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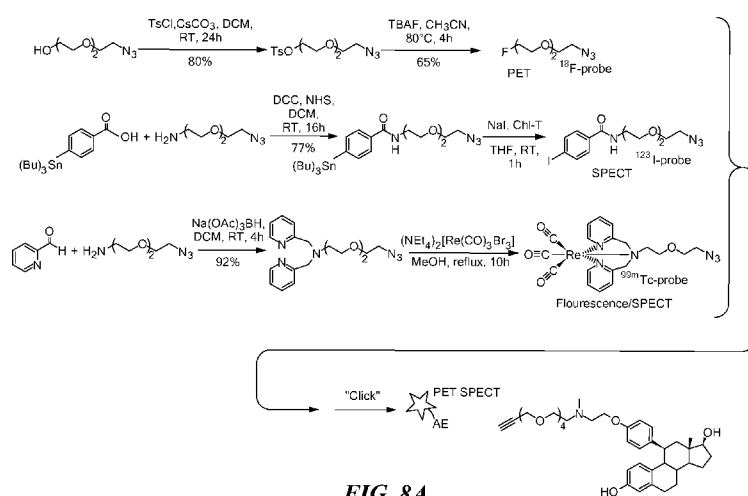
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[Continued on next page]

(54) **Title:** ESTROGEN RECEPTOR IMAGING AGENTS**FIG. 8A**

(57) **Abstract:** Compounds useful for molecular imaging of cells expressing estrogen receptors are provided. Also provided are intermediates for making the compounds and methods of making the compounds using a modular convergent strategy. Further, methods of making the intermediates are described, as well as methods of diagnosing disease in a subject by using the compounds as imaging agents.



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TITLE OF THE INVENTION

ESTROGEN RECEPTOR IMAGING AGENTS

5

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the priority of U.S. Provisional Application No. 61/735,184
10 filed December 10, 2012 and entitled "RADIONUCLIDIC AND NONRADIONUCLIDIC
MOLECULAR IMAGING AGENTS FOR THE ESTROGEN RECEPTOR BASED ON 11-
BETA SUBSTITUTED STEROIDAL ANTIESTROGENS", which is hereby incorporated
by reference in its entirety.

15

BACKGROUND

Molecular imaging (MI) enables integration of patient and disease-related information
with anatomical imaging (Hanson, R.N., 2012). With MI one can visualize, characterize, and
measure biological processes at the molecular level in a living organism (Kodibagkar, V. D et
al., 2012; Koolen, B. B. et al., 2012; Misri, R. et al., 2012). Further, use of MI in the
20 diagnosis of a disease results in improved clinical risk assessment, optimization of treatment
and therapy, better patient outcome, and helps inter- and intra-subject comparisons. (Moss, J.
A. et al., 2012; Vavere, A. L. et al., 2012; James, M. L. et al., 2012).

In medicine MI has evolved into two distinctive areas: anatomy-based or structural
imaging, and nuclear medicine (Beyer, T. et al., 2009; Antoch, G., et al., 2009; Mawlawi, O.
25 et al., 2009). Traditional imaging such as computed tomography (CT) and magnetic
resonance imaging (MRI) are now primarily used for extracting anatomical and/or structural
information. On the other hand nuclear medicine, using methods such as positron emission
tomography (PET) and single photon emitted tomography (SPECT)), focusses on molecular
events in living organisms, and thereby provides functional and/or physiological imaging. In
30 oncology the information derived from PET and SPECT imaging is integrated with other
patient specific disease information for diagnosis and treatment (Buck, A. K. et al., 2012;
Morales-Avila, E. et al., 2011; Khalil, M. M. et al., 2011). Table 1 below summarizes the
major differences among the various MI modalities.

Table 1 - Noninvasive in vivo molecular imaging modalities (Buck, A. K. et al., 2012)

Imaging	Modality Energy used	Spatial Resolution (mm)		Acquisition time/frame (s)	Probe mass (ng)	Sensitivity of detection (Mol/l)	Depth of penetration (mm)
		Clinical	animal				
PET	Annihilation	3-8	1-3	1-300	1-100	10^{-11} - 10^{-12}	>300
SPECT	γ -photons	5-12	1-4	60-2000	1-1000	10^{-10} - 10^{-11}	>300
CT	X-rays	0.5-1	0.03-0.4	1-300	-	-	>300
MRI	Rf	0.2-0.1	0.025-0.1	50-3000	10^3 - 10^6	10^{-3} - 10^{-5}	>300
Ultrasound	High Rf	0.1-1.0	0.05-0.1	0.1-100	10^3 - 10^6	-	1-200
BLI	IR	-	3-10	10-300	10^3 - 10^6	10^{-13} - 10^{-16}	1-10
FLI	IR	-	3-10	10-2000	10^3 - 10^6	10^{-9} - 10^{-11}	1-20

Magnetic resonance imaging (MRI) primarily provides information about soft tissue. Although MRI provides high spatial resolution (<1 mm), and has the ability to visualize anatomical, physiological, and/or metabolic information in a single imaging session, it has low sensitivity for biological targets. Therefore, this method has not been tailored to visualize cancer or disease specific disorders (Misri, R. et al., 2012).

X-ray computed tomography or computed tomography (CT scan) is a computer processed X-ray imaging technology that provides anatomical images of the body, which may be manipulated to obtain diagnostic information. CT inherently provides high-contrast and resolution, but exposes the patient to high radiation dose enhancing the risk of DNA or cellular damage. CT methods that provide high signal to noise ratio at lower radiation doses are currently being investigated (Buck, A. K. et al., 2012).

Optical imaging is another modality for molecular imaging, and is based on the detection of light photons and their interaction with the tissue. It has been used mostly in preclinical studies. Two major optical imaging methods are bioluminescence imaging (BLI) and fluorescence imaging (FI). BLI requires cellular expression of the enzyme luciferase, which is incorporated into the DNA of animals used for modeling of any given disease. BLI is most useful *in vivo* in small animal disease models, e.g., mouse models, since the depth sensitivity of the BLI is only 1-2 cm (Alhasan, M. K., et al. 2012). Fluorescent imaging (FI) is designed to look at the cell surface distribution of fluorescent signals. It is used in both live and fixed cells, and does not require any substrate. In this method fluorochromes are conjugated to peptides or antibodies, and binding of the conjugates to targets on the surface of cells provides information about target expression. Fluorescent imaging provides contrast sensitivity that is approximately comparable to radioactivity-based imaging. However, tissue penetration of FI signals is less than that of PET or SPECT. New methods of FI for

improving relative sensitivity and resolution, preparation of newer imaging probes, and better signal-to-noise amplifiers are currently being developed (Kodibagkar, V. D. et al., 2012; Koolen, B. B. et al., 2012; Misri, R. et al., 2012).

Ultrasound imaging (UI) also a molecular imaging technique, and is currently being development as a nano-drug delivery system. UI is performed by utilizing microbubbles, liposomes, or perfluorocarbon emulsions as scaffolds functionalized with different targeting agents. UI offers high spatial resolution (<1mm) and can provide detailed anatomical information for coregistration with other molecular imaging methods. Because of the relatively large size of imaging reagents or particles (>250nm) this technique has limited capacity for tissue penetration, and is used specifically for vascular imaging.

Molecular imaging of estrogen receptor expressing cells and tissues can provide the ability to detect tumors that overexpress the estrogen receptor (ER), having significant positive impact on diagnosis and therapy of ER-dependent cancer, such as breast cancer, in which over-expression of ER plays a major role (Dunn L. et al., 2009; Varghese C. et al., 2007; Schiff, R. et al., 2005). Among patients presenting with breast cancer, approximately 65-75% have primary tumors with elevated ER levels. Currently, receptor status of the tumor is determined via fine needle biopsy and, while minimally invasive, the technique requires removing tumor cells to be analyzed from the body (Higa, G. M. et al., 2009; Allred, D. C. et al., 2009). Because treatment protocols are dependent upon accurate characterization of the tissue sample, alternate methods that non-invasively characterize the entire body for potential distal metastases, and are quantitative, are of interest. MI agents that bind to specific biomarkers can provide important physiological and biochemical information regarding the disease leading to improved treatment plans and improved patient outcomes.

Several non-invasive modalities are currently used to image normal and cancerous breast tissue (Keune, J. D. et al., 2010; DeMartini, W. et al., 2008; van de Ven, S. M. W. Y. et al., 2008; Yu, E. Y. et al., 2007; Czernin, J. et al., 2010; Pantaleo, M. A. et al., 2008). Although many biomarkers associated with tumor sensitivity and invasiveness are being examined using experimental radiotracers, the expression of ER remains one of the most important diagnostic indicators for breast cancer (de Vries, E. F. J. et al., 2007; Schroeder, C. P. et al., 2007; Dittmann, H. et al., 2009). A radiotracer that binds with high affinity and selectivity to the estrogen receptor-alpha (ER α) would yield diagnostic data not provided by any other molecular agent or imaging modality as it would directly indicate the receptor status of suspected lesions. Whole body imaging would detect other ER-expressing tissues which may be secondary lesions. Imaging following chemotherapy (and/or surgery) would

also demonstrate the presence or absence of recurrent ER-responsive disease, as well as the effectiveness of the intervention. Such information is crucial in providing individualized treatment.

Estradiol derivatives used for molecular imaging in clinical studies include
5 radioiodinated molecules, e.g., isomers of 11 β -methoxy-(17 α , 20E/Z)-
[¹²³I]iodovinylestradiol. Among the isomers, the 20Z isomer yields better images of ER(+) human breast tumors than the 20E isomer. Although the diagnostic radioiodinated estradiols were selectively and sensitively detected in both primary and metastatic ER(+) breast cancer,
10 extensive correlation between imaging and clinical outcome has not yet become available (Pysz, M. A. et al., 2010; Yager, J. D. et al., 2006; Benard, F. et al., 2005; Cummins, C. H., 1993).

Steroidal estrogen derivatives with the prosthetic ^{99m}Tc chelates at different positions have been investigated also, although none have yet been clinically approved (Pavlik, E. J. et al., 1990).

15 Estradiol derivatives labeled with ¹⁸F have also been explored, and among the compounds evaluated (FIG. 1), 16 α -[¹⁸F]-estradiol ([¹⁸F]-ES) is currently in clinical use. The 11 β analog, 4-Fluoro-11 β -methoxy-16 α -[¹⁸F]-fluoroestradiol (4FMFES; FIG. 1) tracer also showed favorable biodistribution in small animal studies, and exhibited higher selectivity uptake ratios than that obtained by using [¹⁸F]-ES. The compound 4FMFES is also being
20 evaluated in studies for human biodistribution and dosimetry. Another compound, 7 α -substituted [¹⁸F]-ES, has also been prepared and evaluated as a possible tracer for ER(+) breast tumors. However, the biological data showed no significant improvement on target to non-target uptake ratios compared to other ¹⁸F-ES radiotracers (Cummins, C. H., 1993; Pavlik, E. J. et al., 1990; Ribeiro-Barras, M. J. et al., 1992; Rijks, L. J. M. et al., 1997;
25 Bennink, R. J. et al., 2001).

Although [¹⁸F]-16 α -fluoroestradiol (FES) was developed in the 1980s, its use has been limited to fewer than a thousand patients (Kiesewetter, D. O. et al., 1984; Kiesewetter, D.O., Kilbourn, M. R. et al., 1984; Sundararajan, L. et al., 2007; Kurland, B. F. et al., 2011; Peterson, L. M. et al., 2011; Linden, H. M. et al., 2011; Dehdashti, F. et al., 2009; Gemignani,
30 M.L., et al., 2013). FES is prepared from a simple precursor (2 mg/ run) in an overall radiochemical yield of only approximately 30%. A careful chromatographic separation is required to remove several other radioactive by-products and non-radioactive materials. FES is typically obtained with a specific activity in the 200-1000 Ci/mmol range, appropriate for most clinical applications. This radiosynthetic procedure is not trivial, even with the use of

automated methods (Lim, J. L. et al., 1996; Romer, J. et al., 1999; Oh, S. J. et al., 2007; Kumar, P. et al., 2007; Kumar, P. et al., 2012; Knott, K. E. et al., 2011). Biologically, FES is essentially a mimic for endogenous estradiol and undergoes comparable pharmacodynamic (PD) and pharmacokinetic (PK) processes (Mankoff, D. A. et al., 1997; Jonson, S.D. et al., 5 1998; Bonasera, T. A. et al., 1997; Downer, J. B. et al., 2001; Benard, F. et al., 2001).

FES binds to steroid hormone binding globulin (SHBG) and as much as 45% of FES in circulating plasma is found complexed with SHBG. Therefore, although FES binds to ER in target tissues with a relative binding affinity (RBA) of 80%, it may largely not be accessible to target tissue, thereby impairing uptake of FES uptake in tumors. FES is rapidly 10 cleared by the liver where it undergoes rapid and extensive metabolism such that less than 20% of radioactivity in plasma of patients is parent FES. The metabolites generated contribute to non-specific tissue distribution and accumulation in the liver and gut, and results in high background signal that compromises detection of lesions in adjacent tissues. Nevertheless, eleven completed or ongoing clinical studies are attempting to demonstrate the 15 extent to which FES can contribute to the management of patients with breast cancer (see clinicaltrials.gov). A recent report shows that imaging with FES of patients with primary breast cancer in a preoperative setting can provide valuable information, but significant limitations still remain (Gemignani, M.L. et al., 2013). For example, the Gemignani, M.L. et al., report showed that although the standard uptake value of ^{18}F -FES PET correlated with 20 ER immunohistochemistry expression, it did not correlate with gene expression patients with early breast cancer.

