NOTICE

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A. Review of the Work Performed During the First Contract Period (3/81-3/84).

The research to be performed by the Contractor is described as follows:

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1. Objective

The objective of the work was to conduct the necessary studies required to evaluate the efficacy, potential benefit and role of Bromine-77 labeled steroids in the detection and evaluation of treatment for hormone-dependent tumors.

2. Tasks to be Performed by the Contractor (from contract).

2.1 An initial investigation will concentrate on the radiobromination at Carbon-6 or Carbon-7 in selected simple steroids utilizing the nuclides, Bromine-82 and Bromine-77.

2.2 Conduct analytical spectroscopy of radiolabeled compounds.

2.3 Investigate the biodistribution, toxicology and tumor affinity of labeled agents.

3. Summary of Accomplishments:

3.1 Laboratory and Facilities

Our laboratories, located in the Basic Medical Sciences Building, contain 1300 square feet of work area dedicated to organic chemistry, synthetic radiochemistry and biological studies. The synthetic laboratory has the required equipment for the synthesis of organic compounds: fume hoods, glassware, vacuum pumps, rotary evaporator and facilities for TLC and column

chromatography. In addition, the facilities include a Spectra-Physics 8700 high performance liquid chromatograph equipped with UV/VIS detector, radiation detector and Spectra-Physics 4100 integrator

The Department of Chemistry at the University of New Mexico has allowed us complete access to all its equipment including:

Varian FT-80 NMR spectrometer (HNMR & CNMR)

Infared spectrometers

Finnigan gas chromatograph-mass spectrometer

X-ray diffractometer

The facilities for the biological studies include separate rat storage and surgical rooms which are equipped with refrigerators, freezers and Packard Auto-Gamma Scintillation spectrometer.

Equipping and setting up the laboratory required approximately 15 months during the described contract period. During this time, research was conducted in laboratory space and with equipment donated by the late Dr. Guido Daub of the Department of Chemistry at the University of New Mexico Main Campus and by Dr. Robert Loftfield of the Department of Biochemistry at the UNM Medical Center.

3.2 Radiolabeling of the B-ring.

It was demonstrated by our group that 6-dehydroestradiol-3-acetate could be prepared and that this intermediate reacted rapidly with NCS and $Na^{77}Br$ in methanol to generate the labeled product in high yield. All attempts to remove the protecting acetate group with mild base; however,

resulted in loss of all activity from the molecule. It has been determined that removal of the acetate was accompanied by dehydrohalogenation (-H^{77}Br) which generated a methoxy adduct. (See Publication 4.3)

We concluded from this study that the 7- position, as an sp^3 carbon, may not be suitable for radiolabeling due to the acidity of the neighboring benzylic hydrogen.

Treatment of 17ß- dihydroequilen-3-acetate with NCS/Na^{77}Br in methanol under identical conditions resulted in no radiobromine incorporation. Rather, the addition of "^{77}BrOCH$_3$" across the double bond was followed by a rapid double elimination forming dihydroequilenin-3-acetate. This reaction is of definite synthetic value; however, is not applicable for the incorporation of radiohalogens. (See Publication 4.3)

It was concluded that unless the radiohalogen is attached to an sp^2 carbon to enhance stability or is attached without an adjacent leaving group, radiolabeling the B- ring generally leads to formation of unstable compounds.

The attachment of a halogen to a tc a B- ring sp^2 carbon was earlier demonstrated by Caspi and co-workers. The radiolabeled steroid, 6-iodo-6-dehydroestradiol-[^{127}I] showed good stability and a reasonable <u>in vitro</u> binding affinity.

3.3 Radiolabeling of the C- Ring:

Synthetically available 9, 11-Δestradiol-3-acetate was

reacted with $Na^{77}Br$/NCS in methanol. The resultant radiolabeled adducts were obtained in high radiochemical yield. Further treatment of the major product with base facilitated the removal of the acetate protecting group. The product, bromo-methoxyestradiol-[^{77}Br] was obtained in high radiochemical yield and was ultimately obtained in quantities up to 10 mCi. (See Publication 4.3)

The specific activity was estimated to be greater than 1000 Ci/mmol; however, stability studies showed that over 50% of the Bromine-77 was lost from the molecule within 24 hours.

3.4 Biodistribution studies:

Despite the questionable in vivo stability of bromo methoxyestriadiol-[77Br], its biodistribution study in female rats with mammary tumors was performed. No significant receptor uptake in the uterus or tumor of interest was observed and the distribution of the radioactivity was indicative of free bromide. It was concluded that radiochemical decomposition of the compound had occurred in vivo.

