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New Fluorogenic Substrate for the First Continuous Steroid Sulfatase Assay

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Abstract—The screening for new inhibitors of steroid sulfatase requires an efficient test system. To overcome the shortcomings of the available discontinuous fluorimetric assay, several coumarin-type compounds were investigated as potential new substrates. 3,4-Benzocoumarin 7-*O*-sulfate was found to have appropriate substrate properties for the establishment of the first direct continuous assay of steroid sulfatase.

Steroid sulfatase (estrone sulfatase, E.C. 3.1.6.2.) is involved in the local production of estrogens in normal and malignant breast tissues by catalysing the desulfation of estrone sulfate to estrone. There is increasing evidence that the steroid sulfatase pathway is the major source of estrogens in breast and endometrial tumours.[1] Inhibitors of steroid sulfatase are therefore considered as potential new therapeutic agents for the treatment of estrogen-dependent cancers.

For the evaluation of potential inhibitors three types of steroid sulfatase assays are available: a radiometric assay[2] using the radiolabeled natural substrate ^{3}H-estrone sulfate, colorimetric assays[3] using *p*-nitrophenyl (pNPS) or *p*-nitrocatechol sulfate (pNCS) and a fluorimetric assay[3] using 4-methylumbelliferyl sulfate (**1**; 4-MUS, Scheme 1). The usefulness of the non-radiometric substrates is limited by their relatively high K_M values (736, 680, and 275 μM for pNPS, pNCS, and 4-MUS, respectively as compared to 95 μM for estrone sulfate; A. Billich, unpublished). Furthermore, these are discontinuous assays because at the pH optimum of the steroid sulfatase (7.5) only little change in absorbance or fluorescence intensity is observed. Alkalisation is required to generate the absorbing/fluorescing phenolates of the reaction products; e.g. 4-methylumbelliferone (**2a**; 4-MU, Scheme 1) as the product of 4-MUS shows low fluorescence intensity at pH 7.5, but the phenolate (**2b**) shows a strong signal at pH >10. Therefore, we argued that a continuous assay would require a substrate that yields a phenolic product with a lower pK_a, hence producing a signal at physiological pH.[4]

We envisaged designing coumarin derivatives exhibiting properties that would allow both shortcomings to be overcome, i.e., low binding affinity of the sulfate and insufficient fluorescence of the phenolic cleavage product at physiological pH. The sulfamate analogues of both the natural and the assay substrate (Scheme 2), estrone 3-*O*-sulfamate (**3**) and 4-methylcoumarin 7-*O*-sulfamate (**4**), are irreversible inhibitors of steroid sulfatase.[5] Their relative inhibitory potencies correlate with the ranking of the K_M values of the corresponding sulfates as substrates (**3**: $rIC_{50} = 1$, $K_M = 95$ μM; **4**: $rIC_{50} = 30$, $K_M = 275$ μM; see also Table 1).

Based on these data we decided to first prepare the sulfamate derivatives[6] **5**–**12** of our target molecules (Fig. 1) and to test their inhibitory potencies (Table 1) in order to rapidly screen for the magnitude of the enzyme binding affinity. Only for selected compounds with high inhibitory activities the synthesis of the corresponding sulfates and the evaluation of their usefulness as substrate was planned.

In 1985 Koller and Wolfbeis reported on the preparation of several coumarin-based compounds with pK_a values ≤ 7 and suggested the corresponding sulfates as substrates for continuous assays of aryl sulfatases.[7,8] The most promising compound according to their results was 3-(2-benzothiazolyl)coumarin-7-*O*-sulfate.[8] Following our strategy, we synthesised the corresponding sulfamate **5** and tested it against steroid sulfatase. Compound **5** was found to be at least 250 times less potent than the standard **3** and also considerably less active than the 4-methylumbelliferyl analogue **4** (Table 1). The most likely reason for this finding is the fact that Koller and Wolfbeis used preparations of arylsulfatases A and B in their investigations. These

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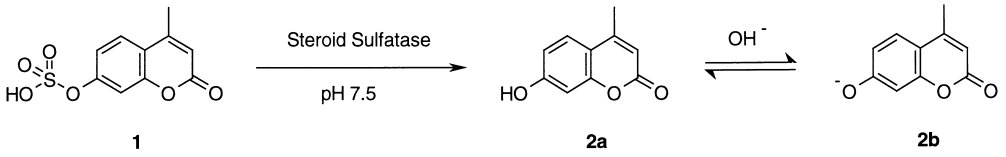
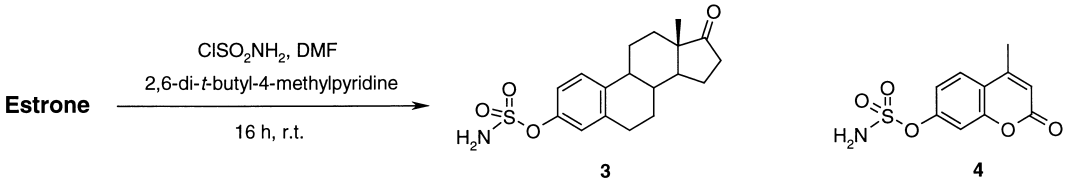


