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Bioorganic & Medicinal Chemistry 12 (2004) 595–601
Bioorganic & Medicinal Chemistry

# A colormetric assay for catechol-*O*-methyltransferase

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Received 7 August 2003; revised 14 October 2003; accepted 22 October 2003

**Abstract**—A series of catechol diazo dyes were synthesized and tested as substrates for the enzyme catechol-*O*-methyltransferase (COMT) with the aim of developing a sensitive HPLC assay method using visible wavelength light detection. A method was developed which allowed for the determination of the two regioisomeric methylated products of the COMT catalyzed reaction of 4-[(3,4-dihydroxyphenyl)azo]benzenesulfonate with *S*-adenosylmethionine (AdoMet). Separation of the assay components was achieved by reverse phase chromatography using an isocratic mobile phase. No pre-preparation of the assay samples was required.


## 1. Introduction

The enzyme catechol-*O*-methyltransferase (COMT) has been implicated in the 'wearing off' effect seen in Parkinson's disease patients who have been treated with L-dopa based medications for an extended period of time. The enzyme inactivates catechol based neurotransmitters by methylating either of the two aromatic hydroxy groups of the catechol substructure.[1] Extensive research has been focused on developing inhibitors of the enzyme,[2–11] which in turn has led to the development of numerous enzyme assays to determine the activity of COMT. The most popular type of assays use radiolabeled AdoMet substrates such as *S*-adenosyl-L-[$^{3}$H methyl] methionine or *S*-adenosyl-L-[$^{14}$C methyl] methionine.[7,12–17] During the course of the enzyme reaction the radiolabel is incorporated into the methylated products. Products and reactants are then separated using solvent extraction methods and the radiolabeled products in the organic phase are quantified using a photo-scintillation device. From the level of products formed the enzyme activities can be determined.

Assays can also be performed without radiolabeled substrates. One example uses HPLC with electrochemical detection.[18,19] Most methods using this setup require sample cleanup procedures before the *O*-methylated products from the COMT reactions can be analyzed. A method by Nissinen et. al. avoids this problem by using 3,4-dihydroxybenzylamine as a substrate.[20] After the reaction with COMT the assay sample is directly injected onto the HPLC machine for analysis. UV detection can also be used in place of electrochemical detection. One such method determines the amount of product formed by determining the amount of SAH produced during the course of the assay.[21] Direct measurement of the methylated product has also been achieved by using methyl 3,4,5-trihydroxybenzoate as the substrate with detection of the product methyl 3,5-dihydroxy-4-methoxybenzoate.[2] A shortcoming with the last method is that problems arise if there are other components in the assay that also have large UV absorbancies (at ~280 nm) that have similar retention times. Products that absorb in an entirely different region of the UV–vis spectrum would be an advantage as interference effects would be diminished. Diazo catechol dyes, which absorb in the visible range of the spectrum, would be good substrates in this regard. A paper by Haghbeen et al. describes the synthesis of a set of diazo catechol dyes and their suitability as substrates for the enzyme monooxygenase.[22] In this paper, we describe the synthesis of a series of diazo catechol dyes which were tested as substrates for the enzyme COMT. In addition these dyes were analyzed to see if they could be incorporated into a new COMT assay.

## 2. Results

Three sets of diazo dyes were synthesized (Fig. 1). One set (**1**) are potential COMT substrates while the other two sets (**2** and **3**) are the expected methylated products

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doi:10.1016/j.bmc.2003.10.038

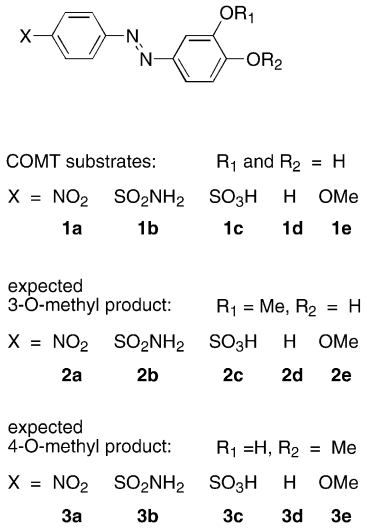
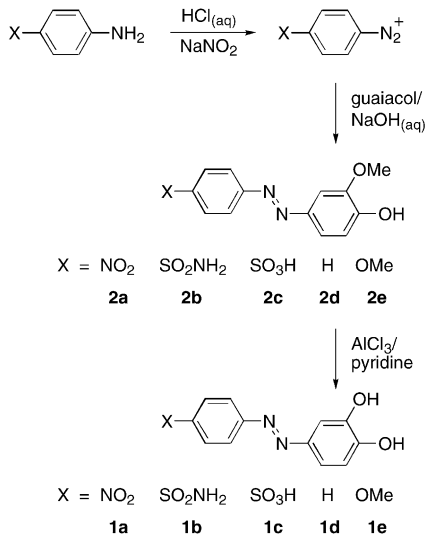



