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Prokaryotic and Eukaryotic Aryl Sulfotransferases: Sulfation of Quercetin and Its Derivatives

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This manuscript is dedicated to Wolf-Dieter "Woody" Fessner on the occasion of his 60th birthday.

Two types of sulfotransferases, namely recombinant rat liver aryl sulfotransferase AstIV and bacterial aryl sulfotransferase from *Desulfitobacterium hafniense*, were used for the sulfation of quercetin, its glycosylated derivatives (isoquercitrin and rutin), and dihydroquercetin ((+)-taxifolin). The rat liver enzyme was able to sulfate only quercetin and taxifolin, whereas the quercetin glycosides remained intact. The *D. hafniense* enzyme sulfated isoquercitrin and rutin selectively at the C-4′ position of the catechol moiety with very good yields. Taxifolin was sulfated at the C-4′ position and a minor amount of the C-3′ isomer was formed. Sulfation of quercetin proceeded preferentially at the C-3′ position, but a lower proportion of the C-4′ isomer was formed as well. A detailed analysis of the kinetics of this reaction is provided and a full structural analysis of all products is presented.

Introduction

Flavonoids are naturally occurring polyphenols, which play a prominent role in the human diet and also in herbal medicines. Flavonoids are generally considered to be potent antioxidants and radical scavengers, but they have a plethora of other biological activities, such as antiallergic, anti-inflammatory, antimicrobial, or anticancer properties. For these reasons, they are now largely used for disease prevention and health improvement in dietary supplements and nutraceuticals.[1,2]

Flavonols are a major class of flavonoids in the human diet and, among them, quercetin[3] and its glycosides, rutin[4] and isoquercitrin,[5] are largely used in dietary supplements; therefore, they are in the focus of investigators. Taxifolin, also denoted as dihydroquercetin,[6] is also used in nutraceuticals, although not as frequently as quercetin.

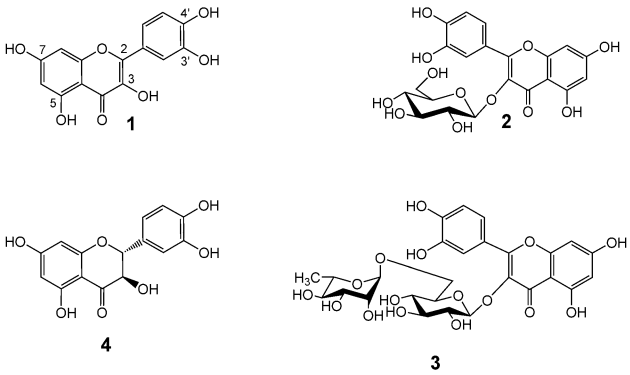

Figure 1. Quercetin (**1**), isoquercitrin (**2**), rutin (**3**), and taxifolin (**4**).

Quercetin (**1**; Figure 1) acts as a strong reducing agent to protect body tissues against oxidative stress and prevents the organisms from various diseases associated with oxidative and radical-mediated misbalances, such as cancer, cardiovascular diseases, or inflammation.[7–10] In nature, it often occurs in the form of glycoconjugates, for example, quercetin 3-*O*-β-D-glucopyranoside (**2**, isoquercitrin) or rutin (**3**, quercetin 3-*O*-rutinoside).

Rutin can be found in consumable parts of plants like apples, onions, berries, tea, wine,[11] red beans, or buckwheat (*Fagopyrum esculentum*);[12,13] it is also typically produced from the Brazilian tree Fava d'anta (*Dimorphandra mollis*). Rutin is widely utilized in the pharmaceutical, nutraceutical, and cosmetic industries as a stabilizer, preserver, and natural coloring agent.[14] The majority of its biological activities (vascular endo-

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thelium protection, anticancer, anti-inflammation, antiasthma, and antimicrobial) are considered to originate from the antioxidant and antiradical properties of rutin.[15] Isoquercitrin is widely distributed as well, but it could not until recently be prepared in sufficient amounts as a result of its low content in plant material.[16] Hence, a new and efficient method for the production of highly pure isoquercitrin (>95%) from rutin by the alkali-tolerant α-L-rhamnosidase from *Aspergillus terreus* was developed.[17–19] Isoquercitrin is a very efficient chemoprotective agent in both in vivo and in vitro experiments against cancer, allergy, cardiovascular diseases, diabetes, and oxidative stress and it displays better bioavailability than rutin and quercetin.[5]

(+)-Taxifolin, isolated from the bark of *Pinus roxburhii, Larix sibirica*, or *Taxus chinensis* var. *mairei*, plays a special role in keeping normal function of the circulatory system, improves immunity, and reduces cancer cell formation.[6]

During the last two decades, flavonoids have become popular as food adjuvants or dietary supplements and their daily intake has increased. As a result of this, their pharmacokinetics and metabolism have been extensively studied.[20,21] Flavonoids are preferably sulfated, glucuronidated, or methylated by sulfotransferases, uridine diphosphate glucuronyl transferases, *N*-acetyl transferases, glutathione *S*-transferases, thiopurin *S*-methyl transferases, and *O*-methyl transferases.[22] Metabolites of phase II of the biotransformation of polyphenols are often sulfates. Sulfation of xenobiotics and eobiotics usually leads to lower toxicity and improved elimination. However, sulfated small molecules could also be of value in therapeutics because of their hydrophilic nature and improved bioavailability. Sulfated quercetin derivatives are also important as authentic standards for metabolic studies. Besides this, some sulfated flavonols, including quercetin and rutin, possess anticoagulant or antiaggregant activity and also substantial antiviral activity.[23] Quercetin sulfates were shown to display some lipid peroxidation inhibitory effect[24] and antiradical activity (in the 2,2-diphenyl-1-picrylhydrazyl assay).[25] Quercetin 3′-*O*-sulfate demonstrated not only inhibition of cyclooxygenase-2 activity but also reduction of the expression of cyclooxygenase-2 messenger RNA.[26,27] Other interesting biological activities (antimicrobial, anti-inflammatory, anti-HIV, antitumor, etc.) of various quercetin sulfates and their derivatives were described in a recent review.[23]

The exact identification of the specific metabolites strongly depends on the analytical procedure used. Only HPLC–MS or MS analyses have been utilized so far for the conjugated-product characterization in the case of quercetin, rutin, and taxifolin. Up to now, no detailed identification of isoquercitrin metabolites (conjugates) has been accomplished.[28–30]

The preparation of authentic, pure, and fully structurally characterized metabolites of polyphenols is of utmost importance for the proper determination of their pharmacological profiles. Commonly used MS techniques seldom provide structural information that allows the determination of the exact structure. This is mainly important for polyphenols, for which regioisomers are usually hard to distinguish. NMR spectroscopy is the only reliable method (besides X-ray spectroscopy) to determine the site(s) of sulfation. However, even here, the task is not trivial because the place of sulfate substitution can be determined only indirectly (no couplings available) from the shifts of the carbon atoms in the aromatic ring. In the literature, there are numerous accounts of controversies concerning the distinguishing and proper assignment of 3′- and 4′-quercetin sulfates (at the catechol moiety). In this work, we have performed a rather complex structural study that has led to the unequivocal determination of the specific structures and possible re-evaluation of former studies.

