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Synthesis of a New Fluorescent Probe Specific for Catechols

Douglas E. Stack,* Anastacia L. Hill, Clark B. Diffendaffer, and Nicole M. Burns

Department of Chemistry, University of Nebraska at Omaha, 6001 Dodge Street, Omaha, Nebraska 68182-0109

destack@unomaha.edu

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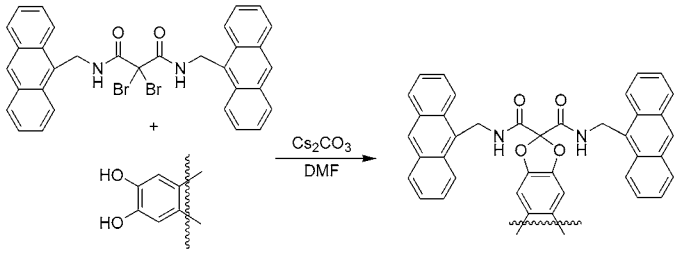
ABSTRACT



The synthesis of a new fluorescent probe, specific for the catechol moiety, has been conducted by preparation of α,α-dibromomalonamides containing an appropriate fluorophore. *N,N′*-Bis-anthracen-9-ylmethyl-2,2-dibromomalonamide reacted with various catechols in the presence of cesium carbonate to generate highly fluorescent derivatives.

The catechol moiety is an important structural unit in many biomolecules. Analytical assay of various catechol-containing substances, such as catecholamines and catechol estrogens, has received considerable attention. High-performance liquid chromatography (HPLC) using electrochemical detection (HPLC-ED),[1] precolumn HPLC fluorescent derivation,[2] and gas chromatography interfaced to mass spectrometry (GC/MS)[3] have all been employed in detecting catechols. We are interested in the detection of catechol estrogen−DNA adducts (CE−DNA) generated by the reaction of catechol estrogen quinones (CE−Q) with DNA bases, namely guanine and adenine.[4] Recent interest in CE−DNA adducts stems from their use in studies aimed at understanding the etiology of hormonal carcinogenesis, and the hope they may be employed as biomarkers for the early detection of estrogen-induced cancers.[5]

Recent publications indicate that production of CE−DNA adducts in vivo occurs at very low levels, ca. at the low femtomolar level.[5d,e] Detection at this level by HPLC−ED was not possible due to the diminished selectivity of HPLC−ED at these levels. Fluorescent derivation using 1,2-diphenylethylenediamine (DPE) has been used for femtomolar detection of catecholamines. Precolumn derivation with DPE requires prior oxidation of the catechol ring system so that condensation of the DPE amino groups with quinone carbonyls can occur.[2] We tried applying the DPE methodology toward CE−DNA adducts with no success. 1-Pyrenesulfonyl chloride was used as a precolumn fluorescent probe for the CE−DNA adduct, 4-OHE_2-1-N7Gua (see Figure 1)

(1) (a) Moleman, P.; Borstrok, J. J. M. *Biog. Amines* **1985**, *3*, 33. (b) Musso, N. R.; Vergassola, C.; Pende, A.; Lotti, G. *J. Liq. Chromatogr.* **1990**, *13*, 1075.

(2) (a) Mitsui, A.; Nohta, H.; Ohkura, Y. *J. Chromatogr. Field* **1985**, *344*, 61. (b) Ohkura, Y.; Nohta, H. *Trends Anal. Chem.* **1992**, *11*, 74.

(3) Castagnetta, L. A.; Granata, O. M.; Arcuri, F. P.; Polito, L. M.; Rosati, F.; Cartoni, G. P. *Steroids* **1992**, *57*, 437.

(4) (a) Stack, D. E.; Byun, J.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. *Chem. Res. Toxicol.* **1996**, *9*, 851. (b) Cavalieri, E. L.; Stack, D. E.; Devanesan, P. D.; Todorovic, R.; Dwivedy, I.; Higginbotham, S.; Johansson, S. L.; Patil, K. D.; Gross, M. L.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10937.

