Synthesis of per-sulfated flavonoids using 2,2,2-trichloroethyl protecting group and their factor Xa inhibition potential

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Received 28 September 2004; revised 30 November 2004; accepted 30 November 2004
Available online 28 December 2004

Abstract—The synthesis of per-sulfated flavonoids, organic compounds with multiple sulfate groups, is challenging. We present here a two-step synthesis of fully sulfated flavonoids in high overall yields using the 2,2,2-trichloroethyl moiety as a protecting group. The two-step synthesis results in exclusive formation of the per-sulfated product in contrast to common sulfating agents that yield differentially sulfated mixture of compounds. Most per-sulfated flavonoids studied are activators of antithrombin for accelerated inhibition of factor Xa, a key enzyme of the blood coagulation cascade. As a group the per-sulfated flavonoids possess a range of factor Xa inhibition potential.

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1. Introduction

Organic molecules possessing sulfate moieties are increasingly gaining importance as modulators of physiological or pathological function. These functions include inhibition of clotting,1–6 viral infection,7–12 and cancer.13,14 Yet, the number of multiple sulfated synthetic, organic molecules available for investigation remains small, primarily because of difficulties in synthesis and isolation. As the structural diversity in sulfated organic molecules increases, better modulators are expected.

Our work has focused on designing sulfated molecules that activate antithrombin, a plasma glycoprotein and a member of the serpin superfamily of proteins.15,16 Antithrombin is a major regulator of the blood coagulation cascade and it performs this function by inhibiting thrombin and factor Xa, two key proteinases of the clotting cascade.15,16 Antithrombin inhibits these enzymes relatively slowly under physiological conditions, however, this inhibition can be greatly accelerated in the presence of fully sulfated flavanoids and flavonoids.1,3–5

A central aspect of these new designed synthetic antithrombin activators and other sulfate-containing biologically active molecules is the requirement of high sulfate content. Typically sulfation of alcoholic or phenolic compounds is performed at the very end of a synthetic scheme because they radically alter the physical properties of the organic molecule. Sulfate groups greatly reduce the hydrophobic character of the molecule thereby, eliminating organic solvent solubility and introducing water solubility. Further, the small size of typical organic scaffolds generates major challenges in purification of these multiple sulfated organic molecules.

An added complication in sulfation of multiple functionalized alcoholic or phenolic groups is the possibility of numerous products resulting from incomplete sulfation. Although in principle, it should be possible to drive the reaction to completion using a huge excess of the...
sulfating reagent, in practice a Gaussian distribution of products is typically observed. Thus, for an organic skeleton with five potential sulfation sites, a total of 31 mono-, di-, tri-, tetra-, and penta-sulfated species are possible, of which tri- and tetra-substituted products are generally formed in greater proportion. A primary reason for this pattern is the lowering of nucleophilicities of remaining –OH groups following introduction of successive sulfate groups on a small organic skeleton. Additionally, adjacent sulfate groups are likely to create charge repulsion making further sulfation more difficult.

Numerous sulfation reagents and conditions are reported in literature. For example, sulfur trioxide complex with pyridine is extensively used for preparing sulfated oligosaccharides \(^{17-19}\) and sulfated polymeric natural sugars, including sulfated hyaluronic acid \(^{20}\), chitosan \(^{21}\), \(\beta\)-glucan \(^{22}\), dextran \(^{23}\), galactomannan \(^{24}\), fucan \(^{25}\), and curdlan \(^{26}\). In contrast, sulfation of serine and tyrosine residues in peptides has been achieved with sulfur trioxide complex with DMF or trimethylamine \(^{27,28}\). Sulfation of organic skeletons with phenolic sulfur trioxide complex with DMF or trimethylamine \(^{27,28}\) was reported using the triethylamine–sulfur trioxide complex \(^{30}\).

Recently, Liu et al. reported the use of trichloroethyl sulfonate (TCE) group, which can be removed in a selective manner using catalytic hydrogenation, to achieve mono-sulfation of an estrone derivative \(^{30}\). To test whether TCE protecting group paves a cleaner way for per-sulfation, we studied sulfation of flavones containing varying number of phenolic groups. Our work shows that TCE protection and deprotection of flavonoids is an excellent way of generating per-sulfated products. In addition, the method possesses several other important advantages, including ease of purification and identification, which are unsurpassed in comparison to traditional sulfating methods. Finally, the sulfated flavonoids display interesting factor Xa inhibition properties.

