DOI: 10.1002/adsc.201200564

# Sulfation of Various Alcoholic Groups by an Arylsulfate Sulfotransferase from *Desulfitobacterium hafniense* and Synthesis of Estradiol Sulfate

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Received: July 6, 2012; Published online: December 4, 2012

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201200564.

**Abstract:** Bacterial arylsulfate sulfotransferases (AST) are enzymes that catalyse the transfer of a sulfate group from *p*-nitrophenyl sulfate (*p*-NPS) to a phenolic acceptor molecule. By screening of the NCBI protein database a gene coding for an AST was found in *Desulfitobacterium hafniense*. After expression the enzyme was purified and characterised. This AST efficiently sulfates various acceptor molecules (estrone, estradiol, enkephalin and non-phenolic alcohols) using *p*-NPS as sulfate donor. The purified AST has a pH optimum of 9.6, it is stable in the presence of 10% of DMSO, and depending on the conditions it has a melting temperature of up to 47 °C. Surprisingly, and in great contrast to all other known bacterial ASTs, this enzyme was able to use a variety of non-phenolic alcohols as sulfate acceptor. Because of these properties, this unique enzyme is a promising tool for biotransformation processes, providing a green and simple method to specifically sulfate compounds without need for functional group protection.

**Keywords:** biocatalysis; green chemistry; regioselectivity; steroids; sulfation

## Introduction

Sulfate monoesters are of great importance to many biological systems. Sulfation (also: sulfurylation) of a molecule changes many physico-chemical properties of the acceptor molecule, such as charge, size, and importantly, solubility. In the synthesis of these compounds sulfotransferases are involved that catalyse the transfer of a sulfuryl group to a hydroxy or amine moiety on various molecules including small-molecule drugs, steroids, hormones, carbohydrates, and proteins.[1,2] Sulfate conjugation in mammals is a major pathway for the biotransformation of phenolic and catechol drugs and forms a detoxification route in the liver.[3] Recent studies have implicated roles of sulfotransferases in a number of disease states including entry of HIV,[4,5] chronic inflammation,[6] and various forms of cancer.[7] Because of this great biological relevance, there is growing interest in the synthesis of sulfated molecules.[8]

Sulfated compounds are in general synthesised by the pharmaceutical/chemical industry using complexes of sulfur trioxide ($SO_3$) with tertiary amines or amides. However, use of this reactive reagent suffers from numerous disadvantages such as harsh reaction conditions and lack of reaction selectivity. Additionally, in order to prevent side reactions of labile functionalities and to enhance the chemo- or regioselectivity of the overall reaction, these functionalities have to be protected.[9–11] Enzymatic methods for sulfation of compounds under mild conditions may have clear advantages and the possibility to use enzymes for this purpose has been studied.[12–14]

Two families of sulfotransferase enzymes can be distinguished for the transfer of a sulfate group from a donor molecule to the hydroxy group of an acceptor molecule (for a review, see ref.[1]). In eukaryotes, a family is found that uses 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the sulfate-donor molecule for the sulfation of for example, sugars, hormones and antibiotics. Use of these enzymes is problematic since PAPS is very expensive and has to be regenerated, introducing another enzyme and sulfate donor in the system and further these sulfotransferases are in gen-

  

eral donor- and substrate-specific.[1] The other family of sulfotransferases found in bacteria uses phenolic sulfate esters such as *p*-nitrophenyl sulfate or *p*-acetylphenyl sulfate as a sulfate-donor. The bacterial sulfotransferase [arylsulfate sulfotransferase; A(S)ST, EC 2.8.2.22] was originally discovered in the bacterium *Eubacterium* A-44 from human intestine[15,16] and more recently many more bacteria[17–21] have been found to contain PAPS-independent aryl sulfotransferases (see ref.[2] for a review). The biological role of these bacterial ASTs has not been elucidated. Only for the recently discovered AST from *Streptomyces* sp. MK730-62F2 a physiological role was shown. This enzyme acts as a sulfotransferase in liponucleoside biosynthesis: in this organism it is responsible for the formation of sulfated liponucleoside antibiotics.[22]

Kinetic studies on AST from *Eubacterium* A-44 demonstrated a ping-pong bi-bi reaction mechanism.[23] A crystal structure of the homodimeric AST from the uropathogenic strain *E. coli* CFT073 in the presence of various sulfuryl donors[24] showed that the sulfate group is bound transiently to a catalytic histidine residue. This mechanism bears resemblance to the mechanism of transphosporylation catalysed by a group of acid phosphatases. During transfer of a phosphate donor to a phosphate acceptor molecule by these enzymes an activated enzyme phospho-histidine intermediate is formed.[25,26] Interestingly, earlier site-directed mutagenesis studies on AST from *Enterobacter ambigenus* implied transient sulfurylation of a tyrosine residue.[19] Different classes of AST can be discerned,[27] possibly having different reaction mechanisms (also see Results and Discussion section).

The bacterial ASTs have been much less characterised than the eukaryotic PAPS-dependent sulfotransferases. However, the ASTs are highly interesting for biotransformation purposes because of the lower cost and higher stability of *p*-NPS compared to PAPS. Since the enzymes also have a broader substrate specificity[12] these enzymes may be potentially useful in the selective and mild sulfation of many compounds such as peptides, steroids and carbohydrates. Here we report successful cloning and expression of the *astA* gene from *Desulfitobacterium hafniense* and explored its donor and acceptor specificity.