(5) (a) Devanesan, P.; Santen, R. J.; Bocchinfuso, W. P.; Korach, K. S.; Rogan, E. G.; Cavalieri, E. *Carcinogenesis* **2001**, *22*, 1573. (b) Devanesan, P.; Todorovic, R.; Zhao, J.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. *Carcinogenesis* **2001**, *22*, 489. (c) Todorovic, R.; Devanesan, P.; Higginbotham, S.; Zhao, J.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. *Carcinogenesis* **2001**, *22*, 905. (d) Cavalieri, E. L.; Devanesan, P.; Bosland, M. C.; Badawi, A. F.; Rogan, E. G. *Carcinogenesis* **2002**, *23*, 329. (e) Cavalieri, E. L.; Li, K.-M.; Balu, N.; Saeed, M.; Devanesan, P.; Higginbotham, S.; Zhao, J.; Gross, M. L.; Rogan, E. G. *Carcinogenesis* **2002**, *23*, 1071. (f) Cavalieri, E. L.; Rogan, E. G.; Chakravarti, D. *Cell. Mol. Life Sci.* **2002**, *59*, 665.

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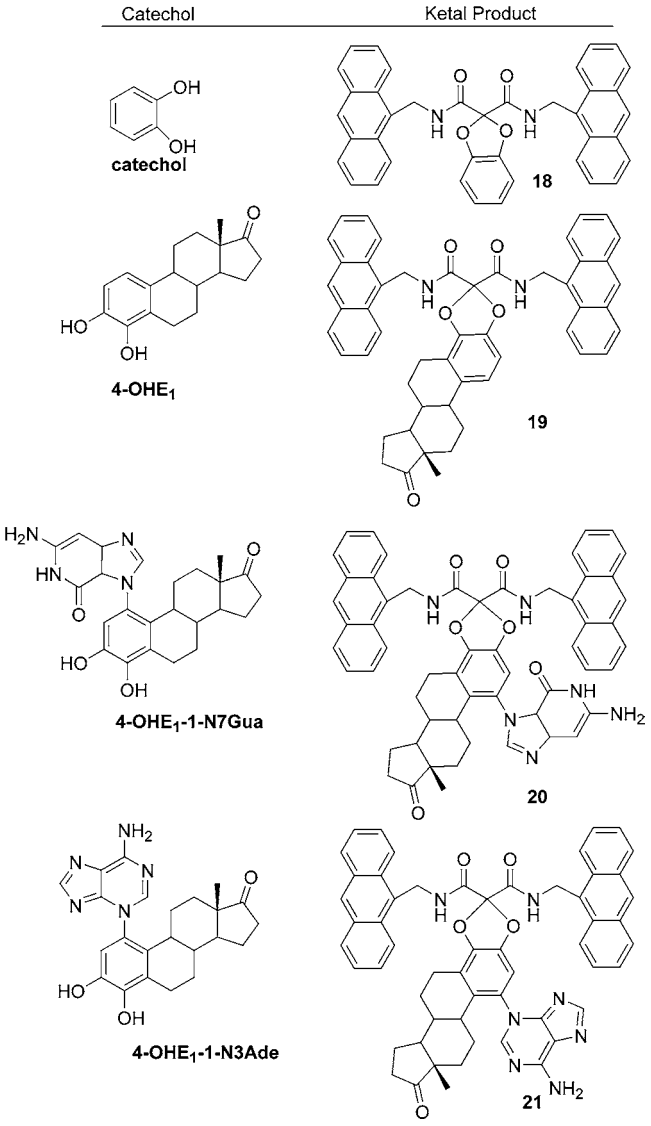


Figure 1.

and was successful at detecting this adduct in rat tissue from animals exposed to high levels of CE−Q.[6] These adducts were characterized by fluorescence line narrowing (FLN) spectroscopy, but $\pi-\pi$ interactions between the fluorophore and the purine ring system of guanine caused the FLN spectra to lack the sharp detail usually generated by this fluorophore. In addition, the high reactivity of sulfonyl chloride with other cellular nucleophiles required several HPLC separations to isolate the derivatized product from a complex mixture of HPLC signals. To improve the fluorescence detection of catechols we sought to design a fluorescent probe specific for the catechol moiety by producing a structure that would bind with both catechol oxygens on a single carbon to generate a ketal linkage. Attaching both oxygens to a single structure should improve sensitivity by providing a more rigid linkage to the substrate thus reducing fluorescence quenching.