We have recently developed preparatory methods for the chemoenzymatic synthesis of sulfated flavonoids[31] with two types of aryl sulfotransferases (ASTs). We used recombinant mammalian sulfotransferase IV from rat liver (EC 2.8.2.1) to catalyze the transfer of a sulfate group from phenolic sulfate esters to a phenolic acceptor substrate by employing the 3′-phosphoadenosine-5′-phosphosulfate (PAPS) cofactor system.[32,33] Fundamental problems, such as optimization of recombinant expression, poor stability of this enzyme, and PAPS regeneration, were addressed in detail in our study. The use of the mammalian liver enzyme should also lead to authentic mammalian metabolites, that is, the proper regioisomers.

Another sulfotransferase, namely the bacterial aryl sulfotransferase from *Desulfitobacterium hafniense,* previously proved to be suitable for the preparatory syntheses of certain aryl sulfates and its regioselectivity seems to be rather close to the mammalian enzyme.[31,34] In this case, a cheap sulfate donor, for example, *p*-nitrophenylsulfate (*p*-NPS), can be used.

We report herein the fully characterized sulfated flavonoids quercetin, taxifolin, isoquercitrin, and rutin, which were prepared by the bacterial aryl sulfotransferase from *D. hafniense*. Sulfated molecules prepared in this way can be considered as the phase II metabolites if the equivalent mammalian (preferably liver) enzyme is used. We have compared these sulfated products with the products obtained from aryl sulfotransferase IV from rat liver. A detailed NMR study supports the structure elucidations.

Results and Discussion

Polyphenolic substances are easy targets for conjugation reactions and these are the major biotransformation pathways leading to their excretion.[20] Quercetin is methylated, sulfated, and glucuronidated to form its major human metabolites: 3′-*O*-methylquercetin (isorhamnetin), quercetin 3-*O*-glucuronide, quercetin 3′-*O*-sulfate, and 3′-*O*-methylquercetin-3-*O*-glucuronide.[35,36] Isoquercitrin and rutin are deglycosylated to quercetin, conjugated and then methylated. There are, however, some indications that isoquercitrin can be absorbed intact and it could thus enter the circulation and undergo appropriate biotransformation reactions.[5]

Sulfation with aryl sulfotransferase AstIV from rat liver

Enzyme-activity optimization was tested with chaperone G7 (GroEL/GroES) coexpression in the strains BL21(DE3)Gold,

BL21(DE3)plyS, and Origami (DE3). However, no significant improvement of the enzyme activity and stability was reached. Therefore, we have followed our procedure[31] with whole-cell *Escherichia coli* BL21(DE3)Gold transformed with AstIV.

We tested the sulfation of all four quercetin derivatives with aryl sulfotransferase AstIV from rat liver, expressed recombinantly in *E. coli*, with the aim of verifying whether compounds **1**–**4** are substrates for this mammalian aryl sulfotransferase; if the results were positive, we wanted to isolate the respective sulfates, which should be identical to the authentic mammalian metabolites.

The sulfation reaction was tested with AstIV from rat liver by following a recently published procedure.[31] We have employed a modified semipreparatory sulfation method with transformed living *E. coli* cells expressing AstIV. This method overcame two major problems involved in the use of the isolated enzyme: 1) poor isolation yields and low stability of the pure enzyme and 2) the need for the expensive and labile cofactor 3′-phosphoadenosine-5′-phosphosulfate (PAPS), which is intrinsically present in the *E. coli* cells. AstIV is able to catalyze the parallel regeneration of PAPS by using *p*-NPS as a sulfate donor (see Figure 6).

Taxifolin was the only derivative that was accepted by AstIV as a substrate and the sulfated products were obtained. Isolated taxifolin monosulfate, tentatively assigned to be taxifolin 3′-*O*-sulfate (**4b**; see Figure 2 and 6), was characterized only by MS as a result of its paucity (1 mg). We proposed the structure of **4b** because another isolated product was identified (with MS and NMR spectroscopy) to be quercetin 3′-*O*-sulfate (**1b**), which is a presumed product of **4b** oxidation. Taxifolin is very sensitive to air oxidation at neutral and alkaline pH values (reaction at pH 7.5). Herein, we used metabolically active *E. coli* cells with preserved respiration involving cytochromes (and other redox enzymes), which may also cause enzymatic oxidation of taxifolin.

To avoid the oxidation potentially caused by cytochromes in the whole-cell catalyst, the respiration inhibitor sodium azide (1 mM) was added to the reaction mixture. We observed that the sulfation reaction was not affected by respiratory inhibition by the azide and both products were still formed. We could not distinguish whether the oxidation of the taxifolin skeleton occurred in the form of intact taxifolin or in the form of its sulfate. However, by taking into account sulfation experiments with pure quercetin, which gave no isolable product (see below), we assume that taxifolin was the primary substrate for the sulfation and only the sulfated product underwent oxidation to the respective quercetin sulfate. Sulfation at the 3′-position of the catechol moiety of quercetin and clearly also taxifolin seems to be typical for mammalian sulfotransferases because quercetin 3′-*O*-sulfate was isolated as a urinary metabolite, for example, in rats.[37]

If quercetin was tested as the substrate in the AstIV-catalyzed reaction, consumption of *p*-NPS accompanied by the formation of *p*-nitrophenol (*p*-NP) gave a clear indication that the sulfation reaction had occurred. However, despite our endeavors, no sulfated product was isolated. We assume that substrate **1** was probably sulfated but then was subsequently metabolized by the cells; hence, the sulfation reaction could only be proved indirectly.

Table 1. Rat liver AstIV substrate specificity towards quercetin derivatives.

Substance	Formation of *p*-NP	Isolated product(s)	Product structure
taxifolin	●[a]	●	taxifolin 3′-*O*-sulfate; quercetin 3′-*O*-sulfate
quercetin	●	not found	–
isoquercitrin	–[a]	–	–
rutin	–	–	–

[a] – indicates that this compound was not a substrate for AstIV; ● indicates isolated sulfated product(s) and/or the release of *p*-NP.

The quercetin glycosides rutin (**3**) and isoquercitrin (**2**) were not substrates for AstIV because no formation of *p*-NP was observed and also no sulfated products were identified (Table 1).

Sulfation with aryl sulfotransferase from *D. hafniense*

AstIV from rat liver indicated well the substrate specificity of the mammalian enzyme for phase II biotransformation. However, this did not afford preparatory amounts of the various metabolites. To overcome this, we used aryl sulfotransferase from *D. hafniense*, which is more stable, available in larger quantities, and has broader substrate specificity.

We performed a series of new transformations aimed at increasing the specific activity of the enzyme. The best results were obtained with BL21(DE3)Gold *E. coli* and yielded a specific activity of 28500 U mg^{-1}, which is approximately two orders of magnitude higher than the specific activity reported previously.[34] We found that, because of the high enzyme stability and lack of sulfatase activity, the purification of the protein, as in the work by Hartog and co-workers.[34], was not required. Therefore, we used a mere lysate of the transformed *E. coli*, which proved to be even better than the purified enzyme. As a result of the considerably higher activity of the AST used, we were able to substantially shorten (by approximately 20 times) the reactions times relative to those in the previous studies.[34]

With AST from *D. hafniense*, we were able to obtain sulfated products (Figure 2) in very good yields and in large amounts (Table 2). This enabled a detailed structural analysis of the products and also further biological tests.

