RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

16α-[^{77}Br]Bromoestradiol-17β: A High Specific-Activity, Gamma-Emitting Tracer with Uptake in Rat Uterus and Induced Mammary Tumors

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16α-[^{77}Br]Bromoestradiol-17β (Compound 1) has been synthesized by radiobromination of estrone enoldiacetate. Tissue uptake studies performed 1 hr after administration of Compound 1 to immature or mature female rats showed uterus-to-blood ratios of 13, with nontarget tissue-to-blood ratios ranging from 0.6 to 2. Co-administration of unlabeled estradiol caused a selective depression in the uterine uptake with no effect on nontarget tissue uptake. In adult animals bearing adenocarcinomas induced by DMBA (7,12-dimethylbenz(a)anthracene), tumor-to-blood ratios of 6.3 were obtained, this uptake also being depressed in animals treated with unlabeled estradiol. The studies demonstrate that Compound 1 has suitable binding properties and sufficiently high specific activity so that its uptake in estrogen target tissues in vivo is mediated primarily by the estrogen receptor. Furthermore, they suggest that this compound may be suitable for imaging human breast tumors that contain estrogen receptors.

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The estrogen receptor, a specific, high-affinity binding protein present in estrogen target tissues, is thought to be the principal mediator of the action of estrogens in these tissues. Estrogen receptors are also present in many human breast tumors, and there is currently intense interest in the use of the estrogen receptor content of a tumor as the basis for selecting the most favorable course of therapy (*1,2*).

Through selective uptake by these high-affinity binding proteins, estrogens at physiological concentrations are known to be concentrated several fold over blood levels in estrogen target tissues—e.g., uterus, vagina, pituitary, hypothalamus (*3–5*), and mammary tumors (*6–9*). Thus the estrogen receptor provides a potential mechanism by which a suitable gamma-emitting estrogen could be concentrated in a receptor-containing tissue or tumor and thus act as an imaging agent. This would provide a noninvasive assay of the dynamics of estrogen binding by the tissue or tumor. The achievement of high selectivity through a receptor-mediated uptake process requires a gamma-emitting estrogen that has high affinity for the estrogen receptor, that does not have excessive binding to nonreceptor proteins, that has reasonable metabolic stability, and that can be prepared in high specific activity. In a recent review (*10*) these characteristics have been discussed in greater detail, and earlier work in this area by ourselves and others has been summarized.

In this report, the synthesis of 16α-[^{77}Br]bromoestradiol-17β in two steps from estrone enoldiacetate is described. Unlabeled 16α-bromoestradiol-17β has been shown to have high affinity for the estrogen receptor (by competitive radioreceptor binding assay), and is predicted not to exhibit excessive nonreceptor binding (*10,11*). The radiobrominated material we have obtained has sufficiently high specific activity to show highly se-

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lective, receptor-mediated uptake by the uteri of immature and adult rats, and by mammary adenocarcinomas induced by DMBA (7,12-dimethylbenz(a)anthracene) in adult rats. Estrogen receptor interaction and selective tissue uptake of 16α-[^{125}I]iodoestradiol-17β has recently been described by Hochberg (*12,13*) and others (*14*).

MATERIALS AND METHODS

Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl and stored over molecular sieves (0.4 nm). Lithium aluminum hydride was dissolved in THF by stirring at room temperature for 3 hr under a nitrogen atmosphere, followed by pressure filtration through a Schlenk filter. The solution was collected in an Erlenmeyer flask equipped with a three-way stopcock and ground-glass stopper. Standardization with a gas burette to measure evolved hydrogen gave a concentration of 1.2 *M*. Bromine-77 was either produced in the Washington University 52-in. cyclotron as previously described (*15*) or was obtained as a spallation product from Los Alamos Scientific Laboratory (*16*) for use in the synthesis development work. All of the animal studies reported here were carried out using the spallation-produced radionuclide. The purity of estrone* was ascertained before use by TLC, melting-point determination, and nuclear magnetic resonance. All standard steroidal compounds (unlabeled halogenated estrogens, intermediates, and byproducts) were prepared or obtained as described previously (*11*).