Imaging of estrogen receptor (ER) in vivo using ER binding radiopharmaceuticals can be used for determining the ER expression status during tumor staging. Fluorine-18, Iodine-123 and other cyclotron- produced radionuclides have been used to label ER binding ligands 25 to develop such in vivo radioimaging probes (Pomper, M. G. et L., 1990; Kiesewetter, D. O. et al., 1984; VanBrocklin, H. F. et al., 1993; VanBrocklin, H. F. et al., 1994; Landvatter, S. W. et al., 1983; French, A. N. et al., 1993; French, A. N., Wilson, S. R. et al., 1993; LaFrate, A. L. et al., 2009; Bergmann, K. E. et al., 1994; Hostetler, E. D. et al., 1999).

Some of these radioligands, for example 16α - ^{18}F - 17β -estradiol (^{18}F -FES), have been 30 evaluated clinically for the imaging of hormone dependent breast tumor and predicting the responsiveness of the tumor to antiestrogen drugs (Seimbille, Y. et al., 2002; Mortimer, J. E. et al., 1996). So far, none of the established ligands has been approved for routine clinical use as a breast cancer diagnostic reagent. Estrogen imaging agents with the radionuclide $^{99\text{m}}\text{Tc}$ for SPECT have also been reported (Bigott, H. M. et al., 2005; Luyt, L. G. et al., 2003;

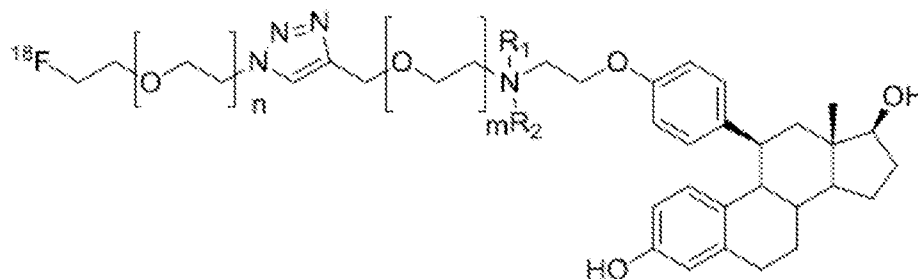
Skaddan, M. B.; Wust, F. R.; Jonson, S. et al., 2000). However, most of the reported compounds have displayed suboptimal target tissue selectivity, possibly due to their lipophilicity, or rapid metabolism. In addition, these radioligands were synthesized by inefficient linear approaches, leading to products that were difficult to purify, leading to suboptimal yield and purity (Huang, L. et al., 2010; Nayak, T. K. et al., 2008).

Because many of the radioligands that have been developed still exhibit low receptor (estrogen receptors ER α , ER β) binding affinity and non-ER regulated uptake, there is a need to develop ER-targeted radioligands having high specific activity (radioactivity per unit mass of the radioligand >1 Ci/mmol, high specific receptor binding affinity, low non-specific binding), and appropriate metabolic and clearance characteristics, both to characterize the tumors and to predict or determine their response to anti-hormonal therapy.

SUMMARY OF THE INVENTION

Provided herein are compounds useful for molecular imaging of cells expressing estrogen receptor, intermediates for making the compounds, methods of making the compounds and intermediates, and methods of diagnosing, prognosing, or treating a disease in a subject by administering the compounds.

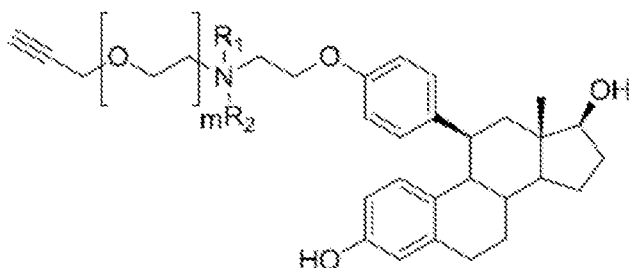
One aspect of the invention is a compound according to formula (I),



(I)

where n is a number in the range of 1-10; m is a number in the range of 4-10; R¹ is H or a C₁₋₃ alkyl group; and R² is either absent, or selected from a group consisting of H and a C₁₋₃ alkyl group. As used herein, a “number” in a chemical formula refers to a whole number, unless otherwise specified.

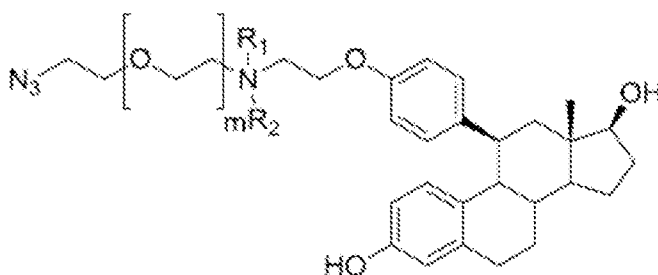
Another aspect of the invention is a compound according to formula (II),



(II)

- 5 where m is a number in the range of 4-10; R¹ is H or a C₁₋₃ alkyl group; and R² is either absent, or selected from a group consisting of H and a C₁₋₃ alkyl group.

Another aspect of the invention is a compound according to formula (III),

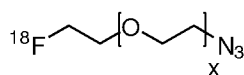


(III)

- 10 where m is a number in the range of 4-10; R¹ is H or a C₁₋₃ alkyl group; and R² is either absent, or selected from a group consisting of H and a C₁₋₃ alkyl group.

Another embodiment of the invention is a process for the preparation of a compound according to formula (I), including the step of reacting a compound according to formula (II)

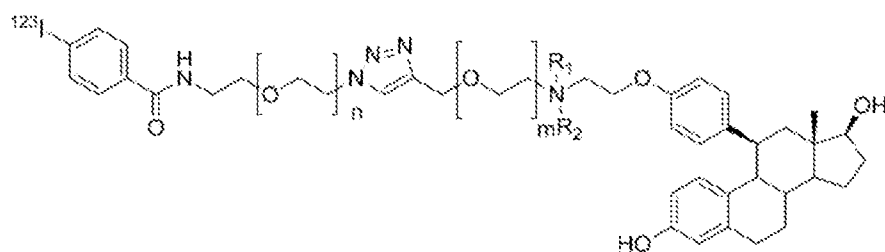
- 15 with a compound according to formula (IV) using a Huisgen [3 + 2] cycloaddition reaction,



(IV)

where x is a number in the range of 1-10.

- 20 Yet another embodiment of the invention is a compound according to formula (V)



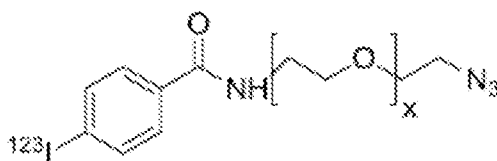
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(V)

where n is a number in the range of 1-10; m is any number in the range of 4-10; R^1 is H or a C_{1-3} alkyl group; and R^2 is either absent, or selected from a group consisting of H and a C_{1-3} alkyl group.

10

A related embodiment of the invention is a process for the preparation of a compound according to formula (V) including the step of reacting a compound according to formula (II) with a compound according to formula (VI) using a Huisgen [3 + 2] cycloaddition reaction,



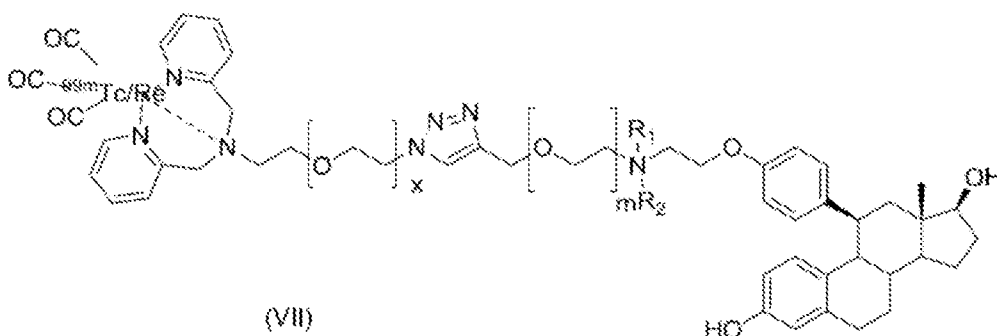
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(VI)

where x is a number in the range of 1-10.

Another aspect the invention is a compound according to formula (VII),

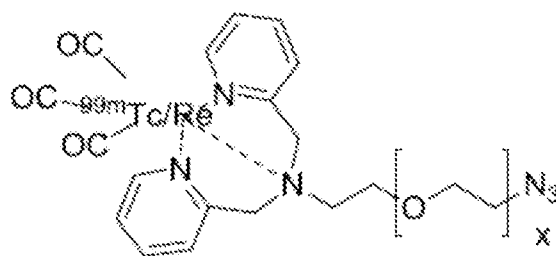
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(VII)

where x is a number in the range of 1-10; m is a number in the range of 4-10; R¹ is H or a C₁₋₃ alkyl group; and R² is either absent, or selected from a group consisting of H and a C₁₋₃ alkyl group.

A related embodiment of the invention is a process for the preparation of a compound according to formula (VII) including the step of reacting a compound according to formula (II) with a compound according to formula (VIII) using a Huisgen [3 + 2] cycloaddition reaction,



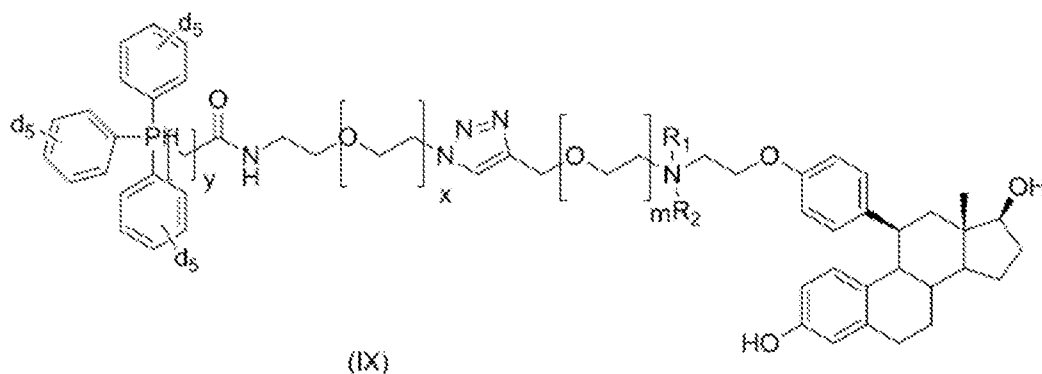
(VIII)

where x is a number in the range of 1-10.

Another aspect of the invention is a pharmaceutical composition containing any of the compounds described above and a pharmaceutically effective carrier or diluent.

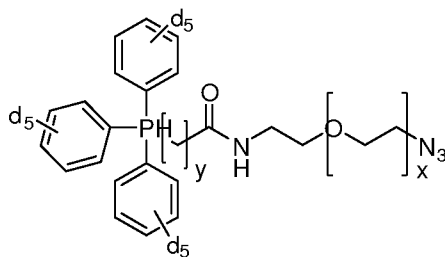
Another aspect of the invention is a method of diagnosing a disease in a subject including detecting a cell expressing estrogen receptors by contacting any of the compounds having the formula (I), (V), or (VII) to the cell, and imaging the cell with positron emission tomography (PET) or single photon emission computed tomography (SPECT). In a related embodiment the cell contains or expresses an estrogen receptor. For example, the disease can be breast cancer or endometrial cancer. In a further related embodiment the subject has received anti-cancer treatment, and the method is used for determining the effectiveness of the treatment or the progress of the disease.

Another aspect of the invention is a compound according to formula (IX),



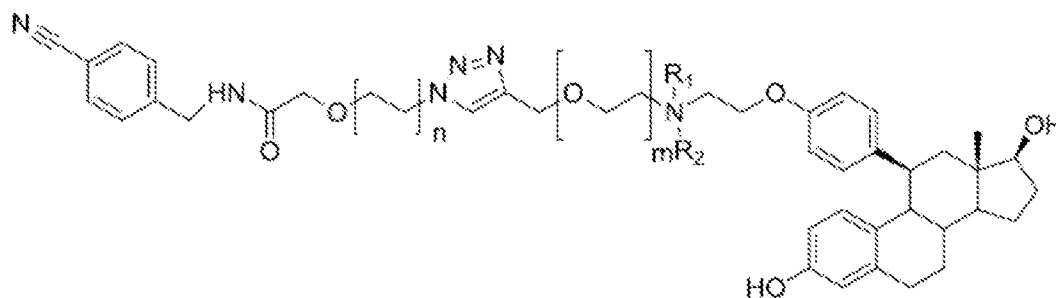
where m is a number in the range of 4-10; each of x and y is independently a number in the range of 1-10; R^1 is H or a C_{1-3} alkyl group; d is deuterium; and R^2 is either absent, or
 5 selected from a group consisting of H and a C_{1-3} alkyl group.