**Figure 1.** Diazo dye COMT substrates and expected products.




**Scheme 1.** Synthesis of guaiacol diazo dyes.

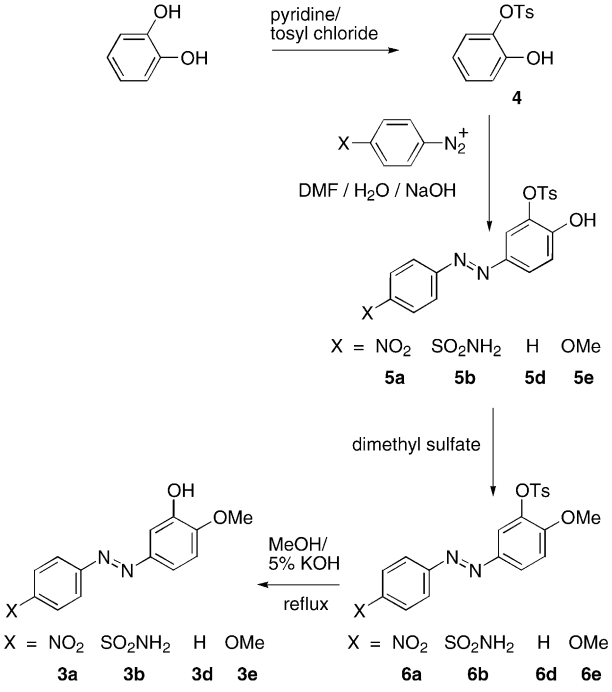
after the reaction of **1** with COMT. Compounds **2** and **3** were used as authentic sample standards to determine product retention times for HPLC analysis.

The synthesis of dyes **1b** and **1d** have been previously described.[22] Using the same methodology, diazo dyes **1a**, **1c** and **1e** were synthesized. Fortuitously, the intermediates in the synthesis are the 3-methylated catechol diazo dyes (**2**) that were required as product standards. Thus, compounds **2a**, **2c** and **2e** were made by the direct coupling of diazotised aniline derivatives to guaiacol (Scheme 1).

Compounds **1a**, **1c** and **1e** were synthesized by the removal of the 3-*O*-methyl group from **2a**, **2c** and **2e** using pyridine and $AlCl_3$ dissolved in chloroform. In the case of **2e** it was found that some of the 4′-*O*-methyl group was also removed resulting in lowered yields of **1e**. By replacing chloroform with toluene the amount of **1e** produced was increased.

The synthesis of the dyes **3(a, b, d, e)** is as shown in Scheme 2. The tosyl ester of catechol **4** was prepared by the reaction of tosyl chloride with catechol in pyridine. The coupling of **4** with the appropriate phenyldiazonium derivatives gave the 3-*O*-tosyl catechol diazo dye precursors. The 4-*O*-hydroxy groups were then methylated to give compounds **6(a, b, d, e)**. Detosylation of these compounds gave the 4-*O*-methyl dyes **3(a, b, d, e)**.

The synthesis of the 4-*O*-methylsulfonic acid dye **3c** is as shown in Scheme 3. The synthesis of the starting material *N*-acetyl 4-aminobenzenesulfonyl chloride has been previously described in a book article by Blatt et al.[23] *N*-Acetyl 4-aminobenzenesulfonyl chloride was dissolved into pyridine and reacted with phenol to form the phenyl ester **7**. The acetate group of **7** was hydrolyzed by heating **7** in a dilute solution of HCl under reflux to give **8**. Compound **8** was then transformed into the diazonium salt and reacted with **4** to form the diazo dye which was subsequently methylated to give compound



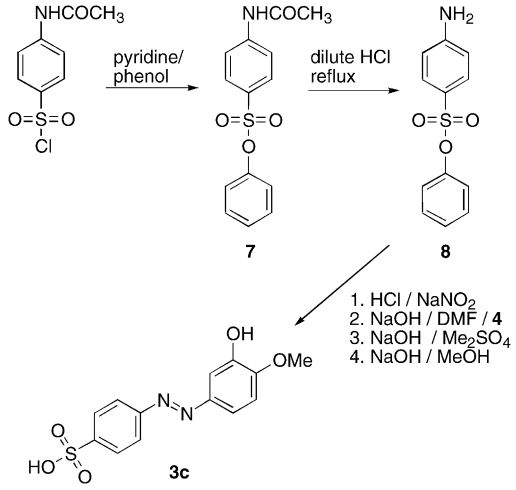

**Scheme 2.** Synthesis of 4-*O*-methyl 3,4-dihydroxyazobenzene derivatives.

**6c**. The tosyl and phenyl groups of compound **6c** were removed to give the final product **3c**.

## 3. Discussion

The buffers used in the mobile phase for the HPLC analysis of the COMT assay reactions were chosen to give good resolution between the substrate and product(s) of the assay. For maximum sensitivity the UV–vis detector was set to scan at the wavelength corresponding to the absorption maxima of the substrate and product dyes being analysed. The resulting chromatograms of the assay mixtures are shown in

Figure 2. In the case of substrate **1a**, the expected products were not detected. For the reaction of **1b** with COMT the resulting chromatogram of the assay shows three peaks. Compound **1b** was found to elute at 9.7 min and the enzyme products **2b** and **3b** were found to elute at 16.4 min and 17.9 min, respectively. Some peak tailing was observed which did not disappear with higher concentrations of TFA in the mobile phase.




