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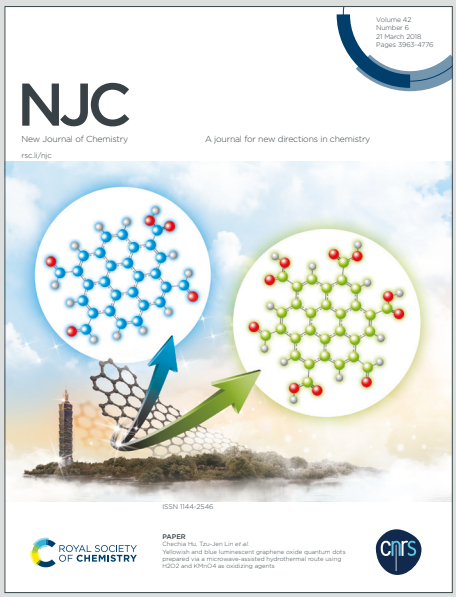

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Bisaurones - Enzymatic Production and Biological Evaluation

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ABSTRACT: The *Trametes versicolor* laccase catalyzed oxidation of chalcone butein afforded four dimers of aurone sulfuretin (*i.e* two regioisomeric pairs of diasteromers, **1** - **4**) as the major products. The formation of the dimers was explained by a two step process involving initial cyclization of butein into aurone sulfuretin, followed by combining of two molecules of sulfuretin. The coupling process occurred between 2,10-double bond of one molecule of sulfuretin and (3′,4′) catechol group of the another to yield dimeric structure. This was confirmed by the experiment involving laccase catalyzed oxidation of sulfuretin yielding the same dimeric bisaurones. Compounds **1**, **3** and **4**, were isolated using semipreparatve HPLC and characterized by detailed analysis of NMR, MS, IR, and UV-Vis data. The structure of compound **2**, isolated as a mixture containing *ca*. 25% of **1** was proposed by comparison of ^{1}H NMR data to **1** and by LC-ESIMS analyses. The starting chalcone butein and products of the biocatalytic transformation, aurone sulfuretin and sulfuretin dimers **1**, **3** and **4** were evaluated for cytotoxic and antioxidative properties *in vitro* using healthy human fibroblasts (MRC5) cell line. Biotransformation products showed lower cytotoxicity but higher antioxidative properties. *C. coggygria* bark methanol extract rich in butein and sulfuretin was also biotransformed by laccase. Transformed extract exhibited significantly improved antioxidative activities.

Keywords: aurones, laccase-catalyzed oxidation, sulfuretin dimers, NMR analysis

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1. Introduction

Aurones belong to the family of flavonoids together with flavones, flavonols, flavanones, flavanols, flavanonols, anthocyanins and chalcones. The name aurone comes from the latin name for gold (aurum) refering to the mostly golden color of aurones due to the planar structure and strong resonance and delocalization of electrons. From all flavonoids only aurones possess a five-membered C-ring and they are the smallest flavonoid group in nature with diverse biological activities,[1,2] antimicrobial,[3] antimalarial,[4] antileishmanial,[5] including antiinflammatory,[6] anti-Alzheimer[7,8] and cytotoxic and anticancer[9] properties. Sulfuretin is an aurone with catechol moiety in the B-ring and 6-OH group in the A-ring. It is a well known constituent of plants of genera *Rhus*[10] and *Cotinus*.[11,12]

In the previous studies aurones, especially sulfuretin, exhibited wide range of biological activities. Shin et. al found that sufuretin has anti-inflammatory properties, as it reduces the production of nitric oxide (NO) and PGE2 induced by lipopolysaccharide (LPS).[13] It was also found, in that study, that C-6 hydroxy-substituted aurones were the more potent inhibitors of PGE2 production, while C-6 methoxy substituted aurones were the more potent inhibitors of NO production. Another anti-inflammatory study of sulfuretin showed that the anti-inflammatory effect of sulfuretin in LPS treated RAW 264.7 macrophages is associated with the suppression of NF-κB transcriptional activity via the inhibitory regulation of IKKβ phosphorylation.[14] Antimutagenic activity of sulfuretin and other flavonoids from *Rhus verniciflua* has been presented in the work of Park et al.[15] Sulfuretin and 2-benzyl-2,3′,4′,6-tetrahydroxybenzo[b]furan-3(2*H*)-one from *Toxicodendron vernicifluum* showed promising antiproliferative activities against human tumour cell lines A549, SK-OV-3, SKMEL-2, and HCT-15 with IC_{50} values of 7.43-26.84 μM. Sulfuretin dose-dependently reduced the production of NO and tumour necrosis factor-a (IC_{50} 23.37 μM).[16] A bioassay guided fractionation (based on the induction of quinone reductase (QR) in cultured Hepa 1c1c7 mouse hepatoma cells to monitor chromatographic fractionation) by Jang and co-workers led to the isolation of six potential cancer chemo-preventive constituents from the seeds of *Dipteryx odorata* (tonka bean). Among these, two of the compounds were 6-hydroxyaurones, analogues of sulfuretin and 6,4′-dihydroxy-3′-methoxyaurone. The mouse mammary organ culture (MMOC) bioassay confirmed these as potential cancer chemo-preventive agents.[17] Reduction of quinones by enzyme quinone reductase (QR), also known as quinone oxidoreductase, is an important detoxification pathway, which prevents oxidative cellular

damages by conversion quinones to hydroquinones. Hence, QR is biomarker in the chemoprevention of cancer.[18]