High-pressure liquid chromatography was performed using μ-porasil† or Partisil PXS 10/25‡ columns for analytical separations, or a Partisil M-9‡ magnum column for preparative scale separations. The columns were eluted with a linear gradient of ether (20–75%) in hexane, and the eluant was monitored with a variable-wavelength ultraviolet detector set at 275 nm, and a sodium iodide scintillation detector. All uv and radioactivity peaks were identified by co-injection with authentic samples.

All reactions were conducted under a nitrogen atmosphere in glass culture tubes with magnetic stirring, and solutions were transferred using a nitrogen-purged syringe. Extraction solvents were removed by nitrogen purge with gentle heating. Radioactivity was determined using either a sodium iodide well counter or a dose calibrator.

16α-[^{77}Br]bromo-3-acetoxy-1,3,5(10)estratriene-17-one. Synthesis of 16α-[^{77}Br]bromo-3-acetoxy-1,3,5(10)estratriene-17-one (Compound **2**) was based on a modification of published procedures for the preparation of the unlabeled compound (*11* and references cited therein), as outlined in Fig. 1. Estrone 3-acetate 17-enolacetate (*11*) (0.20 mg) was dissolved at room temperature in THF (0.03 ml) and diethyl ether (0.02 ml), buffered with 0.10 ml freshly prepared potassium acetate 85% acetic acid buffer (2.81 g KOAc in 50 ml, 85% HOAc), in a glass culture tube with rapid magnetic stirring. To the clear solution was added 2–6 mCi Na^{77}Br in dilute NaOH solution, followed by 30% hydrogen peroxide solution (0.10 ml) and glacial acetic acid (0.05 ml). Incorporation of radioactivity was monitored by removal at half-hour intervals of 2-μl aliquots, which were partitioned between 1.0 ml each of diethyl ether and distilled water; each phase was then assayed for radioactivity. Upon reaching 65–80% organic incorporation (~3 hr), the reaction was quenched with 1.5 ml distilled water and extracted with three 1-ml portions of diethyl ether. Solvent was removed under a gentle stream of nitrogen, and an aliquot of the mixture was analyzed by HPLC on a Partisil PXS 10/25 column.‡

16α-[^{77}Br]bromo-1,3,5(10)estratriene-3,17β-diol (Compound 1). Reduction of compound **2** was carried out as outlined in Fig. 1. Compound **2** was taken up in THF (1 ml) under an atmosphere of dry N_2 and chilled to −78°C in a dry-ice/acetone bath. One milliliter of a 1.2 *M* solution of lithium aluminum hydride in THF was added by syringe, in slow drops with rapid magnetic stirring. After 10 min, the reaction was quenched by the dropwise addition of 1.5 ml of a 1:1 solution of THF:ethyl acetate (pre-chilled to −78°C). After another 5 min, the dry-ice:acetone bath was removed; the reaction mixture was allowed to warm to 0° in an ice-water bath, and quenching was completed by the addition of 10% HCl and distilled water. The reaction mixture was extracted with three 1-ml aliquots of ethyl acetate; following solvent removal, the material was analyzed and purified by HPLC on a Partisil M-9 column.‡

In vivo studies. Compound **1** was separated by HPLC, evaporated to dryness, and dissolved in 50 μl ethanol. Before injection, a mixture of 0.9% NaCl and rat serum (1:1) was added to the solution to prevent adsorption of the radiolabeled estrogen to the syringe. The final injectate contained ≤1% ethanol by volume.

Tissue uptake studies were carried out in immature (25 day) Sprague-Dawley female rats and mature (110–130 day) Sprague-Dawley female rats bearing mammary tumors (induced by intravenous injection of 5 mg DMBA at 50 days). The rats were injected either intravenously or intraperitoneally with 2–150 μCi Compound **1** and killed 1 hr after i.v., or 2 hr after i.p., injection. Samples of blood and 11 tissues were measured, weighed, and assayed for radioactivity. In subsequent experiments, immature and mature rats were given a simultaneous injection of 15 μg unlabeled estradiol to block receptor-mediated uptake.

A mature, Sprague-Dawley, female rat bearing DMBA-induced mammary adenocarcinoma was injected with 150 μCi Compound **1**. Scintillation images were obtained at 1 hr after injection using a pinhole collimator.