2. Methods

2.1. Materials

Human antithrombin and factor Xa were from Hematology Technology (Essex Junction, VT). Spectrozyme FXa was from American Diagnostic (Greenwich, CT). Flavonoids 1–6 were from Indofine (Somerville, NJ) and were used as such. Palladized charcoal (10%), 2,2,2-trichloroethanol, sulfuryl chloride, ammonium formate, and HPLC solvents were from Sigma-Aldrich (Milwaukee, WI).

2.2. Synthesis of the protecting group

2,2,2-Trichloroethyl chlorosulfonyl chloride (TCE-Cl) was synthesized by dropwise addition of sulfuryl chloride to 2,2,2-trichloroethanol (1:1 molar ratio) in diethyl ether in the presence of pyridine (1:1 molar ratio) at \(-78^\circ C\). Pyridine hydrochloride formed in the reaction was filtered and the product purified using vacuum distillation (bp 32 °C at 0.4 mm).

2.3. Reverse phase HPLC analysis

Reverse phase HPLC of TCE protected (1p–6p) and sulfated (1s–6s) flavonoids was performed using a C-18 analytical column YMC–ODC-HQ (4.6 × 250 mm, Waters) on a Shimadzu 10Avp HPLC system. The column was pre-equilibrated with buffer A consisting of acetonitrile–H₂O (50:50 v/v) solvent. Analysis was performed using a linear gradient of buffer A to buffer B consisting of acetonitrile–H₂O (95:5) in 25 min at a flow rate of 0.5 mL/min and monitoring UV absorbance at 279 nm.

2.4. General synthesis of TCE-protected flavones 1p–6p

To a stirring solution of flavone (1 mmol), triethylamine (2–4 mmol per –OH group), and DMAP (1 mmol per –OH group) in anhydrous THF (15 mL), a solution of TCE-Cl (2–4 mmol per –OH group) in anhydrous THF (5 mL) was added dropwise at ambient temperature over 15 min. The solution was stirred overnight, after which the reaction mixture was washed with 0.5 M HCl, 5% (w/v) K₂CO₃, brine and water, and then dried over anhydrous Na₂SO₄. Removal of solvents in vacuo resulted in a solid containing the TCE-protected flavone and TCE dimer. The mixture was fractionated on silica gel column using 1% EtOAc in petroleum ether.

1p: \(^1H\) NMR (CDCl₃): \(\delta 4.93 (s, 2H, –CH₂)\), 4.95 (s, 2H,
The protected flavone (1 mmol) was dissolved in 2 mL anhydrous THF to which was added 2 mL MeOH. To this stirring solution under nitrogen was added 10 wt.% of 10% Pd/C and NH₂HCO₃ (6 mmol per -OH group). The reaction was continuously monitored on RP-HPLC. Following completion of reaction, the mixture was centrifuged and the supernatant filtered on Whatman filter paper containing Celite. Evaporation of solvent gave a solid to which was added ethanol and the solution allowed to stand overnight at room temperature. The precipitated per-sulfated product was collected by filtration. 1s: ¹H NMR (DMSO-d₆ + D₂O): 6.61 (s, 1H, H3), 7.27 (m, 1H, H5'), 7.45 (dd, 1H, 3J = 7.8 Hz, 4J = 1.5 Hz, H6'), 7.57 (dd, 1H, 3J = 9.3 Hz, 4J = 2.7 Hz, H7), 7.65 (dd, 1H, 3J = 9.3 Hz, H8), 7.70 (dd, 1H, 3J = 8.25 Hz, 4J = 1.5 Hz, H4'), 7.81 (d, 1H, 3J = 2.4 Hz, H5), 7.33 (d, 1H, 3J = 2.7 Hz, H6), 7.34 (d, 2H, 3J = 9.0 Hz, H2'), 7.97 (d, 2H, 3J = 9.0 Hz, H3'). 2s: ¹H NMR (DMSO-d₆ + D₂O): 6.52 (s, 1H, H3), 7.22 (d, 1H, 4J = 2.4 Hz, H8), 7.30 (d, 1H, 3J = 2.4 Hz, H6), 7.66 (dd, 1H, 3J = 9.0 Hz, 4J = 2.4 Hz, H5'), 7.68 (dd, 1H, 3J = 7.8 Hz, 4J = 2.4 Hz, H2'), 7.12 (dd, 1H, 3J = 7.8 Hz, 4J = 2.7 Hz, H5'), 7.42 (d, 1H, 3J = 7.8 Hz, 4J = 1.8 Hz, H6'), 7.48 (2H, H7 and H8), 7.72 (dd, 1H, 3J = 7.8 Hz, 4J = 1.8 Hz, H4'). 2.5. General procedure for synthesis of per-sulfated flavonoids 1s–6s