## Results and Discussion

### Identification of the *ast* Gene

In the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein), the number of protein sequences that encode (putative) ASTs is growing rapidly. These either have been annotated as AST, or can be found manually using BLAST analyses. Among these, a sequence from the dehalorespiring bacterium *Desulfitobacterium hafniense* can be found, annotated as arylsulfotransferase. This sequence codes for a protein of 628 residues corresponding to a molecular mass of 71.4 kDa. Sequence alignment of ASTs from different organisms shows there might be different classes of AST proteins (an alignment of some examples from both classes are shown in the Supporting Information, Figure S1). One class (Class I) represents proteins mainly from proteobacteria and the sequences show a high similarity. Depending on the amount of disulfide bridges in the protein, and the genomic context of the encoding gene, this class can be further subdivided.[27] The other class (Class II) consists of proteins which show internally less sequence similarity and originate from firmicutes. According to the sequence similarity AST from *D. hafniense* belongs to Class II. The sequence similarity between the two main classes of AST is relatively low (for example, 23% identity between the AST proteins from *Eubacterium* and *E. coli*). Furthermore, the sequences of Class II are about 30 residues longer than Class I sequences. The tyrosine residue identified by Kwon et al.[19] to be essential for enzymatic activity in AST from *Enterobacter ambigenus* is only conserved in the sequences of Class I enzymes. The region of the sequence in which this residue is located is highly non-homologous between the two classes. Another difference that can be observed is the number, and location, of cysteine residues. Class I contains three conserved cysteine residues (not shown), whereas Class II enzymes contain eight, and several cysteines are found that are randomly present. In Class I the cysteines are involved in disulfide bond formation and are important for catalytic activity.[28] Interestingly the spatial arrangement of the active site residues in PAPS dependent sulfotransferases is the same as that in the bacterial ASTs.[29]

### Cloning, Expression and Purification

To determine the functionality of AST, its gene was amplified from *D. hafniense* genomic DNA and cloned in the vector pET26b. The resulting plasmid, pJFT006, was used to transform *E. coli* BL21(DE3) cells. The temperature during induced expression was lowered to 25 °C to reduce the amount of expressed protein appearing as inclusion bodies. Though cloning in pET26b promotes heterologous expression to the periplasm, AST was found in large amounts in the cytoplasm as well. Accordingly, the enzyme was purified from whole cells, by following a conventional purification strategy, as described in the Experimental Section. The gene codes for a protein with a molecular mass of 71.4 kDa and in agreement with this, SDS-PAGE of the purified AST resulted in a single band of about 70 kDa (not shown). This molecular mass is

  

close to that of the subunit[2] of the *E.coli* sulfotransferase.

## Enzymatic Activity, pH Optimum and Temperature Stability

Arylsulfate sulfotransferases require two different substrates for activity, one to act as a sulfate donor, the other as sulfate acceptor. Absence of either acceptor or donor does not result in any measurable activity. Among the different sulfated compounds tested as a donor, only the arylsulfates could be used (see Figure S2, Supporting Information) and the alkylsulfates were inactive. This may have a thermodynamic origin but selectivity of the active site for an aromatic group cannot be excluded. In all further experiments, *p*-nitrophenyl sulfate was used as the sulfate donor. A variety of phenols were tested for their potential as sulfate acceptor for this enzyme. The results, which are in line with the results found previously[15–19,30] are summarised in Table 1. Except for *p*-hydroxybenzoic acid, all phenolic compounds were good sulfate acceptors.

In reactions with phenol and tyramine a sharp, alkaline, pH dependency was found with optimal sulfation activity at pH 9.5–9.8 (Figure 1). The sharp increase in activity suggests that an acid base group with a high $pK_a$ is involved in catalysis and points to the involvement of phenolic OH group (which has a $pK_a$ value of about 10). The involvement of a histidine residue having this value is less likely, since the $pK_a$ for histidine is usually lower.[31] Mozhaev et al. reported that the AST from *Clostridium innocuum* has optimal activity at slightly lower pH (8.0–8.5).[12]

**Table 1.** Phenolic sulfate acceptors. Relative activity of AST with different sulfate acceptors. Reactions were performed in 50 mM Tris-glycine buffer (pH 9.0), with 5 mM of *p*-NPS as the sulfate donor and 5 mM of acceptor.

| Sulfate acceptor | Relative reaction rate[a] |
|---|---|
| phenol | 100, 106[b] |
| resorcinol | 70 |
| *p*-Cl-phenol | 195 |
| *m*-Cl-phenol | 138 |
| dopamine | 540 |
| *p*-cresol | 200 |
| tyramine | 60 |
| 1-naphthol | 50[b] |
| 2-naphthol | 86[b] |
| 4-hydroxybenzoic acid | 0.7[b] |
| 5-hydroxyindole | 74[b] |
| 4-hydroxyindole | 88[b] |
| *p*-aminophenol | 102[b] |
| *o*-aminophenol | 136[b] |
| 3,5-dimethoxyphenol | 33[b] |

[a] Initial reaction rates for different substrates are normalised to the rate of sulfation with phenol (100%).
[b] These transformations were performed in 10% DMSO.

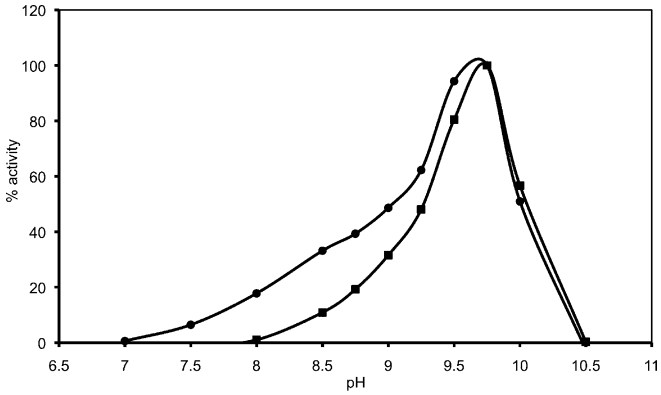


**Figure 1.** Sulfation activity of AST at different pH values, both with phenol (closed circles) and tyramine (closed squares) as a sulfate acceptor. Relative activities are shown. Activity at pH 9.75 is taken as 100%. Reactions were carried out using 5 mM of *p*-NPS and 5 mM of acceptor in 100 mM Tris-HCl at the various pH values.