Table 2. Analytical yields of sulfated products prepared by AST from *D. hafniense*.

Products[a]	Yield [%]
quercetin 3′-*O*-sulfate (**1b**) + quercetin 4′-*O*-sulfate (**1a**) (75:25)	47
taxifolin 4′-*O*-sulfate (**4a**) + taxifolin 3′-*O*-sulfate (**4b**) (80:20)	75
isoquercitrin 4′-O-sulfate (**2a**)	69
rutin 4′-O-sulfate (**3a**)	53

[a] Full details of the procedures for the sulfation of quercetin, taxifolin, isoquercitrin, and rutin with AST from *D. hafniense* are given in appropriate parts of the Experimental Section.

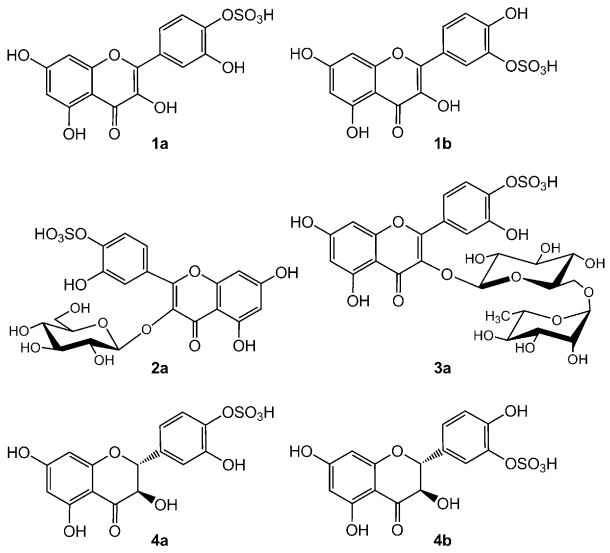

Figure 2. Isolated products sulfated by bacterial AST from *D. hafniense*.

For the preparatory reactions, we faced complex separation problems. The large amounts of *p*-nitrophenol that were formed complicated substantially the separations and the products were often contaminated by this toxic compound. To overcome this, we tried to replace the sulfate donor *p*-NPS with the alternative methyl sulfate, but unfortunately no reaction was observed.

We investigated the possibilities of bulk *p*-NP removal. We found that, in a very narrow pH range of 7.7–7.5, *p*-NP can be selectively removed by extraction with ethyl acetate, whereas the products and unused *p*-NPS remain in the aqueous phase. This pretreated mixture could then be separated by gel chromatography. Preparatory HPLC with an Asahipak column was also employed but was found to be less efficient; it provided lower yields and very poor purity of sulfated products (less than 50%), even after repeated injections. The separation by employing Sephadex LH-20 medium was found to be a convenient method that gave very pure products (>95%) in high yields (Table 2). There is only one slight disadvantage of this method, in that it is rather time demanding. Typically two to seven days were required for complete product purification. Isolated sulfated compounds **1a**–**4b** were fully characterized by MS and NMR spectroscopy analyses (see below and the Supporting Information).

At the pH value used (pH 8.9, as well as pH 7.0), taxifolin (**4**) is very sensitive to oxidation, which leads to the generation of the dehydro derivative (quercetin) and its sulfates. We observed that alkaline pH values and the presence of air oxygen led to the formation of the oxidized products. If the AST from *D. hafniense* is employed in the enzymatic reaction, the oxidation can be avoided by using a strictly inert atmosphere (N_2) and short reaction times. Unfortunately, this procedure cannot be used in the case of the whole-cell biotransformation. To avoid oxidation, the pure enzyme or crude lysate was applied under the inert atmosphere, but the requirement of the PAP(S) cofactor system and instability of AstIV limited the preparative reaction.

Table 3. Comparison of the two AST reactions and the catalyst properties.

Properties	AstIV rat liver	AST *D. hafniense*
PAP/PAPS	yes	no
catalyst	whole cells	lysate
optimum pH value	7	9
optimum temperature [°C]	37	30
reaction time	3 d	4 h
cosolvent	DMSO	acetone
typical yields	0–25% (10 mg)	50–80% (100 mg)
catalyst efficiency [mL of Luria–Bertani media for 1 mg of product]	450	0.1

Table 3 compares the reaction conditions, yields, and efficiency of the two enzymes studied. AstIV from rat liver is not a suitable catalyst for preparatory purposes because the reaction is time demanding, proceeds with low yields and poor efficiency, and causes substrate/product decomposition. On the other hand, this reaction provides important information on the authentic mammalian sulfated metabolites and the acceptance of substrates by this phase II biotransformation enzyme.

AST from *D. hafniense* was identified to be a suitable tool for the sulfation reaction; it is a highly efficient and highly yielding enzyme. Moreover, it was also found that it produces the same sulfated derivatives as the mammalian enzyme, which makes this enzyme applicable for production on a large scale.

Structure identification of the sulfated metabolites

Determination of the exact site of sulfation on the polyphenols is a fundamental issue; however, it is a very intriguing problem. Mass spectrometry can give only a limited amount of information, typically the number of the sulfates in the molecule. NMR spectroscopy does not allow a direct proof of the sulfation site because the sulfate group is not detectable with ^{1}H and ^{13}C NMR spectroscopy. Its position can be determined only indirectly through changes in the chemical shifts of attached and neighboring atoms. This situation is particularly complicated in the case of the catechol moiety that occurs in all of the substrates used. Unfortunately, in some literature sources, there are dubious interpretations of NMR data.[38]

Herein, we are reporting for the first time the complete NMR data of the sulfated compounds. The assignment of individual proton spin systems was accomplished by COSY and then transferred to the carbon atoms by HSQC analysis. HMBC experiments enabled us to assign quaternary carbon atoms and to link partial structures together. Quaternary carbon atoms in the catechol moiety showed the same HMBC coupling patterns in all samples under study (Figure 3). It is clear that the C-3′ and C-4′ carbon

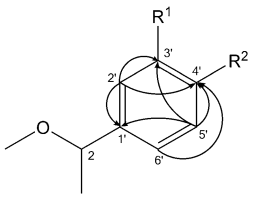

Figure 3. Diagnostic HMBC correlations in the catechol moiety of quercetin derivatives.

atoms can be differentiated by using a diagnostic correlation between the C-4′ carbon atom and the H-6′ proton; thus, all carbon atoms in the catechol moiety can be unequivocally assigned.

The fundamental question was to determine the position of the sulfate group(s). The 3′-OH and 4′-OH hydroxy groups resonate in the sulfates as broad singlets that give no correlation in the HMBC spectrum. Our novel approach for direct and unambiguous assignment was based on methylation of the isoquercitrin sulfate **2a**. The broad signal of the hydroxy group was replaced by an intensive methoxyl singlet that gave clear correlations with the C-2′ and C-3′ carbon atoms in the HMBC spectrum. The position of the methoxyl group in 5,7,3′-tri-*O*-methyl-4′-*O*-sulfo-3-*O*-β-D-glucopyranosyl quercetin (**5a**, prepared from **2a** by diazomethane methylation) was therefore directly assigned at the C-3′ carbon atom, which implied sulfate attachment at the C-4′ position. The parent isoquercitrin sulfate **2a** was thus definitely confirmed as the 4′-*O*-sulfate.