Laccases belong to the multicopper oxidase family, enzymes that catalyze the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water.[19,20] They can be found in bacteria, fungi, lichens, plants, and even oysters and insects. Their ecophysiological roles are determined by the origin and the stage of life of the organism producing them. In plants they are often involved in the process of lignification,[21] as well as natural product biosynthesis such as justicidin B, matairesinol, and sesamin.[22-24] In organic synthesis, laccases have been employed for the oxidation of a variety of functional groups,[25-29] coupling of phenols and steroids,[30-32] construction of carbon-nitrogen bonds[33] and in the synthesis of complex natural products. The substrate specificity of laccases can be overcome by addition of chemical redox mediators, such as 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS).[34,35]

Recently, new multicopper oxidase from *Thermothelomyces thermophila* capable of biocatalytic transformation of 2′,3,4-trihydroxychalcone to aurone as a sole product has been reported.[36] In order to get more information regarding the use of laccase enzymes in the biotransformation of chalcones to aurones, a study of oxidation of butein using *Trametes versicolor* laccase was undertaken for the first time.

2. Results and Discussion

2.1. Enzymatic oxidation

Laccase catalyzed oxidation of butein was carried out at 50 ^{o}C in NaOAc buffer solution using the procedure given in Experimental section. After 2 h reaction time HPLC analyses of the reaction mixture revealed the expected cyclisation product sulfuretin (t_R = 13.44 min) and unreacted butein (t_R = 18.53 min), as well as additional reaction products eluting at retention times t_R > 25 (six peaks, **1** - **4**) (Figure S1, Supporting Information). After the prolonged reaction time (20 h) the chromatogram mostly contained peaks of **1** - **4** and a trace of sulfuretin, whereas the peak of butein was absent (Figures S2, S3, Supporting Information). LC/ESIMS Analysis indicated the presence of the same peak at *m/z* 537 $[M - H]^-$, corresponding to molecular for compounds **1** - **4**, dimeric products of sulfuretin with molecular formula $C_{30}H_{18}O_{10}$. The proposed dimerisation of sulfuretin was confirmed in the

reaction carried out under the same conditions, using *T. versicolor* laccase and sulfuretin as a substrate, resulting in the same dimers. In this way, oxidative cyclization and coupling of aurones were in accordance with previously reported data for the laccase activity.[19] Additionally, if 4-hydroxy chalcone is used in the reaction with laccase, the formation of aurones is not possible. Then 2,3-dihydrobenzofurans (DHB), well known constituents of lignans, neolignans, alkaloids and isoflavonoids, are expected.[37]

2.2. Structure elucidation

Compounds **1** – **4** were isolated as yellow amorphous substances using semipreparatve reverse phased HPLC. Whereas the samples of **1**, **3** and **4** were sufficiently pure, the sample of the minor product **2** contained *ca.* 25% of the closely eluting **1**. All isolated dimers exhibited very similar spectroscopic data, almost identical ^{1}H and ^{13}C NMR spectra (Table 1) as well as the UV spectra of sulfuretin type.

The full assignment ^{1}H and ^{13}C spectra of **1** - **4** (Table 1) was mostly based on characteristic multiplicities and chemical shifts, as well as the extensive 2D NMR measurements, such as COSY, NOESY, HSQC and HMBC of **1**, **3** and **4** (presented in the Supplementary data). In the ^{1}H NMR spectra of **1** - **4**, two sets of signals were observed, both corresponding to sulfuretin type subunits **S** and **Sa** (see Figures S6 and S7, Supporting Information). The presence of **S** was unambiguously verified by two three proton NMR patterns typical for 6-mono and 3′,4′-oxygenated aromatic rings (A and B, respectively) same as in sulfuretin. The ^{1}H NMR data of A-ring in **S**, *i.e.* H-4 (δ_H 7.61 - 7.63, *d*, J = 8.5 Hz), H-5 (δ_H 6.70 - 6.71, *dd*, J_1 = 8.5 Hz, $J_2 \geq$ 1.5 Hz) and H-7 (δ_H 6.74 - 6.76, *d*, $J \geq$ 1.5 Hz,) were very similar to those in sulfuretin (Figures S6, S8, S16, and S25, Supporting Information). At the same time, all ^{1}H signals of the B ring, *i.e.* H-2′ (δ_H 7.69 - 7.73, *d*, $J \sim$ 1.5 - 2.0 Hz), H-5′ (δ_H 7.08 - 7.11, *d*, J = 8.5 Hz) and H-6′ (δ_H 7.49 - 7.55, *dd*, J_1 = 8.5 Hz, $J_2 \sim$ 1.5 - 2.0 Hz) exhibited downfield shift of $\Delta\delta_H \sim$ 0.2 ppm in comparison with the chemical shift of the same protons in sulfuretin. These downfield shifts suggested connection of the second dimer unit via *ortho* OH groups of the B ring. At the same time, chemical shift of the vinylic H-10 (δ_H 6.76 - 6.77, *s*) was similar to that in sulfuretin. Subunit **Sa** also contained two characteristic sulfuretin type three spin systems, such as H-4a (δ_H 7.27 - 7.34, *d*, J = 8.5 Hz), H-5a (δ_H 6.47 - 6.48, *dd*, J_1 = 8.5 Hz, J_2 = 2.0 Hz) and H-7a (δ_H 6.36 -6.39, *d*, J = 2 Hz) in ring A, and H-2′a (δ_H 6.88 - 6.92, *d*, $J \sim$ 1.5 - 2.0 Hz), H-5′a (δ_H 6.63, *d*, J = 8.5 Hz) and H-6′a (δ_H 6.74 - 6.77, *dd*, J_1 = 8.5 Hz, $J_2 \sim$ 1.5 - 2.0 Hz,) in ring B (Figures S8, S16, and S25, Supporting Information). All signals of the aromatic protons of **Sa** were considerably shifted to the higher