RESULTS

Synthesis of Compound 1. The quantity of the estrone derivative used (200 μg) provides a reasonable compromise between achieving an adequate rate of bromination (65–80% complete within 3 hr) while enabling

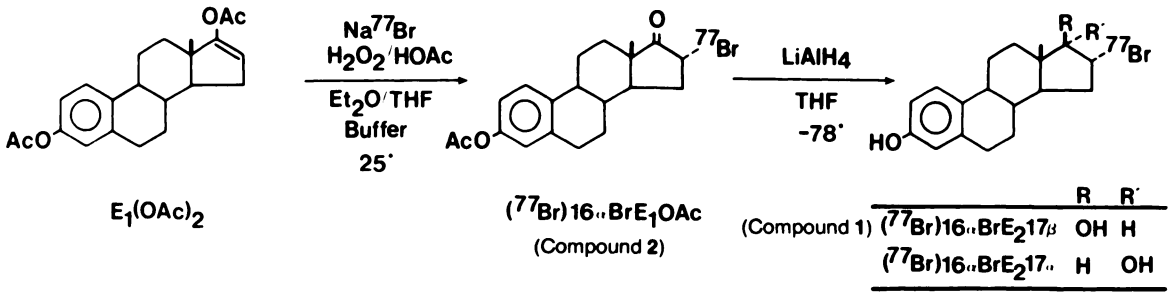

FIG. 1. Reaction scheme for the synthesis of Compound 1.

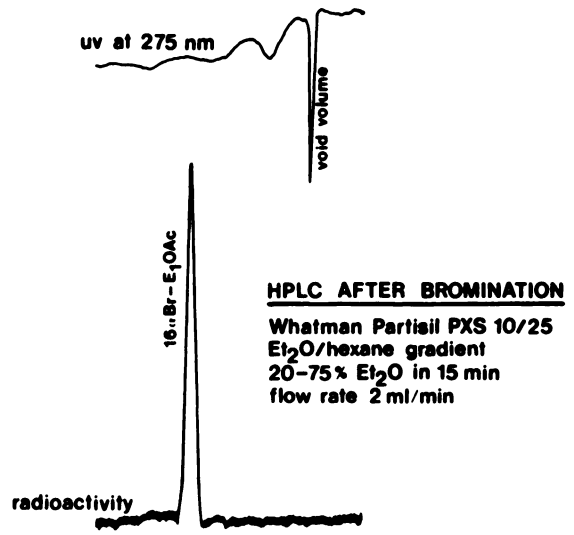

FIG. 2. High-pressure liquid chromatographic analysis of reaction mixture following radiobromination of estrone-3-acetate 17-enol-acetate. Compound 2 elutes at 753 sec.

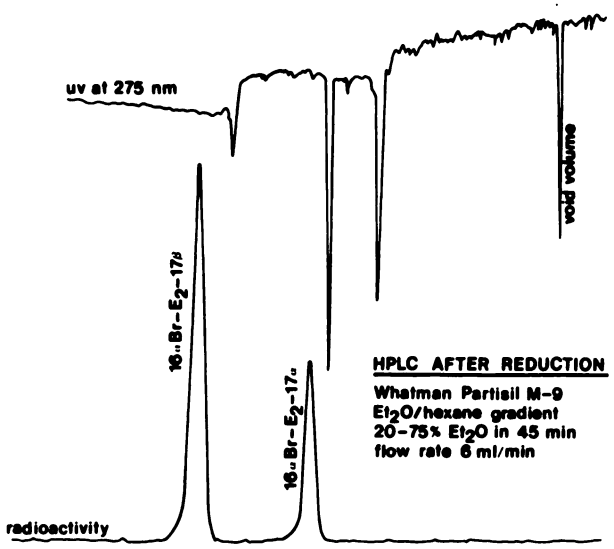

FIG. 3. High-pressure liquid chromatographic analysis of reaction mixture following reduction of Compound 2. Elution times are 1214 sec for the -17α epimer and 1664 sec for Compound 1.

purification of the final product by HPLC. Analysis of the radiobromination reaction mixture by HPLC on a Partisil PXS 10/25 column shows a single radioactive peak (Fig. 2). This material was identified as Compound **2** by its coelution with authentic material (data not shown).

Treatment of Compound **2** with a filtered solution of lithium aluminum hydride in THF for 10 min at −78°C, followed by low-temperature quench of excess hydride with ethyl acetate, effects both reduction of the 17-ketone and reductive deacetylation of the phenol. The use of other reductants ($Zn(BH_4)_2$, $NaAlH_2(OCH_2CH_2OCH_3)_2$ or $LiB(C_2H_5)_3H$, B_2H_6), or more prolonged or more vigorous conditions resulted in excessive debromination either by direct reductive debromination, producing estrone, or by hydrogenolytic debromination, giving a mixture of estradiol-17β and estradiol-17α.