The ^{13}C NMR data of isoquercitrin 4′-*O*-sulfate (**2a**) were then compared with those of the mixture of two quercetin monosulfates (**1a** and **1b**). The chemical shifts of the minor component correspond to quercetin 4′-*O*-sulfate, whereas the major component agrees with the previously published quercetin 3′-*O*-sulfate (Table 4).[38] The mixture of quercetin sulfates **1a** and **1b** served also as a reference for the structure elucidation of the taxifolin and rutin sulfates (**4a**, **4b**, and **3a**; Tables 4 and 5). This simple and unequivocal method can be thus applied with certain care to the structural analysis of analogous compounds carrying a monosulfated catechol moiety.

Table 4. ^{13}C NMR data of flavonoid sulfates in CD_3OD (150.95 MHz, T = 25 °C).[a]

	4′-*O*-Sulfates [ppm]				3′-*O*-Sulfates [ppm]	
Atom	**5a**	**2a**	**1a**	**3a**	**1b**[38]	**1b**
2	156.22	158.46	146.97	158.65	147.0	147.45
3	138.65	136.61	138.56	136.50	137.6	137.80
4	175.95	179.95	177.88	179.85	177.4	177.70
5	162.37	163.42	162.81	163.37	162.5	162.74
6	97.61	100.33	99.68	100.43	99.3	99.64
7	166.85	166.60	166.13	166.68	165.7	165.94
8	94.18	95.10	94.79	95.25	94.4	94.82
9	160.70	158.91	158.62	158.96	158.2	158.51
10	109.88	106.20	104.92	106.11	104.5	104.86
1′	128.84	129.46	130.38	129.42	124.3	124.59
2′	115.76	119.62	118.00	119.81	123.8	124.07
3′	152.63	150.28	150.53	150.17	141.3	141.63
4′	145.36	143.98	143.04	144.01	152.6	152.86
5′	122.99	122.54	123.86	122.65	118.2	118.52
6′	122.67	123.56	121.00	123.48	127.4	127.72

[a] Data from carbohydrate moieties not given (for full data, see Tables S5 and S7 in the Supporting Information).

The comparison of the carbon chemical shifts of different flavonoids with their sulfates revealed several characteristic changes in the catechol moiety. All 4′-*O*-sulfates display a downfield shift for the C-3′ carbon signal and an upfield shift for the C-4′ signal relative to those for the parent compound; by contrast, an upfield shift of the C-3′ carbon signal and a downfield shift for the C-4′ signal were detected in the 3′-*O*-sulfates (Table 6).

Table 5. ^{13}C NMR data of flavonoid sulfates in [D_6]DMSO (150.95 MHz, T = 30 °C).

	4′-*O*-Sulfates [ppm]		3′-*O*-Sulfates [ppm]	
Atom	**1a**	**4a**	**1b**	**4b**
2	145.91	82.67	146.17	82.67
3	136.66	71.51	136.06	71.51
4	176.16	197.47	175.98	197.47
5	160.78	163.37	160.78	163.37
6	98.34	96.21	98.30	96.21
7	164.24	167.27	164.09	167.27
8	93.56	95.17	93.49	95.17
9	156.34	162.46	156.20	162.46
10	103.16	100.38	103.08	100.38
1′	126.89	133.89	122.29	133.89
2′	116.33	116.93	122.67	116.93
3′	148.55	148.87	140.88	148.87
4′	142.74	141.25	151.25	141.25
5′	122.12	122.65	117.26	122.65
6′	119.19	119.24	124.97	119.24

Table 6. Relative changes in the ^{13}C NMR chemical shifts in the catechol moieties, expressed as δ_C (sulfate)−δ_C (parent compound).

	3′-*O*-Sulfates [ppm]		4′-*O*-Sulfates [ppm]			
Atom	**1b**[a]	**4b**[a]	**1a**[a]	**4a**[a]	**3a**[b]	**2a**[b]
1′	0.28	0.36	4.88	5.80	6.02	6.11
2′	7.54	7.70	1.20	1.53	1.81	1.77
3′	−4.21	−4.40	3.46	3.88	4.08	4.09
4′	3.51	3.89	−5.00	−4.56	−6.06	−6.17
5′	−2.75	1.75	2.11	7.47	6.31	6.24
6′	9.33	5.51	3.55	−0.18	−0.40	0.07

[a] In [D_6]DMSO at T = 30 °C. [b] In CD_3OD at T = 25 °C.

Table 7. Relative changes in the ^{1}H NMR chemical shifts in the catechol moiety, expressed as δ_H (sulfate)−δ_H (parent compound).

	3′-*O*-Sulfates [ppm]		4′-*O*-Sulfates [ppm]			
Atom	**1b**[a]	**4b**[a]	**1a**[a]	**4a**[a]	**3a**[b]	**2a**[b]
2′	0.352	0.398	−0.001	0.100	0.104	0.029
5′	0.094	0.104	0.495	0.414	0.587	0.587
6′	0.308	0.367	0.037	0.144	0.035	0.049

[a] in [D_6]DMSO at T = 30 °C. [b] In CD_3OD at T = 25 °C.

Although the proton NMR spectra of individual flavonoids were not directly comparable, the common diagnostic features for their 3′- and 4′-*O*-sulfates were also observed. Regioisomers can be differentiated with downfield-shifted H-2′ and H-6′ proton signals in 3′-*O*-sulfates and a downfield-shifted H-5′ proton signal in 4′-*O*-sulfates (Table 7).

The proton chemical shifts of both quercetin 3′- and 4′-*O*-sulfates (Figure 4) are in accordance with the proton spectrum published by Jones et al.[37]

Our assignment of quercetin 4′-*O*-sulfate (**1a**) is in disagreement with the study of Duenas et al. in 2012.[38] Their structure assignment of the quercetin monosulfates in the chromato-

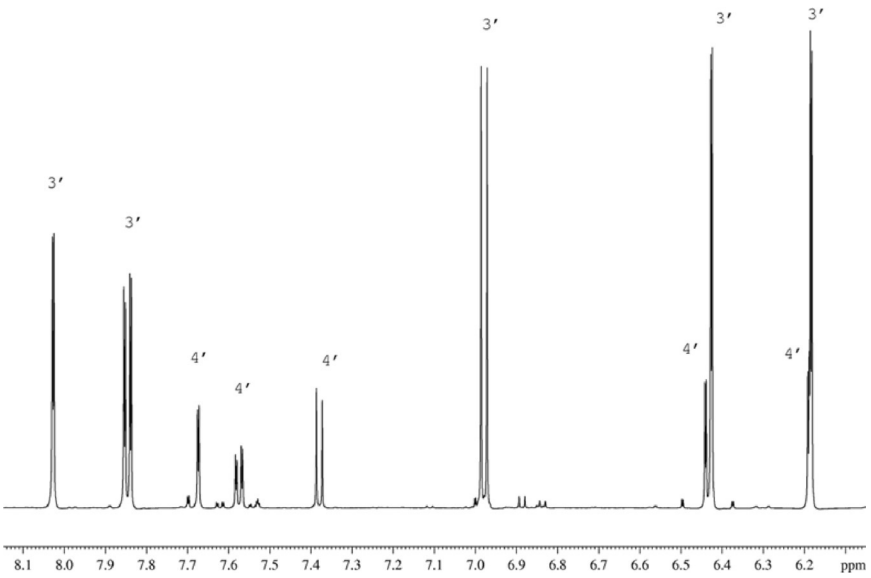

Figure 4. Expanded ^{1}H NMR spectrum of the mixture of quercetin 3′-*O*-sulfate (**1 b**) and quercetin 4′-*O*-sulfate (**1 a**) in [D_6]DMSO.

grams was based mainly on elution characteristics published by Jones et al.[37] The NMR data of the monosulfate that was assigned by Duenas et al.[38] as quercetin 4′-*O*-sulfate displayed neither up- nor downfield changes in the proton and carbon chemical shifts with respect to quercetin. This observation contradicts our carbon and proton NMR data and also the proton spectrum published in the original work by Jones et al.[37] Therefore, we regret to state that the structure assignment of quercetin 4′-*O*-sulfate in the study of Duenas et al.[38] was incorrect.

