Sulfation made easy: A new versatile donor for enzymatic sulfation by a bacterial arylsulfotransferase

Aloysius F. Hartog, Ron Wever*

Biocatalysis and Bio-organic Chemistry, Van't Hoff Institute for Molecular Sciences, University of Amsterdam, Science Park 904, 1090 GD Amsterdam, The Netherlands

ABSTRACT

An efficient and versatile donor for the sulfation by a bacterial arylsulfotransferase of various phenolic acceptor molecules is reported. Most studies in the past used toxic p-nitrophenyl sulfate as a sulfate donor for sulfation by this enzyme. However both the donor and p-nitrophenol are difficult to remove from the sulfated products. This new donor N-hydroxysuccinimide sulfate is easy to synthesize and has the advantage that at pH values above 7 it hydrolyzes to N-hydroxysuccinimide which is a safe compound and can easily be removed. As examples we demonstrated the formation of sulfated resveratrol and synthesized efficiently 3-sulfo-17-β-estradiol and bisphenol A bisulfate. It is likely that many other phenolic compounds are sulfated using this donor.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Sulfated compounds are widespread and include proteins, steroids, polysaccharides and assorted metabolites. In many cases sulfation regulates their bioactivity and there is increasing interest in designing sulfated pharmaceutical agents [1–3]. Sulfated molecules are difficult to synthesize chemically since their solubility changes dramatically upon introduction of the sulfate group, rendering them water soluble and difficult to purify. In addition, when labile functionalities are present, or when dealing with polyfunctional substrates, protecting groups are usually required [4,5]. Moreover under acidic conditions most monosulfate esters are unstable [6] limiting chemical procedures at low pH. Various chemical strategies have been reported for synthesis. A convenient procedure is the synthesis of protected sulfate monoesters followed by deblocking the sulfate diester with an appropriate strong nucleophile [6]. This procedure has been successfully employed also in the synthesis of Fmoc-protected tyrosine cassettes [7]. Although the yields are high, this is a two-step procedure in which first the protected monoester has to be synthesized. More recently sulfated phenolic compounds were synthesized by an improvement of the method describes by Kawai et al. [8]. A sulfur-trioxide complex is used as a donor in dry dioxane at low temperatures after

which water is added and the mixture is neutralized with diethylamine. The compounds are subsequently purified on silica gel and or a cation-exchange column [9]. An elegant route to sulfated peptides using Fmoc-fluorosulfated tyrosine has also been reported [10]. After incorporating the fluorosulfated tyrosine residue into peptides using a Fmoc-based solid-phase peptide synthetic strategy the fluorosulfated tyrosine peptides are transformed into sulfotyrosine peptides. Sulfated compounds have also been synthesized enzymatically using the sulfotransferases which catalyze the transfer of a sulfuryl group from an activated donor to acceptors like hormones, proteins and carbohydrates [11–13]. These mainly eukaryotic sulfotransferases exclusively use 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as a sulfuryl donor. Though an enzymatic regeneration system for PAPS has been developed this adds complexity to the procedure requiring a strict control of conditions [14]. More recently [15–17] a more cheaply synthetic procedure for PAPS has been described which allows its direct use as a sulfate donor. Unfortunately the PAPS-dependent enzymes are highly specific for the acceptor molecules, which limits their application [2,13].

Bacterial arylsulfotransferases form another class of enzymes that are able to sulfate steroids, polyphenols and peptides [18–27]. These versatile enzymes catalyze sulfuryl transfer from a phenolic sulfate to an OH group and have broad substrate specificity though in general only aromatic OH groups are sulfated. However, sulfation at a slow rate of a variety of non-phenolic alcohols by the bacterial enzyme from *Desulfitobacterium hafniense* has also been
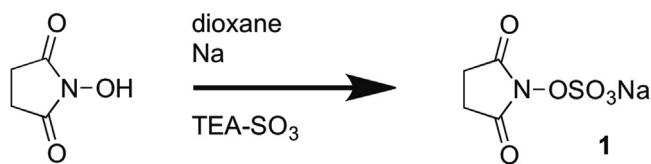
Fig. 1. Scheme of the synthesis of *N*-hydroxysuccinimide-sulfate (**1**).

observed [24]. Recently it was demonstrated [28] that by substrate engineering a number of carbohydrate derivatives could be sulfated by this bacterial enzyme. However, a broad applicability of the enzymatic sulfation is hindered by the rather cumbersome purification of the sulfated product to remove toxic *p*-nitrophenol and the donor *p*-NPS limiting the application of the enzymatic system in the synthesis of products with a pharmaceutical application.