A related aspect of the invention is a process for the preparation of a compound according to formula (IX) including the step of reacting a compound according to formula (II) with a compound according to formula (X),



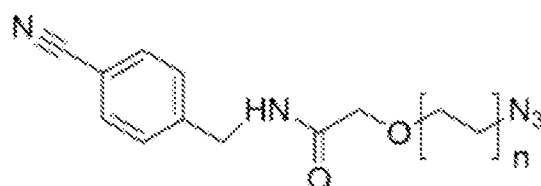
using a Huisgen [3 + 2] cycloaddition reaction, where each of x and y is independently a number in the range 1-10, and d is deuterium.

According to another aspect the invention is a compound according to formula (XI),



where n is a number in the range of 1-10; m is a number in the range of 4-10; R¹ is H or a C₁₋₃ alkyl group; and R² is either absent or selected from the group consisting of H and a C₁₋₃ alkyl group.

A related aspect of the invention is a process for the preparation of a compound according to formula (XI) including the step of reacting a compound according to formula (III) with a compound according to formula (XII), n being a number in the range of 1-10,



(XII)

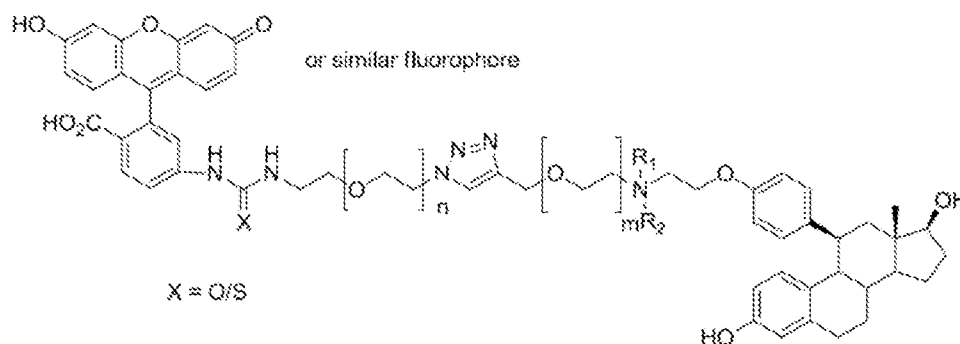
10 using a Huisgen [3 + 2] cycloaddition reaction.

A related aspect of the invention is a pharmaceutical composition including any of the compounds (IX) or (XI) and a pharmaceutically effective carrier or diluent.

Another related aspect of the invention is a method of diagnosing a disease in a subject including detecting a cell by contacting any of the compounds (IX) or (XI), and
15 imaging the cell in vitro with Raman spectroscopy. For example, the disease is breast cancer or endometrial cancer. In related embodiments the cell comprises an estrogen receptor. For example, the subject has received anti-cancer treatment, and the method is used for determining effectiveness of the treatment.

A further embodiment of the invention is a compound according to the formula

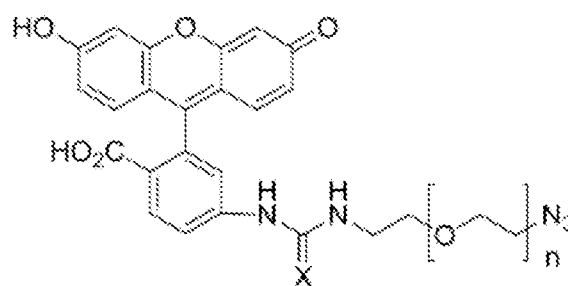
20 (XIV),



(XIV)

where n is a number in the range of 1-10; m is a number in the range of 4-10; X is O or S R¹ is H or a C₁₋₃ alkyl group; and R² is either absent or selected from a group consisting of H and a C₁₋₃ alkyl group.

- 5 An aspect of the invention is a process for the preparation of a compound according to formula (XIV) including the step of reacting a compound according to formula (II) with a compound according to formula (XV),

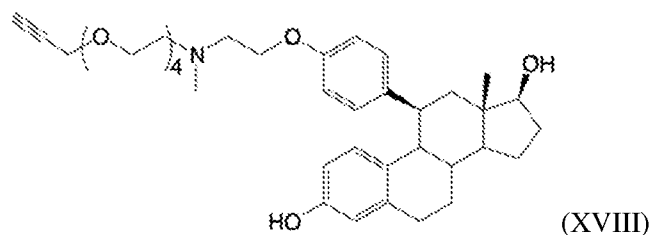


10 (XV)

using the Huisgen [3 + 2] cycloaddition reaction, wherein R¹ is either absent, or selected from a group consisting of H and a C₁₋₃ alkyl group, X is O or S, and n is a number in the range of 1-10.

- 15 A related aspect of the invention is a method of diagnosing a disease in a subject including detecting a cell in vitro by contacting the compound (XIV) and imaging the cell with fluorescence spectroscopy. For example, the cell contains or expresses an estrogen receptor. For example, the disease can be breast cancer or endometrial cancer.

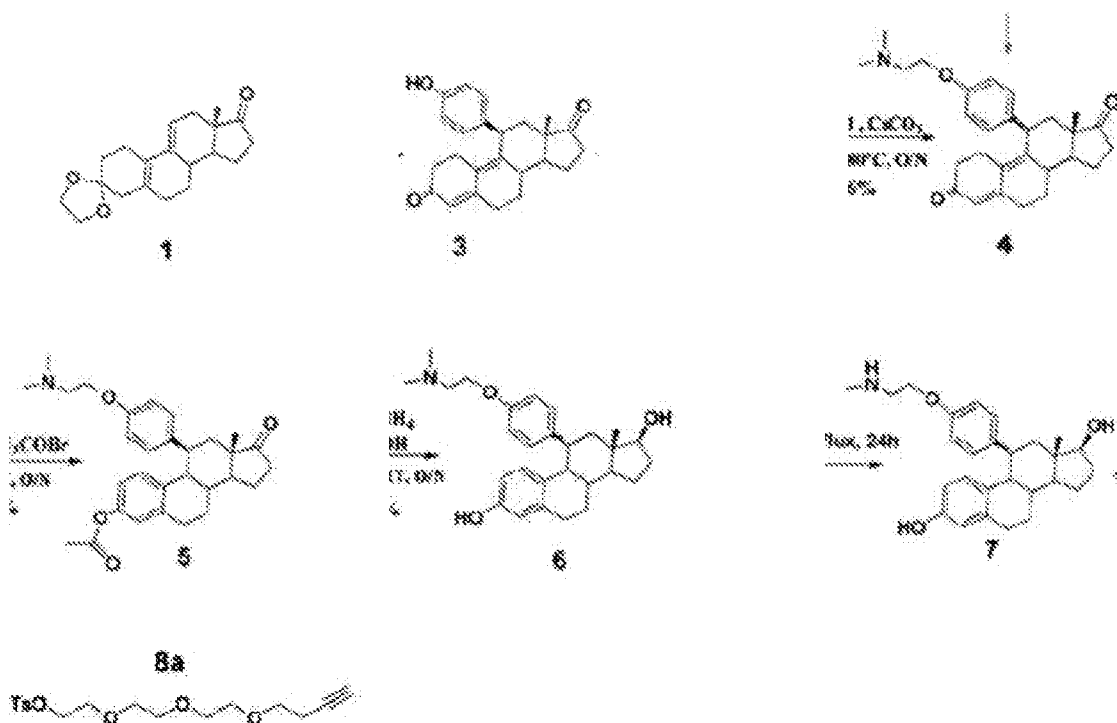
- Another aspect of the invention is a process for the preparation of a compound
20 according to formula (XVIII)



(XVIII)

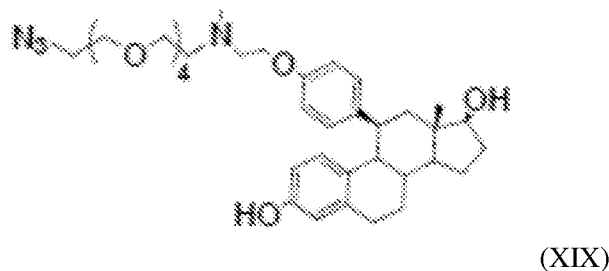
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including the steps of transforming *estra-5(10),9(11)-diene-3,17-dione 3-ethylenedioxy ketal (1)* into *11 β -(4-Hydroxy-phenyl)-estra-4,9-diene-3,17-dione (3)* by reacting **(1)** initially with a peroxide reagent to give an epoxide, and treating the product obtained with the Grignard reagent derived from trimethylsilyloxyphenyl bromide to obtain compound **(3)**, alkylating **(3)** with dimethylaminoethyl chloride to obtain compound **(4)**, aromatizing **(4)** with acetyl bromide and acetic anhydride to obtain compound **(5)**, reducing and saponifying **(5)** to obtain compound **(6)**, demethylating **(6)** with alpha chloroethyl chloroformate to obtain compound **(7)**, and reacting **(7)** with reagent **(8a)**,

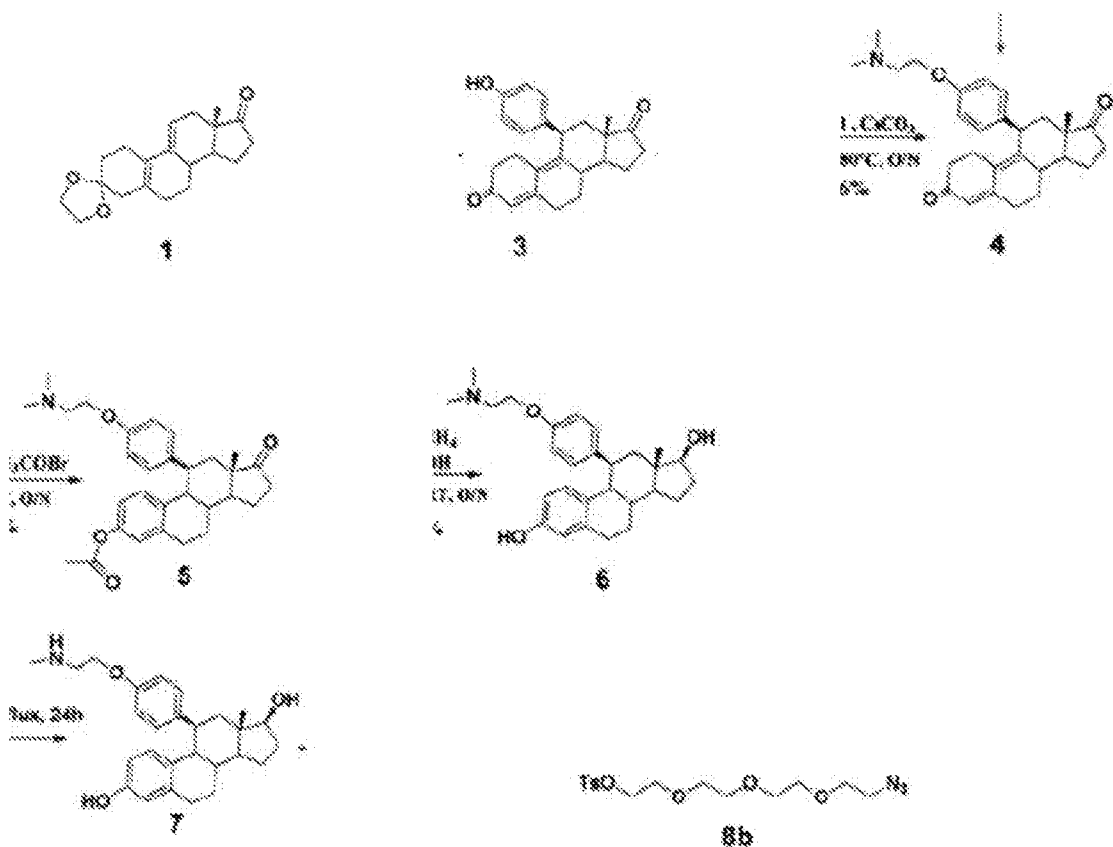


thereby obtaining the compound according to formula (XVIII). See Fig. 2.

Yet another aspect of the invention is a process for the preparation of a compound according to formula (XIX),

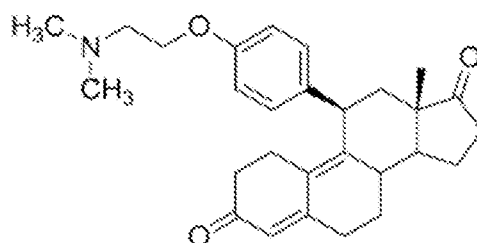


including the steps of transforming estradiol-5(10),9(11)-diene-3,17-dione 3-ethylenedioxy ketal (1) into 11 β -(4-Hydroxy-phenyl)-estra-4,9-diene-3,17-dione (3), by reacting (1) initially with a peroxide reagent to give an epoxide, and treating the product obtained with the Grignard reagent derived from trimethylsilyloxyphenyl bromide to obtain compound (3), alkylating (3) with dimethylaminoethyl chloride to obtain compound (4), aromatizing (4) with acetyl bromide and acetic anhydride to obtain compound (5), reducing and saponifying (5) to obtain compound (6), demethylating (6) with alpha chloroethyl chloroformate to obtain compound (7), and reacting (7) with reagent (8b)



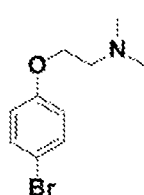
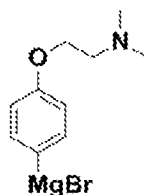
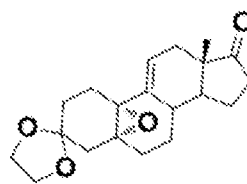
thereby obtaining the compound according to formula (XVIII). See Fig. 2.