3.5 Radiolabeling of the A- Ring:

In our laboratory, we have prepared 1-bromoestradiol, 1-bromoestradiol-[^{82}Br], and 1-bromoestradiol-[^{77}Br] from estrone by the chemistry described in Publication 4.4.

Presently, there are several procedures in the literature which describe the preparation of A- ring halogenated steroids, but they only include halogenation in the 2- and/or 4-

positions. The reasonable chemical stability of the radiohalogenated derivatives in the 2 and 4 positions of the estrone skeleton suggested that 1-bromoestradiol-[77Br] would be equally as stable.

The synthetic difficulties for the preparation of 1-Bromoestradiol were overcome by placement of an amino group in the 4-position, which directed electrophilic bromination to the 1-position. Subsequent deamination and demethylation then led to the desired product. Stability studies of the labeled compounds showed no decomposition over a 48 hour period.

3.6 In vitro and in vivo Studies with the 82Br and 77Br analogs of 1-Bromoestradiol.

Binding affinity studies with 11 were performed in July 1983 by Dr. William Eckleman. The receptor binding affinity that was observed was less than expected (ie. 8% of estradiol). Recent biodistribution studies by our group using 1-bromoestradiol-[^{77}Br] in mature female rats with mammary tumors showed a 3:1 uterus to blood ratio. Further studies during the duration of the present contract will include the biodistribution of 1-bromoestradiol-[77Br] in immature female rats.

4. Publication and Patent Activities for the First Contract Period:

4.1 Hylarides MD, Wilbur DS, Mettler FA Jr.B-Ring Aromatization of Estrogen Derivaties." Steroids, 41, 657 (1983)
Hylarides MD, Mettler F.A.Jr. Wilbur DS. Studies Toward

B-Ring Radiobrominations of Estrogens. Abstract presented at the Society of Nuclear Medicine 28th. annual meeting, June, 1982.

4.2 Mettler FA Jr., Williams AG, Christie JH, Baldwin MH, Hylarides MD, Schott MC, Suitra ZV, Wanak PM, Rodgers RS, O'Brien HA. Biodistribution and Comparataive Tumor Imaging with Copper-67 Citrate and Gallium-67 Citrate, submitted for publication in J Nuclear Medicine.

4.3 Hylarides MD, Wilbur DS, Mettler FA Jr., "Radiobromination of the B- and C- Ring of Estradiol, using No-carrier Bromine-77" J. Radiolabeled Compounds and Radiopharmaceuticals, 22, 433 (1985)

4.4 Hylarides MD, Wilbur DS, Mettler FA Jr., Synthesis of 1-Bromoestradiol; Incorporation of Radiobromine. Manuscript in preparation for the J. organic Chemistry.

5. Patents Pending (developed under the contract).

5.1 Hylarides MD, Mettler FA Jr. A New Procedure for the Rapid Deamination of Aromatic Amines. Patent Awarded.

5.2. Hylarides MD, Mettler FA Jr. Synthesis of 1-Bromoestradiol. Patent application.

B. Summary of Progress

During the current contract period Dr. Mark Hylarides, a synthetic biochemist, has been successfully recruited and a dedicated synthetic radiolabeling laboratory has been established and is fully equipped and operational.

The tasks outlined in the current contract have been largely completed 2.1 through 2.3. Several compounds have been labeled but were found to be unstable and other labeling sites have been pursued. This has resulted in the development of an exceptionally stable compound which is currently being modified to improve receptor binding affinity.

The contract has resulted in the development of new modifications of known synthetic processes. The major limitation has been "up-time" of the pi meson beam at LANL; however, during the "down-time" periods, research was conducted using Bromine-82 rather than Bromine-77.

C. Summary of the work performed during the second contract period (3/84 - present).

The research performed by the contractor is described as follows:

1. Objective:

The objective of the work was to continue our research in the area of halogenated steroids and to investigate their potential utilization for the detection and visualization of hormone-dependent tumors.

2. Tasks to be performed by the contractor (from contract).

2.1 Devise radiochemical methods for the incorporation of radiohalogens into the 1-, 7- and 11-positions of the steroid nucleous.