In our search for compounds that were sulfated by the bacterial arylsulfotransferase we observed [24] that when *N*-hydroxysuccinimide (**1**) was added to a solution of *p*-nitrophenol sulfate in the presence of the enzyme that the concentration of *p*-nitrophenol sulfate decreased. We concluded that sulfate transfer might have occurred and decided to synthesize the sulfate ester of NHS as a reference compound and to test its application as a sulfate donor for the enzyme.

2. Experimental

2.1. HPLC analysis and NMR

All chemicals used were purchased from Sigma-Aldrich. Reaction progress was monitored by HPLC. Samples from the reaction mixture were diluted 100 times with acetonitrile/water (1:1), and 25 μL was injected onto a Nucleosil 100-5C-18HD column (plus guard column), equilibrated with $NH_4H_2PO_4$ buffer (pH 6.3, 25 mM) containing TBAP (ion-pairing reagent, tetrabutylammonium phosphate, 5 mM) and 5% acetonitrile (buffer A). The HPLC dual pump system was an Agilent 1100 system with a 0–80% gradient (37 min run, 0.4 mL min^{-1} flow rate) of acetonitrile (90% aq.), containing TBAP (5 mM) (buffer B). The UV absorbance of the compounds was monitored at 210 and 300 nm. Peak areas of the detected substrates and products were used to calculate the degree of sulfate transfer. ^{1}H and ^{13}C NMR spectroscopy was carried out with a Bruker NMR instrument at 400 or 500 MHz. Samples for NMR measurements were dissolved in [D$_6$]DMSO.

2.2. Heterologous overproduction and purification of AST

The AST was expressed and purified using a method developed earlier with minor modifications [24,25].

2.3. Chemical synthesis of N-hydroxysuccinimide-sulfate (**1**)

Esters of NHS are widely used in peptide synthesis, in the synthesis of *N*-acylamino acids and acetylated NHS has been used as acetate donor for the acetylation and derivatization of amino groups in peptides [29–31]. However, there is hardly any literature on the synthesis of the NHS-sulfate ester (1-(sulfooxy)-2,5-pyrrolidinedione, CAS 127007-81-2). Only a patent [32] describes its synthesis (outlined below in Fig. 1) and its application as a cross linker of cellulose fibers.

0.5 g of a mixture of sodium hydride (NaH) in mineral oil (60%) was washed 2 times with petroleum ether. The solid was dispersed in 8 mL dry dioxane under nitrogen and 1.15 g (10 mmol) of *N*-hydroxysuccinimide was added wise at 20 °C. After 1 h 16 mL dry dioxane with 2 g (11 mmol) triethylamine-SO$_3$ was added drop

wise and the solution was stirred for 24 h. 10 mL of water was added drop wise and the dioxane removed by evaporation. The 10 mL water phase was extracted twice with 10 mL ethyl acetate and water was removed by evaporated to almost complete dryness. 20 mL of methanol was added resulting in a precipitate that was removed and discarded. After standing white crystals were formed in the solution, these were collected on a glass filter and washed with methanol. HPLC, IR, and ^{1}H and ^{13}C NMR identified this as NHS sulfate (sodium salt, 0.93 g).

2.4. Enzymatic synthesis of 3-sulfo-17-β-estradiol (**3**) using NHS-sulfate as donor

17-β-Estradiol (**2**) (0.024 g, 0.088 mmol) was dissolved in 600 μL acetone and 5 mL Tris-glycine (100 mM, pH 9) was added. To the milky solution 500 μL of 12 U/ml AST was added. The resulting solution (1 unit/ml, 1.6 μM AST, 15 mM 17-β-estradiol) was stirred and NHS-sulfate Na salt (0.021 g, 0.1 mmol) was added (Fig. 2). The addition was repeated 3 times every hour. After 4 h acetic acid was added to pH 6 and the reaction mixture evaporated to almost dryness. The residue was solubilized in 20 mL methanol and 5 g silica gel was added. After evaporation to almost dryness the silica gel was poured onto a silica column and the silica gel column was eluted with acetonitrile (0.5% acetic acid/5 to 10% methanol). NHS eluted first followed by (**3**). Due to complete hydrolyses of NHS-sulfate at pH > 7 only NHS was found in the first fractions. TLC and HPLC were used to check the fraction and these were pooled and evaporated to complete dryness. Pure **3** (0.028 g, 68% isolated yield) was obtained.