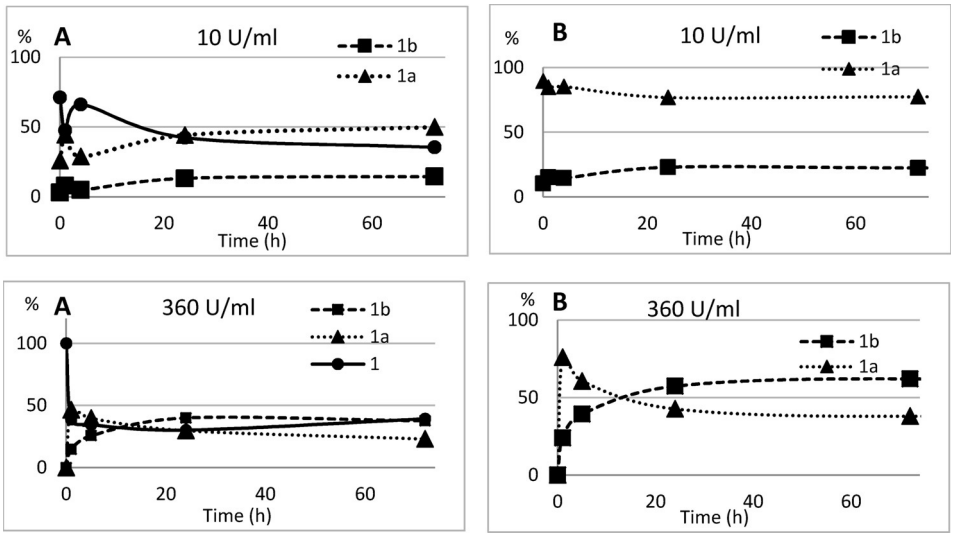

Figure 5. Kinetics of regioisomeric quercetin sulfate formation catalyzed by AST from *D. hafniense*. Quercetin (**1**; 100 mg, 0.332 mmol; Sigma) was dissolved in acetone (1.5 mL). *p*-NPS (5 mL, 100 mM), AST from *D. hafniense* (0.067 or 3 mL; corresponding to 10 or 360 U ml^{-1} of the reaction mixture), and tris(hydroxymethyl)aminomethane (Tris)–glycine buffer (to a final volume of 18 mL; 100 mM, pH 8.9) were added to the substrate solution and the mixture was incubated at $T = 30\,^{\circ}C$ under nitrogen. The reaction progress was monitored by HPLC method B and by NMR spectroscopy and expressed as A) a percentage of all quercetin derivatives in the reaction mixture and B) a percentage of the quercetin monosulfates. ■: Quercetin 3′-*O*-sulfate (**1 b**); ▲: quercetin 4′-*O*-sulfate (**1 a**); ●: quercetin (**1**).

Kinetic studies

During the preparation of this manuscript, the sulfation of quercetin (among other compounds) by AST from *D. hafniense* was published by van der Horst et al.[39] However, in their hands, the formation of only quercetin 4′-*O*-sulfate was observed. With the knowledge that they used a much less active enzyme, to deal this discrepancy, we performed a kinetic study of quercetin sulfation with reaction mixtures of 10, 36, 180, and 360 U mL^{-1} AST enzymatic activity for $t = 1$, 4, 24, and 168 h. With our original, optimized HPLC method B, we were able to separate the quercetin monosulfates and determine their proportion and also the relative conversion. We found that quercetin 3′-*O*-sulfate is the major reaction product only with 360 U mL^{-1} enzymatic activity and reaction times greater than 4 h. Low enzymatic activity (for example, 10 U mL^{-1}; Figure 5) always led to preferential production of quercetin 4′-*O*-sulfate and lower conversion, but both regioisomers were always found in the reaction mixture (Figure 5 and Figures S2 and S14 in the Supporting Information).

On the other hand, careful examination of the NMR data (chemical shifts and signal integration) in the supplementary material to the work of van der Horst et al.[39] revealed that approximately 15% of quercetin 3′-*O*-sulfate was also present in their product, which was declared in the paper to be pure quercetin 4′-*O*-sulfate. The signals of the quercetin 3′-*O*-sulfate in their spectra were erroneously ascribed to *p*-NP and *p*-NPS, which were, however, absent in the spectra.

The kinetic study was also performed in the same manner with taxifolin, but in this case, taxifolin 4′-*O*-sulfate was the major reaction product, together with less than 20% of the 3′-*O*-sulfate, irrespective of the reaction length and enzyme activity (data not shown).

To elucidate whether the enzyme from *D. hafniense* was able to catalyze the transfer of the sulfate group between the 3′- and 4′-positions of the quercetin molecule, we incubated a mixture of quercetin 3′-*O*-sulfate (**1b**) and quercetin 4′-*O*-sulfate (**1a**; 75:25 ratio) in Tris–glycine buffer in the presence of the enzyme for 24 h. The proportion of the regioisomers did not change during whole incubation time (samples at $t=0$, 1, 4, and 24 h; data not shown). Subsequently, this mixture of quercetin sulfates (as potential sulfate donors) was incubated in the presence of AST from *D. hafniense* with an equimolar amount of pyrocatechol (as a potential sulfate acceptor). In this case, we found, by using HPLC method C, that quercetin sulfates and pyrocatechol quickly disappeared from the reaction mixture, whereas quercetin and sulfated pyrocatechol were formed. Already after 1 hour, 65% of the quercetin sulfates were desulfated and 61% of the pyrocatechol was sulfated. After one week, only 6% of the initial amount of quercetin 3′-*O*-sulfate and 12.5% of the 4′-*O*-sulfate remained in the reaction mixture (Figure S15 in the Supporting Information). The proportion of the regioisomers in this reaction mixture progressively changed from 70:30 ($t=0$), through 64:36 ($t=1$ h) and 66:34 ($t=4$ h), to 52:48 ($t=166$ h). This result suggests that quercetin 3′-*O*-sulfate is desulfated slightly preferentially. The various reactants were determined by co-chromatography with authentic samples, by UV spectra of respective peaks and by NMR spectroscopy confirmation.