2.2 Determine the chemical and in vivo stability of the resultant radiohalogenated products.

2.3 Investigate the biodistribution, toxicology and tumor affinity of the radiolabeled agents.

2.4 Predict regional substituent and conformational effects of steroids on receptor binding affinities.

3. Summary of Accomplishments:

3.1 Laboratories and Facilities:

During this contract period, an additional synthetic laboratory was set-up and equipped. This laboratory measures approximately 200 square feet and has a fumehood, glassware and general equipment for routine organic synthesis. This space is used exclusively for "cold" synthesis.

3.2 Personnel

A post-doctoral research associate (Dr. Alberto Leon) was hired and worked in that capacity under this contract from 1984 to 1985. During the period of October of 1985 to October 1986 Dr. Leon beacme a faculty member at the Radiology Department and collaborated with Drs. Hylarides and Mettler in the completion of this contract. Since Dr. Hylarides departure (October 1986),

Dr.Leon was in charge of the completion of this contract.

3.3 Organic Synthesis and Radiolabeling

During the last 3 years, we have found it necessary to shift the emphasis of this research toward the organic synthesis aspects of the proposed investigation. Due to the availability of accurate in vitro test of the binding affinities of the these compounds, it is more efficient to synthesize "cold" derivatives, evaluate them in vitro, and then radiolabel only the compounds that show very good promise. Another reason for this change was the fact that the radioisotopes of interest to our project are no longer available free of cost from the Los Alamos facility.

3.4 Synthesis and studies of A-ring substituted estradiol analog.

After sucessfully synthesizing and radiolabeling the 1-position of estradiol, we decided to study the structure/activity relationship of the 1-halogenated estradiol derivatives. To that effect we prepared the 1-fluoro, 1-chloro and 1-iodoestradiol in addition to the already prepared 1-bromoestradiol.

In vitro binding assays performed in our laboratories indicated that there is a distinctive correlation between the size of the halogen in the 1-position and the obtained binding affinity. This values ranged from about 5% for 1-iodoestradiol to over 40% for 1-fluoroestradiol. (See Papers Presented, 5.7)

The 17-ethynyl derivatives were also prepared. These compounds show in vitro binding affinities similar to that of the parent compounds. (See Papers Presented, 5.7)

X-ray crystallographic studies were performed on 1-fluoro and 1-bromoestradiol and these results were compared to previousx-ray studies on estradiol. In the 1-halogenated compounds, the intramolecular distance between the oxygen attached to carbon-3 and the oxygen attached to carbon-17 was very similar to that of estradiol. Bond lengths (other than the carbon-halogen bond) were very similar in each compound and their conformational characteristics were almost identical. One can conclude that the observed binding affinity is directly related to the "bulkyness" on the substituent on the 1-position. Electronic effects, exhibited by the different halogens, appear to have little influence on the observed binding affinity.
(See Papers Published, 4.5)

3.5 synthesis of B- and C-Ring Analogs:

Due to the problems encountered when an acetate protecting group was used at the 3-position in the labeling of the 6-7 double bond (see first period report), an alternative protective group was sought. It was determined that if an acid labile group such as tetranydiophranyl (THP) was used, the addition of Br-Ome proceeded smoothly and deprotection was possible by addition of diluted acid to the reaction mixture.(See Papers Published 4.4)

The product of this reaction was identified via high field NMR as 6β-methoxy-7δ-bromoestradiol and it showed a relatively low binding affinity <u>in vitro</u>.

Attempts to synthesize the Δ^7-7-bromoestradiol analog by acid treatment failed. Treatment of the 7-hydrazone derivative with

Br_2 also failed to produce the desired product. Several routes were tried for the synthesis of 7-substituted estradiol derivatives with only modest success.

Direct preparation of the 7-halogenated analogs was attempted using the 7-hydroxy derivative and a variety of other analogs and the elimination product was always the major component of the reaction mixtures.

Similar attempts were conducted using C-ring analogs with very little success.

3.6 Synthesis of D-Ring Analogs:

In light of the reported high binding affinities that other investigators have found using D-ring halogenated estradiol derivatives, the emphasis of our synthetic efforts were concentrated in this area toward the end of the contract period.

Our research group developed several efficient ways to functionalize the 15-position of the steroid reaction and we already synthesized and tested several compounds.

The first series of analogs that we prepared were the 15-vinyl bromides in both the 3-unprotected form and the 3-methoxy derivaties.

These compounds were prepared using Δ^{15}-analogs as starting materials via a bromination/dehydrohalogenation sequence developed in our laboratories. (See Papers Published, 4.6 and Papers Presented 5.6)

The 3-phenol derivative showed the expected high chemical stability and it was envisioned that it could be easily labeled via exchange reactions. Unfortunately the relative binding affinity was only 25% that of estradiol.