The reduction product, obtained in 95–98% radiochemical yield, consists of a 3:1 mixture of radiobrominated 16α-bromoestradiol-17β and 16α-bromoestradiol-17α, as determined by HPLC on Partisil M-9 (Fig. 3). These compounds were identified by their coelution with authentic samples of the -17β and -17α compounds (data not shown). The epimeric alcohols are cleanly separated, allowing the isolation of Compound **1** in 45–60% overall radiochemical yield, and the -17α epimer in 15–20% yield. There are no other radioactive peaks, and no impurities are detectable by ultraviolet absorbance under the peak corresponding to Compound **1**.

Note that under the chromatographic conditions used, Compound **1** is also separated cleanly from estrone enoldiacetate, estradiol-17α and -17β, 16β-bromoestrogens, and the 16-chloroestrogens and estrone. The epimeric estradiols could be byproducts of the reduction if any debromination is indeed taking place; they could also be products of reduction of unreacted estrone enoldiacetate. The chloroestrogens are potential byproducts from the oxidative bromination reaction using spallation-produced bromine-77, since this $Na^{77}Br$ solution contains chloride that is added during separation of the radiobromide from the target (*16*).

The most critical separation involves estrone, which elutes immediately ahead of Compound **1** (cf. Fig. 3). Estrone is produced when the bromination product mixture (which contains excess, unreacted estrone enoldiacetate) is reduced with lithium aluminum hydride and quenched nonprotonolytically (with ethyl acetate). While baseline separation between estrone and Compound **1** can be achieved using a highly efficient HPLC column, suboptimal performance that leads to tailing of the estrone peak can cause contamination of Compound **1** by estrone. In recent studies (data not shown) we have found that cautious protonolysis of the reduction mixture by the slow addition of tert-butanol at −78°C minimizes the production of estrone, presumably by selective protonation of the estrone enolate, which is then reduced further to estradiol by the remaining hydride reagent.

Tissue uptake selectivity in immature rats. Ten immature rats were injected by tail vein with 2–20 μCi Compound **1**, and tissue samples were removed and assayed 1 hr after injection; six rats were injected intraperitoneally with the tracer and killed after 2 hr. The data presented in Fig. 4 show the average percentage injected dose per gram for various tissues, and their ratios of counts per gram to cpm/g of blood for five rats injected i.v. with 20 μCi Compound **1**. The 2 μCi and 20 μCi i.v. doses distributed identically, and the same doses, delivered i.p., showed no significant differences from the i.v. regarding tissue-to-blood ratios.

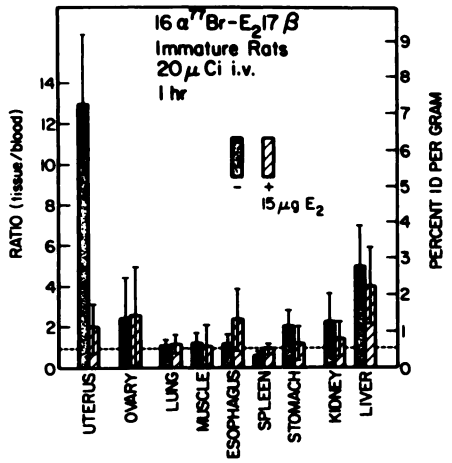

FIG. 4. Tissue uptake selectivity of Compound 1 in immature Sprague–Dawley female rats: mean and range for five rats injected i.v. with 20 μCi of tracer with and without 15 μg unlabeled estradiol, and killed 1 hr after injection.