2.5. Enzymatic synthesis of bisphenol A 4,4′-bisulfate (**5**) using NHS-sulfate (**1**) as a donor

Bisphenol A (**4**) (0.046 g, 0.2 mmol) was added to 14 mL Tris-glycine (100 mM, pH 9). To the milky solution 700 μL of 47 U/ml AST was added. The resulting solution (2.2 unit AST/ml) was stirred and (**1**) (0.035 g, 0.16 mmol) was added. The addition of (**1**) was repeated 5 times every hour. After 2 h the colorless solution clarified. Each additions of (**1**) was followed by the addition of 0.05 g solid Tris to keep the pH between pH 8.5 and 9. After 6 h the reaction was stopped by the addition of acetic acid and (**5**) was purified by silica gel chromatography as described for (**3**). Pure **5** (0.055 g, 70% isolated yield) was obtained.

3. Results and discussion

First *E*-resveratrol, 17-β-estradiol (**2**) and bisphenol A (2,2-bis (4-hydroxyphenyl) propane) (**4**) were tested as acceptors in the enzymatic sulfation with the NHS-sulfate as a donor. The first two compounds have previously been sulfated by the arylsulfotransferase using *p*-nitrophenol sulfate. Bisphenol A is a well-known endocrine-disrupting chemical [33]. After exposure the latter compound is secreted in its sulfated form by the human body. As shown before the sulfation of *E*-resveratrol is complex since it has three OH groups that may become sulfated resulting in the formation of mono, di- and trisulfated resveratrol [25]. On a small scale (1 mL) enzymatic sulfation of resveratrol was tested by the daily addition of NHS-sulfate. After 3 days resveratrol was converted completely into its sulfated forms. The HPLC traces in the Supporting information shows this conversion in detail. Similarly on a small scale (1 mL) 17-β-estradiol (**2**) and bisphenol A (2,2-bis (4-hydroxyphenyl) propane) (**4**) were completely sulfated enzymatically (not shown) in 4–6 h by adding each hour one equivalent of solid NHS-sulfate (Na salt) keeping the pH at 9 by addition of solid Tris.
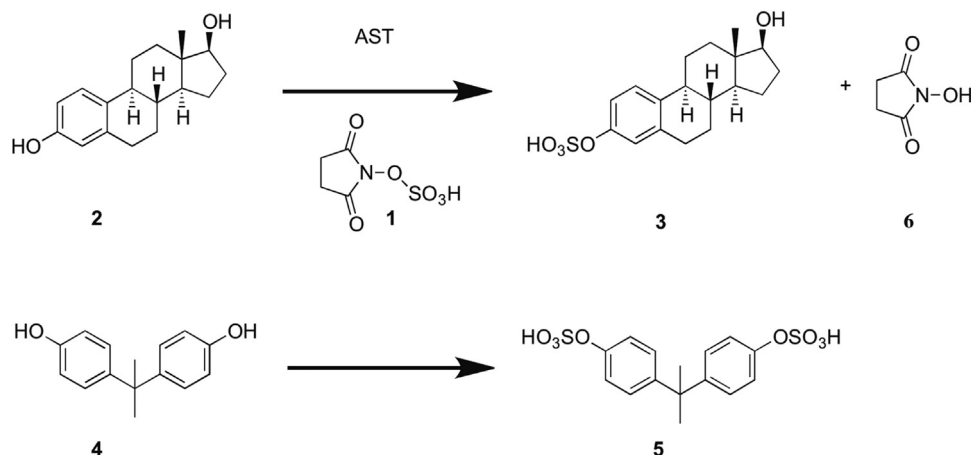
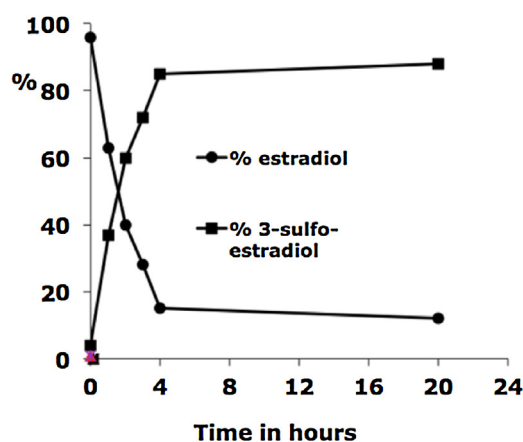**Fig. 2.** Enzymatic synthesis of 3-sulfo-17-β-estradiol (yield 68%) and bisphenol A 4,4′-bisulfate (yield 70%).

Fig. 3. The sulfation of 0.088 mmol 17-β-estradiol (**2**) catalyzed by 1.2 U/ml AST was performed in 6 mL Tris-glycine (100 mM, pH 9.0) by subsequent additions of 0.1 mmol of NHS-sulfate (**1**). Reaction progress was monitored by HPLC and measuring the UV absorbance at 210 nm.