field in comparison with the chemical shift of the same protons in **S**. The biggest upfield shift among them ($\Delta\delta_H$ ~ - 0.8 ppm) exhibited H-2′a and H-6′a. These upfield shifts could be rationalized in terms of the absence of the conjugated 2a,10a-vinyl group. This is also in accordance with a significant upfield shift H-10a (*s*), resonating at δ_H 5.12 - 5.17 ($\Delta\delta_H$ ~ - 1.6 ppm) as well as the the downfield shift of carbonyl carbon C-3a (δ_C 193.7) in **Sa** compared to C-3 (δ_C 184.6) in **S**. This, together with the chemical shifts of the directly attached carbon C-10a (δ_C 78.0 - 78.3) as well as the adjacent C-2a (δ_C 102.1 -102.5) indicated the lack of double bond and the attachment of these carbons to additional oxygens (Figures S9, S10, S11, S17, S18, S19, S26, and S27, Supporting Information). The above evidence is compatible with formation of two dimeric regioisomers **A** and **B** obtained by attachment of **S** and **Sa,** either through O(4′) – C-10a and O(3′) – C-2, or O(3′) – C-10a and O(4′) – C-2 bonds, respectively (Figure 1). The HMBC correlation H-10a/C-3′ observed in **3** and **4** indicated **B** structure for these compounds, whereas the assignment of **A** structure for **1** was based on H-10a/C-4′ correlation (Figures S13, S21, and S30, Supporting Information). Dimeric products **A** and **B** each exist as a pair of diastereomers with respect to the relative configuration at positions C-2a and C-10a of 1,4-benzodixane ring such as **1** and **2** (**A**), an **3** and **4** (**B**). Negative-mode HRESIMS of **1**, **3**, **4** exhibited a $[M - H]^-$ molecular ion peaks at *m/z* 537.0825, 537.0829, 537.0819, respectively, and were consistent with the molecular formula $C_{30}H_{18}O_{10}$. HSQC, COSY and NOESY spectra confirmed proposed structures (Supporting Information).

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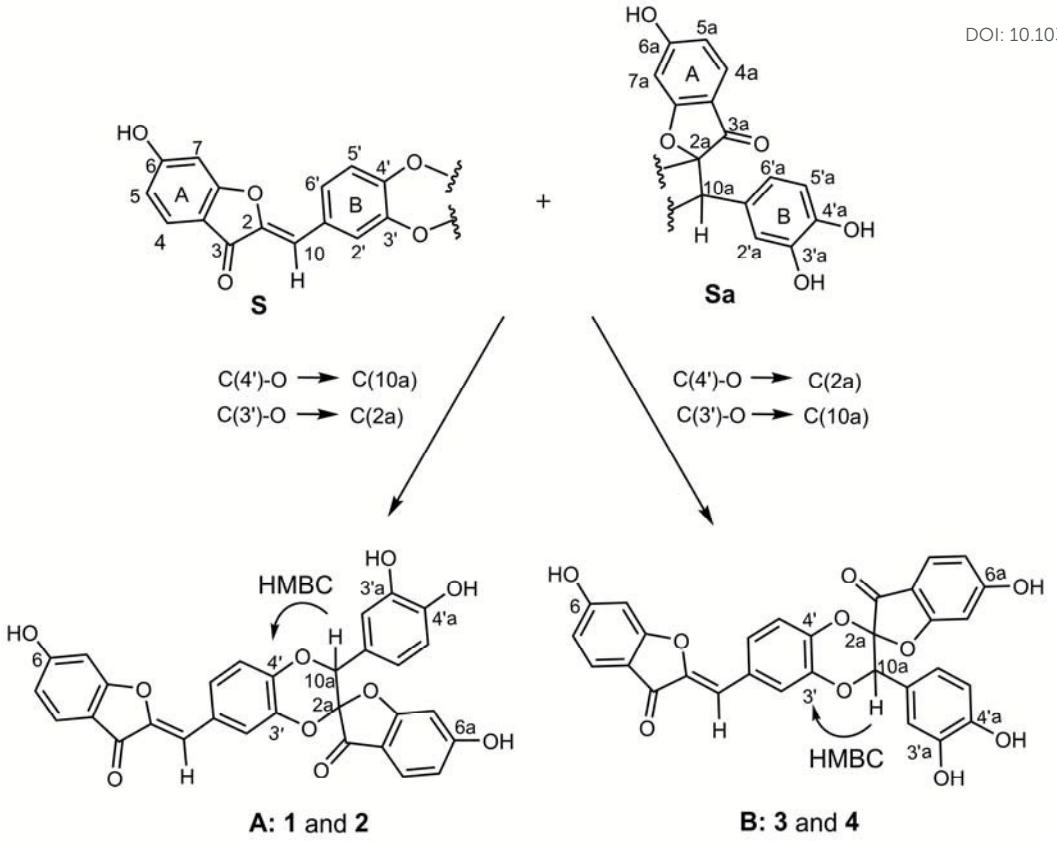

Figure 1. Sulfuretin type subunits **S** and **Sa** identified in dimers **1** – **4**; HMBC correlations H-10a/C-4′ detected in **1**, and H-10a/C-3′ in **3** and **4**; compounds **1** and **2**, as well as **3** and **4** are diasteromeric pairs with respect to the relative configuration at C-2a and C-10a.