Conclusions

We have tested two types of sulfotransferases, namely recombinant rat liver aryl sulfotransferase AstIV and bacterial aryl sulfotransferase from *Desulfitobacterium hafniense*, for the sulfation of quercetin (**1**), its glycosylated derivatives (isoquercitrin (**2**) and rutin (**3**)), and dihydroquercetin ((+)-taxifolin (**4**)). The rat liver enzyme was able to sulfate only quercetin and taxifolin, whereas the quercetin glycosides remained intact. The *D. hafniense* enzyme sulfated isoquercitrin and rutin selectively at the C-4′ position of the quercetin moiety in very good yields. Taxifolin was sulfated at the C-4′ position and a minor amount of the C-3′ isomer was formed. The sulfation of quercetin proceeded preferentially at the C-3′ position, but a lower proportion of the C-4′ isomer was formed as well.

We propose the methylation of flavonoid sulfates as a novel approach for the direct and unequivocal determination of the position of sulfates (and possibly also other groups that lack direct interactions) in polyphenols. The replacement of the hydroxy group signals by easy detectable methoxyl singlets enables the methoxyl groups to be located based on the HMBC correlations and allows clear deduction of the site of sulfate attachment.

The AST from rat liver is not a convenient catalyst because it is time demanding and gives poor yields with a limited spectrum of substrates. Isoquercitrin and rutin were not substrates for the AST from rat liver. On the other hand, the bacterial AST from *D. hafniense* was identified as a perfect tool for the biotransformation of the whole array of flavonoids; it is stable, highly efficient, and high yielding (50–80%). The isolation and full characterization of the sulfated products from both ASTs indicated that their sulfated products were identical. The sulfates can, therefore, be used as authentic standards in further metabolic studies.

Experimental Section

NMR spectroscopy

NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (600.23 MHz for ^{1}H, 150.94 MHz for ^{13}C) and Bruker Avance III 700 MHz spectrometer (700.13 MHz for ^{1}H, 176.05 MHz for ^{13}C; compound **5a**). The residual signals of solvents (DMSO: $\delta_H=2.500$ ppm, $\delta_C=39.60$ ppm; CH_3OH: $\delta_H=3.330$ ppm, $\delta_C=49.30$ ppm) were used as internal standards. NMR experiments: ^{1}H NMR, ^{13}C NMR, gradient COSY, gradient HSQC, and gradient HMBC were performed by using the manufacturer's software. ^{1}H NMR and ^{13}C NMR spectra were zero filled to fourfold data points and multiplied by a window function before Fourier transformation. A two-parameter double-exponential Lorentz–Gauss function was applied for ^{1}H NMR spectra to improve the resolution and line broadening (1 Hz) was applied to get a better ^{13}C NMR signal-to-noise ratio. Chemical shifts are given in the δ scale with digital resolution that justifies the reported values to three (δ_H) or two (δ_C) decimal places, respectively.

Mass spectrometry

Mass spectra in the negative-ion mode were measured with the LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source. The samples were dissolved in methanol and introduced into the mobile-phase flow (methanol/water, 4:1; 100 μL min^{-1}) by using a 2 μL loop. The spray voltage, capillary voltage, tube lens voltage, and capillary temperature were 4.0 kV, −16 V, −120 V, and 275 °C, respectively.

The ESI mass spectrum of trimethyl derivative **5a** was acquired with a Waters Micromass ZMD mass spectrometer (Micromass UK Ltd., Manchester, UK; direct inlet; cone voltage: 20 V; source voltage: 4.3 kV).

Analytical HPLC–photodiode array (PDA)

All analytical HPLC analyses were performed with the Shimadzu Prominence System (Shimadzu, Kyoto, Japan) consisting of a DGU-20A mobile-phase degasser, two LC-20AD solvent delivery units, a SIL-20AC cooling autosampler, a CTO-10AS column oven, and the SPD-M20A diode-array detector. Chromatographic data were collected and processed with Shimadzu Solution software at a rate of 40 Hz and a detector time constant of 0.025 s.

Method A: The Chromolith Performance RP-18e monolithic column (100×3 mm internal diameter; Merck, Germany) coupled with a guard column (5×4.6 mm; Merck, Germany) was used. Mobile phases: acetonitrile/water/formic acid (80/20/0.1, v/v/v; phase A) and acetonitrile/water/formic acid (5/95/0.1, v/v/v; phase B). Gradient: 0–4 min 7→40% A, 4–6 min 40% A, 6–7 min 40→7% A, 7–8 min 7% A. Flow rate: 1.5 mL min^{-1} at 25 °C. The PDA data were acquired in the 200–450 nm range and the 285 nm (**4**) and 370 nm (**1**, **2**, **3**) signals were extracted, respectively.

Method B: Separation of quercetin sulfates was achieved on a Kinetex 5 μm pentafluorophenyl core–shell silica column (150×

4.6 mm; Phenomenex, USA) thermostated at $T=40\,^{\circ}C$ and equipped with a guard column (5×4.6 mm; Merck, Germany). Mobile phases: water/trifluoroacetic acid (100/0.1, v/v; phase A) and methanol/water (80/20, v/v; phase B). Linear gradient: 0–25 min 40→80% B. Flow rate: 0.8 mL min^{-1}. The PDA data were acquired in the 200–450 nm range and the 370 nm signal was extracted.

Method C: Separation of taxifolin sulfate(s) was achieved on a Kinetex 5 μm pentafluorophenyl core–shell silica column (150×4.6 mm; Phenomenex, USA) equipped with a guard column (5×4.6 mm; Merck, Germany). Mobile phases: water/trifluoroacetic acid (100/0.1, v/v; phase A) and methanol/water (80/20, v/v; phase B). Binary gradient elution: 0–16 min 45→50% B, 16–18 min 50→70% B, 18–22 min 70% B. Flow rate: 0.8 mL min^{-1}. The mobile phase was filtered through a 0.45 μm nylon membrane filter (Whatman, USA). The PDA data were acquired in the 200–450 nm range and the 285 nm signal was extracted.

Preparatory HPLC was performed on a 20×300 mm Asahipak GS-310 20F column (Shodex, USA). The system consisted of a Shimadzu LC-8A pump, a SPD-20 A dual wavelength detector, a FRC-10A fraction, and a CBM-20A controller connected to a computer. Separations were performed with methanol at $T=25\,^{\circ}C$. Flow rate: 5 mL min^{-1}. Detection was at 254 and 369 nm.

Aryl sulfotransferase from rat liver

AstIV from rat liver (the plasmid bearing *astIV* was kindly provided by Prof. L. Elling, RWTH Aachen University, Germany) was expressed as described in our previous work[31] with the following modifications. To support the enzyme folding and to enhance the activity of AstIV, the cotransformation with the chaperone G7 (GroEL/GroES) was applied for the *E. coli* strains BL21(DE3)Gold, BL21(DE3)plyS, and Origami (DE3). However, no significant improvement of the enzyme activity and stability was observed. Freshly prepared whole cells, as well as crude lysate or purified AST enzyme, were prepared with some modifications in accordance with Ref. [31] and tested in the biotransformation experiments. Only the freshly prepared whole cells were able to catalyze the sulfation reaction (see Figure 6).