Fig. 4. The sulfation of 0.2 mmol bisphenol A (**4**) catalyzed by 2.2 U/ml AST was performed in 15 mL Tris-glycine (100 mM pH 9.0) by subsequent additions of 0.15 mmol of NHS-sulfate (**1**). Reaction progress was monitored by HPLC and measuring the UV absorbance at 210 nm.

This successful approach prompted us to synthesize 3-sulfo-17-β-estradiol (**3**) and bisphenol 4,4′-bisulfate (**5**) with NHS-sulfate (**1**) as a sulfate donor. Fig. 3 gives the transformation of estradiol (**2**) and bisphenol A (**4**) by the enzyme and NHS-sulfate as a sulfate donor.

Fig. 3 shows the time course of the conversion of estradiol (**2**) and the formation of its sulfated form (**3**) by the bacterial enzyme.

In this synthesis of 3-sulfo-17-β-estradiol (**4**), NHS-sulfate (**1**) was added step wise in near stoichiometric amounts every hour to the sulfate acceptor. Notably super stoichiometric amounts of the donor are needed to completely sulfate estradiol. This is due to hydrolysis since when NHS-sulfate was incubated at pH 9 in the absence of the enzyme, HPLC data showed that after 1 h already 50% of the donor was hydrolysed. The conversion and subsequent purification resulted in pure (**3**) (0.028 g, 68% isolated yield) according to HPLC and NMR data. Fig. 4 shows similar results in the conversion of bisphenol into its sulfated form. Apparently, once monosulfated bisphenol is formed from poorly soluble bisphenol it is rapidly converted into the disulfated bisphenol. This also explains the linear formation (Fig. 4) of this compound in time.

Due to hydrolysis of NHS-sulfate (**1**) again super stoichiometric amounts of the donor had to be added to complete the conversion. Pure (**5**) (0.055 g, 70% isolated yield) was obtained. It is interesting to note that NHS-sulfate is also able to sulfate p-NP by AST (not shown).
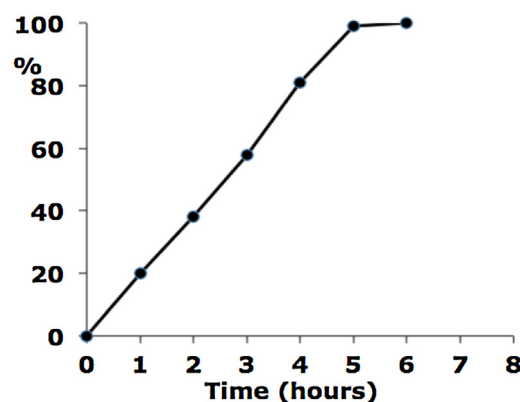
4. Conclusion

We have demonstrated that N-hydroxysuccinimide-sulfate can be used as sulfate donor in the enzymatic sulfation of E-resveratrol, 17-β-estradiol and bisphenol A. We also synthesized 3-sulfo-17-β-estradiol and bisphenol A 4,4′- bisulfate using this enzymatic procedure and characterized these compounds by ^{1}H and ^{13}C NMR. In the procedures used previously [24], removal of the donor p-nitrophenol sulfate or the formed p-nitrophenol was cumbersome. With this new donor the yield of formation of 3-sulfo-17-β-estradiol is higher and it has the advantage that a single silica gel column is sufficient to obtain pure product. Furthermore N-hydroxysuccinimide-sulfate easily hydrolyzes at the optimal pH value for enzymatic activity (pH 8–9) and compared to p-nitrophenol N-hydroxysuccinimide is much safer in use and much less toxic.

Conflict of interest

The authors declare no conflict of interest

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2016.04.007.