**Scheme 3.** Synthesis of diazo COMT substrate **3c**.

The assay containing **1c** as the substrate was analyzed by HPLC and the resulting chromatogram of the assay showed three well-resolved and symmetrical peaks. Compound **1c** was found to elute at 5.4 min and the enzyme products **2c** and **3c** were found to elute at 8.2 min and 8.9 min, respectively. The assay containing **1d** as the substrate was analyzed by HPLC and the resulting chromatogram showed two peaks. Compound **1d** was found to elute at 6.6 min and the product **3d** was found to elute at 12.9 min. The assay containing **1e** as the substrate was analyzed by HPLC and the resulting chromatogram showed two peaks. Compound **1e** was found to elute at 9.3 min and compound **3e** was found to elute at 9.3 min. The absence of peaks corresponding to **2d** and **2e** would indicate that COMT exclusively methylates at the 4-*O*- position when substrates **1d** and **1e** were used.

Of the five dyes used in the COMT assays, **1c** and the corresponding products **2c** and **3c** had the shortest elution times and also gave the most symmetrical peaks with no tailing in HPLC analysis. In addition, this

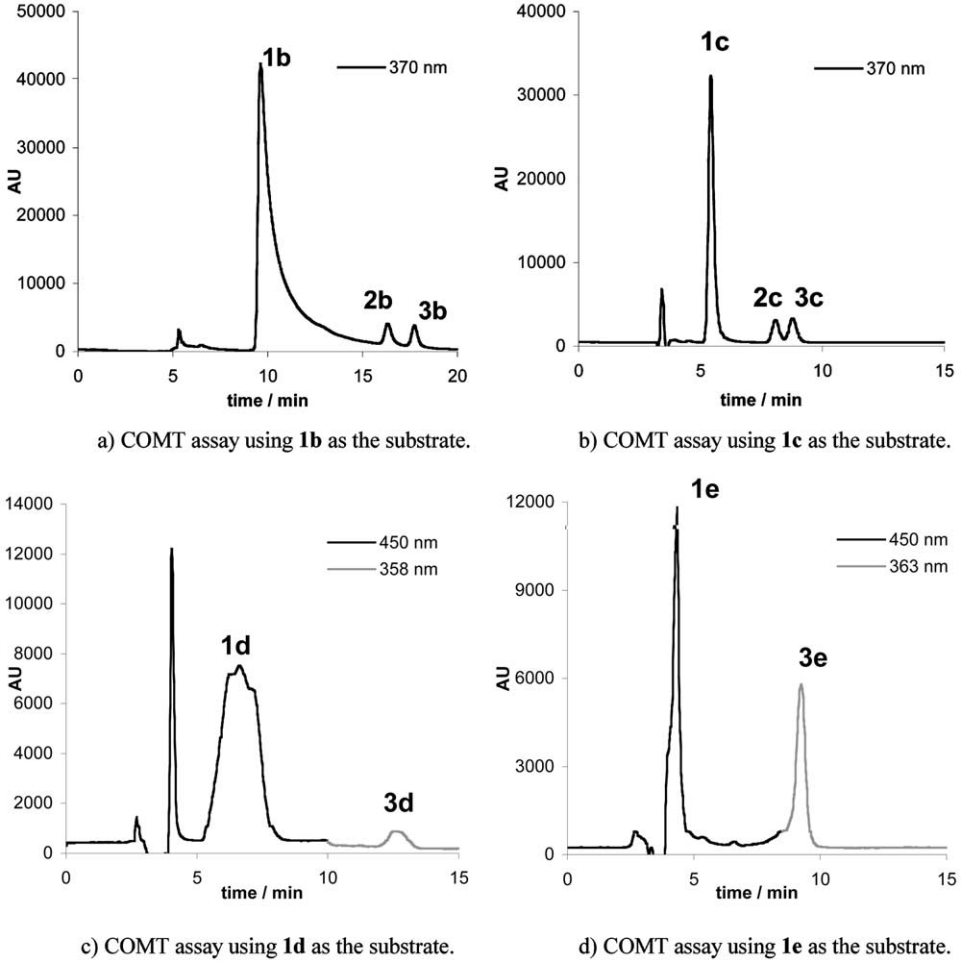



**Figure 2.** HPLC chromatograms of COMT reactions with **1b**–**1e**.

compound was also the only water soluble dye which meant an organic co-solvent was not required in the enzyme assay. These results indicated that **1c** was the most suitable substrate for a COMT assay. The fact that **1c** produces both the 3- and 4-*O*-methylated products was not considered to be overly detrimental as the enzyme activity can be determined from the total amount of both products in the assay.

The assay using **1c** as the substrate, was then optimized by varying the incubation time and AdoMet concentration. Time dependence experiments were performed to determine the optimal incubation time for the COMT assay and to ensure a constant rate with time. A series of reaction assay solution containing the substrate **1c** were set up and during the 2 h of the experiment an assay solution was quenched with 4 M perchloric acid every 15 min and analyzed by HPLC. A plot of velocity (calculated as the sum of nmol of **2b** and **2c** produced per mg of protein per min) versus time showed that a linear relationship existed for reaction times up to one h.

The kinetics of the enzyme reaction were studied by running assays using various concentrations of **1c**. AdoMet was used at a concentration of 2 mM (saturation kinetics) in the assay solution. The resulting two product peaks (**2b** and **2c**) were converted from area into velocity values. Maximum product formation was approached when the concentration of **1c** was approximately 150 μM. This concentration can be taken as the saturating concentration under these assay conditions. $V_{max}$ and $K_m$ values were found to be 2.85 (nmol product/min/μg protein)$^{-1}$ and 41 μM, respectively, by Lineweaver–Burke double reciprocal plots. The $K_m$ value for **1c** is within the range of $K_m$ values that have been determined for a range of COMT substrates.[3,7,17,24] The limits of detection for this assay were 150 and 255 pmol in a 30 μL injection (signal to noise ratio of 10) for the enzyme products **2b** and **2c**, respectively.