The assignment of compound **2** to regioisomer **A** was tentative, due to a small quantity of **2**, contaminated with *ca*. 25% of **1** (Figure S34, Supporting Information). The HMBC measurement was not undertaken. The **A**-type structure, diastereomeric to **1** was the most probable possibility for this dimer and proposed according to the similar HPLC pofiles (peak areas) of **3** and **4**, and according to the similar ^{1}H NMR spectrum to **1**.

It should be noted that the minor differences of the 1D and 2D NMR spectra between diasetereomers **1** and **2**, as well as **3** and **4**, were not sufficient to enable evaluation of the relative stereochemistry at stereogenic centers C-2a and C-10a in these compounds.

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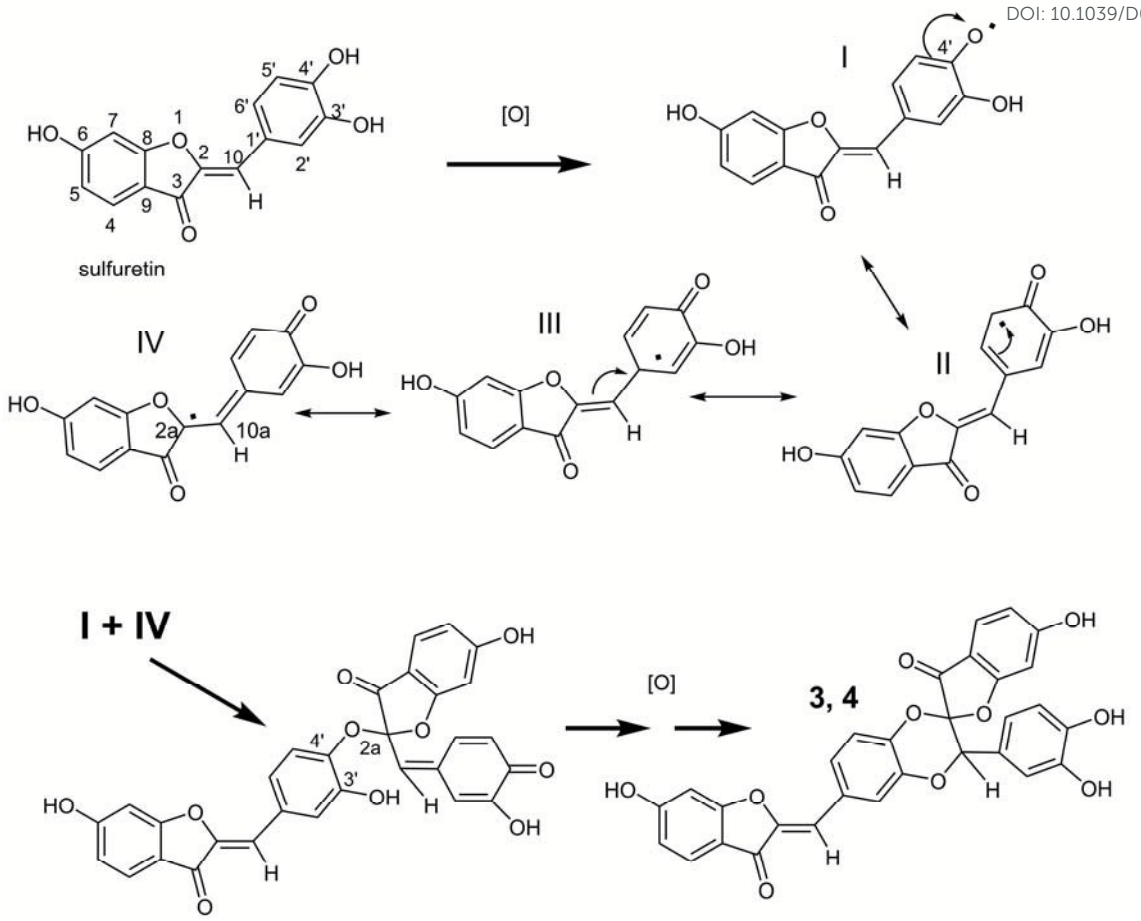

Figure 2. One of the proposed mechanisms of dimers **3** and **4** oxidative formation

One of the proposed mechanisms of dimers **3** and **4** (as the most dominant) formation include: 1) formation of 4′-sulfuretin- and 2a-sulfuretin radicals in the reactions of single-electron oxidations; 2) coupling of two radicals to form intermediate dimer; 3) formation of the 3′-dimer radical; 4) coupling with the 10a-dimer radical (Figure 2). Another possible mechanism implies formation of *ortho*-quinone of one molecule of sulfuretin which reacts with the double bond of another sulfuretin in Diels-Alder reaction of cyclization. Both of these and few additional possible mechanisms are given in the mechanistic study of the biomimetic synthesis of diastereoisomers flavonolignan.[38]

The preposition why **3** and **4** were the most dominant products is that they were produced by the combination of the most stable intermediate radicals I (*para* oxygen radical) and IV (tertiary, stable radical), as presented in Figure 2.