Since many potential substrates for AST are poorly soluble in water, the use of organic solvents is often required to guarantee adequate substrate concentration. A range of organic solvents was used to study their effect on the catalytic activity. Addition of 10% ethanol, methanol and methylene chloride reduced the enzymatic activity to 50–65%. In DMF, THF, dioxane and acetone the activity of the enzyme decreased to 30–34% of the original activity. However, 10% DMSO had no effect on the activity and even in 25% of DMSO, the remaining activity was still 73%. Therefore in general for example, as in Table 2, 10% DMSO was used to ensure substrate solubility. For prolonged incubations, and for preparative catalytic reactions, 10% acetone was used instead of DMSO because of its easy removal.

For synthetic applications the AST should be sufficiently stable and therefore the thermostability of AST was investigated at different pH values and in the absence or presence of substrates. The enzyme was incubated exactly 5 min at a given temperature after which the activity was measured. From a plot of the remaining activity against the incubation temperature, the midpoint of the denaturation curve ($T_m$, melting temperature) was determined (Figure 2). A melting temperature of 39.2 °C was found at the near optimal pH of 9.0 that increased to 41.7 °C at pH 8 (not shown).

At both higher (pH 9.75) or lower pH (6.5) values the melting temperatures decreased to 30.9 °C and 36.8 °C, respectively. The presence of a high concentration of salt (500 mM NaCl) at pH 6.5 had hardly any effect on the melting temperature ($\Delta T_m = 0.9$ °C). The stability of AST in prolonged catalytic incuba-

  

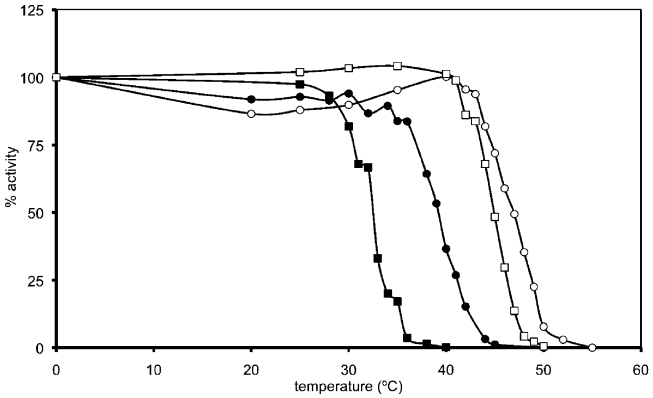


**Figure 2.** Thermostability of AST. Thermal denaturation curve of arylsulfate sulfotransferase at pH 9.0 in the absence and presence of acetone and *p*-nitrophenyl sulfate. Samples were incubated for 5 min at the given temperatures. pH 9.0 (closed circles), containing 5 mM *p*-nitrophenyl sulfate (open circles), 10% acetone (closed squares), 10% acetone and 5 mM *p*-nitrophenyl sulfate (open squares). Remaining activity was measured at standard assay conditions.

tions containing acetone was investigated by assessing the melting temperature of AST in the presence of *p*-NPS and 10% acetone at pH 9.0. As is evident from Figure 2, the addition of 5 mM *p*-NPS causes a significant increase in melting temperature, whereas 10% of acetone results in a decreased melting temperature ($T_m = 46.9\,°C$ and 32.5 °C, respectively). When both *p*-NPS and acetone were present in the incubation mixture, the destabilising effect of acetone was diminished and the melting temperature ($T_m = 44.9\,°C$) was considerably higher than that without the addition of either.

## Sulfation of Steroids and Complete Conversion of Estrone

Sulfotransferases and sulfated steroids are of considerable toxicological, pharmacological and pharmaceutical interest and therefore the sulfation of a set of steroids as substrates for AST was studied. As Figure 3 shows steroids that have an aromatic 3′-OH were good sulfate acceptors. This test was based on the formation of a yellow colour due to the formation of *p*-NP, when the steroid was incubated with *p*-NPS and AST. As a control, hydrolysis of *p*-NPS was monitored in the absence of the steroid and in the presence of the enzyme, however, hydrolysis of the *p*-NPS did not occur under those conditions and thus the aryl sulfotransfer reaction was strictly dependent on the presence of the steroid. No measurable activity was detected with steroids like nandrolone and desogestrel that lack the 3′- hydroxy group. This also shows that the 17′-OH group is not sulfated.