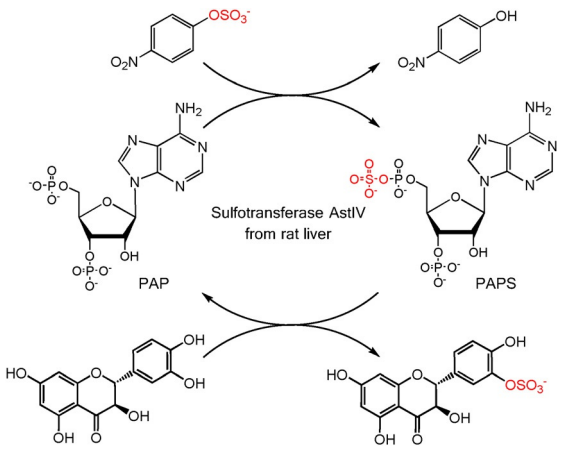

Figure 6. Sulfation of (+)-taxifolin (**4**) and PAPS regeneration, (both) catalyzed by sulfotransferase AstIV from rat liver.

Taxifolin sulfation by aryl sulfotransferase from rat liver

(+)-Taxifolin (**4**; 52 mg, 0.171 mmol; Amagro, Czech Republic) and *p*-NPS (35 mg, 0.136 mmol) were dissolved in DMSO (3 mL). Whole *E. coli* cells (2 g wet weight) expressing recombinant aryl sulfotransferase from rat liver resuspended in potassium phosphate buffer (pH 7.5, 17 mL) were added. The reaction mixture was incubated in the bench-top 5404 R rotary shaker at $T=37\,^{\circ}C$ for 24 h, then the cells were centrifuged (30 min, 5000 rpm; Eppendorf, Germany) and a new portion of fresh cells (2 grams, wet weight, resuspended in potassium phosphate buffer) was added (15 mL; 3 times in 3 days). The reaction was monitored by HPLC method A and the product was purified after 72 h by preparative HPLC by employing an Asahipak GS-310 20F column (Shodex, USA) with isocratic elution with 100% methanol. The fraction containing the putative sulfated product(s) was evaporated, dissolved in 80% methanol (1 mL), and loaded onto a Sephadex LH-20 column (15 g dry weight; 12 mm internal diameter) packed and eluted with 80% methanol. The fractions were analyzed by HPLC method A and the fractions containing the products **4b** and **1b** were collected and evaporated in vacuo at $T=45\,^{\circ}C$. Taxifolin sulfate (**4b**) was obtained as a yellowish solid (1 mg, 1.5%). The structure was characterized only by HRMS (*m/z* calcd for $C_{15}H_{11}O_{10}S$: 383.00784; found: 383.00677; see Figure S11 in the Supporting Information). Quercetin 3′-*O*-sulfate (**1b**) was obtained as a yellowish solid (8 mg, 12%). The structure was characterized by HRMS (*m/z* calcd for $C_{15}H_9O_{10}S$: 380.99219; found: 380.99126; see Figure S10 in the Supporting Information) and by NMR spectroscopy (For ^{1}H and ^{13}C NMR data, see Tables S1 and S2 in the Supporting Information). Complete assignment of all NMR signals was accomplished by a combination of gradient COSY, gradient HSQC, and gradient HMBC experiments.

To avoid substrate oxidation (of taxifolin to quercetin), tentatively thought to be caused by cytochromes, an inhibitor of the respiration, NaN_3 (1 mM final concentration), was added to the reaction mixture and the reaction was repeated, which yielded the same products.

Substrates **1**, **2**, and **3** were subjected to the above-described procedure for taxifolin; however, no sulfated products were obtained.

Aryl sulfotransferase from *D. hafninense*

Expression of the AST enzyme was performed as described by van der Horst et al.[34] with the following amendments. The plasmid containing the AST gene (kindly provided by Dr. van der Horst, University of Amsterdam, The Netherlands), was retransformed into various *E. coli* strains: BL21, BL21(DE3)Gold, and BL21(DE3)plyS (Table 8). Bacterial cultures were grown in Luria–Bertani medium with kanamycin and appropriate second antibiotics (according to the *E. coli* strain used) for the better selection and maintenance of the plasmids. The purity of the enzyme was confirmed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis analy-

Table 8. Transformation of AST from *D. hafninense* into various expression systems.

Strain	Activity [U mL^{-1}] at $T=37\,^{\circ}C$	at $T=30\,^{\circ}C$
BL21	0	N.D.[a]
BL21(DE3)plyS	12	N.D.[a]
BL21(DE3)Gold	105	2170

[a] N.D.: not determined.

sis,[40] which showed a single band of 70 kDa (Figure S13 in the Supporting Information). The crude lysate of the most active clone (*E. coli* BL21(DE3)Gold) was used in the enzymatic reactions. The AST was always prepared fresh and standard enzymatic assays were performed at $T=37$ or 30 °C (Table 8). The activity of AST was expressed as released *p*-NP at 400 nm (extinction coefficient at 400 nm: 0.2488 $cm^2\,\mu mol^{-1}$). One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 mmol *p*-NP per minute. The activities were corrected for nonenzymatic *p*-NP release. The specific activity of the recombinant AST from *D. hafniense* was 28500 U mg^{-1} (with 2-naftol as the acceptor).

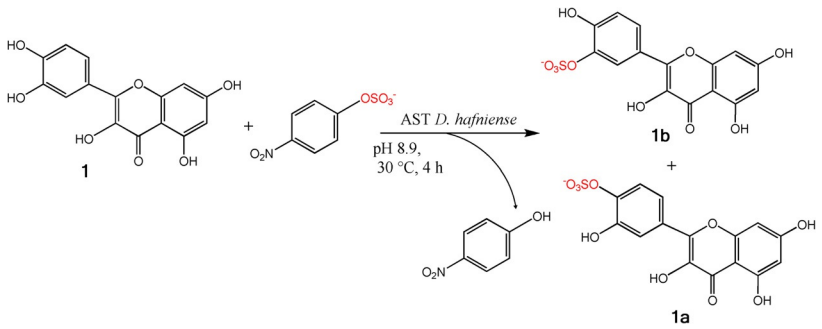

Figure 7. Sulfation of quercetin (**1**) catalyzed by sulfotransferase from *D. hafniense*.

Quercetin sulfation with aryl sulfotransferase from *D. hafniense*

Quercetin (**1**; 150 mg, 0.498 mmol; Sigma) was dissolved in acetone (2 mL). 100 mM Tris–glycine buffer (pH 8.9; 20 mL), *p*-NPS (Sigma–Aldrich; 130 mg, 0.50 mmol), and AST from *D. hafniense* (2 mL, 180 U ml^{-1} of the reaction mixture) were added to the substrate solution and the mixture was incubated for 4–5 h at $T=$ 30 °C under nitrogen. The reaction progress was monitored by HPLC or by TLC (ethyl acetate/methanol/HCO_2H, 4:1:0.01). The reaction was terminated by short heating to > 90 °C and the reaction mixture was halved by evaporation in vacuo so that all organic solvents were removed. The pH value was adjusted to 7.5–7.7 and then *p*-NP and residual starting materials were removed by extraction (3×50 mL EtOAc). The aqueous phase (15 mL) containing the sulfated product was evaporated and the residue was dissolved in 80% methanol (2 mL) and loaded onto a Sephadex LH-20 column (30 g dry weight, 3 cm internal diameter) packed and equilibrated with 80% aqueous methanol. The elution typically takes 2–7 days. The fractions were analyzed by TLC (EtOAc/MeOH/HCO_2H, 4:1:0.01, v/v/v) and the fractions containing the respective products were collected and evaporated in vacuo at $T=45$ °C.