Scheme 1. Substrate (**1**; 4-MUS) and product (**2a,b**; 4-MU and its phenolate) of the standard fluorimetric assay.



Scheme 2. Structures of the irreversible steroid sulfatase inhibitors **3** and **4** and synthesis of **3** as example for the general synthesis of sulfamates (DBMP = 2,6-di-*t*-butyl-4-methylpyridine).

Table 1. Relative inhibitory potencies (rIC_{50}) of sulfamates **3–12** against recombinant human steroid sulfatase[9]

Comp.	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
rIC_{50}[a]	1	30	>250	48	11	>250	3.5	5	>160	3

[a]Compounds **5**, **9**, **10** were tested using a colorimetric assay, compounds **3**, **4**, **6**, **7**, **8**, **11**, **12** using a fluorimetric assay.[3] The substrate (5 mM of pNCS or 0.5 mM of 4-MUS) was incubated with steroid sulfatase (50 nM or 1.5 nM, respectively) in the presence of graded concentrations of inhibitors at pH 7.5 and 37 °C for 60 min. Then, 0.2 M NaOH was added and absorption at 492 nm or fluorescence intensity ($\lambda_{ex} = 355$ nm, $\lambda_{em} = 460$ nm) was measured, respectively. The inhibitory activities of the test compounds (IC_{50}) were calculated using nonlinear regression (software Grafit) and expressed as relative IC_{50} values normalised to the standard estrone sulfamate (**3**): $rIC_{50} = (IC_{50}$ of test compound$)/(IC_{50}$ of **3**$)$.

sulfatases show only ca. 30% homology to steroid sulfatase and their natural substrates—galactose sulfates in sulfolipids and glucosaminoglycans—are structurally very different from estrone sulfate, the natural substrate of steroid sulfatase.

Assuming that the benzothiazolyl substituent was sterically too demanding for tight binding to the enzyme we investigated the 3-phenyl substituted coumarin derivatives **6–8**. Sulfamates **6** and **7** displayed inhibitory activities in the same range as 4-methylumbelliferyl sulfamate (**4**). In contrast, analogue **8** with the sulfamate moiety in position 6 of the coumarin element was substantially less active than **4**, **6** and **7**. This finding confirmed that substitution by the oxygen functionality at position 7 of the coumarin core structure is required,[1] which is rationalised by considering the coumarin 7-*O*-sulfamates as AB-ring mimics of estrone sulfate. Next we explored structures more similar to the steroidal skeleton of estrone and estrogens: the natural product coumestrol, known to exhibit fluorescence at physiological pH, and the benzocoumarin analogue. Sulfamoylation of coumestrol under standard conditions[6] yielded one main product, the monosulfamate **9**. The assigned structure was confirmed by spectral and biological comparison with the two monosulfamate derivatives **10** and **11** which were synthesised from the monohydroxy precursors. As shown in Table 1, coumestrol monosulfamate **9** and coumestan 3-*O*-sulfamate (**10**) were nearly as potent as the standard estrone sulfamate (**3**) in inhibiting steroid sulfatase. In contrast, the regioisomeric coumestan derivative **11** was substantially less active, thus, again confirming the importance of the 7-hydroxycoumarin motif in steroid sulfatase inhibitors. Interestingly, the benzocoumarin analogue **12** also showed high potency being only 3-fold less potent than **3**. Therefore, the benzocoumarin core structure