The uterus-to-blood ratio in immature rats at 1 hr is 13.0 ± 3.4. Most of the nontarget tissues (lung, kidney, spleen, stomach, esophagus, and muscle) show levels not significantly different from that in the blood; however, organs that are involved in metabolism and excretion of steroids (e.g., liver and intestines) have higher levels. The percent injected dose per gram of large and small intestine was highly variable because only a segment of the intestines was counted; hence, these results are not included with the data presented in Figs. 4 and 5. Since in rats the principal route of excretion of most estrogens is through the feces, the high intestinal levels are not unexpected; similar results have been described for estradiol and a series of tritium-labeled halogenated nonsteroidal estrogens (*17*). That the high uptake seen

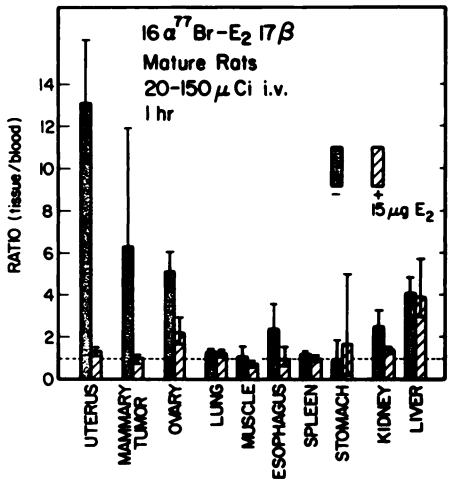

FIG. 5. Tissue uptake selectivity of Compound 1 in mature Sprague–Dawley female rats bearing DMBA-induced mammary adenocarcinoma: mean and range for four rats injected i.v. with 20–150 μCi of tracer, with and without 15 μg unlabeled estradiol, and killed 1 hr after injection.

in the uterus is mediated by a high-affinity, limited-capacity uptake system is demonstrated by the fact that co-administration of an excess of unlabeled estradiol causes a selective depression of the uptake by the uterus; the uterus-to-blood ratio decreases from 13.0 ± 3.4 to 1.95 ± 1.24. Uptake in the other organs is not affected significantly by the presence of unlabeled estradiol.

Tissue uptake selectivity in mature rats. Four mature rats bearing DMBA-induced mammary adenocarcinoma were injected (femoral vein) with 20–150 μCi Compound **1** in the presence and absence of 15 μg unlabeled estradiol. Eight tumors were removed from the four rats injected with the radiotracer alone, and seven tumors were assayed in the four rats that also received unlabeled estradiol. The tissue distribution of radioactivity, determined after 1 hr, is presented in Fig. 5 as the ratio of tissue counts per gram to blood counts per gram.

As with the immature female rats, the uterus shows high uptake that is selectively depressed by an excess of unlabeled estradiol; the uterus-to-blood ratio decreases from 13.1 ± 2.7 to 1.29 ± 0.20 in the presence of 15 μg estradiol. Nontarget tissues show low uptake that is unaffected by excess estradiol. Note particularly that mammary tumors show average uptake ratios of 6.27, and that tumor uptake is also selectively depressed (to an average ratio of unity) by treatment with excess estradiol. In mature but not in immature rats, the ovaries show enhanced uptake of Compound **1** that appears to be receptor-mediated.

Mammary tumor imaging. Figure 6 shows gamma-camera images, obtained using a pinhole collimator at 1 hr after injection of 150 μCi of Compound **1**, of a mature Sprague–Dawley female rat bearing DMBA-induced mammary adenocarcinoma. The image on the left covers the head and torso, showing increased uptake in the neck and abdominal regions. The center image is a magnified view of the neck area, and that on the right illustrates two adjacent areas of uptake in the abdomen. At necropsy, one tumor was found in the neck, and there

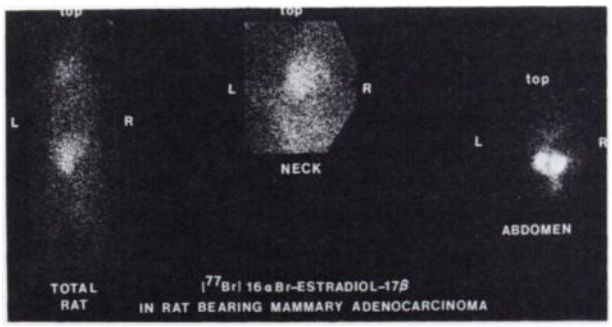

FIG. 6. Scintillation images of a mature Sprague–Dawley female rat bearing DMBA-induced mammary tumors in neck and abdomen. Images were obtained using pinhole collimator, 1 hr after injection of 150 μCi Compound 1.

were two adjacent tumors in the abdominal region where uptake was observed in the image.