Another aspect of the invention is a process for the preparation of a compound according to formula (XX)



(XX)

including the steps of: preparing 2-(4-bromophenoxy)-N,N-dimethylethanamine (**1**) by reacting 4-bromophenol with 2-dimethylaminoethyl chloride, converting (**1**) to (4-(2-(dimethylamino)ethoxy)phenyl)magnesium bromide (**2**) by treating with reagents including Mg, and iodine, and reacting (**2**) with 3,3-Ethylenedioxy-5(10)- α -epoxy-estr-9-ene-17-one (**3**) in the presence of CuI and acetic acid

**1****2****3**

thereby obtaining the compound according to formula (XX). See Fig. 2.

Yet another aspect of the invention is a kit for preparing a compound according to any of Formulas I, V, VI, VII, or VIII. The kit can include, for example a labeling moiety and instructions for reacting a compound according to any of Formulas II, III, or XI and the labeling moiety to provide a compound according to according to any of Formulas I, V, VI, VII, VIII, or IX. The labeling moiety can be, for example, a compound according to any of Formulas IV, VI, or VIII.

Still another aspect of the invention is the use of a compound according to any of Formulas I, V, VI, VII, VIII, or IX as an imaging agent for estrogen receptors in a cell, tissue, or a mammalian subject.

Another aspect of the invention is the use of a compound according to any of Formulas II, III, or XI to prepare an imaging agent for estrogen receptors.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the structures of a set of ^{18}F -radiolabeled estradiols previously described
5 (Bennink, R. J. et al., 2001; Pomper, M. G. et al., 1990; Kiesewetter, D. O. et al., 1984; VanBrocklin, H. F. et al., 1993; VanBrocklin, H. F., 1994; Seimbille, Y. et al., 2002).

FIG. 2 is a diagram of a synthetic scheme for alkynyl and azido 11β -antiestrogen precursors.

FIG. 3 is a comparison of NMR spectra and thin layer chromatograms associated with
10 the syntheses of the intermediate **4** (FIG. 2) using a first approach (lower panel) and using a second (alternate) approach (upper panel).

FIG. 4 is a diagram of a scheme for convergent synthesis of ^{18}F -radiolabeling of 11β -substituted antiestrogen.

FIG. 5 is a diagram of a scheme for synthesis of ^{18}F radiolabeled 11β substituted
15 estradiol showing variation in “click” chemistry conditions.

FIG. 6 is a diagram showing eight different “click” reactions (right) performed, and radiochromatographic purifications (left) for the synthesis of ^{18}F radiolabeled 11β substituted estradiol.

FIG. 7A shows preparative radio HPLC (high performance liquid chromatography)
20 purification of the ^{18}F radiolabeled 11β substituted estradiol product, under different chromatographic conditions. Peaks corresponding to the product and reactants are indicated by arrows. FIG. 7B shows radio HPLC traces confirming the assignment of peaks in the purification shown in FIG. 7A.

FIG. 8 is a diagram showing attachment of different imaging modalities to the alkynyl
25 11β -antiestrogen precursor of FIG. 2 using “click” chemistry.

FIG. 9 is a diagram showing synthesis of a lipophilic ^{18}F radiolabeled 11β antiestrogen in addition to an amphiphilic ^{18}F radiolabeled 11β antiestrogen.

FIG. 10 is a proton NMR spectrum of demethylated material.

FIG. 11 is a proton NMR spectrum of material with propargyl tetraethylene glycol
30 linker.

FIG. 12 is a carbon-13 NMR spectrum of material with propargyl tetraethylene glycol linker.

FIG. 13 shows an LC-MS scan showing retention times of starting material, tetraethylene glycolated product, and N,N-dimethyl standard.

FIG. 14 is a high resolution mass spectrum of propargylated tetraethylene glycolated material.

FIG. 15 shows high resolution mass spectra of N-demethylated material.

FIG. 16 shows an HPLC trace indicating the purity of propargyl tetraethylene glycolated material.

FIG. 17 shows an HPLC trace indicating the purity of propargyl tetraethylene glycolated material using a different solvent system than in FIG. 16 or FIG. 18.

FIG. 18 shows an HPLC trace indicating the purity of propargyl tetraethylene glycolated material using a different solvent system than in FIG. 16 or FIG. 17.

FIG. 19 is a proton NMR spectrum of the depicted N-fluoropropyl derivative.

FIG. 20 shows an expanded proton NMR spectral region for the N-fluoropropyl derivative.

FIG. 21 is an expanded proton NMR spectral region for the N-fluoropropyl derivative.

FIG. 22 is a carbon-13 NMR spectrum for the N-fluoropropyl derivative.

FIG. 23 shows LC-MS traces for the N-fluoropropyl derivative.

FIG. 24 shows a high resolution mass spectrum for the N-fluoropropyl derivative.

FIG. 25 is a proton NMR spectrum of the depicted N-demethylated antiestrogen.

FIG. 26 is a diagram showing the variation in the conditions for synthesizing ^{18}F -estradiol substituted at the 11β -position.

FIGS. 27A and 27B show mass spectra of the compounds depicted above the respective spectra. FIG. 27C is an HPLC analysis of the progress of reaction, and shows peaks corresponding to the reactant ^{18}F -PEG₃-N₃, and the products ^{18}F -11 β -FES and ^{19}F -11 β -FES. FIG. 27D shows a preparative HPLC purification of the product obtained as a result of the “click” reaction between the reactants ^{18}F -PEG₃-N₃ and HC $\equiv$ C-11 β -ES, the alkynyl 11 β -antiestrogen precursor. Upper trace shows UV absorption and lower trace shows radioactivity.

FIG. 28 shows optimization of conditions of the “click” reaction for the synthesis of ^{18}F radiolabeled 11 β antiestrogen using the reactants ^{18}F -PEG₃-N₃ and the alkynyl and 11 β -antiestrogen precursor of FIG. 2.

FIG. 29A is a graph of the pharmacokinetics of ^{18}F radiolabeled 11 β antiestrogen (^{18}F -11 β -FES) showing half-life measurements of the compound in the blood. FIG. 29B is a table of different formulations used in the pharmacokinetics measurements shown in FIG. 29A. FIG. 29C is a graph of the biodistribution of ^{18}F -11 β -FES. FIG. 29D is a comparison of the

biodistribution of ^{18}F -16 α -FES (left bar of each pair, Kiesewetter (1984)) and ^{18}F -11 β -FES of the present invention (right bar of each pair).

FIG. 30A is a graph of the biodistribution of ^{18}F -11 β -FES in a breast cancer model of a xenograft of MCF7 cells in nu/nu mice. FIG. 30B is a graph of comparison of the biodistribution of ^{18}F -11 β -FES in wild type mice (left bar of each pair) and mice having the MCF7 xenograft (right bar of each pair). FIG. 30C shows a summary of the data shown in FIG. 30B.

FIG. 31 is an image of a 2 hour dynamic PET scan of a C57Bl/6 mouse administered with ^{18}F -11 β -FES. The image shows that within 3 minutes post injection the majority of activity is in the liver (2nd image from left), from which it quickly moves to the intestines (3rd image from left). At approximately 15 minutes post injection (far right image), a significant amount of activity has accumulated in the bladder.

DETAILED DESCRIPTION OF THE INVENTION

Described herein are estrogen receptor (ER) targeted, 11 β -aryl estradiol-based molecular imaging (MI) agents having high specific activity, high specific receptor binding affinity, and metabolic and clearance characteristics that render them useful as imaging agents for mammalian subject, including human subjects.

Previous structure activity relationship (SAR) studies of steroidal estrogens have indicated that small substituents at the 11 β -position conferred agonist activity, usually with an increase of binding affinity (Dao et al., 2012, and Hanson, R. N.; Hua, E. et al., 2012, and references therein). Alkyl or heteroalkyl groups beyond 3-4 atoms in length have been shown to impart ER antagonist properties, often without a significant loss of binding affinity (Hanson, R. N.; Hua, E. et al., 2012, and references therein). Analysis of the properties of anti-estrogen-doxorubicin bioconjugates has shown that the 11 β -aryl estradiol-based antiestrogens possessed several key features as potential candidates for MI for (ER+)-breast cancer (Dao et al., 2012). The 11 β -aryl estradiol-based antiestrogens (AEs) retained high ER affinity, expressed ER antagonist properties, showed selectivity for ER-expressing cancer cells, and a linker between the steroid and doxorubicin improved pharmaceutical properties and allowed convergent attachment of different terminal groups.

The 11 β -aryl group of the EAs of the present invention occupies a secondary pocket of the ER that extends to the surface. In the antagonist conformation, the 4'-position of the aryl group accesses the solvent-exposed space, thereby permitting further conjugation with

the imaging moieties of the present invention without interference with receptor binding specificity.

The present invention employs a convergent strategy that allows the imaging tag component (imaging moiety), and the 11 β -aryl estradiol-based antiestrogen component
5 having a complementary linker at the 4'-position of the aryl group, to be prepared separately and then linked with an orthogonal ligation. Further, the preparation of labeled 11- β substituted estradiols described herein is modular. The imaging moiety and the 11- β substituted estradiol precursor are independently made with high yields and purity.

As a first step toward synthesis of 11 β substituted estradiol-imaging conjugates
10 having a radiohalogenated moiety, a high affinity alkynyl 11 β -phenyl substituted estradiol component (**9a**) was prepared. Separately, an exemplary radio imaging label component, fluorinated triethylene azide (**8b**), was prepared. The compound, alkynyl 11 β -phenyl substituted estradiol (**9a**), was prepared by two methods, both of which are shown in FIG. 2. The precursor was synthesized from estra-5(10),9(11)-diene-3,17-dione 3-ethylenedioxy
15 ketal (**1**) and transformed into the key intermediate 11 β -(4-Hydroxy-phenyl)-estra-4,9-diene-3,17-dione (**3**). An alkylation step was used to prepare the precursor 11 β -[4-(2-dimethylamino-ethoxy)-phenyl]-estra-4, 9-diene-3, 17-dione (**4**).

Initially the dimethylaminoethoxy group was introduced after the 11 β phenyl group to
synthesize the intermediate (**4**). In an alternate approach the side chain was introduced prior
20 to the Grignard reaction. In this route, 4-bromophenol was alkylated with dimethyl chloroethylamine to give 4-bromo-(2-dimethylaminoethoxy) benzene. The Grignard reaction was carried out as the next step to produce the intermediate (**4**). The alternate approach offered a number of advantages: the synthesis of the steroidal portion was shortened by one
step; the yield of the product was higher, and the separation/isolation process was easier
25 (FIG. 3). Further, the 4-bromo phenol starting material is significantly less expensive than the 1-bromo-(4-trimethylsilyloxy) benzene, and reaction using the alternate approach was more reproducible than reaction according the initial scheme. Starting from the intermediate
(**4**), the alkynyl antiestrogen was obtained as illustrated in the synthetic scheme shown in
FIG.2. The molecular imaging probe, such as an azido fluorinated ligand, can be attached
30 using the "click" chemistry of azide-alkyne Huisgen cycloaddition employing copper or ruthenium as a catalyst (FIG. 4, and Hanson, R. N. et al., 2012; Dao, K.-L. et al., 2012; Hanson, R. N.; Hua, E. et al., 2012; Adam, H. J. et al., 2012; Dao, K.-L. et al., 2013; Haddad, T. et al., 2012; Hanson, R. N.; Hua, E.; Adam, H. J. et al., 2012; Hanson, R. N.; Kirss, R. et al., 2012; Hanson, R. N.; McCaskill, E. et al., 2012; Olmsted, S. L. et al., 2012).

The preparation of the fluorinated component proceeded in good overall yield. The synthesis began with the ω -chloro triethylene glycol derivative which underwent displacement with sodium azide to give **(10)** (FIG. 4). Tosylation gave intermediate **(11)**, which was treated with tetra-*n*-butylammonium fluoride (TBAF) to give the fluorinated precursor **(12)**. Conversion to the final product **15** was achieved in good yields using classical “click” conditions. All of the intermediates and final products were characterized by IR, NMR, and LC-MS. The convergent modular approach proved to be a successful strategy for assembling the target anti-estrogen (AE).