## 4. Conclusion

The diazo dye 4-[(3,4-dihydroxyphenyl)azo]benzene sulfonate (**1c**) was shown to be a good substrate for COMT. As **1c** is readily water soluble, assay conditions could dispense with the use of an organic co-solvent. It was the preferred substrate of the range of dyes tested. After reaction, a sample of the assay mixture could be injected directly onto an HPLC column for the analysis and quantification of the dye products, **2c** and **3c**. Interference effects from the assay solvents, buffer, enzyme and the co-factor AdoMet are markedly reduced as none of these components absorb light in the visible region. No pre-purification of the assay sample is necessary prior to HPLC analysis. This factor and the short retention times of the dyes and good resolution by HPLC would facilitate the rapid analysis of numerous samples, which is especially advantageous for kinetic studies of COMT. The preferred substrate **1c** and product dye standards were easily prepared in high yields. The assay method using the diazo dye is inexpensive in comparison to the methods using radioactive substrates while offering comparative sensitivity.

## 5. Experimental

Melting points were recorded on a Gallenkamp capillary melting point apparatus and are uncorrected. Elemental analyses were performed at the Campbell Microanalytical laboratory, Otago University. $^1$H NMR and $^{13}$C NMR spectra were measured on a Varian Unity Inova 300 MHz spectrometer. The δ values are reported in ppm downfield from TMS. Column chromatography was performed with silica gel (Sorbsil, partical size 32–63 μM). AdoMet as the p-toluenesulfonate salt and COMT were purchased from Sigma$^{TM}$. Guaiacol, aniline, 4-methoxyaniline, 4-sulfanilamide, 4-aminobenzenesulfonic acid and 4-nitroanaline were purchased from Aldrich. Solvent and inorganic bases were generally sourced.

### 5.1. 4-[(4-Nitrophenyl)azo]-catechol (1a)

Into a two neck flask was placed **2a** (1 g, 3.7 mmol), $CHCl_3$ (100 mL) and anhydrous $AlCl_3$ (4.34 g, 0.033 mol). The mixture was stirred under nitrogen for 30 min whilst adding distilled pyridine (10 mL, 0.12 mol). The reaction vessel was then refluxed in an oil bath for 24 h. Distilled water (100 mL) and concd HCl (10 mL, 0.11 mol) was added to hydrolyse the product. The crude product was then isolated by vacuum filtration and recrystallised from ethanol (60 mL) and HCl (10mL of 1 mol L$^{-1}$). (0.2 g, 20%): mp 230 °C; $^1$H NMR (DMSO-$d_6$) 8.48 (2H, d, $J$ = 9.0 Hz), 8.06 (2H, d, $J$ = 9.0 Hz), 7.56 (1H, dd, $J$ = 2.4, 8.4 Hz), 7.48 (1H, d, $J$ = 2.4 Hz), 7.06 (1H, dd, $J$ = 2.4, 8.4 Hz). Anal. calcd for $C_{12}H_9N_3O_4 \cdot 1/2H_2O$: C, 53.57; H, 3.76; N, 15.67. Found C, 53.52; H, 3.65; N, 15.33. HRMS $m/z$ MH$^+$, 260.0674. calcd for $C_{12}H_{10}N_3O_4$ 260.0671.

### 5.2. 4-[(3,4-Dihydroxyphenyl)azo]benzenesulfonic acid (1c)

A mixture of **2c** (1.15 g, 3.25 mmol), $AlCl_3$ (4.34g, 32.5 mmol) and $CHCl_3$ (100 mL) was stirred for 30 min under a nitrogen atmosphere. Following the addition of pyridine (10.28 g, 0.13 mol) the mixture was stirred for a further hour then heated under reflux for 23 h. The addition of 30 mL of water resulted in the formation of a solid red precipitate. Concentrated HCl was added until the solid redissolved. The chloroform layer was removed and then concentrated HCl was added to the aqueous layer until an orange precipitate formed. The precipitate was filtered off and redissolved in a minimum amount of water. Sodium bicarbonate was slowly added to the solution until the sodium salt precipitated. The salt was filtered off and recrystallized from water to give the final product as flaky yellow crystals. (0.24 g, 23%): mp 260–265 °C (dec); $^1$H NMR ($D_2O$) 7.65 (2H, d, $J$ = 8.8 Hz), 7.45 (2H, d, $J$ = 8.8 Hz), 6.99 (1H, dd, $J$ = 2.8, 8.5 Hz), 6.77 (1H, d, $J$ = 2.8 Hz), 6.37 (1H, d, $J$ = 8.5 Hz). Anal. calcd for $C_{12}H_9N_2O_5SNa$: C, 45.57; H, 2.87; N, 8.86; S, 10.14. Found C, 45.39; H, 2.84; N, 8.87; S, 9.92.