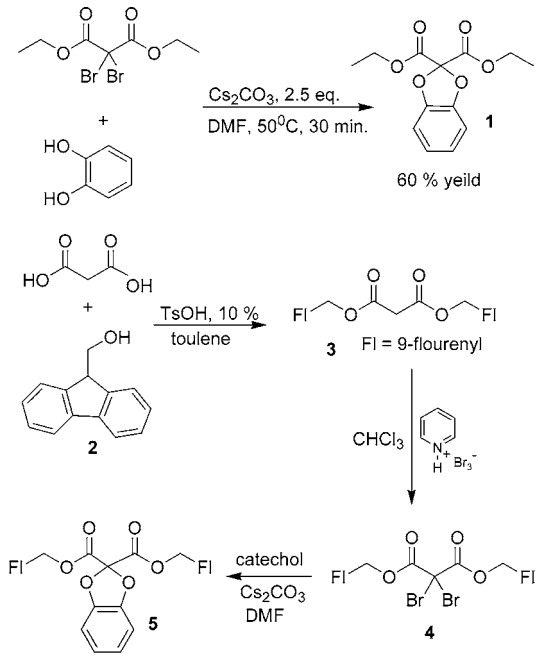
(6) Jankowiak, R.; Zamzow, D.; Stack, D. E.; Todorovic, R.; Cavalieri, E. L.; Small, G. J. *Chem. Res. Toxicol.* **1998**, *11*, 1339.

Increases in selectivity could also be observed if the vicinal dinucleophile reacted preferentially to the probe (versus nonvicinal dinucleophilic species) and/or the fluorescent properties of the resulting product differed when compared to nonvicinal dinucleophiles. Herein, we describe several different approaches to fluorescence labeling of catechols with the hope of generating a probe capable of detecting CE−DNA adducts at endogenous levels.

In designing an effective fluorescent probe for catechols, the following structural features were thought important. First, both catechol oxygens should react with the same atom; thus, a geminal disubstitued carbon with activated leaving groups was sought. Second, no stereocenter should be created in the reaction since CE−DNA adducts are chiral, and generation of an additional stereocenter would produce a mixture of diastereomers. Finally, the substitution reaction should take place in good yield under reasonable experimental conditions. Dibromomalonates fit these criteria. The reaction conditions needed to couple these electrophiles with phenolic nucleophiles were developed using the commercially available ethyl dibromomalonate.

Ethyl dibromomalonate reacts with catechol, in the presence of cesium carbonate in DMF at 50 °C, to generate the ketal product **1** in less than 1 h (Scheme 1). Employing DBU

Scheme 1

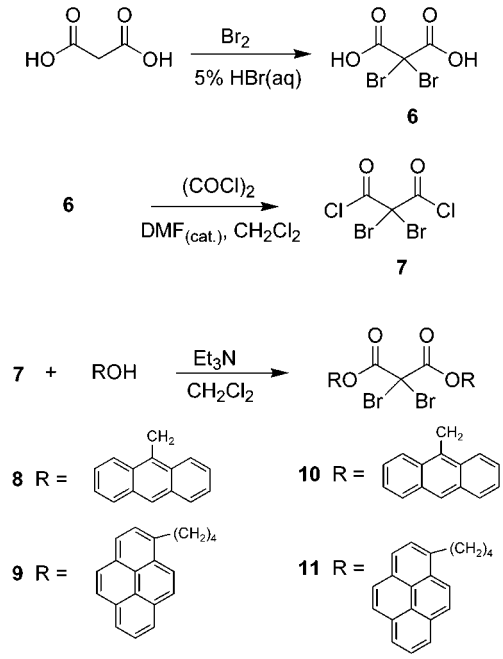


as a base in acetonitrile also generated **1**, but in lower yield. The attachment of a fluorophore to malonic acid was then accomplished first by ester formation using 9-fluorenemethanol to produce the diester **3** (Scheme 1). Bromination of the diester produced **4**, which coupled with catechol in a manner similar to ethyl dibromomalonate to produce the ketal **5**. Ketal **5** displayed poor fluorescent properties at room temperature, and was not effective as a sensitive fluorescence probe for catechol or CE. The reasons for reduced quantum

yields of **5** have not yet been established. Quenching could be due to certain conformational effects, or resonance energy transfer (RET) between fluorene emission and catechol absorption. Molecular dynamics calculation on **5** indicated that the ester linkage provides substantial conformational flexibility at room temperature.