The antithrombin inhibition of factor Xa in the presence of sulfonated flavonoids 1s–6s was determined using a single time point method. The inhibition reactions were carried out in PEG20K-coated cuvettes at 25 °C. Activator 1s–6s (10 or 50 μM) was incubated at 25 °C with antithrombin (1 μM) in 20 mM sodium phosphate buffer containing 20 mM NaCl, 0.1 mM EDTA, and 0.1% PEG8000 at pH 6.0. Factor Xa (30 nM) in MES buffer, pH 6.0, was then added. The inhibition reaction was allowed to proceed for 600 s, following which the residual enzyme activity was determined by following the amidolysis of substrate Spectrozyme Rx (100 μM) in 20 mM sodium phosphate buffer containing 100 mM NaCl, 0.1 mM EDTA, and 0.1% PEG8000 at pH 7.4. The initial slope of absorbance at 405 nm in the presence of the activator was compared with that in its absence to obtain the percent inhibition of factor Xa. Each experiment was done in duplicate.

2.6. Mass spectrometric analysis of TCE protected (1p–6p) and deprotected (1s–6s) flavonoids

EISI-MS (positive ion mode) of TCE—protected flavones 1p–6p was performed in high-resolution mode using Micromass QTOF-Ultima mass spectrometer (Waters Corporation, Milford, MA). Sample was dissolved in acetonitrile and 10 μL was injected into the Q-TOF. Sodium TFA was used as a calibrant. EISI-MS (negative ion mode) of per-sulfated flavones 1s–6s was performed using a Micromass ZMD 4000 single quadrupole mass spectrometer (Waters Corp., Milford, MA). Each sulfated flavones in acetonitrile–water (1:1) was infused at 10 μL/min for a 1 min period during which data was acquired in cumulative MCA mode. Mass scans were obtained in the range 200–800 a.m.u at a scan rate of 400 a.m.u/s. Ionization conditions were optimized for each compound to maximize ionization of each molecule. The capillary voltage was varied between 3.0 and 4.0 V, while the cone voltage usually ranged from 30 to 65 V. The remaining ionization parameters remained constant, the extractor voltage was 4.0: the Ref lens voltage was 0.1 V, the source block temperature was 100 °C and the desolvation temperature was 120 °C.

2.7. Accelerated inhibition of factor Xa by sulfated flavonoids 1s–6s

The antithrombin inhibition of factor Xa in the presence of sulfonated flavonoids 1s–6s was determined using a single time point method. The inhibition reactions were carried out in PEG20K-coated cuvettes at 25 °C. Activator 1s–6s (10 or 50 μM) was incubated at 25 °C with antithrombin (1 μM) in 20 mM sodium phosphate buffer containing 20 mM NaCl, 0.1 mM EDTA, and 0.1% PEG8000 at pH 6.0. Factor Xa (30 nM) in MES buffer, pH 6.0, was then added. The inhibition reaction was allowed to proceed for 600 s, following which the residual enzyme activity was determined by following the amidolysis of substrate Spectrozyme Rx (100 μM) in 20 mM sodium phosphate buffer containing 100 mM NaCl, 0.1 mM EDTA, and 0.1% PEG8000 at pH 7.4. The initial slope of absorbance at 405 nm in the presence of the activator was compared with that in its absence to obtain the percent inhibition of factor Xa. Each experiment was done in duplicate.

3. Results and discussion

We selected flavones 1–6 (Fig. 1) that possess three to five phenolic groups. Flavones 1 and 2 contain three –OH groups, while flavones 3 and 4, and 5 and 6 possess
four and five -OH groups, respectively. As a group they represent small organic molecules with the end-to-end length of ~7–8 Å. Thus, per-sulfation of these molecules is expected to generate high charge density species. Although the placement of -OH groups is varied, there are five instances of adjacent hydroxyls (flavones 1, 3–6). Flavone 6 with three consecutive phenolic groups at positions 3’·4’·5’ is extremely interesting because it represents a structure with highest charge density. In addition, sulfation of such moieties is nearly impossible with sulfur trioxide complexes.31

6,2’,3’-Trihydroxy flavone (1) was used as test molecule to study the applicability of TCE as a sulfating agent. Newly synthesized 2,2,2-trichloroethyl chlorosulfonyl chloride (TCE-Cl) was reacted with the flavone in anhydrous THF at room temperature in the presence of DMAP and triethylamine as base (Scheme 1). At a TCE-Cl molar excess of less than 1.5-fold per available hydroxyl group, the reaction resulted in multiple products as assayed using reverse phase HPLC (not shown). However, when the molar excess of TCE-Cl was increased to two-fold per available hydroxyl group, the reaction resulted in a single compound, the most nonpolar product formed. ESI mass spectrometric analysis of the product showed molecular ion peaks (M + H)+ between 900.681 and 908.667 m/z in positive mode with an isotopic pattern characteristic for nine chlorine atoms.