### 5.3. 4-[(4-Methoxyphenyl)azo]-catechol (1e)

$AlCl_3$ (4.34g, 32.5 mmol), **2e** (0.84 g, 3.25 mmol) and toluene (100 mL) were mixed together and stirred for 30 min under a nitrogen atmosphere. Dried pyridine (10.28 g, 130 mmol) was added and the mixture was heated under reflux for 2 h, then stirred for a further 12 h under a nitrogen atmosphere. During the course of the reaction a yellow precipitate formed which turned red upon the addition of 2 M HCl (40 mL). The solution containing the red precipitate was dissolved in ethyl acetate and methanol (1:1 50 mL). The organic layer was separated and rotary evaporation of the solvent gave the crude product as a purple tar. Recrystallization from ethyl acetate and toluene gave the final product as purple crystals. (0.29 g, 31%): mp 45–46 °C; $^1$H NMR ($CDCl_3$) 7.87 (2H, d, $J$ = 9.0 Hz), 7.50 (1H, dd, $J$ = 2.2, 8.3 Hz), 7.46 (1H, d, $J$ = 2.2 Hz), 7.02 (1H, d, $J$ = 8.3 Hz), 7.00 (2H, d, $J$ = 9.0 Hz), 5.68 (1H, br s), 5.52 (1H, br s), 3.90 (3H, s). Anal. calcd for $C_{13}H_{12}N_2O_3 \cdot 11/4H_2O$: C, 58.52; H, 5.48; N, 10.50. Found: C, 58.81; H, 5.18; N, 10.58.

### 5.4. General procedure for the preparation of diazo derivatives of guaiacol (2)

A cooled (5 °C) solution containing $NaNO_2$ (2.1 g, 0.03 mol) and water (50 mL) was slowly added to a cooled solution of the appropriate aniline derivatives (0.03 mol), 6M HCl (10 mL) and water (50 mL) to form the corresponding diazonium salt.

The diazonium salt solution was then added drop-wise to a cooled (5 °C) solution of 2-methoxyphenol (3.7 g, 0.03 mol) dissolved in dilute sodium hydroxide (1.2 g in 50 mL) to give the diazo dye. The resultant mixture was stirred for a further 2 h.

**5.4.1. 4-[(4-Hydroxy, 3-methoxyphenyl)azo]benzenesulfonate disodium salt (2c).** The dye **2c** was prepared from 4-aminobenzenesulfonate using the procedure above. To precipitate the dye from the reaction mixture the solution was acidified with concentrated HCl. The precipitate was filtered off and recrystallised from 1M NaOH solution to give compound **2c** as dark red chunky crystals. (5.21 g, 49%): mp 258–262 °C (dec); $^1$H NMR($D_2O$) 7.84 (2H, d, $J$ = 9.0 Hz), 7.63 (2H, d, $J$ = 9.0 Hz), 7.33 (1H, dd, $J$ = 2.1, 8.4 Hz), 7.22 (1H, d, $J$ = 2.1 Hz), 6.82 (1H, d, $J$ = 8.4 Hz), 3.76 (3H, s). Anal. calcd for $C_{13}H_{10}N_2O_5SNa_2$: C, 44.32; H, 2.86; N, 7.95; S, 9.10. Found: C, 44.66; H, 3.12; N, 7.86; S, 9.09.

**5.4.2. 4-[(4-Methoxyphenyl)azo]-2-methoxyphenol (2e).** The dye **2e** was prepared from *p*-anisidine using the procedure above. To precipitate the dye from the reaction mixture the solution was acidified with concentrated HCl. The precipitate was filtered off and recrystallised from water and ethanol to give compound **2e** as dark blue chunky crystals. (6.51 g, 84%): mp 104–106 °C; $^1$H NMR ($CDCl_3$) 7.89 (2H, d, $J$ = 8.7 Hz), 7.56 (1H, dd, $J$ = 2.4, 8.4 Hz), 7.50 (1H, d, $J$ = 2.4 Hz), 7.05 (1H, d, $J$ = 8.4 Hz). 7.01 (2H, d, $J$ = 8.7 Hz), 5.91 (1H, s), 4.00 (3H, s), 3.9 (3H, s). Anal. calcd for $C_{14}H_{14}N_2O_3$: C, 65.10; H, 5.46; N, 10.85. Found: C, 65.37; H, 5.65; N, 11.06.

### 5.5. General procedure for the preparation of 4-*o*-methylated catechol diazo dyes (3)

The appropriate diazo dye derivative was dissolved into 5% KOH w/w methanol solution and heated under reflux for 30 min. A 5% aqueous HCl solution was added and the solvent is removed by rotary evaporation to give the crude product.

**5.5.1. 4-Nitro [(4-methoxy, 3-hydroxyphenyl)azo]benzene (3a).** The dye **3a** was prepared from **6a** using the procedure described above. Recrystallization from chloroform and hexane gave the product as red needle-like crystals. (98 mg, 37%): mp 167–169 °C; $^1$H NMR ($CDCl_3$) 8.37 (2H, d, $J$ = 9.0 Hz), 7.99 (2H, d, $J$ = 9.0 Hz), 7.65 (1H, dd, $J$ = 2.4, 8.7 Hz), 7.57 (1H, d, $J$ = 2.4 Hz), 7.03 (1H, d, $J$ = 8.7 Hz), 5.75 (1H, s), 4.03 (3H, s). Anal. calcd for $C_{13}H_{11}N_3O_4$: C, 57.14; H, 4.06; N, 15.38. Found: C, 57.15; H, 3.95; N, 15.26.