To avoid fluorescence quenching seen with ketal **5**, we sought a new fluorophore with a longer wavelength emission and a more rigid structure. Thus, we synthesized anthracenyl esters of dibromomalonic acid. The synthetic order had to be modified due to the propensity of 9-substituted anthracenes toward bromination at the 10-position.[7] Thus, malonic acid was first brominated with Br_2 in aqueous HBr, followed by formation of diacid chloride **7** (Scheme 2). Ester formation

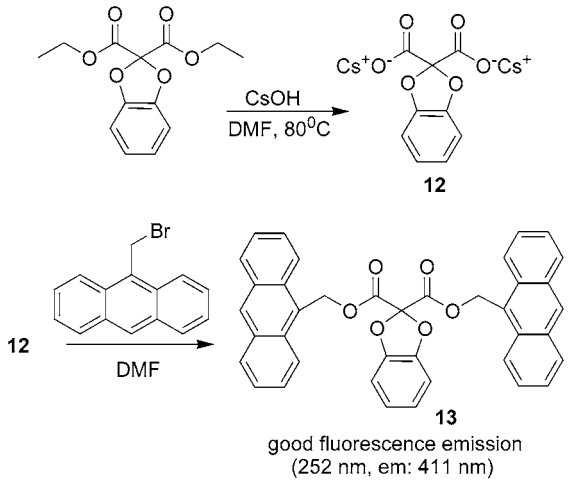
Scheme 2



was then completed by reacting **7** with an excess of 9-anthracenemethanol or 1-pyrenebutanol, from **10** and **11**, respectively. These esters reacted differently toward catechol when compared to diethyl dibromomalonate and **4**. Instead of ketal formation, the alcohols **8** and **9** were recovered in quantitative yield when probes **10** and **11** were reacted with catechol in DMF in the presence of Cs_2CO_3.[8] The mechanism causing hydrolysis of ester **10** and **11** has not been determined, but we observed that (1) probes **10** and **11** hydrolyze slowly in DMF in the presence of Cs_2CO_3 alone, (2) mixing catechol and the probe alone without Cs_2CO_3 does not cause ester hydrolysis, (3) reactions done in the dark still produced **8** and **9**, and (4) varying the carbon chain length between the *gem*-dihalide carbon and the fluorophore, i.e., probe **10** vs probe **11**, did not prevent hydrolysis of the ester. While dibromomalonates have been employed as brominating agents,[9] and while both anthracene and pyrene undergo facile bromination, the probes seem to be stable in DMF under in the presence of a mild base. Also, substitution of geminal dibromides with chloride leaving groups did not prevent ester hydrolysis.[10]

Before designing a new probe that circumvented the problem of hydrolysis, we sought to make the ketal product of probe **10** indirectly to determine its fluorescent properties. Scheme 3 shows the production of the ketal product

Scheme 3



containing anthracene fluorophores. The diethyl malonate ketal was produced as before (Scheme 1), but then the ketal product was subjected to base hydrolysis using CsOH to form the dicarboxylate **12**. The hydrolysis was monitored via HPLC and was complete after 1 h at 80 °C. The reaction mixture was then treated with an excess of 9-bromomethylanthracene which produced the very fluorescent product **13**. While this procedure demonstrated that malonate ester containing two anthracene fluorophores would display good fluorescent emission, this process required additional steps in the derivation process and yields were low when compared to direct reaction of catechol with ethyl dibromomalonate.

To prevent the occurrence of probe hydrolysis, we turned to dibromomalonamides containing anthrancene moieties. Scheme 4 shows the synthetic sequence that produced *N*,*N*′-bis-anthracen-9-ylmethyl-2,2-dibromomalonamide. Synthesis of 9-aminomethylanthracene from 9-bromomethylanthracene has been accomplished by the Delépine reaction.[11] We found this procedure sensitive to workup conditions, and yields

(7) Duan, S.; Turk, J.; Speigle, J.; Corbin, J.; Masnovi, J.; Baker, R. J. *J. Org. Chem.* **2000**, *65*, 3005.

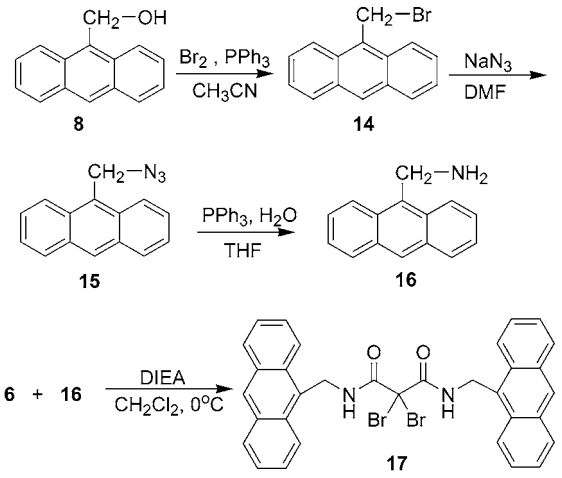
(8) Quantitation of the alcohol products was done via HPLC analysis using authentic standards.