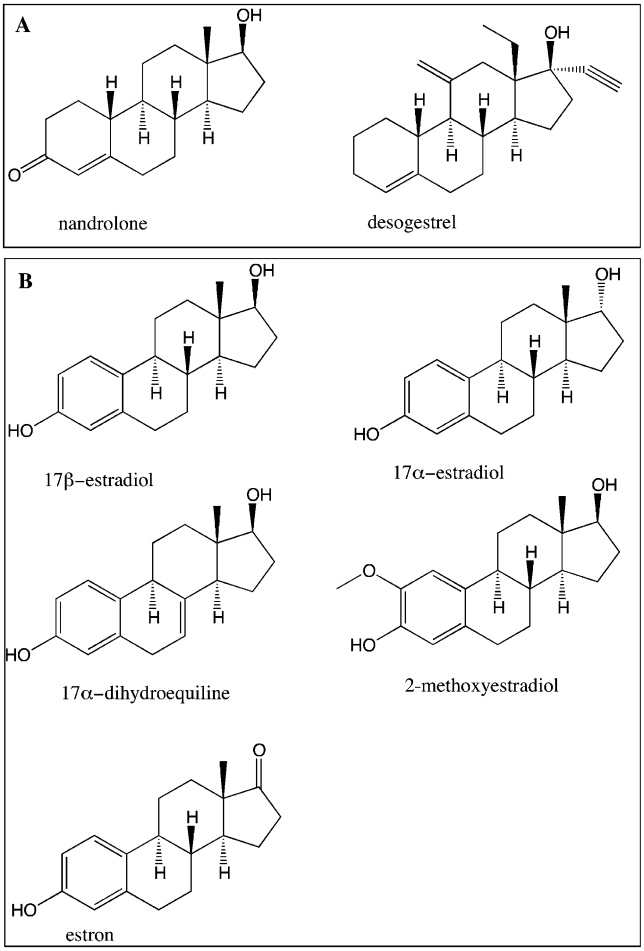


**Figure 3.** Steroids used in this study. The compounds in panel B are good sulfate acceptors for AST, whereas the compounds depicted in panel A do not accept sulfate from AST. Reactions were performed in 50 mM Tris-glycine buffer (pH 9.0), with 5 mM of *p*-NPS as the sulfate donor and 5 mM of acceptor. Reaction progress was judged by the increase of absorption at 410 nm in the reaction mixture, indicative of *p*-NP formation.

In addition to this qualitative test of sulfation of steroids the extent of sulfation of estrone was determined quantitatively. For this, the *p*-NPS and estrone were incubated in equimolar amounts (5 mM) and the reaction was followed by HPLC over time (Figure 4). While estrone and *p*-NPS gradually disappeared, two new products were simultaneously formed, which as judged by the elution time are *p*-nitrophenol and estrone sulfate, respectively. After 24 h 90% of the estrone was sulfated. When another aliquot of 5 mM *p*-nitrophenyl sulfate was added the residual estrone was sulfated within 2 h and less than 1% remained. This demonstrates that the enzyme is stable during the incubation, also under turnover conditions.

To test if the sulfation reaction using AST could be up scaled, a 100-mg scale conversion of 17β-estradiol was performed. This also allows the isolation and

  

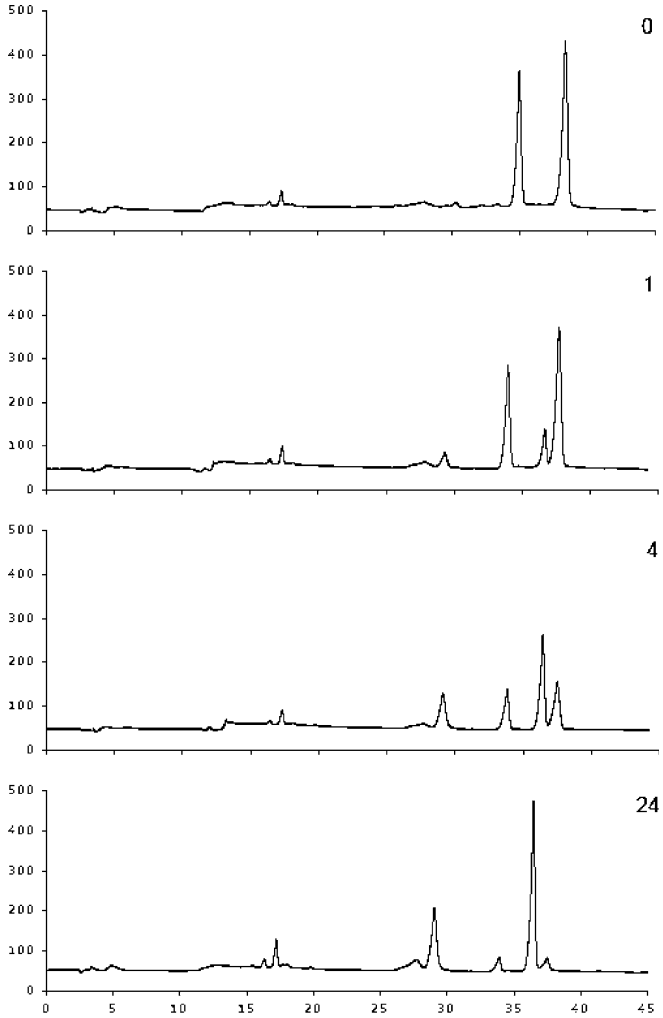


**Figure 4.** Sulfation of estrone followed by HPLC. The conversion of estrone to estrone-sulfate and *p*-NPS to *p*-NP as followed by HPLC. At the time intervals given, samples were taken and analysed by HPLC. The following peaks could be identified: 28.1 min *p*-NP; 33.4 min *p*-NPS; 36.2 estrone sulfate and at 37.3 min estrone. *Reaction conditions:* 0.1 unit $mL^{-1}$ AST, 5 mM *p*-NPS, 5 mM estrone in 100 mM Tris-HCl (pH 8.0).

analysis of the reaction products. After 24 h about 80% of the 17β-estradiol was converted, but longer incubation even for another day or addition of 0.25 μM AST and 1 mM *p*-NPS did not result in additional conversion. The isolated yield of the final product (17ß-estradiol 3-sulfate as a sodium salt) was 79 mg (57%) and, after analysis by NMR and HPLC, was calculated to be >90% pure (see the Supporting Information, Figure S4 for the $^1$H NMR spectrum of 17ß-estradiol 3-sulfate). The impurities consisted of *p*-NP, *p*-NPS, and less than 1% 17ß-estradiol.