The synthetic developments realized during this project allowed us to place other functionalities in the 15-position and test their influence in the resultant binding affinity.

Competitive binding affinity measurements were conducted in the 15-hydroxy,-methoxy, allyloxy and benzyloxy. The obtained results were compared with previously gathered data concerning the 14, 15 and 15-15-bromoestradiol. (See Papers Presented 5.7)

This analysis allowed us to determine that the size of the substituent at the 15-position is probably the determinant factor in the affinity of these compounds for estrogen receptor proteins.

As a consequence of the above mentioned findings, the final facets of this project were focused in the synthesis of the 15 - fluoroestradiol and the study of its properties. Preliminary results indicated that this compound can be synthesized using synthetically available 15-hydroxyestradiol as the starting material upon reaction with diethylaminosulfurtrifluoide (DAST).

4. Papers Published

4.1 Hylarides, M.D., Leon, A.A., Mettler, F.A., Wilbur, D.S. Synthesis of 1-Bromoestradiol, J.Org. Chem., 49, 2744 (1984).

4.2 Hylarides, M.D., Leon, A.A., Mettler, F.A. Synthesis of 1-Chloroestrdiol, Steroids, 43, 219 (1984).
Hylarides, M.D., Buksa, P.L., Mettler, F.A., Wilbur, D.S. Radiolabeling of the B- and C-Ring of Estradiol Using No-Carrier-Added Bromine-77, J. Label. Compd. Radiopharm., 22, 443 (1985).

4.3 Hylarides, M.D., Leon, A.A., Mettler, F.A., Wilbur, D.S.Radiobromination of the 1-Position of Estradiol Using No-Carrier-Added Bromine-77, J. Label. Compd. Radiopharm., 22, 437, (1985).

4.4 Leon, A.A., Mettler, F.A., Hylarides, M.D. Preparation of the B-Ring Halogenated Estradiol Derivatives, Steroids, 48/5-6 395 (Nov.-Dec. 1986)

4.5 Hylarides, M.D., Mettler, F.A., Leon, A.A. Molecular Conformational Study of 1-Bromo and 1-Fluoroestradiol Using X-Ray Crystallography and Correlation of their Relative Binding Affinities to Estrogen Receptors, Acta Cryst, in press.

4.6 Leon, A.A., Dillon, M.P., Mettler, F.A., Hylarides, M.D.Preparation of 15-Bromo-Δ15-Estradiol. Organic Preparations and Procedures International, in press.

5. Papers Presented:

5.1 Hylarides, M.D., Mettler, F.A., Wilbur, D.S., "Studies Toward B-Ring Radiobromination of Estrogens" Scientific Paper - Presented at Society of Nuclear Medicine Meeting, Miami Beach, FL, June 1982.

5.2 Hylarides, M.D., Mettler, F.A., Wilbur, D.S., "Synthesisof 1-Bromoestradial; Incorporation of Bromine-82 and Bromine-77", Scientific Paper - Presented at National American Chemical Society Meeting, St. Louis, MO, April 1984.

5.3 Hylarides, M.D., Leon, A.A., Mettler, F.A. Wilbur, D.S., "Studies Toward B- and C-Ring Radiohalogenation of Estradiol", Scientific Paper - Presented at the regional American Chemical Society Meeting, Albuquerque, New Mexico, May 1984.

5.4 Hylarides, M.D., Leon, A.A., Mettler, F.A., Wilbur, D.S. "Further Studies Toward the Radiobromination of the B- and C-Ring of Estradiol", Scientific Paper - Presented at the 1984 International Chemical Congress of Pacific Basin Societies, Honolulu, HI, December 1984.

5.5 Leon, A.A., Hylarides, M.D., Mettler, F.A., "Synthesis of 1-Chloroestradiol", Scientific Paper - Presented at the 1984 International Chemical Congress of Pacific Basin Societies, Honolulu, HI, December 1984.

5.6 Leon, A.A., Mettler, F.A., Hylarides, M.D., "Synthesis of 15-Vinyl Bromide Estradiol Derivatives", Scientific Paper - Presented at the National American Chemical Society Meeting, Chicago, IL, September 1985.

5.7 Leon, A.A., Mettler, F.A., Hylarides, M.D., "Preparation of 15-Substituted Estradiol Derivatives. Comparison of their Relative Estrogen Receptor Binding Affinities - Presented at the 193rd National American Chemical Society Meeting, Denver, CO. April 1987.

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