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TITLE: Determination of Catechol Estrogen Adducts by High-Performance Liquid Chromatography: Establishing Biomarkers for the Early Detection of Breast Cancer

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The metabolism of estrogen produces reactive electrophiles, catechol estrogen quinones (CE-Q). CE-Q have been shown to be genotoxic, reacting with DNA to produce CE-DNA adducts. In order to determine the correlation between formation of CE-Q and breast cancer, an analytical protocol that can measure CE-DNA adducts at ultra-low, endogenous levels in breast tissue is being developed. The synthesis of novel fluorescent probes specific to the catechol moiety was the focus of this year’s work. These fluorescent probes will allow HPLC analysis of CE metabolites and CE-DNA adducts at the femtomolar level. Starting from anthracene, dichloro-di-(9-anthryl)methane was synthesized in three steps. The dibromo analog is also under production. In addition, probes based on flurocene have been generated so that derivation of catechol structures leads to a spiro ring system. The production of a spiro ring system was also explored using the commercially available dichlorodiphenylsilane. The synthesis of gram quantities of 4-hydroxyestrone and 4-hydroxyestradiol was also accomplished in year 1. The ultimate goal of this research is the development of a biomarker for the early detection of breast cancer.
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**INTRODUCTION:**
The metabolism of estrogen to procarcinogenic catechols has been hypothesized as an initiation step in the development of breast cancer [1-4]. Specifically, the over expression of 4-hydroxylase activity has been observed in organs prone to estrogen-induced tumors [5-8]. The role of 4-hydroxyestradiol (4-OHE$_2$) and 4-hydroxyestrone (4-OHE$_1$) metabolites with the increase occurrence of estrogen-induced tumors is still not clear. Hypothesis regarding redox-cycling [9-13] and oxidation to electrophilic quinones have been examined [3,4]. Since oxidation of catechol estrogens (CE) to catechol estrogen quinones (CE-Q) has been shown to lead to CE-DNA adducts [4], we seek to develop an analytical technique that can measure these adducts at biologically meaningful, endogenous levels. The purpose of developing this assay is to examine whether CE-DNA adducts are present in breast cancer tissue. This would be a first step in understanding the etiology of breast cancer as it relates to estrogen metabolism; specifically, the role of 4-hydroxylase activity in the increase occurrence of estrogen-induced cancers. The scope of this method development involves the production of fluorescent probes, specific for the catechol moiety, so that ultra, low-level detection of these adducts can be accomplished. With the proper development of fluorescent probes, this assay would not only be very sensitive, but selective towards the oxidatively liable CE-DNA adducts.

**BODY:**
Task 1 in our statement of work, "Task 1. Develop an extraction procedure for the isolation of CE-adducts, CE and MPEM from rat mammary tissue. (Months 1-14)." was not are initial focus in year 1 of this grant. Instead, we decided to concentrate on Task 2, "Task 2. Develop an HPLC analytical procedure, via pre-column fluorescence derivatization, for the femtomolar detection of CE-adducts, CE and MPEM in human breast tissue. (Months 15-30)." This was done since Task 1 relied heavily on the acquisition of a new high performance liquid chromatograph (HPLC) for which matching funds were not available for the summer research period. Since this work is being conducted in an undergraduate institution, the summer research period is when the maximum work effort (via undergraduate research assistants) can be devoted to the tasks outlined in the statement of work. The development of different fluorescent probes, the initial focus of Task 2, does not rely on state-of-the-art HPLC equipment. Since the purchase of a new HPLC system has now been implemented, Task 1 will become the focus of our work sometime during the course of year 2. Thus, the research described below will summarize our synthetic efforts in developing several fluorescent probe candidates for accomplishing Task 2.

Initially, we sought to use a fluorescent probe that was commercially available, m-dansylaminophenyldboronic acid (DABA). However the use of DABA was found not to be acceptable for reverse phase HPLC conditions. The reason lies in the equilibrium of binding to phenolic hydroxy groups. The equilibrium in Figure 1 can be 'push' to the side of the derivatized product if water is removed during the course of the reaction. Under conditions of reverse phase HPLC, the DABA probe is hydrolyzed back to the boronic acid leaving the catechol underviatized and non-fluorescent. Thus, a novel fluorescent probe capable of irreversible binding in the presence of water had to be synthesized.