To synthesize 11 β -AE radiolabeled with ^{18}F as shown in the structure in FIG. 5, a variety of different “click” conditions were investigated to determine the best method to convert the alkynated AE precursor into the radiolabeled “click” products. Because of the short half-life of ^{18}F radionuclide, it is necessary that the conversion method be fast and proceed in high yield. The Table in FIG. 6 summarizes the “click” reactions that were carried out with a pre-automated synthesizer (Center for Systems Biology, Mass General Hospital, Boston, MA). As the results in the radioHPLC trace (FIG. 5) indicate, the in situ “click” reaction in conditions 4, 7, and 8 gave the best radiochemical yields for the conversion of ^{18}F -PEG- N_3 to the ^{18}F -AE radioligand. However, method 4 involved an additional ligand, bathophenanthroline disulfonic acid disodium salt (BPDS) in a mixture of DMSO/ H_2O (50:50), potentially resulting in more impurities requiring HPLC purification. Although, the radiochemical yield for method 7 was lower, the reaction conditions are much simpler, and only one catalyst copper (I) tetrakis(acetonitrile)copper(I) hexafluorophosphate was used. In reaction 8, 40 μL of 80mM BPDS was added to the scale up reaction to ensure that adequate amount of Cu^{+1} was formed, and was available for catalyzing throughout the “click” reaction. Indeed, this method gave 95% conversion and produced isolated yields ranging from 9.73mCi to 9.12mCi. All the reactions in FIG. 5 used microwave synthesis conditions (temp 60°C, power 30 watts, and 5 minutes), except method 7 in which a 10 min. irradiation time was used.

Use of MeCN: H_2O (50:50) with 0.1% TFA as the solvent for HPLC purification resulted in elution of the radiolabeled antiestrogen product at 5 minutes, followed closely by ^{18}F -PEG- N_3 at about 6 minutes. Changing the eluting solvent to MeCN: H_2O (37.5:62.5) with 0.1%TFA resulted in improved separation, with the ^{18}F -radiolabeled 11 β -substituted antiestrogen eluting at about 10min, ^{18}F -PEG- N_3 eluting at about 7.5min, and the alkyne precursor eluting at 22minutes (UV HPLC trace).

Results of preparative radioHPLC of the ^{18}F labeling “click” product and radioHPLC analysis of the separated compounds demonstrated that 11 β -AE described herein (**9a**; FIG. 4) can be radiolabeled with ^{18}F radionuclide using click chemistry with the azido-PEG- ^{18}F ligand (**12**). The method of synthesis gave a sustainable high activity conversion, and the HPLC purification scheme indicated that the labeling compound is 100% pure (FIG.7) and is suitable for *in vivo* imaging.

In addition to the ^{18}F oligoethylene glycol azide (**12**) used to prepare the ER-targeted radioimaging probe ^{18}F 11 β -AE (FIG. 5), a series of reagents that can be used for other imaging modalities (FIG. 8), were coupled (“clicked”) to the 11 β -AE intermediate described herein (**9a**) or to other targeting groups. For example, a convergent approach of making a prosthetic metallated $^{99\text{m}}\text{Tc}$ /Re chelate that can be directly clicked on the alkynyl (**9a**) or the azido (**9b**) 11- β substituted estradiol (FIG. 2) is described (FIG. 8). The synthesis of the final click product depends on optimal conditions, especially the Cu(I) catalyst ligands and solubility of the reagents in the reaction solvents. The $\text{Re}(\text{CO})_3$ -11 β AE was synthesized and characterized.

It is important to note that the “click” chemistry depicted in Fig. 8 is entirely reversible. That is, any of the labeling moieties depicted can terminate in either an azido group and be reacted with a non-labeled 11 β -AE precursor terminating in an alkynyl group, or any of the labeling moieties can terminate in an alkynyl group and be reacted with a non-labeled 11 β -AE precursor terminating in an azido group. The invention contemplates each such possible combination, and the corresponding methods of synthesizing an ER imaging agent using each and every such combination.

11 β -aryl estradiol-based antiestrogens labeled with Raman active moieties as MI agents are also described herein. Surface-enhanced Raman scattering (SERS) spectroscopy in combination with optical microscopy yields a novel noninvasive and label-free method to assess and image cellular processes based on their biochemical changes (Yigit, M. V. et al., 2011; Qian, X. et al., 2008). The advantage of using Raman spectroscopy over conventional optical imaging method is that it affords minimal sample preparation, high sensitivity to small intracellular fluctuations, as well as high spatial resolution. Raman spectra from within the cell reflect the biochemical composition found within the laser focal volume of approximately 0.3- 1.3 m^3 in size, which is determined by the diffraction limit of the applied laser light. Using the spectral parameters of a cell’s components, it is possible to image cellular organelles such as the nucleus, chromatin, mitochondria, and lipid bodies at the

resolution of conventional microscopy without the use of external labels or dyes (Yigit, M. V. et al., 2011; Chernenko, T. et al., 2009; Kneipp, J. et al., 2009).

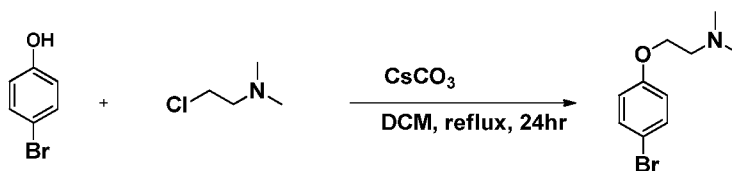
In general, the technique is based on identification of molecular vibrations that are characteristic of distinct functional group in a molecule. The functional groups used as the Raman active moiety for coupling to 11 β -aryl substituted estradiol herein are deteuerated triphenylphosphine (dTPP) and cyano benzyl derivatives (FIG. 8). Vibrational spectra are obtained by illuminating the specimen either with infrared radiation or laser light in the visible range of the spectrum. This method of obtaining vibrational spectra is based upon the inelastic scattering of photons and is known as the Raman effects (Alhasan, M. K. et al., 2012; Pysz, M. A. et al., 2010; Chernenko, T. et al., 2009). The dTPP and azido cyano benzyl labels (FIG. 8) were coupled to the alkynyl and azido 11 β -substituted estradiol described herein (FIG. 2) using click conditions to form *in vitro* Raman imaging probes for ER hormone dependent breast cancer cells. The probes were characterized and results obtained indicate that the Raman labels are nontoxic, stable, do not suffer from photobleaching, and possess a distinctive and specific Raman signature at near 2100cm⁻¹. In the local optical field of the labels SERS also provides sensitive information about the immediate molecular environment of the label, e.g. dTPP in the cell, thereby allowing imaging the native cellular constituents and organelles.

Described herein also is an exemplary traditional fluorescent probe, fluorescein isothiocyanate (FITC), modified with complementary oligo ethoxy glycol (OEG) linker (FIG. 8), which is subsequently attached to 11 β AE (**9a** FIG. 2) to form a fluorescent probe for ER imaging. Such probe is useful in the measurement of cellular binding uptake and/or cellular internalization of the designed ligands by ER.

EXAMPLES

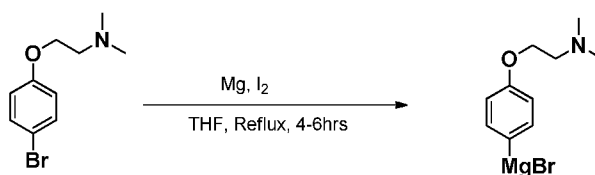
Example 1. Modification of Steroid precursor - (11R,13S)-11-(4-(2-(dimethylamino)ethoxy)phenyl)-13-methyl-7,8,11,12,13,14,15,16-octahydro-1H-cyclopenta[a]phenanthrene-3,17(2H,6H)-dione (intermediate **4**; FIG. 2)

Synthesis of 2-(4-bromophenoxy)-N, N-dimethylethanamine:



To a solution of 4-bromophenol (6.8 g, 40 mmol) in DCM (150 mL) CsCO_3 (19.5 g, 100 mmol) was added, and mixture was allowed to stir at room temperature for about 1 hour. Next, 2-dimethylaminoethyl chloride hydrochloride (5.6 g, 40 mmol) was added to the mixture. The reaction was stirred at reflux for approximately 24 hours. The mixture was then filtered and the filtrate was concentrated to yield a clear oily crude material. The crude material was purified using silica gel chromatography (100% DCM then 1-10% MeOH in DCM) to obtain pure 2-(4-bromophenoxy)-N,N-dimethylethanamine (4 g, 16 mmol, 42% yield). ^1H NMR (500 MHz, CHLOROFORM-d) δ 7.33 (d, $J = 8.79$ Hz, 2H), 6.78 (d, $J = 8.79$ Hz, 2H), 3.99 (t, $J = 5.37$ Hz, 2H), 2.64 - 2.88 (m, 2H), 2.31 (br. s., 6H). ^{13}C NMR (100 MHz, CHLOROFORM-d) δ 158.1, 132.3, 116.6, 66.4, 58.3, 46.1.

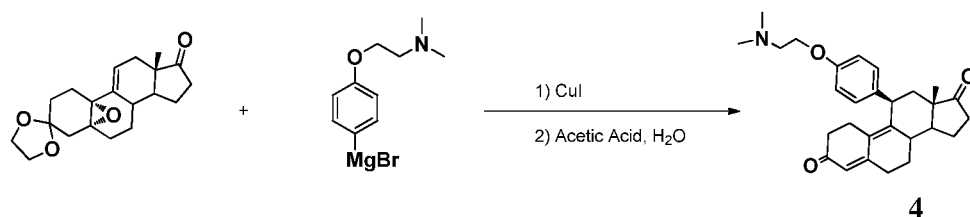
Synthesis of (4-(2-(dimethylamino)ethoxy)phenyl)magnesium bromide:



Glassware for the synthesis was rinsed with DCM and acetone, and dried in an oven overnight at 113 °C. The apparatus for the reaction was assembled and flame dried with a propane torch. Magnesium turnings (3 g, 125 mmol) were placed into a three-necked flask and kept overnight in an oven heated to 113 °C. After it was cooled, the apparatus was placed under argon atmosphere. Distilled anhydrous THF (50 mL) was then added to the three-necked flask followed by the addition of a small granule of iodine which immediately turned the color of the contents of the flask to orange. The mixture was allowed to stir for roughly 12 minutes followed by the addition of 2-(4-bromophenoxy)-N,N-dimethylethanamine (1 ml dissolved in 30 mL of THF) in 5 mL aliquots every 20 minutes until half of the 2-(4-bromophenoxy)-N,N-dimethylethanamine (4 g, 16 mmol) was added to the mixture. The mixture was heated to about 65 °C. Next, the remainder of the 2-(4-bromophenoxy)-N,N-dimethylethanamine was added in 0.5 mL aliquots every 20 minutes. As a result, the contents first became greenish yellow, and then went to lighter yellow and

finally became greenish smoky grey. The reaction mixture was heated for another hour and then cooled to room temperature.

Synthesis of (11R,13S)-11-(4-(2-(dimethylamino)ethoxy)phenyl)-13-methyl-
 5 7,8,11,12,13,14,15,16-octahydro-1H-cyclopenta[a]phenanthrene-3,17(2H,6H)-dione:



3,3-Ethylenedioxy-5(10)- α -epoxy-estr-9-ene-17-one (2.014 g, 6.1 mmol) was dissolved in anhydrous THF (15 mL) under argon. Copper (I) iodide (0.160 g, 0.840 mmol) was added to the solution at -10 °C and stirred for 15 min. Freshly prepared Grignard
 10 reagent, (4-(2-(dimethylamino)ethoxy)phenyl)magnesium bromide, was added dropwise in 5.0 mL aliquots. The reaction was gradually warmed to ambient temperature and stirring was continued for 16 hours. The reaction was quenched by the addition of ammonium chloride (0.8 g, 15 mmol) in 35 mL of water and 35 mL of EtOAc at 10 °C. The organic layer was washed with water (2x 35 mL). The organic solvent was removed under reduced pressure and
 15 the resulting residue was dissolved in a mixture of acetic acid (14 mL) and water (6 mL). The resultant mixture was warmed at 50-60 °C for 1.5 hours, after which it was diluted with ethyl acetate (20 mL). The solution was neutralized by the addition of saturated aqueous sodium bicarbonate. The organic layer was separated, washed with brine solution, dried over magnesium sulfate and evaporated to dryness to give a crude, yellow oil. Purification using
 20 silica gel column chromatography (70:30 hexane/ethyl acetate) afforded the desired product (2.00 g, 76%) as yellow solid: ^1H NMR (CDCl_3 , 400 MHz): δ 0.53 (3H,s), 4.38 (1H, d, J = 6.9), 5.78 (1H, s), 6.71 (2H, d), 6.97 (2H,d). ^{13}C NMR (CDCl_3 , 100MHz): δ 14.17, 21.76, 25.99, 27.06, 30.68, 35.02, 36.87, 38.14, 38.34, 39.71, 47.53, 50.77, 115.53, 122.82, 128.30, 129.83, 135.52, 145.62, 155.59, 155.99, 197.54, 217.44; m.p.248°C.

25

Example 2. Pharmacokinetics and biodistribution analysis of ^{18}F -11 β -FES

Pharmacokinetics measurements were carried out after administration of ^{18}F -11 β -FES to C57 BL/6 mice (FIG. 29 A and B). A number of co-solvents, dimethyl sulfoxide (DMSO, 5%), 4% 1:1 Solutol: dimethyl acetamide (DMA), and ethanol (8%) were tested. A 36%

reduction in terminal concentration of ^{18}F -11 β -FES was found when ethanol was used as a co-solvent. Biodistribution measurements showed that other than kidney, liver, blood and feces, which are associated with excretion, the highest concentration of ^{18}F -11 β -FES was found in the uterus (an ER expressing organ) (FIG. 29C). A comparison of the
5 biodistribution of ^{18}F -11 β -FES and ^{18}F -16 α -FES (Kiesewetter, J. Nucl.Med., 1984, vol. 25, 1212) is shown in FIG. 29 D.