Table 1. Reaction yield and molecular ion peaks in positive ion ESI-MS spectrum of TCE-protected flavonoids 1p–6p

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Yield in protection (%)</th>
<th>Positive ion ESI-MS profile</th>
<th>[M + 1]+</th>
<th>Isotopes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found (rel int) (m/z)</td>
<td>Calculated (m/z)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1p</td>
<td>90</td>
<td>902.671 (71)</td>
<td>C2H15O2S3Cl3ClY</td>
<td>7</td>
</tr>
<tr>
<td>2p</td>
<td>95</td>
<td>904.668 (100)</td>
<td>C2H15O2S3Cl3ClY</td>
<td>3</td>
</tr>
<tr>
<td>3p</td>
<td>95</td>
<td>906.666 (83)</td>
<td>C2H15O2S3Cl3ClY</td>
<td>4</td>
</tr>
<tr>
<td>4p</td>
<td>57</td>
<td>902.676 (52)</td>
<td>C2H15O2S3Cl3ClY</td>
<td>2</td>
</tr>
<tr>
<td>5p*</td>
<td>82</td>
<td>1130.539 (84)</td>
<td>C2H15O2S3Cl3ClY</td>
<td>2</td>
</tr>
<tr>
<td>6p</td>
<td>92</td>
<td>1130.539 (84)</td>
<td>C2H15O2S3Cl3ClY</td>
<td>2</td>
</tr>
</tbody>
</table>

*Sodium adducts, rather than free ions, were detected. The adducts correspond to mono-sodiated ions, for example, C2H15O2S3Cl3ClYNa.

Figure 1. Structure of flavonoids: Per-sulfation was studied in two steps—TCE protection of flavonoids 1–6 to give 1p–6p, followed by hydrogenation to yield 1s–6s. Substituents R2, R3, R4, and R5 through R7 are either –H or –OG, where G is –H for native flavonoid, is –SO3, –SO2OCH2CCl3 for TCE-protected flavonoids 1p–6p, or is –SO3·NH4+ for per-sulfated flavonoids 1s–6s.
HPLC profile of at room temperature, essentially following Liu et al. Selective hydrogenation of the TCE group was enhancing the ease of identification. The number of TCE groups and the base skeleton, thus the idea of using ammonium formate as a mild hydrogen source to selectively hydrolyse the SO2–O bond instead of the ArO–SO2 bond is appealing for two reasons: (i) it eliminates HCl formed during the reaction, thereby preventing strongly acidic conditions in which per-sulfated flavonoids are unstable, and (ii) it introduces NH4+ as a counter-ion, which is known to ease the MS analyses of highly sulfated molecules using electrospray ionization. In contrast, Na+ counter-ions typically introduced in reactions of SO3 complexes give highly complex sodium adducts of polysulfated molecules that are difficult to analyze. Thus, ESI-MS analyses following TCE-deprotection of flavonoid 1s gave ions at 510.9, 526.8, 543.9, and 560.7 m/z values corresponding to molecular ions with three –OSO3 groups and either 0, 1, 2 or 3 NH4+ counter-ions, respectively (Table 2). Likewise, peaks corresponding to \([\text{M}–n\text{H}+(n–1)\text{NH}_4]^–\), where \(M\) corresponds to the free acid (–OSO3H form), were detected for each of the TCE-deprotected flavonoid 2s–6s, except for 5s for which mixed molecular ions containing both Na+ and NH4+ were observed (see Table 2). This suggested exclusive formation of per-sulfated flavonoids. The isolated yield of the sulfated flavonoids 1s–6s range from 48%–78%, which is 1.5- to 2-fold greater than typical isolated yields obtained using SO3 complexes with amines. Most importantly, the ability to sulfate three adjacent –OH groups, as in 6, greatly enhances the applicability of this methodology in synthesis of this important class of biologically active compounds.