The production of a new fluorescent probe specific for the catechol moiety requires the consideration of several issues. Quantum yields for fluorescent molecules are highest in ridged systems with minimal conformational flexibility near the fluorescent emitting portion of the molecule [14]. This is why the DABA probe was sought as a replacement to earlier fluorescent markers used by us on CE and CE-DNA adducts [15]. The binding of both phenol hydroxy groups onto a single atom generates a new ring system with reduced conformational flexibility when compared to the binding of two separate fluorescent probes (Figure 2). Thus, the design of our new probe was conducted with several features in mind. First, a highly fluorescent
fluorophore was selected to maximize sensitivity. Second, the fluorophore should be attached to an atom bearing two electrophilic leaving groups so that reaction with both nucleophilic phenol hydroxy groups could take place leading to the formation of a five-membered ring. Third, the atom possessing the two electrophilic leaving groups could not be prochiral because formation of a new chiral center would produce a mixture of diastereomeric products (since the estrogen ring system is chiral), complicating separation and quantitation of CE-DNA adducts. Fourth, the reaction with phenols should take place in near quantitative fashion, under mild conditions, in solvents conducive to reverse phase HPLC. We have explored several structures that can satisfy these requirements.

Scheme 1 shows the synthetic steps used to produce the first fluorescent probe we believe will meet requirements stated above. Dibromo-di-(9-anthryl)methane should produce a highly reactive probe with intense fluorescent emission. The synthesis of this molecule started from anthracene which was bromonated by N-bromosuccinimide (NBS) in anhydrous dimethylformamide (DMF) to furnish 9-bromoanthracene (1) in good yield. In situ lithiation of (1) in the presence of N,N,N,N-tetraethylurea produces the symmetrical ketone (2). The chloronation of (2) with \( \text{PCl}_5 \) produced dichloro-di-(9-anthryl)methane, (3), in 70% yield. The reaction of (3) with the catechol ring system will be conducted when the fluorescence detector and corresponding HPLC arrives later this summer. Since the dichloro compound (3) may not possess sufficient reactivity to couple with catechols, the production of the dibromo analog has been undertaken. Reduction of (3) with mossy Zn in NaOH generated the hydrocarbon (4). We will bromonate (4) at the benzylic position with NBS to produce (5) early in year 2. Since anthracene is one of the more sensitive fluorophores known, we expect these probes will allow detection of catechol ring systems at the femtamolar level.

The synthesis of probe (5) was first attempted by direct lithiation of anthracene by n-BuLi. This route was to afford a preformed organolithium compound which could react with the commercially available 9-anthracenecarboxylic acid. However, the reaction of n-BuLi with anthracene produced the addition product, 9-butyl-9,10-dihydroanthracene. Even lithiation of (1) with n-BuLi followed by reaction with 9-anthracenecarboxylic acid afforded no ketone product. Thus the route depicted in Scheme 1 was chosen as an alternative.

Since fluorescence is affected by conformational rigidity, a fluorescent probe which results in the formation of a spiro adduct is being pursued. Scheme 2 shows our reaction of fluorene with NBS to produce 9,9-dibromofluorene. We will react this dibromomide with catechols to produce the derived structure (6). The ridged spiro junction could increase the quantum yield of this fluorophore.

The reaction of dichlorodiphenylsilane in the presence of catechol was undertaken to afford the spiro product (7). The fluorescence properties, as well as stability towards hydrolysis, will be investigated. Dichlorodiphenylsilane was chosen since it was commercially available, and it would have the added advantage of not possessing any significant fluorescent properties. An effective fluorescence derivation strategy involves formation of a new fluorophore from non-fluorescent starting materials. This reduces background interference from the fluorescent probe which is usually applied in excess during the derivation process.