Example 3. Biodistribution of ^{18}F -11 β -FES in an animal model of breast cancer

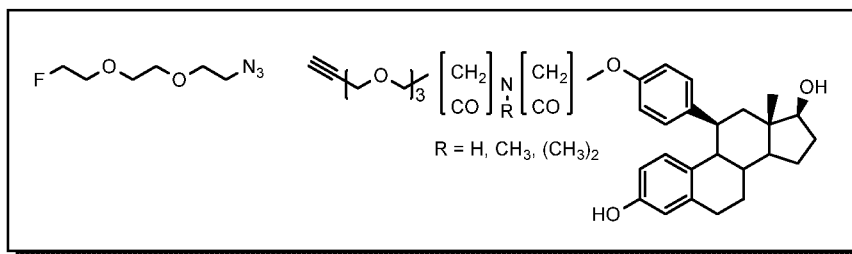
Biodistribution of ^{18}F -11 β -FES was also measured in Nu/Nu mice implanted with a
10 xenograft of MCF7 breast cancer cells. ER expressing tumors and uterus were each observed to accumulate substantial amounts of ^{18}F -11 β -FES, which was higher than in any other tissue/material not associated with excretion, with the exception of bone (FIG. 30 A and C). Similar results were obtained when the biodistribution of ^{18}F -11 β -FES in the tissues of Nu/Nu mice implanted with breast cancer xenograft was compared with that in the tissues of
15 wild type C57BL/6 mice (FIG. 30 B and C).

Example 4. PET scan of ^{18}F -11 β -FES in C57BL/6 mouse

A PET scan was carried out on a C57BL/6 mouse administered with ^{18}F -11 β -FES. A 2 hour dynamic PET scan of the mouse is shown in FIG. 31. Within 3 minutes post injection
20 the majority of activity was found to be localized in the liver (second panel from the left), from which the label was observed to quickly localize to the intestines (third panel from the left). A significant amount of activity was observed to accumulate in the bladder approximately 15 minutes post injection (right panel).

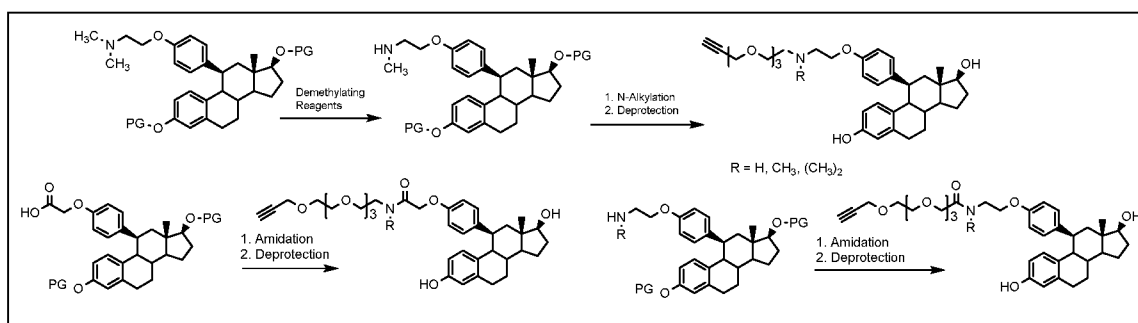
25 Example 5. F-11 β -AE with optimized N-linker

An ER-specific MI compound should have high uptake and retention within target tissues (e.g., uterus and tumor), lower concentration in blood and nontarget tissue, and increased renal clearance. The single factor most likely to influence those parameters is the basicity/hydrophobicity of the nitrogen. Unlabeled N-modified F-11- β intermediates shown
30 below (right) are candidate molecules that have modification of the linker to yield reduced values of cLogP.



Substitution on the N-group includes secondary to quaternary and carbonyl groups on either side (amide). Synthesis of these unlabeled F-11 β -AE derivatives is illustrated below.

5



Subsequent ligation to fluorotriethylene glycol azide provides the final compounds as the unlabeled derivatives. The final compounds are all more hydrophilic than the F-11 β -AE described herein by 0.5-2.5 log units. Analysis of the binding, cell penetration and metabolic properties is carried out to indicate the influence various substituents on the nitrogen has on biological properties. Based upon the results, one or more compounds are selected for evaluation as their [^{18}F]-analogs.

The relative binding affinity (RBA) of the compounds is determined using a competitive binding assay with the full-length recombinant ER- α and ER- β from PanVera (Madison, WI). Because many breast cancer cells express both subtypes, the binding affinity of the alkynyl precursor and final F-11 β -AE is determined for both subtypes. F-11 β -AE described herein and E2 are used as controls.

Efficacy studies for the synthesized F-11 β -AE compounds are performed using a cell-based inhibition ER α trans-activation assay. The assay uses heLA, HepG and MCF-7 cells transfected with plasmids encoding ER (or empty vector) using lipofectamine-2000 (Invitrogen). The cells are treated with increasing concentrations of the compounds or controls, followed by harvesting at 20-24 h. Evaluation for luciferase activity using the Luciferase Assay Kit (Promega) provides data from which the efficacy (IC₅₀) is determined

Compounds having cLogP values in the 1.5-4.5 range are selected for labeling and use as imaging agents.

In vitro metabolic evaluation of representative ^{18}F -11 β -AE is performed as follows. In vitro metabolism measurements utilizes the rat hepatocyte culture, as previously described (Jonson et al., 1999). Rodents do not have steroid hormone binding globulin (SHBG) or Human serum albumin (HAS) and conditions are adjusted to account for this. The compounds of interest are labeled using click chemistry (FIG. 2) and added (100 μL , 1.0 mCi) to the cells (3.9 mL). After a brief swirling to initiate the process, an aliquot (0.1 mL) is removed, transferred to a 0.5 mL centrifuge tube containing ethanol (0.1 mL). The T0 sample is vortexed, sonicated, and centrifuged to separate supernatant from the cell pellet. Additional aliquots are removed and processed in a similar fashion at predetermined time intervals. The supernatant (20 μL) is spotted on TLC plates and developed with an appropriate solvent system. Air dried plates are scanned using an automated radio-TLC scanner and the metabolites/parent compound radiotracer peaks are integrated to determine metabolic disposition. The effect of SHBG on metabolism is determined by repeating the previous experiments but adding human SHBG (80 μg , 1 mg/mL in buffer) to the isolated hepatocytes prior to the addition of the ^{18}F -11 β -AE derivative. The control is the hepatocyte culture with just addition of the buffer solution. The effects of HSA, for which the antiestrogen may have affinity, are examined similarly.

The in vivo bioistribution, pharmacokinetic and metabolic stability measurements of the [^{18}F]-F-11 β -AE synthesized in this Example are performed as follows. The labeled compounds are evaluated initially in normal female mice to determine uterine uptake (%ID/g) and selectivity (T/NT). Imaging also provides data regarding non-target disposition and major clearance (hepatic vs renal) pathways. Each compound is compared to ^{18}F -FES. Uptake of high doses of estradiol demonstrates specific ER binding in target tissues. Based upon the preliminary studies, the compounds with the best uterine uptake values, highest selectivity (uterus to nontarget tissue) and clearance pathway (renal > hepatic) compared to FES is evaluated in implanted breast tumor models. Tissue distribution, selectivity and clearance are determined. Procedures for both normal and tumor bearing animal studies are well established. The results are analyzed to evaluate the effect of RBA, cLogP, and other effects of the substituents on each of the biological parameters associated with effective imaging. The compound(s) with the best properties are evaluated more extensively for their in vivo metabolism.

Metabolism in vivo of the [18F]-F-11 β -AE is evaluated using normal female rats. Groups of rats are sacrificed at 30, 60 and 120 minutes post administration of the radiotracer. Blood is separated to give protein bound and free components and each is evaluated by radio-TLC to determine the number and type of radiolabeled species. Liver and bladder are examined to determine the number and type of labeled compounds present in both at each time point. The values are compared to results found with FES. In rats, FES is rapidly metabolized to more polar species. By 60 minutes after injection, less than 15% of circulating radioactivity is due to [18F]-FES; the remainder is metabolites. It has been reported that injection of blood from rats obtained 2 hours after injection into different rats showed that the metabolites did not accumulate in ER-rich tissues (Mathias, C. J. et al., 1987). The extent to which the 11 β -substituent modifies the metabolism of the radiotracer is determined. The structural modifications is expected to lead to an uptake in the target tissue, comparable to FES (FIG. 4). Reduction of the cLogP values is expected to reduce hepatic uptake and clearance with faster elimination via renal excretion. This produces lower blood levels and less colonic background. Faster blood clearance lowers retention in nontarget tissues, resulting in improved T/NT values. Reduced in vivo metabolism also leads to better pharmacokinetic properties.

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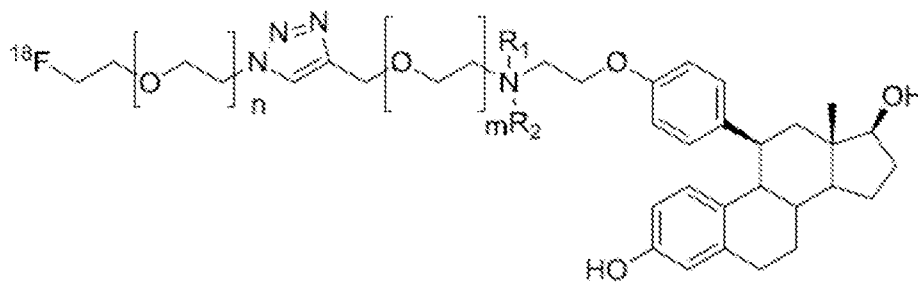
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What is claimed is:

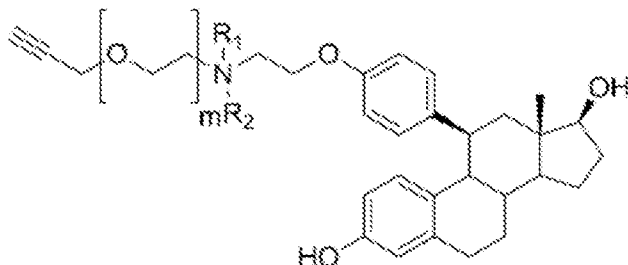
1. A compound according to formula (I)



(I)

wherein n is a number in the range of 1-10; m is a number in the range of 4-10; R^1 is H or a C_{1-3} alkyl group; and R^2 is either absent, or selected from the group consisting of H and a C_{1-3} alkyl group.

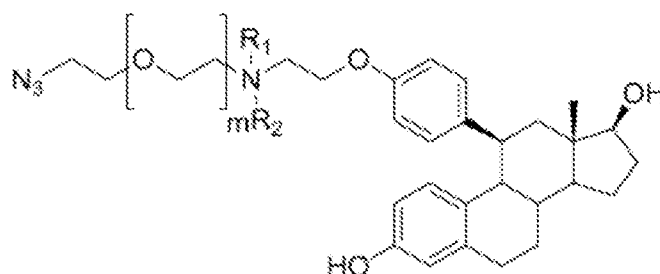
2. A compound according to formula (II)



(II)

wherein m is a number in the range of 4-10; and R^1 is H or a C_{1-3} alkyl group; and R^2 is either absent, or selected from the group consisting of H and a C_{1-3} alkyl group.

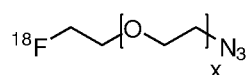
3. A compound according to formula (III)



(III)

wherein m is a number in the range of 4-10; R^1 is H or a C_{1-3} alkyl group; and R^2 is either absent, or selected from the group consisting of H and a C_{1-3} alkyl group.

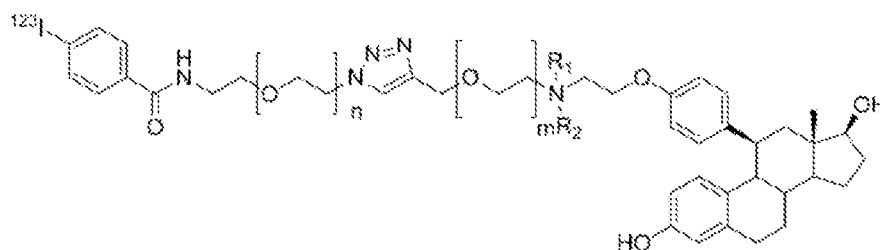
4. A process for the preparation of a compound according to formula (I) of claim 1 comprising the step of reacting a compound according to formula (II) of claim 2 with a compound according to formula (IV) using a Huisgen [3 + 2] cycloaddition reaction,



(IV)

wherein x is a number in the range of 1-10.

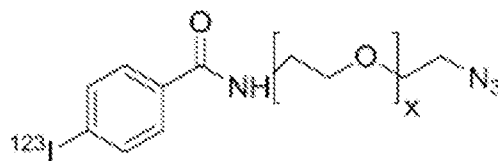
5. A compound according to formula (V)



(V)

wherein n is a number in the range of 1-10; m is any number in the range of 4-10, R^1 is H or a C_{1-3} alkyl group; and R^2 is either absent, or selected from the group consisting of H and a C_{1-3} alkyl group.