DISCUSSION

A two-step sequence for the synthesis of Compound **1** has been described; it is efficient and convenient and produces a material with sufficiently high specific activity for studying selective uptake mediated by the estrogen receptor. Studies in immature and mature rats reveal a pattern of tissue uptake with the characteristics expected for a process mediated by the estrogen recpetor: uptake by the uterus, and by mammary tumors of mature animals, that is high and is selectively depressed when animals are treated with an excess of unlabeled estradiol. The uterus-to-blood ratios of ~13 that are seen with this compound at 1 hr are comparable to those reported for estradiol and other related labeled estrogens (*3–5,17*), and appear to be at least as good as those found with [^{125}I]16αiodoestradiol-17β (*14*). It is possible that higher ratios could be obtained at earlier or later times, but this has not yet been investigated. Preliminary data using extraction techniques show that >98% of the radioactivity present in the blood at 1 hr is organically bound.

Equivalent uterine uptake selectivites are seen in both immature and mature rats, even though uterine receptor concentrations in immature rats (~30 n*M*) are about three times those in mature rats (~10 n*M*) (*18*). This observation, together with the fact that the extraction of circulating estradiol by the uterus is known to be high (*19*), suggests that the uptake of Compound **1** by the uterus is a flow-limited process.

It is also apparent that Compound **1** exhibits selective uptake by the ovaries of mature, but not immature, rats. In the rat ovaries there are estrogen receptors that appear to be associated with the granulosa cells (*20,21*). In immature rats, the concentration of unoccupied receptors in the ovary is less than 10% of that in the uterus (*20*). In mature rats, however, granulosa cells constitute a larger fraction of the ovary; thus, one would expect higher receptor concentrations and greater tissue uptake of estrogens by mature ovarian tissue.

A large proportion (~90%) of DMBA-induced rat mammary adenocarcinomas are known to be ovary-dependent (*22*), and the receptor content of these tumors is in the range of 3 to 4 n*M* (*18*). We have found that the tumor-to-blood uptake ratios (6.3) were less than those of the uterus (13), which is consistent with the lower receptor content of the tumors. Uptake by the tumors showed considerable variation, however, and we note that the tumors that visually appeared to be most extensively vascularized were those that showed the most pronounced uptake.

Although no ultraviolet-absorbing material was observed to elute with Compound **1** upon its final purification by HPLC, a direct measurement of the specific activity of the tracer has not been made. However, since the minimum level of bromoestradiol detectable by uv is ~1 μg, and ~4 mCi Compound **1** were purified in a single preparative HPLC run, we can estimate a minimum specific activity of ~1400 Ci/millimol based on this minimum detectable uv absorbance. On the basis of the following information, another estimate of its minimum specific activity can be made. In a mature tumor-bearing rat that received 150 μCi of Compound **1**, there was no apparent diminution in the uterus-to-blood or tumor-to-blood uptake ratios relative to the animals that were given either 2 or 20 μCi. Since the experiments with the rat receiving 150 μCi Compound **1** were performed 2 days after those using the 2-μCi doses, this actually represents a 150-fold difference in the specific activities of the tracer. The concentration of unoccupied (cytoplasmic) estrogen receptors in mammary tumor tissue is approximately 1–3 pmole/g (*18*); thus, if one makes the conservative assumption that the lack of diminution of uptake selectivity at the highest dose indicates no greater than 33% occupancy of the estrogen receptor at that dose, then the tumor uptake of 470 nCi/g observed in this animal must be occupying less than 0.33–1.0 pmoles of receptor. Hence, Compound **1** must have a specific activity of at least 470–1420 Ci/mmole.

In conclusion, a gamma-emitting, brominated estrogen has been prepared, having sufficiently high specific activity to demonstrate selective uptake into estrogen target tissues and mammary tumors in rats. This selective uptake has the characteristics of a high-affinity, limited-capacity uptake system and is presumed to be due to the estrogen receptor. The convenient preparation of this Compound **1**, its high target selectivity, and the scintigraphic images of mammary tumors in rats obtained using it suggest that Compound **1** may be useful as an agent for imaging human breast tumors or metastases that contain estrogen receptors, and for providing a dynamic assay of estrogen uptake by these tumors in vivo.

FOOTNOTES

* Sigma Chemical Co., St. Louis, MO.
† Waters Associates, Inc., Milford, MA.
‡ Whatman Inc., Clifton, NJ.

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