**5.5.2. 4-[(3-Hydroxy, 4-methoxyphenyl)azo]benzene sulfonamide (3b).** The dye **3b** was prepared from **6b** using the procedure described above. Recrystallization from ethanol and water gave the product as small orange crystals. (80 mg, 30%): mp 229–231 °C; $^1$H NMR (DMSO-$d_6$) 9.66 (1H, s), 8.03 (2H, d, $J$ = 9.0 Hz), 7.99 (2H, d, $J$ = 9.0 Hz), 7.58 (1H, dd, $J$ = 2.7, 8.7 Hz), 7.54 (2H, s), 7.40 (1H, d, $J$ = 2.7 Hz), 7.20 (1H, d, $J$ = 8.7 Hz), 3.93 (3H, s). Anal. calcd for $C_{13}H_{13}N_3O_4S$: C, 50.80; H, 4.26; N, 13.68; S, 10.43. Found: C, 50.83; H, 4.15; N, 13.62; S, 10.45.

**5.5.3. 4-[(4-Methoxy, 3-hydroxyphenyl)azo]benzenesulfonate sodium salt (3c).** The dye **3c** was prepared from **6c** using the procedure described above. Recrystallization from water gave the product as orange flaky crystals. (88 mg, 29%): mp 276–279 °C (dec); $^1$H NMR ($D_2O$) 7.87 (2H, d, $J$ = 9.0 Hz), 7.74 (2H, d, $J$ = 9.0 Hz), 7.14 (1H, dd, $J$ = 2.5, 8.3 Hz), 6.98 (1H, d, $J$ = 2.5 Hz), 6.93 (1H, d, $J$ = 8.3 Hz), 3.78 (3H, s). Anal. calcd for $C_{13}H_{11}N_2SO_5Na$: C, 47.27; H, 3.36; N, 8.48; S, 9.71. Found: C, 46.97; H, 3.51; N, 8.44; S, 9.66.

**5.5.4. 3-Hydroxy, 4-methoxyazobenezene (3d).** The dye **3d** was prepared from **6d** using the procedure described above. Recrystallization from ethanol and water gave the product as small orange crystals. (0.27 g, 90%): mp 84–86 °C; $^1$H NMR ($CDCl_3$) 7.88 (2H, d, $J$ = 9.0 Hz), 7.57 (1H, dd, $J$ = 2.5, 8.5 Hz), 7.55 (1H, d, $J$ = 2.5 Hz), 7.50 (2H, m), 7.44 (1H, m), 6.98 (1H, d, $J$ = 8.5 Hz), 5.72 (1H, s), 3.98 (3H, s). Anal. calcd for $C_{13}H_{12}N_2O_2$: C, 68.40; H, 5.30; N, 12.28. Found: C, 68.49; H, 5.52; N, 12.30.

**5.5.5. 3-Hydroxy-4,4′-dimethoxyazobenzene (3e).** The dye **3e** was prepared from **6e** using the procedure described above. Recrystallization from methanol and water gave the product as flaky gold crystals. (80 mg, 26%): mp 171–172 °C; $^1$H NMR ($CDCl_3$) 7.88 (2H, d, $J$ = 9.0 Hz), 7.51 (2H, d, $J$ = 9.0 Hz), 7.01 (2H, d, $J$ = 9.0

Hz), 6.97 (1H, d, $J=9.0$ Hz), 5.68 (1H, s), 3.99 (3H, s), 3.90 (3H, s). Anal. calcd for $C_{14}H_{14}N_2O_3$: C, 65.10; H, 5.46; N, 10.85. Found: C, 65.29; H, 5.51; N, 11.10.

**5.5.6. Catechol mono *O*-*p*-toluenesulfonate (4).** Catechol (4.44 g, 40 mmol), *p*-toluenesulfonyl chloride (9.53g, 50 mmol) and pyridine (9.5 mL) were mixed together in a 100 mL conical flask and heated on a water bath for 15 min with occasional stirring. Cold water (100 mL) was added and the mixture stirred until a precipitate formed. The precipitate was extracted with chloroform and washed with 1 M HCl to remove any dissolved pyridine. Rotary evaporation under reduced pressure of the dried ($Na_2SO_4$) chloroform solution gave the crude product. Purification by recrystallization from acetone and water gave the final product as small white cubic crystals. (4.84 g, 46%): mp 90 °C; $^1$H NMR ($CDCl_3$) 7.76 (2H, d, $J=8.1$ Hz), 7.34 (2H, d, $J=8.1$ Hz), 7.14 (1H, m), 7.01 (1H, d, $J=7.8$ Hz), 6.77 (2H, m), 6.00 (1H, s), 2.47 (3H, s). Anal. calcd for $C_{13}H_{12}NSO_4$: C, 59.07; H, 4.57; S, 12.13. Found: C, 58.98; H, 4.65; S, 12.11.

**5.5.7. General preparation for the 4-*O*-Methyl, 3-*O*-tosyl catechol diazo dyes (6).** A cooled (5 °C) solution made up of the appropriate aniline derivative dissolved in concd HCl (5 mL) and water (50 mL) was prepared. To this is slowly added a cooled (5 °C) solution of $NaNO_2$ (1 molar equivalent) dissolved in water (15 mL). The resulting mixture was stirred for an hour and then added drop-wise to a stirred cooled (5 °C) solution of catechol mono *p*-toluenesulfonate (4) (1 molar equiv), DMF (40 mL), NaOH (1 molar equiv) and water (5 mL) to bring about the formation of the dye. The mixture was stirred for a further hour and then 5% aqueous HCl was added to precipitate the dye, which was then extracted with $CH_2Cl_2$ (60 mL). To this solution was added NaOH (2 molar equiv), tetrabutylammonium bromide (0.1 molar equiv), water (60 mL) and dimethylsulfate (5 molar equiv). The mixture was then stirred vigorously under a nitrogen atmosphere for 24 h. The $CH_2Cl_2$ layer was then separated and the solvent removed by rotary evaporation under reduced pressure. Residual DMF was removed by bulb to bulb distillation to give the crude product.