References

[1] R.A. Al-Horani, U.R. Desai, Tetrahedron 66 (2010) 2907–2918.
[2] K.G. Bowman, C.R. Bertozzi, Chem. Biol. 6 (1999) R9–R22.
[3] Fl. Nodari, F.F. Hsu, X. Fu, T.F. Holekamp, L.F. Kao, J. Turk, T.E. Holy, J. Neurosci. 25 (2008) 6418–6470.
[4] S.D. Taylor, A. Desoky, Tetrahedron Lett. 52 (2011) 3353–3357.
[5] B. Guilbert, N.J. Davis, M. Pearce, R.T. Aplin, S.L. Flitsch, Tetrahedron: Asymmetry 5 (1994) 2163–2178.
[6] L.S. Simpson, T.S. Widlanski, J. Am. Chem. Soc. 128 (2006) 1605–1610.
[7] M.J. Stone, R.J. Payne, Acc. Chem. Res. 48 (2015) 2251–2261.
[8] N. Kawai, Y. Fujibayashi, S. Kuwabara, K. Takao, Y. Ijuin, S. Kobayashi, Tetrahedron 56 (2000) 6467–6478.
[9] V.P. Martins Gomes, C. Torres, J.E. Rodríguez-Borges, F. Paiva-Martins, J. Agric. Food Chem. 43 (2015) 9565–9571.
[10] W. Chen, J. Dong, S. Li, Y. Liu, Y. Wang, L. Vroon, P. Wu, K.B. Sharpless, J.W. Kelly, Angew. Chem. Int. Ed. 55 (2016) 1835–1838.
[11] E. Chapman, M.C. Bryan, C.-H. Wong, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 910–915.
[12] J.D. Mougous, R.E. Green, S.J. Williams, S.E. Brenner, C.R. Bertozzi, Chem. Biol. 9 (2002) 767–776.
[13] E. Chapman, M.D. Best, S.R. Hanson, C.-H. Wong, Angew. Chem. Int. Ed. 43 (2004) 3526–3548.
[14] M.D. Burkart, M. Izumi, E. Chapman, C.H. Lin, C.-H. Wong, J. Org Chem. 65 (2000) 5565–5574.
[15] X. Zhou, K. Chandarajoti, T.Q. Pham, R. Liu, J. Liu, Glycobiology 21 (2011) 771–780.
[16] X. Zhou, T.R. O'Leary, Y. Xu, J. Sheng, J. Liu, J. Biocatal. Biotrans. 30 (2012) 296–308.
[17] J. Liu, R.N. Linhardt, F.Y. Avci, E.M. Munoz, J. Chen, U.S. Patent, (2012) Application 0322114 A1.
[18] Y. Kobashi, D. Fukaya, H. Kim, T. Akao, T.S. Takebe, Arch. Biochem. Biophys. 245 (1986) 537–539.
[19] B. Delhom, G. Alvaro, G. Caminal, J.L. Torres, P. Clapes, Biotechnol. Lett. 18 (1966) 609–614.
[20] V.V. Mozhaev, Y.L. Khmelnitsky, F. Sanchez-Riera, J. Maurina-Brunker, R.A. Rosson, A.D. Grund, Biotechnol. Bioeng. 78 (2002) 567–575.
[21] G. Malojcic, R.L. Owen, J.P. Grimshaw, R. Glockshuber, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 19217–19222.
[22] G. Malojcic, R. Glockshuber, Antioxid. Redox Signal. 13 (2010) 1247–1259.
[23] G. Malojcic, R.L. Owen, R. Glockshuber, Biochemistry 53 (2014) 1870–1877.
[24] M.A. Van der Horst, J.F.T. Van Lieshout, A. Bury, A.F. Hartog, R. Wever, Adv. Synth. Catal. 354 (2012) 3501–3508.
[25] M.A. Van der Horst, A.F. Hartog, R. El Morabet, A. Marais, M. Kircz, R. Wever, Eur. J. Org. Chem. (2015) 534–554.
[26] I. Ayuso-Fernández, M.A. Galmés, A. Bastida, E. García-Junceda, ChemCatChem 6 (2014) 1059–1065.
[27] K. Purchartová, K. Valentová, H. Pelantová, P. Marhol, J. Cvacka, L. Havlcek, A. Krenková, E. Vavrková, D. Biedermann, C.S. Chambers, V. Kren, ChemCatChem 7 (2015) 3152–3162.
[28] A.F. Hartog, R. Wever, Adv. Synth. Catal. 357 (2015) 2629–2632.
[29] G.W. Anderson, J.E. Zimmerman, F.M. Callahan, J. Am. Chem. Soc. 86 (1964) 1839–1842.
[30] Y. Lapidot, S. Rappoport, Y. Wolman, Y.J. Lipid, Research 8 (1967) 142–145.
[31] V.P. Demushkin, E.I. Budowsky, FEBS Lett. 22 (1972) 89–90;
A. Chakraborty, F.E. Regnier, J. Chromatogr. A 949 (2002) 173–184.
[32] R.A. Jewell, Method for crosslinking individualized cellulose fibres, (1993) WO9314261 A1.
[33] K. Okuda, T. Fukuuchi, M. Takiguchi, S. Yoshihara, Drug Metab. Dispos. 39 (2011) 1696–1703.