy)phenyl)-2-(5-(2,3-dihydroxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)acetamide 2b*:* Reddish solid, 21 mg (41 µmol, 44 %). mp: decomposition at 230°C. ^{1}H-NMR (300 MHz, ($^{2}H_6$)acetone): δ (ppm) = 5.02 (2H, s, C*H*$_2$), 6.84-7.13 (7H, m, C*H*$_{Ph}$), 7.33-7.46 (2H, m, C*H*$_{Ph}$), 7.66-7.70 (2H, m, C*H*$_{Ph}$), 8.21 (1H, s, C*H*), 9.61 (1H, br, N*H*). ^{13}C-NMR (75.5 MHz, ($^{2}H_6$)acetone): δ (ppm) = 47.7 (CH_2), 119.0, 120.4, 120.6, 121.0, 121.1, 122.0, 125.3, 125.4, 127.4, 129.9, 130.6, 135.8, 146.3, 147.2,

153.4, 157.7, 164.2, 168.0, 195.4. UV-vis (H_2O): λ_{max} = 405 nm. MS (EI^+) *m/z* calculated for $C_{25}H_{20}N_2O_5S_2Na$ $[M+Na]^+$: 515.07, found 515.07.

4.2.2.5. (Z)-N-(4-(4-Chlorophenoxy)phenyl)-2-(5-(2,4-dihydroxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)acetamide 3*:* Reddish solid (32 mg, 62 µmol, 69 %). mp: 240-242°C. ^{1}H-NMR (300 MHz, ($^2H_6$)acetone): δ (ppm) = 5.01 (2H, s, C*H*$_2$), 6.65-6.69 (2H, m, C*H*$_{Ph}$), 6.97-7.01 (4H, m, C*H*$_{Ph}$), 7.33-7.40 (3H, m, C*H*$_{Ph}$), 7.68-7.71 (2H, m, C*H*$_{Ph}$), 8.15-8.16 (1H, m, C*H*), 9.68 (1H, br, N*H*). ^{13}C-NMR (75.5 MHz, ($^2H_6$)acetone): δ (ppm) = 47.6, 102.3, 103.7, 108.3, 110.0, 114.7, 118.8, 120.5, 128.2, 130.0, 130.4, 132.2, 132.4, 135.9, 153.3, 157.8, 160.6, 164.9, 168.2, 195.2. UV-vis (H_2O): λ_{max} = 430 nm MS (EI^+) *m/z* calculated for $C_{24}H_{17}ClN_2O_5S_2Na$ $[M+Na]^+$: 535.02, found 535.01.

4.2.2.6. (Z)-2-(5-(3,4 -Dihydroxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)-N-hydroxyacetamide 4*:*Yellow solid (6 mg, 18 µmol, 29 %). mp: decomposition at 270°C. ^{1}H-NMR (300 MHz, ($^2H_6$)acetone): δ (ppm) = 4.88 (2H, s, C*H*$_2$), 7.02-7.07 (2H, m, C*H*$_{Ph}$), 7.16 (1H, d, *J* = 1.8 Hz, C*H*$_{Ph}$), 7.64 (1H, s, C*H*). ^{13}C-NMR (75.5 MHz, ($^2H_6$)acetone): δ (ppm) = 46.8, 117.3, 117.9, 119.6, 126.0, 126.3, 135.0, 146.6, 149.7, 167.9, 167.9, 194.8. MS (EI^+) *m/z* calculated for $C_{24}H_{17}ClN_2O_5S_2Na$ $[M+H]^+$: 348.992, found 348.987.

4.2.2.7. (Z)-2-(5-(3,4-Dihydroxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)-N-acetic acid 5: Yellow solid (143 mg, 45 µmol, 76 %).^{1}H-NMR (300 MHz, ($^2H_6$)acetone): δ (ppm) = 4.86 (2H, s, C*H*$_2$), 7.01(1H, d, *J* = 8.3 Hz, C*H*$_{Ph}$), 7.13 (1H, dd, *J* = 2.1 Hz, *J* = 8.3 Hz, C*H*$_{Ph}$), 7.15 (1H, d, *J* = 2.3 Hz, C*H*$_{Ph}$), 7.69 (1H, s, C*H*). ^{13}C-NMR (75.5 MHz, ($^2H_6$)acetone): δ (ppm) = 44.5, 116.3, 116.9, 118.3, 125.3, 125.4, 134.4, 145.6, 148.9, 166.6, 166.7, 193.5. MS (EI^+) *m/z* calculated for $C_{12}H_9NO_5S_2Na$ $[M+Na]^+$: 333.98, found 333.98.

4.3. Determination of the pKa of derivatives 4 and 5.

The UV/visible spectra of compounds **4** and **5** were recorded between 240 and 580 nm at pH varying from 5.4 to 9. The concentration of the compounds was 10 µM. They were dissolved either in a 50 mM Bis-Tris/HCl buffer (pH 5.4 to 7.3) or in a 50 mM Tris/HCl buffer (pH 7.5 to 9). The pH of the solution was increased by addition of small volumes of a 2M NaOH solution.