## Sulfation of Enkephalin

Previously, some ASTs have been reported to be able to sulfate tyrosine-containing small peptides.[10,20,32] In line with this, also AST from *D. hafniense* was able to sulfate peptides like Leu-enkephalin. Analogous to estrone and estradiol, the reaction with *p*-nitrophenyl sulfate (10 mM) and Leu-enkephalin (10 mM) was followed by HPLC over time. Within 48 h 91% of Leu-enkephalin was sulfated which reached 98% after 6 days of incubation (see the Supporting Information, Figure S3).

## Sulfation of Aliphatic Alcohols

In contrast to all previously reported ASTs, AST from *D. hafniense* was able to use a variety of non-phenolic alcohols as sulfate acceptor. Table 2 shows a number of these.

The qualitative test, in which 100 mM of the acceptor was used, was again based on the formation of a yellow colour due to the formation of *p*-NP. As a control, hydrolysis of *p*-NPS was monitored in the absence of the acceptor and in the presence of the enzyme, however, hydrolysis of the *p*-NPS did not occur under those conditions and thus the aryl sulfotransfer reaction was strictly dependent on the pres-

**Table 2.** Aliphatic sulfate acceptors. The sulfation of various aliphatic alcohols was determined semi-quantitatively. Reactions were carried out in 50 mM Tris-glycine, pH 9.0, with 100 mM of acceptor and 10 mM of donor. Reaction progress was judged by the increase of absorption at 410 nm in the reaction mixture, indicative of *p*-NP formation. +++ corresponds to 50% conversion in 7 days, ++ corresponds to 10% conversion in 7 days, + corresponds to 2–10% conversion in 7 days.

| Sulfate acceptor | Conversion |
|---|---|
| 1-butanol | ++ |
| 2-butanol | +[a] |
| 1-pentanol | ++ |
| 1-octanol | ++ |
| 2-octanol | +[a] |
| 1-phenylethanol | +[b] |
| 2-phenylethanol | +++ |
| dihydroxyacetone | + |
| glycerol | ++ |
| cyclohexanol | ++ |
| furfuryl alcohol | ++ |
| benzyl alcohol | ++ |
| D-glucose | + |
| octyl glucoside | + |
| *N*-hydroxysuccinimide | + |

[a] For these compounds *S*(+) was the preferred isomer.
[b] For these compounds *S*(−) was the preferred isomer.

  

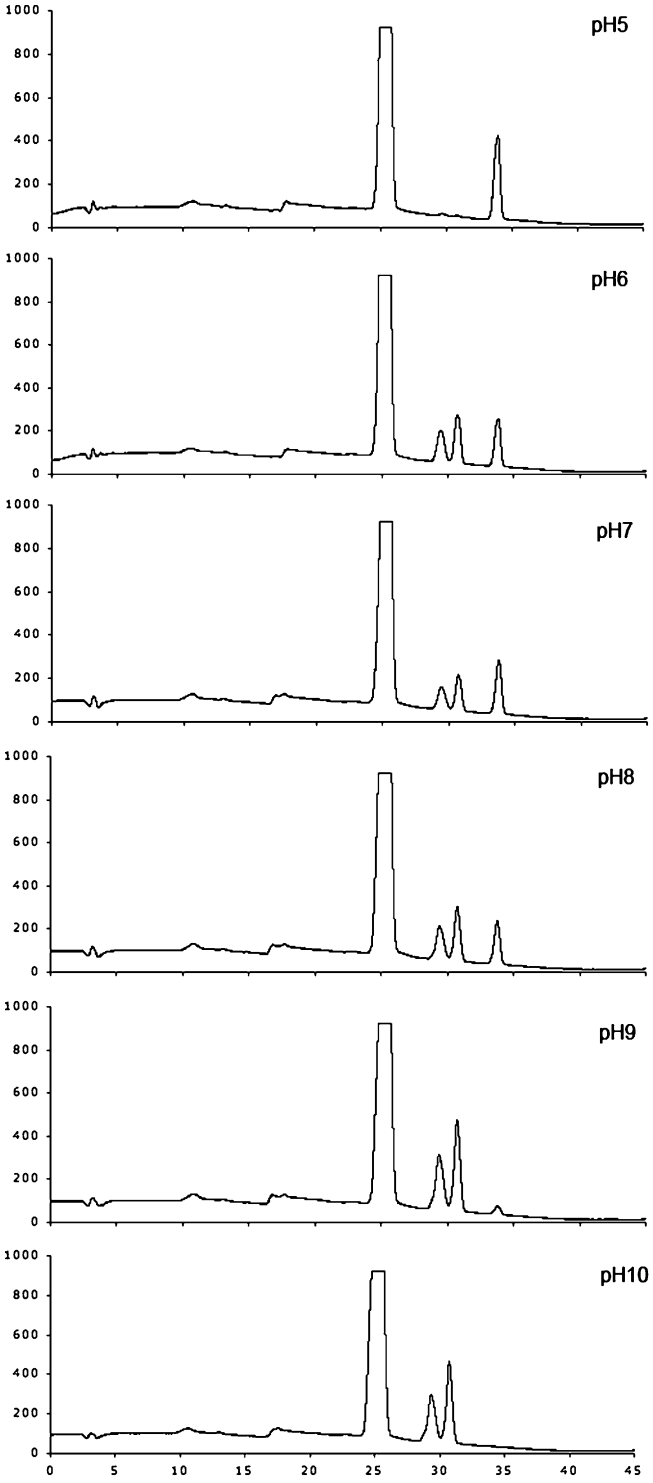


**Figure 5.** Sulfation of 2-phenylethyl alcohol at different pH values, followed by HPLC. Sulfation of 2-phenylethyl alcohol by AST at several pH values. 0.3 unit $mL^{-1}$ AST was incubated with 10 mM *p*-NPS and 100 mM 2-phenylethyl alcohol in 100 mM acetate (pH 5) or Tris-HCl (pH 6, 7, 8, 9 or 10) containing 10% acetone. After 96 h of incubation at 30 °C samples were taken and analysed by HPLC. The peaks eluting at 29.0 min and 33.4 min correspond to *p*-NP and *p*-NPS, respectively. The peak at 31 min has the same elution time as 2-phenylethyl sulfate that was synthesised chemically.

ence of the aliphatic alcohol. It should be noted that the reactions are very slow, requiring days.