(9) (a) Van der Wolf, L.; Pabon, H. J. J. *Recl. Trav. Chim. Pays-Bas* **1977**, *96*, 72. (b) Campbell, J. R.; Clapp, C. H. *Bioorg. Chem.* **1989**, *17*, 281. (c) Le Menn, J. C.; Tallec, A.; Sarrazin, J. *Can. J. Chem.* **1991**, *69*, 761. (d) Mebane, R. C.; Smith, K. M.; Rucker, D. R.; Foster, M. P. *Tetrahedron Lett.* **1999**, *40*, 1459. (e) Coumbarides, G. S.; Dingjan, M.; Eames, J.; Weerasooriya, N. *Bull. Chem. Soc. Jpn.* **2001**, *74*, 179.

(10) The dichloro analogue of **10** was prepared by reacting **10** with an excess of NH_4Cl in DMSO.

(11) Hartmann, M.; Raethe, M. *Z. Chem.* **1979**, *19*, 373.

Scheme 4



N,*N*′-Bis-anthracen-9-ylmethyl-2,2-dibromomalonamide

were variable. Reduction of commercially available 9-cyanomethylanthracene by $LiAlH_4$ did not result in amine formation as 9-cyanomethylanthracene appears to be quite inert to reduction.[12] We found that azide **15**, derived from **14**, reduces cleanly to **16** using PPh_3 in wet THF.[13] The overall yield from **14** to **16** was 92%. To our knowledge, this is the first reported synthesis of 9-aminomethylanthracene from **15** using PPh_3 reduction.[14] When 2.5 equiv of **16** was reacted with diacid chloride **7**, in the presence of diisopropylethylamine, the resulting malonamide **17** was formed in good yield. Reaction of **17** with several catechol structures using the same combination of Cs_2CO_3 and DMF resulted high yields (>90%) of the ketal product with little or no amide hydrolysis.[15] Also, good yields were obtained using several base/solvent combinations, including DBU/CH_3CN, DBU/DMSO, and Cs_2CO_3/DMSO. Ketal formation occurred equally well with the CE estrogen metabolite, 4-OHE_1, and with two known CE−DNA adducts (see Figure 1). Excess probe was sequestered using a polystyrene resin containing thiol groups[16] The CE−DNA adducts derivatives, could be detected at the low femtomolar level from the fluorescent emission at 411 nm (excitation, 252 nm). The development of this probe as an analytical tool for the detection of CE−DNA adducts, including identification using FLN spectroscopy, will be the subject of future experiments.

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Supporting Information Available: Experimental details on the synthesis of probe **17**, identification of **17** via ^1H and ^{13}C NMR data, HPLC analysis conditions, and MS spectra for the ketal products in Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) BH_3 reduction of 9-cyanomethylanthracene has been reported, but yields were less than 10%. See: Copeland, G. T.; Miller, S. J. *J. Am. Chem. Soc.* **1999**, *121*, 4306.

(13) $SnCl_2$ reduction of **15** was also conducted with yields of 65−70%. However, separation of SnO_2 complicated workup when compared to PPh_3 reduction. See: (a) Maiti, S. N.; Singh, M. P.; Micetich, R. G. *Tetrahedron Lett.* **1986**, *27*, 1423. (b) Gee, K. R.; Keana, J. F. W. *Synth. Commun.* **1993**, *23*, 357.

(14) Recuction of **15** with $NaBH_4$ in good yield has been reported: Ostaszewski, R.; Wilczynska, E.; Wolszczak, M. *Pol. J. Chem.* **1999**, *73*, 1725.

(15) Extended reaction time, greater than 12 h, between probe **17** and Cs_2CO_3 did generate small amounts 9-aminomethylanthrancene.

(16) The resin employed was *N*-(2-mercaptoethyl)aminomethylpolstyrene from Novabiochem. The use of amino containing scavenger resins proved unsuccessful. In addition, probe **17** was unreactive toward lysine in experiments designed to remove excess probe. These initial results suggest that dibromo **17** reacts preferential with sulfur and phenolic nucleophiles when compared to nitrogen nucleophiles.