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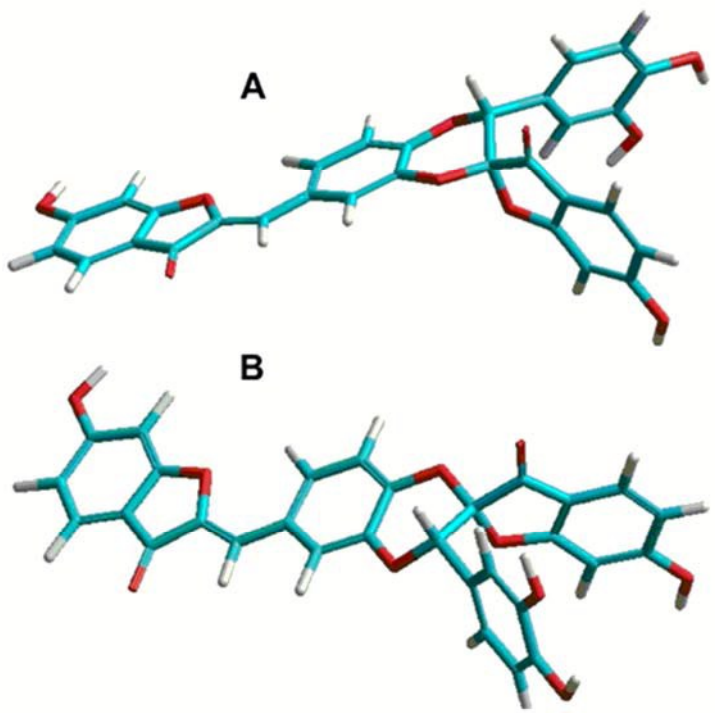

Figure 3. Proposed structures of more stable diastereoisomers of each regioisomer, dimers **1** (A) and **3** (B) (structures obtained for the minimum energy) according to the program HyperChem™ Release 8.0.4 Molecular Modeling System

It is important to emphasize that different products of chalcone transformation depend on the type of enzyme used. If the laccase with high redox potential (Eo) is used, such in the case in this investigation (the Eo of *T. versicolor* laccase is 790 mV), faster cyclization process of butein to sulfuretin is expected and even further oxidative dimerization. In the previous investigation of Zerva and coauthors[36] only the reaction of cyclization of chalcone to aurone occurred and no dimers were found because *T. thermophila* laccase-like multicopper oxidase with the lower Eo (120 mV) was used. In such cases, laccases with the lower redox potential are capable only to form aurone alone with no dimerization.[39] Unlike fungal laccases, over the years it was confirmed that *p*-diphenol oxidases are common in many plant species; however laccases of plant origin have medium redox potential of approximately 400-430 mV.[40]

Since both chalcone butein and aurone sulfuretin have been isolated (in our previous work) in high amounts from the *Cotinus coggygria* methylene chloride-methanol extract (Novakovic et al., 2019) it is assumed that similar oxidase enzyme is present in *C. coggygria*.[12] This is in accordance with the preposition of Maesane and coauthors that rare

aurones could be produced naturally from widely distributed 2′-hydroxychalcones.[41] Additionally, the first laccase was found in 1883 by Joshida in *Rhus vernicifera*[42,43] and genus *Cotinus* is botanically and chemically very similar to genus *Rhus* - few decades ago, genus *Cotinus* with only two species, was segregated from genus *Rhus*. Before that, *C. coggygria* was known as *Rhus cotinus*.[44] Further, in our previous work new auronolignan named cotinignan A was isolated, a conjugate of aurone sulfuretin and lignan sinapyl alcohol. Similar to the present investigation, oxidative coupling of the double bond and catechol group occurred: sulfuretin was catechol group donor but here, sinapyl alcohol served as a double bond donor. This is another potential evidence of existence of laccase like enzyme in the heartwood of *C. coggygria* since laccases are known lignification ezymes.[21] The final confirmation would be an isolation and characterization of this enzyme from *C. coggygria*.

Table 1. ^{1}H and ^{13}C NMR Data of Compounds 1, 3, and 4 Recorded in CD_3OD (500 MHz for ^{1}H and 125 MHz for ^{13}C)[a]

	1		**3**		**4**	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	-	-	-	-	-	-
2	148.8	-	148.9	-	149.1	-
3	184.6	-	184.6	-	184.6	-
4	127.1	7.62 d (8.5)	127.6	7.61 d (8.5)	127.2	7.63 (8.5)
5	114.4	6.70 dd (8.5;1.5)	114.5	6.70 (8.5;1.5)	114.4	6.71 dd (8.5; 2.0)
6	168.8[a]	-	168.8[a]	-	168.8[a]	-
7	99.7	6.74 d (1.5)	99.7	6.74 d (1.5)	99.7	6.76 brs[c]
8	170.3[a]	-	170.3[a]	-	170.3[a]	-
9	114.8	-	114.7	-	114.7	-
10	112.8	6.76 s	112.9	6.76 s	112.5	6.77 s
1'	128.3	-	128.4	-	129.2	-
2'	120.8	7.69 d (2.0)	120.8	7.72 d (1.5)	120.9	7.73 d (2.0)
3'	142.7	-	144.7	-	145.1	-
4'	146.2	-	144.2	-	143.9	-
5'	118.7	7.14 d (8.5)	118.6	7.08 d (8.5)	119.0	7.11 d (8.5)