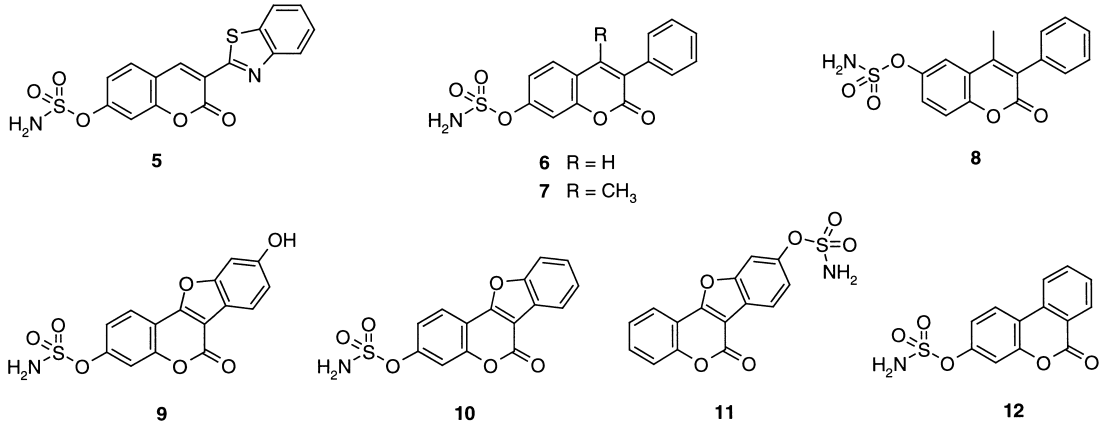
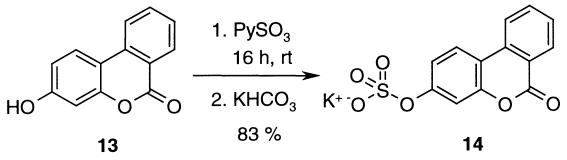


Figure 1. Sulfamates **5–12** of coumarin-based target structures.



Scheme 3. Synthesis of benzocoumarin sulfate **14**.

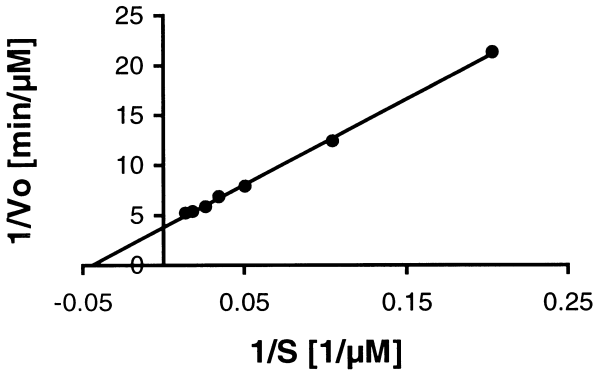


Figure 2. Lineweaver–Burk plot for **14**.

was selected for further investigation, i.e., synthesis and evaluation of the corresponding sulfate.

The sulfate **14** was prepared as outlined in Scheme 3 and the photophysical properties of both the sulfate and the phenolic cleavage product **13** were investigated in detail. Surprisingly, **13** showed low fluorescence at neutral pH, while the sulfate **14** exhibited significant fluorescence quantum yield. The data indicated that a quantifiable intensity change for a fluorimetric assay can be obtained upon sulfate cleavage, but, in contrast to the 4-MUS assay, the enzymatic reaction on **14** results in reduction and not in increase of fluorescence intensity. Kinetic measurements revealed that **14** is recognised as substrate by the steroid sulfatase with a K_M value of 22.5 ± 2.6 µM and a k_{cat} value of 14.4 ± 1.9 min^{-1} (see Figure 2 for a typical Lineweaver–Burk plot; $\lambda_{ex} = 355$ nm, $\lambda_{em} = 460$ nm; pH 7.5; $E_0 = 16$ nM). The K_M value of **14** therefore, is in the range of the natural substrate and more than ten times lower relative to the K_M value of the standard assay substrate 4-MUS (**1**). Moreover, the data proved the validity of our approach to estimate the strength of interaction with the enzyme by testing the inhibitory effect of the corresponding sulfamates. After determination of the necessary correction parameters, the first continuous assay of steroid sulfatase was successfully established using sulfate **14** as substrate (described in detail elsewhere).

In summary, we have discovered 3,4-benzocoumarin 7-*O*-sulfate (**14**) as a new fluorogenic substrate for steroid sulfatase with enzyme kinetic characteristics in the range of the natural substrate. It enables to run a fluorimetric assay of steroid sulfatase in a direct and continuous mode.

References and Notes

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6. Sulfamates **5**–**12** were prepared by reacting the phenolic precursors with 6 equiv of $ClSO_2NH_2$ and 3 equiv of 2,6-di-*t*-butyl-4-methylpyridine in dry DMF for 16 h at rt. After aqueous work-up, the products were purified by chromatography on silica gel. All new compounds were characterised by ^1H NMR, MS and elemental analysis.

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9. Human steroid sulfatase was expressed in CHO cells using a cDNA clone kindly provided by Prof. K. von Figura, Göttingen, Germany. The enzyme was purified from the cells to homogeneity. Details of the expression and purification protocol will be published elsewhere.