**5.5.8. 4-Nitro[(4-methoxy, 3-hydroxyphenyl)azo]benzene 3-*O*-*p*-toluenesulfonate (6a).** The dye **5a** was prepared from 4-nitroaniline and then methylated to give the product **6a** using the procedure described above. Purification by column chromatography (chloroform) followed by recrystallization from hexane and chloroform gave the final product as chunky dark red crystals. (0.67 g, 56%): mp 162 °C; $^1$H NMR ($CDCl_3$) 8.38 (2H, d, $J=9.3$ Hz), 7.98 (2H, d, $J=9.3$ Hz), 7.94 (1H, dd, $J=2.5$, 8.8 Hz), 7.86 (1H, d, $J=2.5$ Hz), 7.81 (2H, d, $J=8.6$ Hz), 7.34 (2H, d, $J=8.6$ Hz), 7.01 (1H, d, $J=8.8$ Hz), 3.70 (3H, s), 2.48 (3H, s). Anal. calcd for $C_{20}H_{17}N_3SO_6$: C, 56.20; H, 4.01; N, 9.83; S, 7.50. Found: C, 56.11; H, 3.98; N, 9.81; S, 7.34.

**5.5.9. 4-[(4-Methoxy, 3-hydroxyphenyl)azo]benzenesulfonamide 3-*O*-*p*-toluenesulfonate (6b).** The dye **5b** was prepared from sulphanilamide and then methylated to give the product **6b** using the procedure described above. Purification by column chromatography (chloroform) followed by recrystallization from hexane and chloroform gave the product as small orange crystals. (1.02 g, 79%): mp 190–191 °C; $^1$H NMR (acetone-$d_6$) 8.09 (2H, d, $J=8.5$ Hz), 8.02 (2H, d, $J=8.5$ Hz), 7.98 (1H, dd, $J=2.5$, 9.0 Hz), 7.79 (1H, d, $J=2.5$ Hz), 7.78 (2H, d, $J=8.5$ Hz), 7.48 (2H, d, $J=8.5$ Hz), 7.25 (1H, d, $J=9.0$ Hz), 6.76 (2H, s), 3.68 (3H, s), 2.48 (3H, s). Anal. calcd for $C_{20}H_{19}N_3S_2O_6$: C, 52.05; H, 4.15; N, 9.11; S, 13.89. Found: C, 51.94; H, 4.24; N, 9.11; S, 13.75.

**5.5.10. 3-Hydroxy, 4-methoxy, 4′-phenoxysulfonylazobenzene 3-*O*-*p*-toluenesulfonate (6c).** The dye **5c** was prepared from 4-phenoxysulfonylaniline (**8**) and then methylated to give the product **6c** using the procedure described above. Purification by column chromatography (chloroform) followed by recrystallization from ethanol and water gave the product as chunky bright red crystals. (2.31 g, 42%): mp 177–178 °C; $^1$H NMR ($CD_2Cl_2$) 7.96 (2H, d, $J=8.9$ Hz), 7.93 (2H, d, $J=8.9$ Hz), 7.93 (1H, dd, $J=2.5$, 9.0 Hz), 7.80 (1H, d, $J=2.5$ Hz), 7.73 (2H, d, $J=8.5$ Hz), 7.33 (2H, d, $J=8.5$ Hz), 7.28 (3H, m), 7.01 (2H, d, $J=8.5$ Hz), 7.00 (1H, d, $J=9.0$ Hz), 3.63 (3H, s), 2.44 (3H, s). Anal. calcd for $C_{26}H_{22}N_2S_2O_7.\frac{1}{2}H_2O$: C, 57.03; H, 4.23; N, 5.11; S, 11.71. Found: C, 56.96; H, 3.86; N, 4.73; S, 11.78.

**5.5.11. 4-Methoxy, 3-hydroxyazobenzene 3-*O*-*p*-toluenesulfonate (6d).** The dye **5d** was prepared from aniline and then methylated to give the product **6d** using the procedure described above. Purification by column chromatography (chloroform) followed by recrystallization from hexane and chloroform gave the product as small orange crystals. (0.64 g, 60%): mp 99–100 °C; $^1$H NMR ($CDCl_3$) 7.86 (2H, d, $J=7.5$ Hz), 7.85 (1H, dd, $J=2.5$, 8.5 Hz), 7.79 (2H, d, $J=8.0$ Hz), 7.77 (1H, d, $J=2.5$ Hz), 7.5 (3H, m), 7.31 (2H, d, $J=7.5$ Hz), 6.96 (1H, d, $J=8.5$ Hz), 3.67 (3H, s), 2.45 (3H, s). Anal. calcd for $C_{20}H_{18}N_2SO_4$: C, 62.81; H, 4.74; N, 7.33; S, 8.38. Found: C, 62.64; H, 4.66; N, 7.20; S, 8.33.