6'	127.7	7.55 dd (8.5; 2.0)	127.1	7.49 dd (8.5; 1.5)	127.4	7.52 dd (8.5; 2.0)
1a	-	-	-	-	-	-
2a	100.2	-	100.5	-	102.1	-
3a	193.7	-	193.7	-	191.8	-
4a	127.1	7.28 d (8.5)	127.1	7.27 d (8.5)	127.7	7.34 d (8.5)
5a	113.9	6.48 dd (8.5;2.0)	114.0	6.47 dd (8.5;2.0)	114.1	6.48 dd (8.5;2.0)
6a	170.0[b]	-	170.2[b]	-	170.3[a]	-
7a	99.4	6.39 d (2.0)	99.4	6.37 d (2.0)	99.4	6.36 d (2.0)
8a	174.3[b]	-	174.3[b]	-	175.7[b]	-
9a	113.3	-	113.2	-	112.5	-
10a	78.3	5.16 s	78.0	5.12 s	78.3	5.17 s
1'a	125.1	-	125.2	-	125.1	-
2'a	116.3	6.88 d (1.5)	116.3	6.90 d (1.5)	116.5	6.92 d (2.0)
3'a	146.2	-	146.2	-	146.3	-
4'a	147.4	-	147.4	-	147.5	-
5'a	116.0	6.63 d (8.5)	116.0	6.63 d (8.5)	116.0	6.63 d (8.5)
6'a	121.2	6.77 dd (8.5; 1.5)	121.2	6.78 dd (8.5; 1.5)	121.4	6.74 dd (8.5; 2.0)

[a,b] signals could be interchangable, [c] signals overlaped (H-7, H-10 and H-6'a)

2.3. Biological activity evaluation

Cytotoxicity of different compounds used in this study was evaluated against two human cell-lines *in vitro* (Table 2). Methanol extract of *C. coggygria*, with butein and sulfuretin as significant constituents, and the same tretated with laccase were added to check the influence of laccase on the activity of the extract. Butein showed considerable anti-proliferative effect against healthy cell line, while it inhibited proliferation of cancer cell line less efficiently. Oxidation product, sulfuretin, was 3-fold less cytotoxic against both cell lines. Further dimerization resulted in decreased cytotoxicity. The least cytotoxic was dimer **4** with IC_{50} values 13- and 9-folds lower in comparison to that of butein (Table 2). Butein has a long history of traditional use in Japan, Korea and China as an analgesic, antibiotic, antithrombotic, anticancer and anti-inflammatory agent and it is considered modern nutraceutical compound.[45,46] The findings from this study are in-line with previously reported anticancer

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properties of butein against the same cell line (A549) whereby the evidence was provided that butein inhibits cyclooxygenase-2 expression and consequently induces apoptosis in these cells.[47] Observed cytotoxicity against normal cell line is contrary to findings of Chowdhury et al. (2005) who found that butein was poorely active against gingival fibroblasts (HGF)[48] or Khan et al. (2012) who reported no cytotoxicity against normal cell lines.[49] Considerable anti-proliferative effect of the *C. coggygria* bark methanol extract has been observed, showing moderate selectivity towards cancer cell line, while laccase-treated extract was 2-4 fold less cytotoxic, which was in line with the observed activity for pure compounds.

Table 2. The cytotoxicity (IC_{50}, µg/mL) of butein and products obtained by biocatalytic using laccase on two human cell lines determined by the MTT assay

	Cell line	
Compound	**MRC5**	**A549**
butein	7.5[a]	12
sulfuretin	22.5	45
1	75[b]	80
3	55	60
4	100	110
C. coggygria extract	25.5	15
Lacc treated extract	50	60

[a] Results represent mean of two independant experiments done in quadriplicate, with standard deviation between 1-5%.

To evaluate the antioxidant potential of butein and derivatives obtained by laccase biotransformation, their possible cytoprotective effect against the generation of ROS induced by hydrogen peroxide were examined in normal human lung fibroblasts MRC5, using the DCF fluorescent probe assay for determination of intracellular reactive oxygen species levels (Figure 4). Cells were pretreated with H_2O_2 and the compounds were added (all at 5 µg/mL) to assess the ability to rescue cells from the generation of ROS (Figure 4). Their activity was compared to that of commercial antioxidant N-Acetyl Cysteine (NAC) at 30 µM (the concentration commonly used in the antioxidative cell assays).

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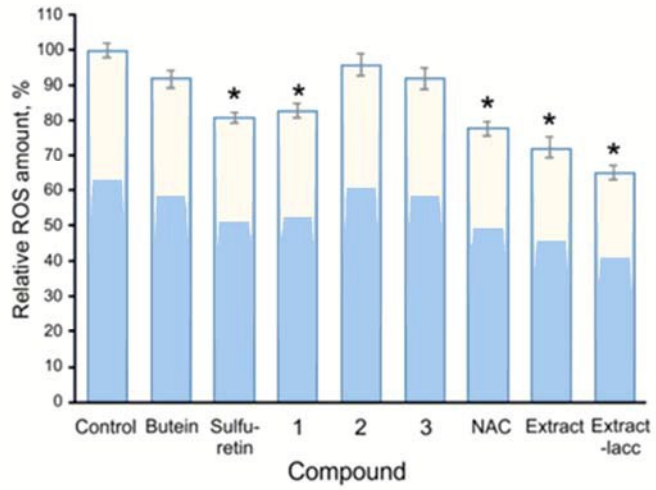

Figure 4. Levels of intracellular ROS species in MRC5 cells determined by DCF fluorescent probe assay in presence of butein and biotransformation derivatives (5 µg/mL). * marks statistical significance in comparison to cells that were not treated with OH-Tyr and derivatives (Control). NAC; 30 µM N-Acetyl Cysteine.