In addition to sulfation of aliphatic OH groups, AST was able to sulfate the *N*-substituted hydroxy group of *N*-hydroxysuccinimide. Of the compounds with non-phenolic hydroxy groups, 2-phenylethyl alcohol was sulfated with the highest rate and its conversion was examined at different pH values and analysed by HPLC (Figure 5).

The pH dependence was similar to that seen in the sulfation of phenol and tyramine as acceptors; higher conversions were observed at increasing pH values. When 10 mM *p*-NPS were incubated with 100 mM 2-phenylethyl alcohol for 4 days at pH 9.0 the sulfate donor was completely desulfated and about 10 mM of sulfated 2-phenylethyl alcohol was formed, as judged from HPLC analysis. This was confirmed using sulfated 2-phenylethyl alcohol which was separately synthesised chemically[33]. Surprisingly, secondary alcohols (Table 2) are also sulfated though the rate is significantly slower. Further when 1-phenylethyl alcohol was used as acceptor, clear preference for the S(−) isomer can be observed.

## Conclusions

We cloned the *astA* gene from *Desulfitobacterium hafniense* and successfully expressed the encoded AST protein. We characterised the biochemical properties of the recombinant protein and we tested the acceptor specificity and found a wide range of not only phenolic, but surprisingly also aliphatic alcohols that can act as sulfate acceptor for this enzyme. The rate of sulfate transfer to aliphatic alcohols is substantially slower than to phenolic alcohols. However, by varying reaction parameters such as enzyme and acceptor concentration, we estimate that this can be significantly improved. Furthermore, enzyme modifications, for example, by using a directed evolution approach, will most likely result in improved rates of sulfate transfer to aliphatic alcohols and this would expand further the scope of the application of this enzyme.

## Experimental Section

### Chemicals

All steroids, including estrone sulfate, were kindly provided by Diosynth (Organon, Oss, The Netherlands). 4-Methylumbelliferyl sulfate and *p*-nitrophenyl sulfate were obtained from Sigma. Octyl sulfate and phenyl sulfate were prepared from the corresponding alcohol using triethylamine-$SO_3$.[33] All other sulfate donors were prepared as previously described.[34] Leu-enkephalin and leu-enkephalin sulfate were obtained from Bachem.

  

## Bacterial Hosts and Vector

The T7 expression vector pET26b was obtained from Novagen. *Escherichia coli* XL-1 blue (Stratagene) was used as an initial host for cloning while *E. coli* BL21 (DE3) (Stratagene) was used as an expression host for the pET-derivative. *E. coli* was grown in TY medium in a rotary shaker at 37 °C. Kanamycin was added to a final concentration of 30 mg mL$^{-1}$.

## Cloning of the *astA* Gene

*D. hafniense* genomic DNA, a kind gift from Krisztina Gabor (Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands), was used as template in a PCR reaction. A primer set (*Bsp*HI and *Sac*I restriction sites underlined) was designed to amplify *astA* by PCR on an iCycler Thermal Cycler (BioRad): 5′-GCGCGCTC*ATGA*ATCCGATCAAAAGTGAACAGAT-3′ and 5′ GCGCGC*GAGCTC*AAGCTGTAATAGACACTCCGGTC-3′. In addition to 10 ng of *D. hafniense* chromosomal DNA and 100 ng of each primer, the 50 mL mixture contained 0.2 mM dNTPs, *Pfu* buffer and 2.5 U *Pfu* DNA polymerase. The PCR product with the expected size was digested and ligated into *Nco*I/*Sac*I-digested vector pET26b, resulting in pJFT006. *E. coli* XL1-blue and BL21 (DE3) were transformed with this plasmid. Sequence analysis of pJFT006 by MWG Biotech AG (Martinsried, Germany) confirmed the correct cloning of the *astA* gene.

## Expression and Purification of AST

An overnight culture of *E. coli* BL21 (DE3) cells with pJFT006 was used to inoculate LB medium containing 50 mg mL$^{-1}$ kanamycin. When the $OD_{600}$ reached 0.5, the cells were induced with 1 mM IPTG and subsequently grown at 25 °C for approximately 16 h. Cells were harvested by centrifugation (4000 × *g* for 10 min.) and resuspended in a 50 mM Tris-HCl buffer (pH 8.0). Cells were lysed by sonication (4 min, output 7, duty cycle 50%) on a Branson sonifier. After removal of the cell debris by centrifugation (20,000 × *g* for 30 min.) Sephacel DEAE was added to the supernatant and stirred for 2 h. After settling the Sephacel was poured into a column, which was washed extensively with 100 mM NaCl in 50 mM Tris (pH 8). The AST was eluted by a stepwise NaCl gradient. Fractions containing AST activity were applied onto a Poros column (Applied Biosystems) using an Amersham Pharmacia FPLC system. The protein was eluted using a linear gradient towards 1 M NaCl. Fractions containing AST activity were pooled, concentrated using Amicon Centricon concentrators, and buffered in 50 mM Bis-Tris (pH 6.0). This sample was applied onto a Q-FF column (GE Healthcare), equilibrated with 50 mM Bis-Tris (pH 6.0). In a linear gradient towards 1 M NaCl, AST eluted between 400 and 500 mM NaCl. The eluted fractions were analysed using SDS-PAGE and used for enzymatic assays and biotransformations.