In addition to the synthetic work outlined above, considerable effort was expended to producing 4-OHE\(_1\) and 4-OHE\(_2\) from estrone and estradiol, respectively. While the synthetic procedures for these synthesis are known, they involve several steps and consume considerable personnel time to generate gram quantities of these catechols. These catechols will be needed for the investigation of fluorescent probes suitable for HPLC analysis, as well as, production of the CE-DNA adducts that will serve as the eventual biomarkers.
KEY RESEARCH ACCOMPLISHMENTS:

- Synthesis of gram quantities of 4-OH-E1 and 4-OH-E2 for production of CE-DNA adducts.
- Establishment of the inappropriate nature of DABA as a fluorescence probe for catechols.
- Synthesis of dichloro-di-(9-anthryl)methane as a potential fluorescence probe for catechol ring systems.
- Synthesis of di-(9-anthryl)methane as a precursor to dibromo-di-(9-anthryl)methane, a more reactive analog to dichloro-di-(9-anthryl)methane.
- Coupling of dichlorodiphenylsilane to catechol to form the siloxy adduct which will be examined for fluorescent properties.

REPORTABLE OUTCOMES:

1) A local grant entitled, “Establishment of Biomarkers for the Early Detection of Breast Cancer”, was submitted and obtained from The University Council on Research, University of Nebraska at Omaha. The amount was $8,300 and was used to match equipment funds needed for a new HPLC purchase.

2) Two Undergraduates, Clark Diffendaffer and Matthew Nammany, were employed full time during the summer of 1998. Their training in synthetic processes and analytical procedures not only provided for summer employment, but also furthered their academic goals relating to careers in health care.

CONCLUSIONS:

We have completed most of the synthetic requirements, which were many, for this project. Now that most of the synthetic prerequisites have been completed, we will be able to start development of the analytical methodology. The time spent producing several fluorescent probes will pay dividends when various analytical approaches are implemented. While our primary goal will be the use of HPLC coupled to fluorescence detection, followed by verification of adducts via mass spectrometry, the production of several fluorescent probes will allow other analytical procedures to be examined. For instance, collaboration with Ryszard Janowiak at Iowa State University has been established so that the derivatized catechol products can be examined by fluorescence line narrowing spectroscopy (FLNS). FLNS was used in our prior work [7] with earlier fluorescent probes. The flexibility of these probes made the identification of CE metabolites from CE-DNA adducts difficult. By producing new fluorescent probes which are more conformationally rigid, the FLNS spectra of these derived adducts could be used to unequivocally verify structure. Dr. Jankowiak is developing the ability to obtain FLNS spectra directly from capillary electrophoresis separations. Thus, our probes could be instrumental in the development of a separation protocol that could quantitate and identify CE metabolites and CE-DNA adducts, simultaneously.

The development of an analytical assay that can measure CE-DNA adducts at endogenous levels is scientifically and medically important for several reasons. First, while the link between increased estrogen exposure and increased rates of breast cancer has been established, the mechanism relating estrogen to cancer is still unknown. While the hormonal properties, i.e. proliferation of cell growth, associated with estrogen certainly is an important area of research, the role of estrogen metabolites as initiators of DNA damage needs to be clarified. In order to establish a link between metabolic production of CE-Q and breast cancer, the CE-DNA adducts produced when these electrophiles bind to DNA must be measured in human tissues. This requires an ultra sensitive analytical process since these CE-DNA adducts...
will no doubt be produced at very low levels. If the detection of CE-DNA adducts in human tissues, and later in fluids (e.g. plasma and urine), establishes a link between breast cancer and the production of these adducts, a new biomarker for the early detection of breast cancer would be at hand. In addition to an effective biomarker, the ability to measure these adducts in different models could led to strategies aimed at prevention of estrogen-induced cancers.

REFERENCES:


**APPENDIES:**

Figure 1. Unfavorable equilibrium of DABA binding with catechols.

Figure 2. Structural features of a successful fluorescent probe.
Figure 3. Fluorescence probe based on fluorecene.

Figure 4. Spiro adduct of catechol and dichlorodiphenylsilane.
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FOR THE COMMANDER:

Encl

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Deputy Chief of Staff for Information Management