Biotransformation products, sulfuretin and dimer **1** as well as bark extract and laccase treated extract, under tested conditions showed positive effect, with laccase treated extract showing the greatest ability to lower the amounts of ROS by 35% in comparison to the control (Figure 4). Sulfuretin and dimer **1** showed a comparable effect to NAC (reduction of about 20%). Previously, butein was shown to scavange DPPH free radicals at concentration values of 43.28 mg/mL.[50] A mechanism for the antioxidant activity of butein towards the DPPH free radical according to which the hydrogen atom at the 4-hydroxyl position in the structure is first abstracted by the DPPH radical. The subsequent removal of a second hydrogen from the 3-hydroxy position, results in the generation of a *ortho* quinone, a class of compounds well-known for their antioxidant potential.[51] The same mechanism could be proposed for sulfuretin and dimers since all of them posseess catechol moiety (B ring).

3. Conclusion

In summary, the four dimers of aurone sulfuretin were isolated and characterized by detailed analysis of NMR, MS, IR, and UV-Vis data after enzyme-catalyzed oxidation of chalcone butein. The starting chalcone butein and obtaind sulfuretin and its dimers **1**, **3** and **4** were evaluated for cytotoxic and antioxidative properties *in vitro* using healthy human fibroblasts (MRC5) cell line. The results showed that biotransformation products possessed lower

cytotoxicity but higher ability to lower the amounts of ROS by 35% in comparison to the control.

4. Experimental

4. 1. General Experimental Procedures

Optical rotations were measured on a Rudolph Research Analytical Autopol IV automatic polarimeter with methanol as solvent and concentration of compounds 1.00 mg/mL. UV spectra were recorded on a GBC Cintra UV/VIS spectrometer with methanol as solvent in concentration range 4-6 × 10^{-5} M, while IR spectra were recorded on a Thermoscientific Nicolet 6700 FT-IR spectrometer. NMR spectra were measured on a Bruker Avance III 500 spectrometer at 500.26 MHz for ^{1}H and 125.80 MHz for ^{13}C, with CD_3OD as a solvent. HRESIMS data were obtained on Agilent 6210 Time-of-Flight LC/MS system equipped with an ESI interface (ESITOFMS). Solvent was methanol, mobile phase was: 0.2% HCOOH/CH_3CN 1:1, 0.2 mL/min. The ESI was operated in a negative mode and nitrogen was used as the drying gas (12 L/min) and nebulizing gas at 350 °C (45 psi). The OCT RF voltage was set to 250 V and the capillary voltage was set to 4.0 kV. The voltages applied to the fragmentor and skimmer were 140 V and 60 V, respectively. Scanning was performed from 100 to 1500 *m/z*. Semipreparative reversed-phase HPLC separation was performed on an Agilent Instruments 1100 series equipped with a DAD (Agilent Technologies, G1315C) using Zorbax Eclipse XDB C18 column (9.4 mm × 250 mm, 5 μm). Solvents for HPLC separation were of chromatographic grade. For the calculation of minimum energy conformation the program HyperChem^{TM} Release 8.0.4 for Windows Molecular Modeling System, Serial No. 12-800-1501799999 was used.

Pure chalcon butein and aurone sulfuretin were isolated from the *C. coggygria* bark.[52] Both compounds, as well as methanol extract of the plant bark were used for the biocatalytic transformation. *T. versicolor* laccase (1.07 U mg^{-1}) was purchased from Sigma Aldrich (St. Louis, MO, USA).

4.2. Biocatalytic transformation of butein, sulfuretin and Cotinus coggygria bark extract

A 25 mL round-bottom flask was charged with butein (10 mg, 0.037 mmol, 7 mM), which was subsequently dissolved in 0.5 mL of MeOH. After the dissolution, 4 mL of NaOAc buffer (0.1 M, pH 4.5) was added under constant stirring. A yellow suspension was formed

and *T. versicolor* laccase (3 mg, 3.2 U), dissolved in 1 mL of the NaOAc buffer, was added. The flask was fitted to a reflux condenser, and the reaction mixture was stirred for 20-24 h at 50 ºC (the temperature has been chosen as an optimum for the activity of *T. versicolor* laccase and to ensure increased solubility of the substrate). Reaction has been monitored every 2 - 4 h by thin layer chromatography. The control reaction containing no enzyme has been performed under same conditions. The mixture was extracted with EtOAc (3 × 10 mL), the combined organic phases were dried under anhydrous Na_2SO_4, filtered, and the solvent removed under reduced pressure. The mass of the crude product was measured to be 13.9 mg after drying under high vacuum. The components of the obtained product mixture were separated and isolated by semipreparative HPLC.

Following the procedure for the biocatalytic dimerization of butein, sulfuretin (16 mg, 0.06 mmol, 7 mM), as well as crude extract of *C. coggygria* bark (10 mg) was transformed by the addition of *T. versicolor* laccase (5 mg, 5.4 U). The mass of the crude product was measured to be 15.5 mg and 8.7 mg, respectively, after drying under high vacuum.