## Enzymatic Assays

Standard enzymatic assays were performed at 30 °C. A 1200 μL quartz cuvette typically contained 50 mM Tris-glycine buffer (pH 9.0), 5 mM of *p*-nitrophenyl sulfate as the sulfate donor and 5 mM of acceptor (usually phenol). After prewarming the cuvette in a water bath for at least 5 min, 50 μL AST extract were added and the release of *p*-nitrophenol was monitored at 405 nm on a Varian Cary50 spectrophotometer. The rate of enzymatic sulfate transfer was calculated from the initial linear part of the kinetic curve, using the molar extinction coefficient for *p*-NP ($e_{405}$ = 16.6 mM$^{-1}$ cm$^{-1}$). One unit of activity is defined as the amount of enzyme catalysing the formation of 1 μmol *p*-NP per minute. All activities were corrected for non-enzymatic release of *p*-nitrophenol.

The pH optimum of the enzyme was determined in a discontinuous assay in Tris-glycine buffer over pH range 4–11 at 30 °C. Enzyme was added to buffered solutions of 5 mM *p*-NPS and 5 mM phenol or tyramine and after 5 min the reaction was stopped by placing the tubes on ice, followed by the addition of NaOH and the absorbance at 405 nm was measured.

For measurements to determine the thermostability, samples were incubated at the indicated temperatures for 5 min. After that, activity was measured as described above.

## HPLC

Enzymatic assays with donor substrates other than *p*-NPS or with a steroid as acceptor substrate were monitored by HPLC. 5 mM of donor substrate were mixed in an Eppendorf tube with 5 mM of acceptor substrate in a 50 mM Tris-glycine buffer (pH 9.0) and incubated at 30 °C in an Eppendorf Thermomixer compact. After 5 min, 50 μL of AST extract were added and samples were taken at regular time intervals. The sample was diluted twenty times in 10% acetonitrile/water and injected on a Nucleosil 100–5 C-18 HD column (plus guard column), equilibrated with 25 mM $NH_4H_2PO_4$ buffer (pH 6.3) containing 5 mM TBAP (tetrabutylammonium phosphate) as an ion-pairing reagent, and 5% acetonitrile. The HPLC dual pump system was equipped with 2 LKB 2150 pumps and a LKB 2152 controller. An injection loop of 50 μL was used. A 0–60% gradient (43 min run, 0.4 mL min$^{-1}$ flow rate) of 90% acetonitrile, containing 5 mM TBAP, was applied. The UV absorbance of the eluate was monitored both at 210 nm (Figure 4 and Figure 5) and at 254 nm using a Pharmacia LKB VWM2141 dual diode array variable wavelength detector. For data collection and integration the Borwin 1.21 software was used. Peak areas of the detected substrates and products were used to calculate the degree of sulfate transfer.

## "Large" Scale Estradiol Sulfation (Reaction Conditions, Product Isolation and Analysis)

To a 100-mL round-bottom flask containing 3.7 mL acetone, 9.25 mL 400 mM Tris/glycine (pH 8.5), 11.95 mL water, 10 mL 40 mM *p*-NPS, 100 mg 17β-estradiol were added and the reaction was started by the addition of 2.1 mL 8.8 μM AST. This resulted in a solution of 37 mL containing 10% acetone/10 mM 17β-estradiol/10.8 mM *p*-NPS and 0.5 μM AST in 100 mM buffer.

This solution was kept at of 30 °C and stirred in a closed round-bottom flask. The reaction was followed by taking samples and analysis by HPLC after dilution in 10% acetonitrile/water (see above). When maximal conversion was reached, the solution was extracted with three times 20 mL $CH_2Cl_2$ which removed acetone, 50% of the *p*-NP and the

  

remaining traces of 17β-estradiol (the purification was adapted from ref.[35]). The water solution was extracted three times with 20 mL *n*-butanol resulting in extraction of 95% of the product and some *p*-NP and *p*-NPS. This organic solution was washed with 5 mL 5% NaCl solution, the organic phase removed and evaporated. The product was washed with ethyl acetate and then solubilised in 3 mL water. The pH was brought to 11 with a small amount of 1 N NaOH. The solution was extracted again with two times 10 mL *n*-butanol, which was evaporated to dryness. The solid product was washed with ethyl acetate and the material was solubilised in 3 mL water. After concentration to 1 mL and cooling a precipitate was obtained which was collected by centrifugation. After discarding the water solution the solid product was dried under vacuum.

## Acknowledgements

*This work was supported in part by the Dutch National Research School Combination (NRSC-Catalysis) and was performed under the ACTS-NWO program Integration of Biosynthesis & Organic Synthesis (IBOS). The former companies Organon and Diosynth which are now MSD (Oss, NL), and Syncom (Groningen, NL) are kindly acknowledged for financial support and Organon and Diosynth for the gift of steroids. We also wish to thank Prof. Dr. D. Kellog (Syncom), Dr. M. Ostendorp (Diosynth, Organon), Dr. M. Huibers, Dr. F.L. van Delft (Radboud University, Nijmegen) for valuable discussions and help.*