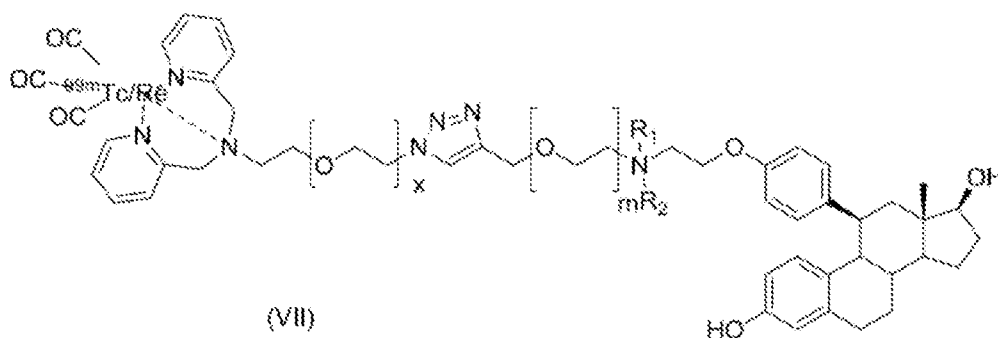
6. A process for the preparation of a compound according to formula (V) of claim 5 comprising the step of reacting a compound according to formula (II) of claim 2 with a compound according to formula (VI) using a Huisgen [3 + 2] cycloaddition reaction,



(VI)

wherein x is a number in the range of 1-10.

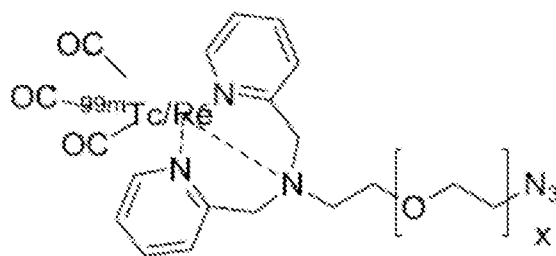
7. A compound according to formula (VII)



(VII)

wherein x is a number in the range of 1-10; m is any number in the range of 4-10, R^1 is H or a C_{1-3} alkyl group; and R^2 is either absent, or selected from the group consisting of H and a C_{1-3} alkyl group.

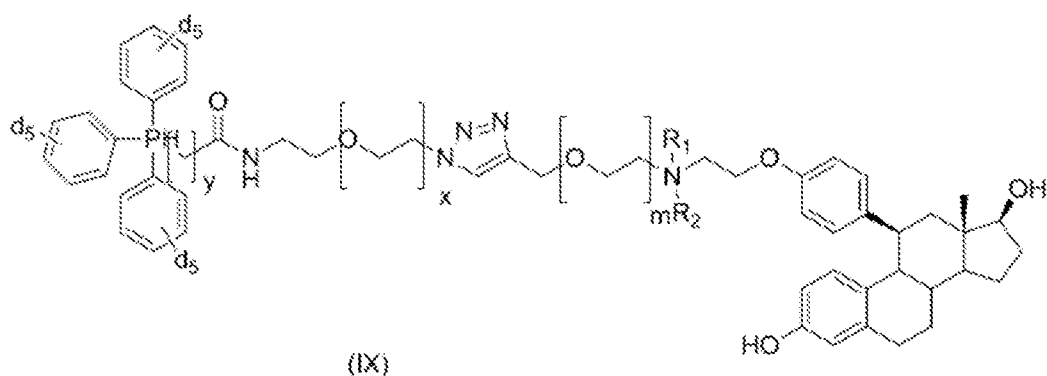
8. A process for the preparation of a compound according to formula (VII) of claim 7 comprising the step of reacting a compound according to formula (II) of claim 2 with a compound according to formula (VIII) using a Huisgen [3 + 2] cycloaddition reaction,



(VIII)

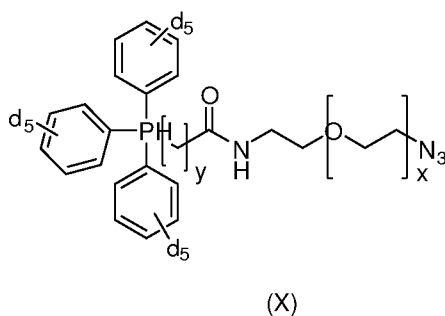
wherein x in the formula (VIII) is a number in the range of 1-10.

9. A pharmaceutical composition comprising the compound according to any of claims 1, 5, or 7, and a pharmaceutically effective carrier or diluent.
10. A method of diagnosing a disease in a subject comprising detecting a cell expressing estrogen receptors by contacting the compound according to any of claims 1, 5, or 7 with the cell, and imaging the cell with positron emission tomography (PET) or single photon emission computed tomography (SPECT).
11. The method of claim 10 wherein the cell comprises an estrogen receptor.
12. The method of claim 11 wherein the disease is breast cancer or endometrial cancer.
13. The method according to claim 12, wherein the subject has received anti-cancer treatment, wherein the method is used for determining effectiveness of the treatment.
14. A compound according to formula (IX)



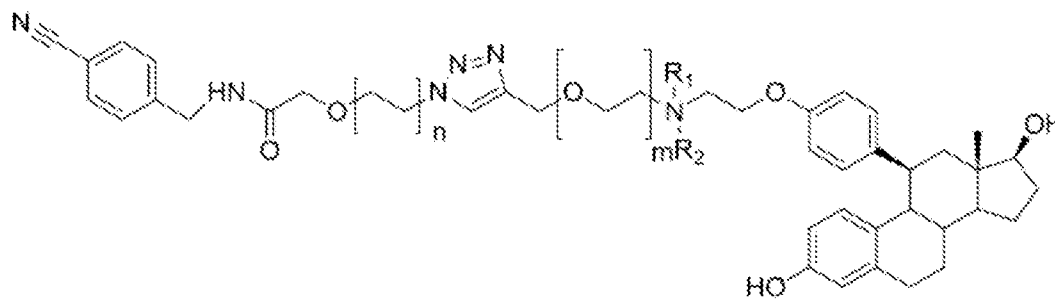
wherein m is a number in the range of 4-10; each of x and y is independently a number in the range of 1-10; R^1 is H or a C_{1-3} alkyl group; d is deuterium; and R^2 is either absent, or selected from the group consisting of H and a C_{1-3} alkyl group.

15. A process for the preparation of a compound according to formula (IX) of claim 14 comprising the step of reacting a compound according to formula (II) of claim 2 with a compound according to formula (X)



using a Huisgen [3 + 2] cycloaddition reaction, wherein each of x and y is independently a number in the range 1-10, and wherein d is deuterium.

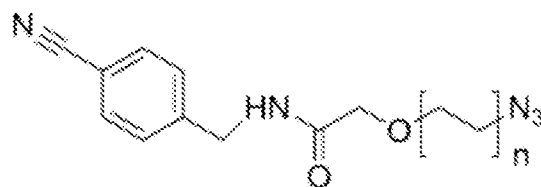
16. A compound according to formula (XI)



(XI)

wherein n is a number in the range of 1-10; m is a number in the range of 4-10; R^1 is H or a C_{1-3} alkyl group; and R^2 is either absent, or selected from a group consisting of H and a C_{1-3} alkyl group.

17. A process for the preparation of a compound according to formula (XI) of claim 16 comprising the step of reacting a compound according to formula (III) of claim 3 with a compound according to formula (XII) wherein n is a number in the range of 1-10,



(XII)

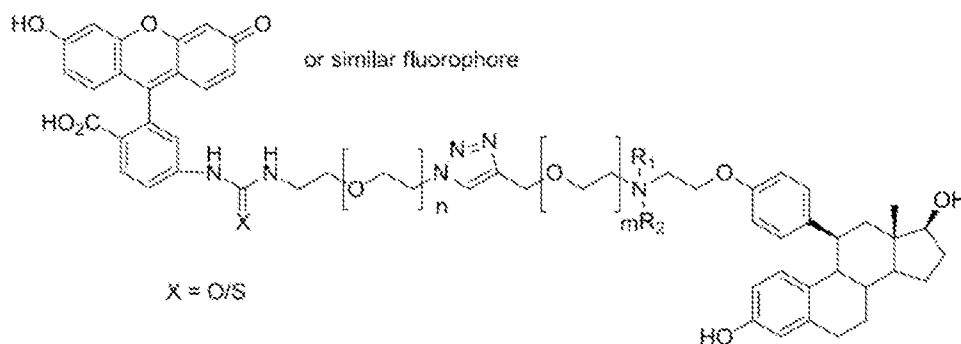
using a Huisgen [3 + 2] cycloaddition reaction.

18. A pharmaceutical composition comprising the compound according to claim 14 or claim 16 and a pharmaceutically effective carrier or diluent.

19. A method of diagnosing a disease in a subject comprising detecting a cell by contacting the compound according to claim 14 or claim 16 with the cell and imaging the cell in vitro with Raman spectroscopy.

20. The method of claim 19 wherein the cell comprises an estrogen receptor.

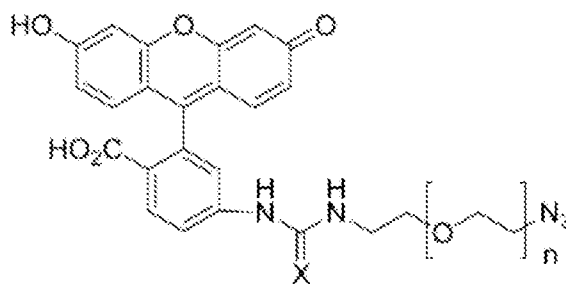
21. The method of claim 20 wherein the disease is breast cancer or endometrial cancer.
22. The method according to claim 19, wherein the subject has received anti-cancer treatment, wherein the method is used for determining effectiveness of the treatment.
23. A compound according to the formula (XIV)



(XIV)

wherein n is a number in the range of 1-10; m is a number in the range of 4-10; X is O or S; R¹ is H or a C₁₋₃ alkyl group; and R² is either absent, or selected from the group consisting of H and a C₁₋₃ alkyl group.

24. A process for the preparation of a compound according to formula (XIV) comprising the step of reacting a compound according to formula (II) of claim 2 with a compound according to formula (XV)



(XV)

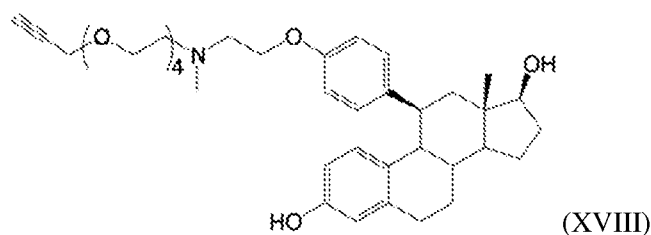
using a Huisgen [3 + 2] cycloaddition reaction, wherein R^1 is either absent or selected from the group consisting of H and a C_{1-3} alkyl group, X is O or S, and n is a number in the range of 1-10.

25. A method of diagnosing a disease in a subject comprising detecting a cell in vitro by contacting the compound according to claim 23, and imaging the cell with fluorescence spectroscopy.

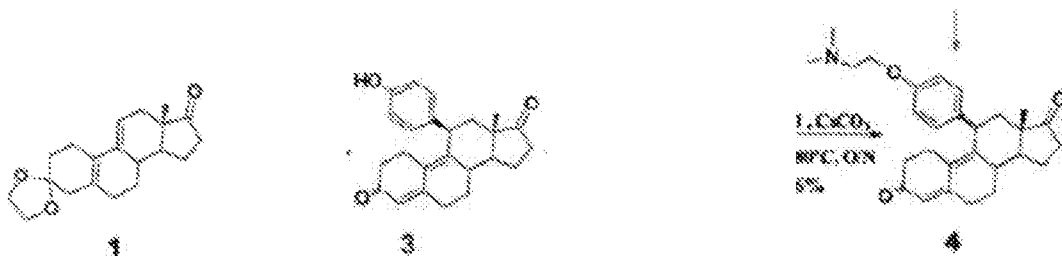
26. The method of claim 25, wherein the cell comprises an estrogen receptor.

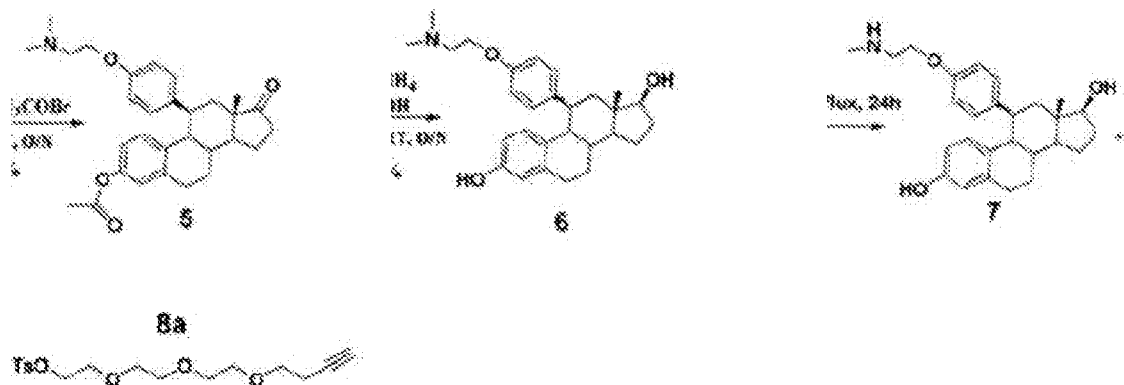
27. The method of claim 26, wherein the disease is breast cancer or endometrial cancer.