4.3. Semi-preparative HPLC isolation of bisaurones

From the ethyl-acetate extract of the biocatalytically transformed butein (totally 10 mg was transformed) by laccase sulfuretin and aurone dimers were isolated using the semi-preparative HPLC using 0.2% HCOOH/CH_3CN solvent system and the following program: 0-20 min, 20-37% CH_3CN; 20-21 min, 37-50% CH_3CN; 21-27 min, 50% CH_3CN; and 27-30 min, 50-100% CH_3CN. The wavelengths of 254, 280 and 320 nm were used for the detection. After isolation aurone dimers were kept at 4 ºC and protected from the light. The isolated amounts were: 1.4 mg (**1**), 0.6 mg (**2**), 4.8 mg (**3**), and 1.9 mg (**4**). For the investigation of biological activity all isolated compounds were purified by reversed phase semi-preparative HPLC using the same program and by NMR, and the purity was over 98%.

4.4. Cytotoxicity assay

Cytotoxicity (anti-proliferative activity) of the methanol extract and pure compounds was determined by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.[53] MRC5 and A549 cells (human lung fibroblast and human lung carcinoma, both obtained from ATCC) were treated with butein, sulfuretin, dimers, as well as with *C. coggygria* bark extract untreated and treated with laccase for 48 h as previously described.[52] MTT reduction assay was carried out and cell proliferation was determined from the absorbance at 540 nm on Tecan Infinite 200 Pro multiplate reader (Tecan Group,

Männedorf, Switzerland). The MTT assay was performed two times in quadruplicate and the results were presented as percentage of the control (untreated cells) that was arbitrarily set to 100%.

4.5. Oxidative stress in human cells

Levels of ROS production in human MRC5 cells were assessed as described previously using DCFH-DA (2′,7′-Dichlorodihydrofluorescein diacetate; Sigma-Aldrich) assay.[54,55] Briefly, MRC-5 cells were seeded at a density of 2.5×10^4/well in a 96-well plate in 100 µL of RPMI 1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 U/mL penicillin (all from Sigma, Munich, Germany) and maintained at 37 °C in a humidified atmosphere in the presence of 5% CO_2 in the dark. After 24 h of incubation, medium was removed and the cells were treated with 1 mM H_2O_2 in serum free RPMI, washed and treated with 5 µg/mL of butein and its derivatives and 5 µM DCFH-DA, both prepared in PBS, at the same time, for a period of 1 h. In this way, the ability to rescue from H_2O_2 stress were assessed.

Fluorecsence of ROS in MRC-5 was measured every 5 min for 3 h, on Tecan Infinite 200 Pro multiplate reader (Tecan Group, Männedorf, Switzerland) using excitation and emission wavelengths of 488 and 515 nm, respectively. Percent of ROS production was calculated according to the following equation: the area under the curve (AUC) was calculated as AUC = [R1/2 + sum ($R2:R_{n-1}$) + $R_n/2$] × CT, where R1 is the fluorescence reading at the initiation of the reaction, R_n is the last measurement, and CT is cycle time in minutes. The AUC was obtained by subtracting the AUC of the blank from that of a sample, expressed as net $AUC=AUC_{sample}-AUC_{blank}$. The blank wells contained cells treated with (DCFH2 -DA). The assay was performed two times in quadruplicate and the results were presented as percentage of the control (treated cells with H_2O_2) that was arbitrarily set to 100%.

4.6. Characterization of the isolated compounds

Compound ***1***: yellow, amorphous solid; $[\alpha]_D^{22}$ +7.0 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 231 sh (4.26), 278 (4.21), 329 sh (4.25), 374 (4.36) nm; IR (KBr) ν_{max} 3294, 2927, 2853, 1678, 1604, 1507, 1456, 1376, 1277, 1198, 1154, 1132, 1105, 1054, 841, 812 cm^{-1}; ^1H

NMR and ^{13}C NMR, see Table 1; HRESIMS *m/z* 537.0825 $[M - H]^-$ (calcd for $C_{30}H_{18}O_{10}$ – H, 537.0822).

Compound ***3***: yellow, amorphous solid; $[\alpha]_D^{22}$ 0 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 231 sh (4.21), 255 sh (4.04), 276 (4.16), 327 sh (4.21), 376 (4.40) nm; IR (KBr) ν_{max} 3371, 2980, 2929, 2849, 1686, 1608, 1525, 1502, 1457, 1374, 1278, 1198, 1156, 1133, 1105, 1059, 981, 958, 844, 815 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 1; HRESIMS *m/z* 537.0829 $[M - H]^-$ (calcd for $C_{30}H_{18}O_{10}$ – H, 537.0822).

Compound ***4***: yellow, amorphous solid; $[\alpha]_D^{22}$ -4.0 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 233 sh (4.15), 254 sh (4.03), 278 (4.16), 327 sh (4.27), 367 (4.35) nm; IR (KBr) ν_{max} 3370, 2929, 2851, 1691, 1609, 1502, 1457, 1371, 1299, 1271, 1158, 1109, 1012, 960, 845, 802 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 1; HRESIMS *m/z* 537.0819 $[M - H]^-$ (calcd for $C_{30}H_{18}O_{10}$ – H, 537.0822).

Associated content

Supporting Information: HPLC chromatograms and NMR spectra of the isolated compounds

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Notes

The authors declare no competing financial interest.

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