The mixture of quercetin 3′-*O*-sulfate (**1b**) and quercetin 4′-*O*-sulfate (**1a**, side product, 25%) was obtained as a yellowish solid (90 mg; 47.5% yield), the identity of which was confirmed by HRMS (*m/z* calcd for $C_{15}H_9O_{10}S$: 380.99219; found: 380.99126; Figure S1 in the Supporting Information). These two compounds were not separable by preparatory HPLC and only partial separation was achieved by analytical HPLC method B (Figures 7 and 8); both regioisomers could be distinguished in the NMR spectra (see Tables S1 and S2 for **1b**, Tables S3 and S4 for **1a**, and Figure S2 in the Supporting Information). The complete assignment of all NMR signals was accomplished by the use of a combination of gradient COSY, gradient HSQC, and gradient HMBC experiments.

Isoquercitrin sulfation with aryl sulfotransferase from *D. hafniense*

Isoquercitrin (**2**; 150 mg, 0.323 mmol), prepared as described previously,[18] was sulfated by using the same procedure as that for quercetin. The reaction progress was monitored by TLC and by HPLC method A. Isoquercitrin 4′-*O*-sulfate (**2a**) was obtained as a yellowish solid (120 mg; 69% yield). The structure was determined by HRMS (*m/z* calcd for $C_{21}H_{19}O_{15}S$: 543.04501; found: 543.04367; Figure S3 in the Supporting Information) and by NMR spectroscopy (see Table S5 and Figure S4 in the Supporting Information).

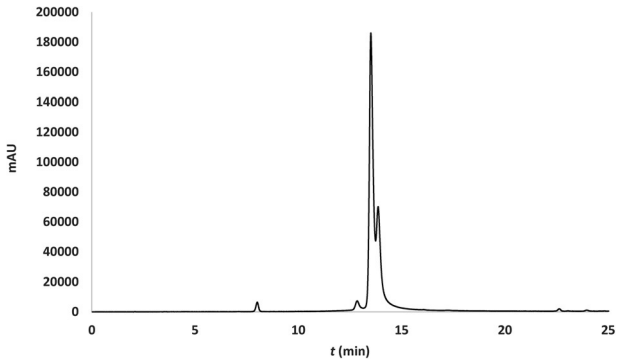

Figure 8. HPLC analysis of quercetin 3′-*O*-sulfate (**1b**; $t_R=13.76$ min), quercetin 4′-*O*-sulfate (**1a**; $t_R=14.07$ min); method B.

Rutin sulfation with aryl sulfotransferase from *D. hafniense*

Rutin (**3**; 150 mg, 0.246 mmol; Sigma–Aldrich) was sulfated by using the same procedure as that for quercetin. The reaction was monitored by TLC and by HPLC method A. Rutin 4′-*O*-sulfate (**3a**) was obtained as a yellowish solid (90 mg; 53% yield). The structure was determined by HRMS (*m/z* calcd for $C_{27}H_{29}O_{19}S$: 689.10183; found: 689.10079; Figure S6 in the Supporting Information) and by NMR spectroscopy (see Table S7 and Figure S7 in the Supporting Information).

Taxifolin sulfation with aryl sulfotransferase from *D. hafniense*

Taxifolin (**4**; 200 mg, 0.66 mmol; Amagro, Czech Republic) was sulfated ($t=4$ h) by using the same procedure as that for quercetin. The mixture of taxifolin 4′-*O*-sulfate (**4a**) and taxifolin 3′-*O*-sulfate (**4b**, side product, approximately 20%) was obtained as a yellowish solid (190 mg; 75.4% yield). The structure was determined by HRMS (*m/z* calcd for $C_{15}H_{11}O_{10}S$: 383.00784; found: 383.00677; Figure S8 and S9 in the Supporting information) and by NMR spectroscopy (see Table S8 for **4a** and Table S9 and Figure S12 for **4b** in the Supporting Information).

Kinetics of regioisomer sulfate formation of taxifolin and quercetin catalyzed by aryl sulfotransferase from *D. hafniense*

Quercetin (**1**; 100 mg, 0.332 mmol; Sigma) and taxifolin (**4**; 100 mg, 0.33 mmol; Amagro, Czech Republic) were separately dissolved in acetone (1.5 mL). *p*-NPS (5 mL, 100 mM), AST from *D. hafniense* (0.067, 0.3, 1.5, or 3 mL; corresponding to 10, 36, 180, or 360 U ml^{-1} of the reaction mixture, respectively), and Tris–glycine buffer (to a final volume of 18 mL; 100 mM; pH 8.9) were added to the substrate solution and the mixture was incubated at $T=30\,^{\circ}C$ under nitrogen. The reaction progress was monitored by HPLC method B and by NMR spectroscopy. The reaction was stopped after 1, 4, and 24 h and, in selected cases, also after 72, 168, and 336 h.

Investigation of potential trans-sulfation activity of aryl sulfotransferase from *D. hafniense*

Two experiments were performed to evaluate the potential trans-sulfation activity of the aryl sulfotransferase from *D. hafniense* owing to its regiospecificity in quercetin sulfation. First, the quercetin sulfates **1b** and **1a** (75:25; 1 mg, 2.5 μmol in 100 mM Tris–glycine buffer (130 μL) at pH 8.9) were incubated with and without the enzyme (180 U ml^{-1} of the reaction mixture). The reaction was stopped at $t=0$, 1, 4, and 24 h and the potential reaction progress was monitored by HPLC method B. Second, a sample of quercetin sulfates **1b** and **1a** (75:25; 3.6 mg, 9 μmol in 100 mM Tris–glycine buffer (600 μL) at pH 8.9) was incubated with an equimolar amount of pyrocatechol (**6**; 1 mg, 9 μmol) and with the enzyme (180 U ml^{-1} of the reaction mixture). The reaction was stopped at $t=0$, 1, 4, 24, and 166 h and the potential reaction progress was monitored by HPLC method C.

Preparation of 5,7,3′-tri-*O*-methyl-4′-*O*-sulfo-3-*O*-β-D-glucopyranosyl quercetin (5 a)

To enable exact structure determination by NMR spectroscopy, compound **2a** was methylated at all free phenolic groups. Isoquercitrin 4′-*O*-sulfate (**2a**, 9 mg) was dissolved in dry methanol and cooled to $T\approx 5\,^{\circ}C$, then a fresh solution of diazomethane (0.7 mL in diethyl ether) was added. The reaction mixture was kept at $T=5\,^{\circ}C$ for 20 min and then evaporated in vacuo with a bath temperature not exceeding $T=35\,^{\circ}C$. 5,7,3′-Tri-*O*-methyl-4′-*O*-sulfo-3-*O*-β-D-glucopyranosyl quercetin (**5a**) was obtained as a yellowish solid (8 mg, 77%). The structure was characterized by MS (ESI$^-$; found: *m/z* 585.82 (100%) [(*M*−H)]$^-$) and by NMR spectroscopy (for ^{1}H and ^{13}C NMR data, see Table S6 and Figure S5 in the Supporting Information). The complete assignment of all NMR signals was accomplished by the use of a combination of gradient COSY, gradient HSQC, and gradient HMBC experiments.

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Keywords: biotransformations · enzyme catalysis · flavonoids · sulfur · transferases

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