## References

[1] E. Chapman, M. D. Best, S. R. Hanson, C. H. Wong, *Angew. Chem.* **2004**, *116*, 3610–3632; *Angew. Chem. Int. Ed.* **2004**, *43*, 3526–3548.
[2] G. Malojcic, R. Glockshuber, *Antioxid. Redox Signaling* **2010**, *13*, 1247–1259.
[3] N. Gamage, A. Barnett, N. Hempel, R. G. Duggleby, K. F. Windmill, J. L. Martin, M. E. McManus, *Toxicol. Sci.* **2006**, *90*, 5–22.
[4] E. G. Cormier, M. Persuh, D. A. D. Thompson, S. W. Lin, T. P. Sakmar, W. C. Olson, T. Dragic, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 5762–5767.
[5] M. Farzan, T. Mirzabekov, P. Kolchinsky, R. Wyatt, M. Cayabyab, N. P. Gerard, C. Gerard, J. Sodroski, H. Choe, *Cell* **1999**, *96*, 667–676.
[6] K. G. Bowman, S. Hemmerich, S. Bhakta, M. S. Singer, A. Bistrup, S. D. Rosen, C. R. Bertozzi, *Chem. Biol.* **1998**, *5*, 447–460.
[7] M. Miksits, K. Wlcek, M. Svoboda, T. Thalhammer, I. Ellinger, G. Stefanzl, C. N. Falany, T. Szekeres, W. Jaeger, *Cancer Lett.* **2010**, *289*, 237–245.
[8] L. S. Simpson, T. S. Widlanski, *J. Am. Chem. Soc.* **2006**, *128*, 1605–1610.
[9] S. D. Taylor, A. Desoky, *Tetrahedron Lett.* **2011**, *52*, 3353–3357.
[10] B. Guilbert, N. J. Davis, M. Pearce, R. T. Aplin, S. L. Flitsch, *Tetrahedron: Asymmetry* **1994**, *5*, 2163–2178.
[11] G. K. Tóth, B. Penke, M. Zarándi, K. Kovács, *Int. J. Peptide Protein Res.* **1985**, *26*, 630–638.
[12] V. V. Mozhaev, Y. L. Khmelnitsky, F. Sanchez-Riera, J. Maurina-Brunker, R. A. Rosson, A. D. Grund, *Biotechnol. Bioeng.* **2002**, *78*, 567–575.
[13] C. H. Lin, G. J. Shen, E. Garcia-Junceda, C. H. Wong, *J. Am. Chem. Soc.* **1995**, *117*, 8031–8032.
[14] C. Niehrs, W. B. Huttner, D. Carvallo, E. Degryse, *J. Biol. Chem.* **1990**, *265*, 9314–9318.
[15] K. Kobashi, Y. Fukaya, D. H. Kim, T. Akao, S. Takebe, *Arch. Biochem. Biophys.* **1986**, *245*, 537–539.
[16] D. H. Kim, L. Konishi, K. Kobashi, *Biochim. Biophys. Acta* **1986**, *872*, 33–41.
[17] M. C. Baek, S. K. Kim, D. H. Kim, B. K. Kim, E. C. Choi, *Microbiol. Immunol.* **1996**, *40*, 531–537.
[18] J. W. Kang, Y. J. Jeong, A. R. Kwon, H. J. Yun, D. H. Kim, E. C. Choi, *Arch. Pharmacal Res.* **2001**, *24*, 316–322.
[19] A. R. Kwon, T. G. Oh, D. H. Kim, E. C. Choi, *Protein Expression Purif.* **1999**, *17*, 366–372.
[20] K. Kobashi, D. H. Kim, T. Morikawa, *J. Protein Chem.* **1987**, *6*, 237–244.
[21] J. W. Kang, A. R. Kwon, D. H. Kim, E. C. Choi, *Biol. Pharm. Bull.* **2001**, *24*, 570–574.
[22] L. Kaysser, K. Eitel, T. Tanino, S. Siebenberg, A. Matsuda, S. Ichikawa, B. Gust, *J. Biol. Chem.* **2010**, *285*, 12684–12694.
[23] D. H. Kim, K. Kobashi, *J. Biochem.* **1991**, *109*, 45–48.
[24] G. Malojcic, R. L. Owen, J. P. Grimshaw, M. S. Brozzo, H. Dreher-Teo, R. Glockshuber, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 19217–19222.
[25] N. Tanaka, Z. Hasan, R-J. Sanders, A. F. Hartog, T. van Herk, R. Wever, *Org. Biomol. Chem.* **2003**, *1* , 2833–2839.
[26] S. de Macedo-Ribeiro, R. Renirie, R. Wever, A. Messerschmidt, *Biochemistry* **2008**, *47*, 929–934.
[27] J. P. Grimshaw, C. U. Stirnimann, M. S. Brozzo, G. Malojcic, M. G. Grutter, G. Capitani, R. Glockshuber, *J. Mol. Biol.* **2008**, *380*, 667–680.
[28] A. R. Kwon, E. C. Choi, *Arch. Pharmacal Res.* **2005**, *28*, 561–565.
[29] T. Teramoto, R. Adachi, Y. Sakakibara, M. C. Liu, M. Suiko, M. Kimura, Y. Kakuta, *FEBS Lett.* **2009**, *583*, 3091–3094.
[30] L. Konishi-Imamura, D. H. Kim, K. Kobashi, *Biochem. Int.* **1992**, *28*, 725–734.
[31] S. P. Edgcomb, K. P. Murphy, *Proteins Struct. Funct. Genet.* **2002**, *49*, 1–6.
[32] B. Delhom, G. Alvaro, G. Caminal, J. L. Torres, P. Clapes, *Biotechnol. Lett.* **1996**, *18*, 609–614.
[33] M. Pogorevc, K. Faber, *Tetrahedron: Asymmetry* **2002**, *13*, 1435–1441.
[34] M. Huibers, *Dissertation*, Univ. Nijmegen, **2009**.
[35] R. Y. Kirdani, *Steroids* **1965**, *6*, 845–853.

  