The spectra depended on the pH of the medium. At low pH the spectra of **4** presented two absorption bands (292 and 410 nm). By increasing the pH, the intensity of the band at 410 nm

decreased with a concomitant formation of a band at 490 nm. Such a phenomenon was observed in the case of **5**.[51] The band at 410 nm was assigned to the neutral form of the catechol moiety, the band at 490 nm to the ionized form of the catechol. The titration in function of the pH gave a pKa of about 7.5 for compound **4**, and about 7.8 for **5** (bands at 406 and 485 nm as found by Ge and Sem).[52] In our assay conditions, the neutral form and the ionized form are accordingly equally present.

4.4. Light scattering analysis of catechol-derivatives.

Light scattering analyses were performed in a Dynapro Nanostar instrument (Wyatt Technology) equipped with a 100 mW laser. The catechol-derivatives **1a**, **4** and **5** solubilized in DMSO were diluted in a 50 mM Tris/HCl buffer pH 7.5 (volume = 500 µL) to final concentrations of 10, 20, 50 and 100 µM. The final concentration of DMSO was 0.4% (v/v). The solutions were centrifuged 5 minutes at 13200 rpm to eliminate eventual solid aggregates. Samples (100 µL) were transferred into disposable cells prior to measurements at 37°C in automated mode. Data were analyzed using manufacturer's software. Buffer with 0.4% DMSO was used as the blank. Mean values were obtained from 10 measurements.

4.5. H-DXR activity.

The assays were performed at 37°C in a 50 mM Tris/HCl buffer pH 7.5 containing 3 mM $MgCl_2$ and 2 mM DTT. The volume was 500 µL. The enzyme was pre-incubated during 2 min with DXP. The enzymatic reaction was initiated by addition of NADPH. The concentrations of DXP and NADPH were 480 µM and 160 µM respectively. The decrease of absorbance at 340 nm due to NADPH oxidation was monitored to determine the initial rates. The retained values were the average of at least two measurements. The relative average deviation must be lower than 4 %.

The protein concentration was determined by the Bradford method using the Bio Rad protein assay and bovine serum albumin as the standard.[53]

4.6. Inhibition of His-tagged DXR.

H-DXR was pre-incubated during 2 min in the presence of the inhibitors at different concentrations and DXP (480 µM). NADPH (160 µM final concentration) was then added to measure the residual activity. The inhibitory potential of the tested compounds was quantified by determining the IC_{50} values. They were obtained by plotting the percentage of residual activity *versus* the Log of inhibitor concentration. The influence of the concentration of Triton X100 (0.01%) on the inhibition capacity of the tested compounds was also performed.

The inhibition mechanism of rhodanine derivatives **4** and **5** was studied by pre-incubating H-DXR with DXP at varied concentrations (96 to 48**0** µM) and at fixed concentrations of inhibitors (1, 4 and 8 µM for **4** and **5**). The enzymatic reaction was initiated by NADPH (final concentration 160 µM).

To test the influence of NADPH on the inhibition, the enzyme was pre-incubated with the inhibitors and NADPH (160 µM) during 2 min. DXP (48**0** µM final concentration) was added to initiate the reaction and determine the residual activity.

4.7. Malate dehydrogenase (MDH) activity.[54,55]

The activity was measured at 37°C in a 50 mM Tris/HCl buffer pH 7.5 by following the reduction of oxaloacetate (OAA) with NADH. The volume was 1 mL. The enzyme was pre-incubated during 4 min in the buffer at 37°C. The enzymatic reaction was initiated by successive addition of OAA and NADH. The final concentrations of OAA and NADH were 250 µM and 160 µM respectively. Initial rates were measured by following the decrease of absorbance at 340 nm due to NADH oxidation with an Uvikon 933 UV-Vis spectrophotometer.

4.8. Inhibition of MDH.

MDH was pre-incubated during 4 min in the presence of the inhibitors at different concentrations. The residual activity was then measured as described above. The assays were carried out at least in duplicate. The compounds have a low solubility in water. Stock solutions were prepared in DMSO. The final concentration of DMSO in the assays was 0.4% (v/v).

The influence of the concentration of NADH on the inhibition was evaluated. The coenzyme was added in the pre-incubation medium at the desired concentration (160 µM). The residual

activity was measured as described above after the addition of oxaloacetate (250 µM final concentration).

The influence of the concentration of Triton X100 on the inhibition was quantified. The detergent was added in the pre-incubation medium at a concentration of 0.01% (w/v). The residual activity was measured as described above.

4.9. Bacterial growth inhibition.

The antimicrobial activity of each inhibitor of H-DXR was tested by the paper disc diffusion method on *Escherichia coli* XL1 Blue.[50] Initially paper discs were impregnated with 400 nanomoles of inhibitors dissolved in DMSO (4 µL). Among the tested compounds, only rhodanine derivative **4** gave a positive result. Variable amounts of the compound (200 to 800 nanomoles) were then tested (Supplementary information Figure S4).

A quantitative study in liquid medium failed because of the formation of red colored, easily detectable aggregates at a concentration of 50 µM.

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