**5.5.12. 4,4′-Dimethoxy, 3-hydroxyazobenzene 3-*O*-*p*-toluenesulfonate (6e).** The dye **5e** was prepared from *p*-anisidine and then methylated to give the product **6e** using the procedure described above. Purification by column chromatography (chloroform) followed by recrystallization from hexane and chloroform gave the product as yellow flaky crystals. (0.81 g, 70%): mp 127–129 °C; $^1$H NMR ($CDCl_3$) 7.86 (2H, d, $J=9.0$ Hz), 7.81 (1H, dd, $J=2.1$, 8.8 Hz), 7.80 (2H, d, $J=8.1$ Hz), 7.74 (1H, d, $J=2.1$ Hz), 7.32 (2H, d, $J=8.1$ Hz), 7.04 (2H, d, $J=9.0$ Hz), 6.95 (1H, d, $J=8.8$ Hz), 3.90 (3H, s), 3.67 (3H, s), 2.47 (3H, s). Anal. calcd for $C_{21}H_{20}N_2SO_5$: C, 61.15; H, 4.89; N, 6.79; S, 7.77. Found: C, 60.93; H, 4.95; N, 6.83; S, 7.68.

**5.5.13. 4-Phenoxysulfonylacetanilide (7).** *N*-acetyl 4-aminobenzenesulfonyl chloride (12.78 g, 55 mmol), phenol (5.17 g, 56 mmol) and pyridine (12.5 mL) were mixed together in a 100 mL conical flask and heated in a water bath for 15 min with occasional stirring. Cold

water (100 mL) was added to the mixture and then stirred until a precipitate formed. The precipitate was extracted with chloroform and then washed with 1M HCl to remove any dissolved pyridine. A second wash with 2M NaOH solution removed any unreacted phenol. Rotary evaporation of the dried ($Na_2SO_4$) chloroform solution gave the crude product. Purification by recrystallization from acetone and water gave the final product as small brown crystals. (7.49 g, 47%): mp 138 °C; $^1$H NMR ($CDCl_3$) 7.76 (2H, d, $J$ = 8.8 Hz), 7.67 (2H, d, $J$ = 8.8 Hz), 7.42 (1H, s), 7.28 (3H, m), 6.98 (2H, dd, $J$ = 1.7, 8.3 Hz), 2.23 (3H, s). Anal. calcd for $C_{14}H_{13}NSO_4$: C, 57.72; H, 4.50; N, 4.81; S, 11.00. Found: C, 57.62; H, 4.67; N, 4.80; S, 10.88.

**5.5.14. 4-Phenoxysulfonylaniline (8).** A mixture of 4-phenoxysulfonylacetanilide (5 g, 17 mmol) in 100 mL of 1M HCl was heated under reflux until the acetanilide derivative dissolved. The product crystallized as the solution cooled. (4.01 g, 94%): mp 134–135 °C; $^1$H NMR ($CDCl_3$) 7.56 (2H, d, $J$ = 8.8 Hz), 7.27 (2H, m), 7.00 (3H, m), 6.64 (2H, d, $J$ = 8.8 Hz), 4.28 (2H, br s).

### 5.6. COMT assays

For all five catechol dyes the following incubation mixture was used to determine the suitability of the dye as a COMT substrate. The buffer used in the assay consisted of 0.2 M $NaH_2PO_4$ and 5 mM $MgCl_2.6H_2O$ with the pH adjusted to 7.45. All of the incubation mixtures were made up to a total volume of 60 μL and consisted of 6 μL of 20 mM AdoMet in buffer solution, 48 μL of 125 Units/mL of COMT in buffer solution, 3 μL of a stock solution of catechol dye and 3μL of buffer solution. For dyes **1a**, **1b**, **1d**, and **1e** the stock solution solvent was made up of a 50/50 mixture of ethanol and water to allow the dyes to completely dissolve. In the case of **1c** the dye was soluble in water. The final concentrations in the assay mixture were AdoMet, 2 mM; COMT, 100 units; catechol dye, 0.150 mM The mixtures were incubated for 1 h at 37°C; the assay was stopped by the addition of 40 μL of 4 M $HClO_4$, then diluted with 200 μL of buffer to give a final volume of 300 μL. A 30 μL aliquot of the assay mixture of the assay was analyzed by HPLC.

The assays containing **1a**, **1d** and **1e** as the COMT substrates were analyzed by HPLC using a mobile phase of buffer (25 mM borate) and methanol (30:70 v/v) adjusted to a pH of 8.5 with a flow rate of 0.75 mL/min. The assays containing **1b** and **1c** as the substrates were analyzed by HPLC using a mobile phase made up of water, methanol and trifluoroacetic acid (TFA) (30:70:0.2 v/v) with a flow rate of 0.75 mL/min.

All analytical HPLC chromatography was performed using a Microsorb- MVTM Column (C18, 5 μm particle size, 4.6×250 mm) in conjunction with a dual wavelength UV–Vis detector. During the course of a chromatography run, the detector was switched between wavelengths depending on the elution time and $\lambda_{max}$ values of the compounds being analyzed. For the assays containing **1b** and **1c** as COMT substrates the detector was set at 370nm for the duration of the HPLC run. For the assay containing **1d** as the COMT substrate the detector was set to 450 nm for the first 10 min and then set to 358 nm for the remainder of the run. For the assay containing **1e** as the COMT substrate the detector was set to 450 nm for the first 8.5 min and then set to 363 nm for the remainder of the run.

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