The acceleration in antithrombin inhibition of factor Xa was studied in 20 mM sodium phosphate buffer, pH 6.0, at 25 °C in the presence of fixed concentration of sulfated activators 1s–6s, as previously reported. A single time point method was used, rather than an extended determination of second-rate order constant, to rapidly assess the activation potential of these novel polysulfates. Small activator-dependent factor Xa inhibition is a function of the amount of antithrombin–small activator in the conformationally activated state, which is further dependent on the binding affinity \((K_D)\) and activation potential \((k_{\text{CAT}})\) of the small molecule activator. Assuming that the activation potential remains nearly equivalent for this series of structurally related compounds, as observed for sulfated flavanoids earlier, the single-time point method primarily reflects the binding affinity of the sulfated activator–antithrombin complex. Thus, greater inhibition of...
factor Xa suggests better binding affinity of the sulfated activator.

Figure 3 shows the profile of factor Xa inhibition potential of sulfated flavonoids 1s–6s (see also Table 2). As a group the sulfated flavonoids exhibit a full range of inhibition potential. Whereas 1s and 2s, with three sulfate groups each, exhibit less than 10% inhibition, 5s and 6s with five sulfate groups exhibit ~90% inhibition (Table 2). Thus, factor Xa inhibition potential appears to be a function of the number of sulfate groups charged on the small skeleton. Although for these subgroups the inhibition appears to be approximately constant, interesting fine structural distinctions exist. For example, 1s and 4s show less potency in comparison to 2s and 3s suggesting a preference for the 5,7,4'-substitution. Further, introduction of 3-OSO_3H in 4s and 5s, in comparison to 1s and 3s, significantly enhances the inhibition potential suggesting the importance of this position. In addition, although both 5s and 6s show nearly equivalent activity at 50 μM, experiments at five-fold lower concentration show that 5s, with 3-OSO_3H group, is better than 6s. It is likely that either 3-OSO_3H interacts directly with antithrombin or that the presence of this group introduces structural constraints on the flexibility of the unicyclic ring, thus enhancing the binding affinity.

Detailed quantitative aspects of sulfated flavonoids 3s, 5s, and 6s interacting with antithrombin are being further investigated.

In conclusion, synthesis of per-sulfated flavonoids was achieved in two simple steps using the TCE protecting group. Overall, good yields were obtained in comparison to traditional direct sulfation methods involving sulfur trioxide complexes. The two-step approach avoided the formation of partially sulfated flavonoid products, thereby obviated tedious purification procedures. Further, the unique ESI-MS profile of TCE-protected intermediate greatly aided rapid characterization of products. Finally, the antithrombin–dependent factor Xa inhibition potential of the sulfated flavonoids is varied and highly structure dependent.

### Acknowledgments

We thank Prof. Michael Hindle of Virginia Commonwealth University for making his mass spectrometry facility generously available to us. In addition, mass spectrometry support was also provided by the Washington University Mass Spectrometry Resource, an NIH Research Resource (P41RR0954). This work was supported by American Heart Association—Mid-Atlan-

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**Table 2. Reaction yields, negative ion ESI-MS spectrum profile and factor Xa inhibition of per-sulfated flavonoids 1s–6s**

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Yield in deprotection (%)</th>
<th>Negative ion ESI-MS profile</th>
<th>Factor Xa inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1s</td>
<td>55</td>
<td>510.9/509.4</td>
<td>0 ± 2c</td>
</tr>
<tr>
<td>2s</td>
<td>40</td>
<td>526.8/526.4</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>3s</td>
<td>52</td>
<td>539.9/539.5</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>4s</td>
<td>78</td>
<td>543.0/543.5</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>5s</td>
<td>48</td>
<td>588.5/589.5</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>6s</td>
<td>65</td>
<td>607.4/605.5</td>
<td>82 ± 4</td>
</tr>
</tbody>
</table>

A formula [M – nH]^- or [M – nH + (n – 1)NH_4]^+ is used to calculate the expected mass, where M corresponds to per-sulfated flavonoid in acid form (~OSO_3H-form).

*b For per-sulfated flavonoid 5, mixed molecular ions containing both Na^+ and NH_4^+ were observed. These include the 1Na^+ + 2NH_4^+, 2Na^+ + 1NH_4^+, and 1Na^+ + 3NH_4^+ ions.

c Error in two independent measurements.

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**Figure 3.** Antithrombin-dependent factor Xa inhibition property of sulfated flavonoids 1s–6s: Factor Xa inhibition was assessed in 20 mM sodium phosphate buffer, pH 6.0, at 25 °C using the single time point method in the presence and absence of fixed concentration of sulfated flavonoids and antithrombin. Error bars represent the variation in percent inhibition observed in duplicate measurements. See Methods for additional details.
References and notes