28. A process for the preparation of a compound according to formula (XVIII)



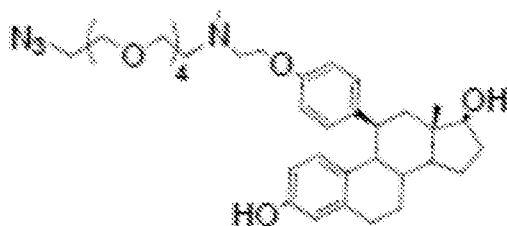
comprising the steps of transforming estra-5(10),9(11)-diene-3,17-dione 3-ethylenedioxy ketal (**1**) into 11 β -(4-Hydroxy-phenyl)-estra-4,9-diene-3,17-dione (**3**) by reacting (**1**) initially with a peroxide reagent to give an epoxide, treating the product obtained with the Grignard reagent derived from trimethylsilyloxyphenyl bromide to obtain compound (**3**), alkylating (**3**) with dimethylaminoethyl chloride to obtain compound (**4**), aromatizing (**4**) with acetyl bromide and acetic anhydride to obtain compound (**5**), reducing and saponifying (**5**) to obtain compound (**6**), demethylating (**6**) with alpha chloroethyl chloroformate to obtain compound (**7**), and reacting (**7**) with reagent (**8a**),





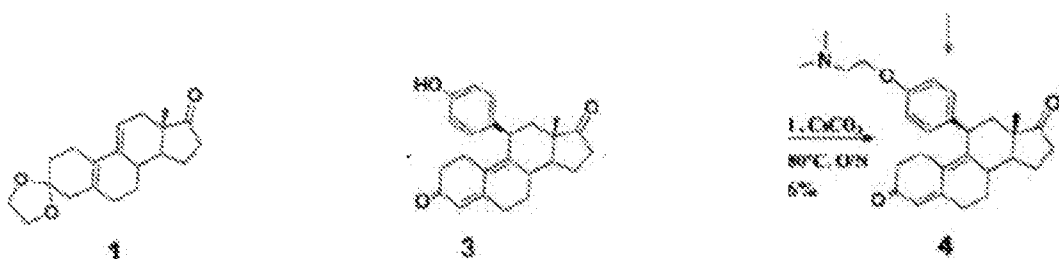
thereby obtaining the compound according to formula (XVIII).

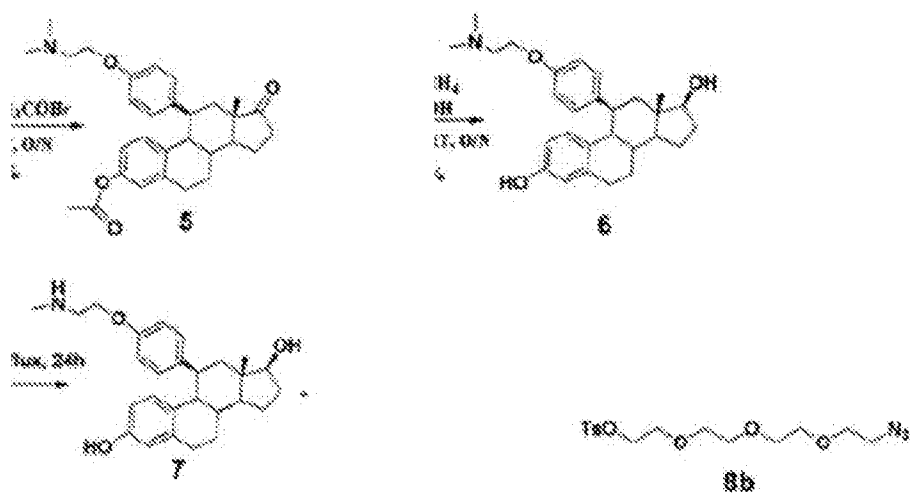
29. A process for the preparation of a compound according to formula (XIX)



(XIX)

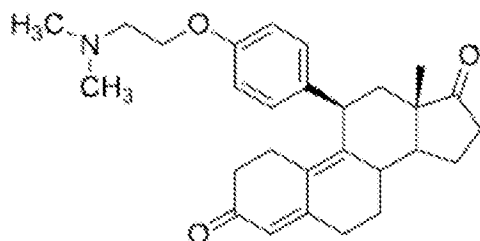
comprising the steps of transforming estra-5(10),9(11)-diene-3,17-dione 3-ethylenedioxy ketal (**1**) into 11 β -(4-Hydroxy-phenyl)-estra-4,9-diene-3,17-dione (**3**), by reacting (**1**) initially with a peroxide reagent to give an epoxide, treating the product obtained with the Grignard reagent derived from trimethylsilyloxyphenyl bromide to obtain compound (**3**), alkylating (**3**) with dimethylaminoethyl chloride to obtain compound (**4**), aromatizing (**4**) with acetyl bromide and acetic anhydride to obtain compound (**5**), reducing and saponifying (**5**) to obtain compound (**6**), demethylating (**6**) with alpha chloroethyl chloroformate to obtain compound (**7**), and reacting (**7**) with reagent (**8b**)





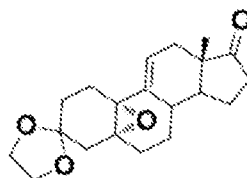
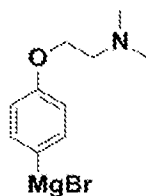
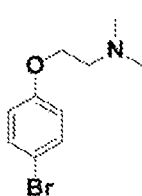
thereby obtaining the compound according to formula (XVIII).

30. A process for the preparation of a compound according to formula (XX)



(XX)

comprising the steps of: preparing 2-(4-bromophenoxy)-N,N-dimethylethanamine (**1**) by reacting 4-bromophenol with 2-dimethylaminoethyl chloride, converting (**1**) to 4-(2-(dimethylamino)ethoxy)phenyl)magnesium bromide (**2**) by treating with reagents comprising Mg, and iodine, and reacting (**2**) with 3,3-Ethylenedioxy-5(10)- α -epoxy-estr-9-ene-17-one (**3**) in the presence of CuI and acetic acid



1**2****3**

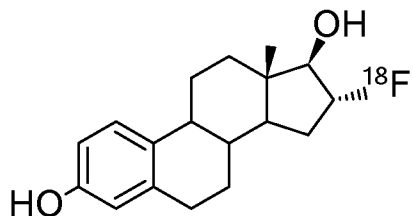
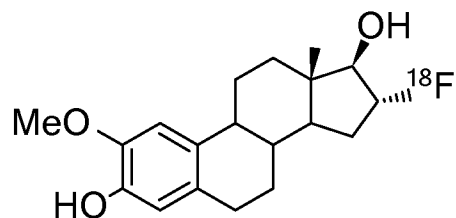
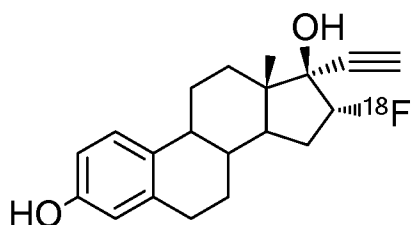
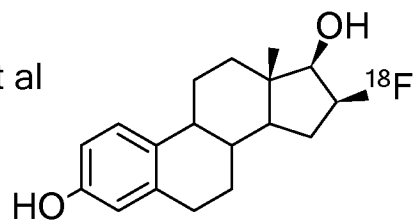
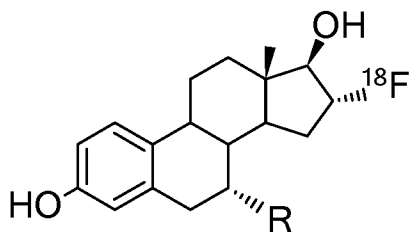
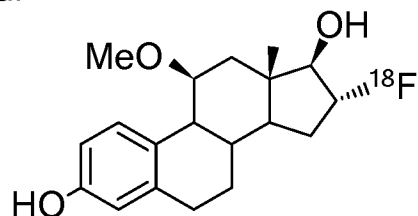
thereby obtaining the compound according to formula (XX).

31. Use of a compound according to claim 1, 5, 7, or 14 as an imaging agent for estrogen receptors in a cell, tissue, or a mammalian subject.

32. Use of a compound according to claim 2, 3, or 16 to prepare an imaging agent for estrogen receptors.

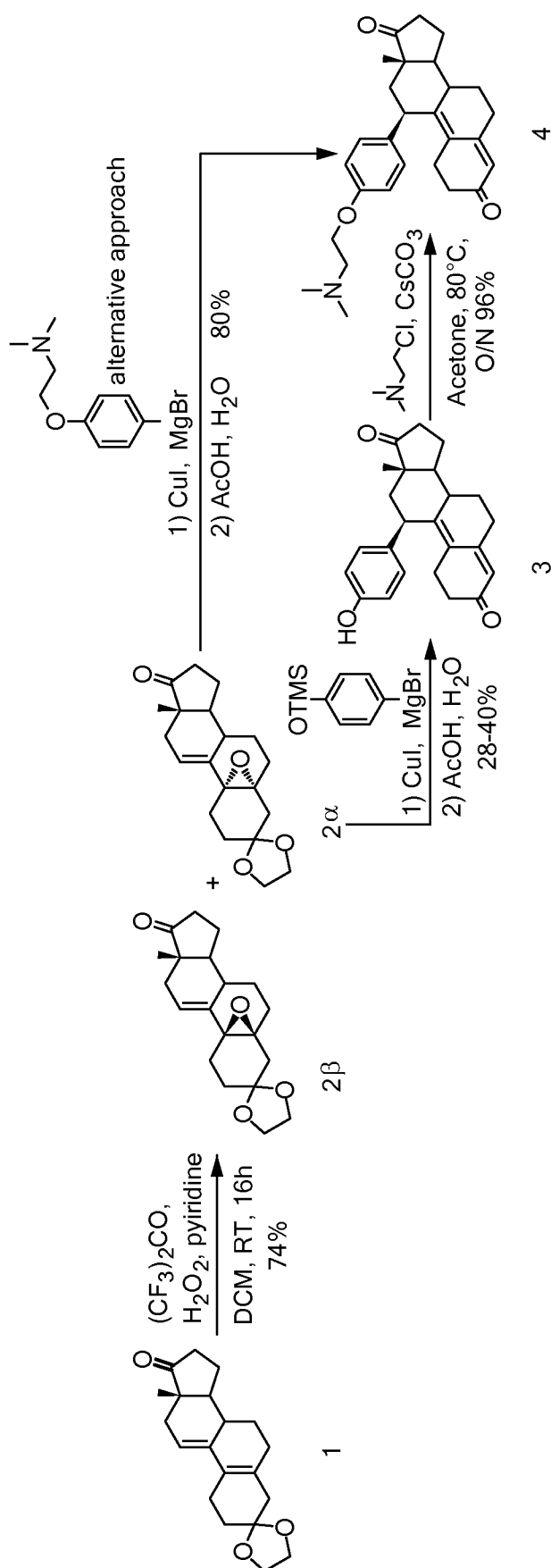
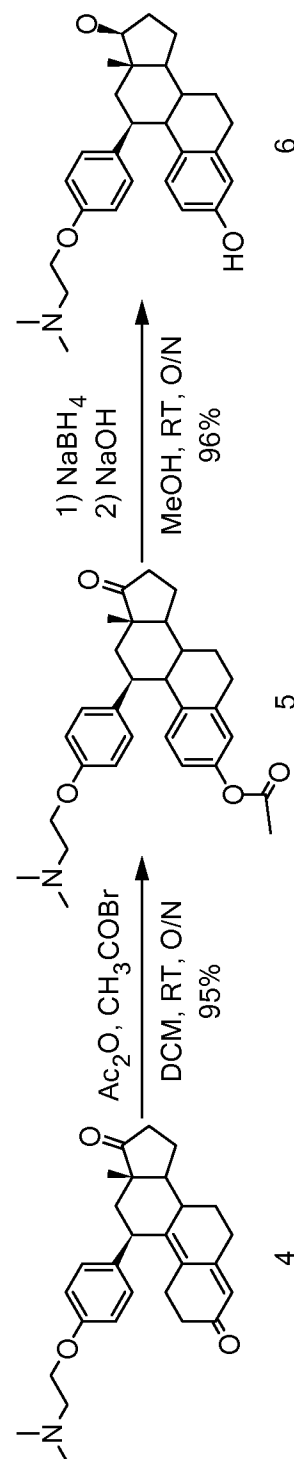
33. A kit comprising a compound according to claim 2, 3, or 16 and a labeling moiety and instructions for reacting the compound and the labeling moiety to provide a compound according to claim 1, 5, 7, or 14.

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16 α [^{18}F]-ES; (ref) Katzanellabogen et al4-Me[^{18}F]-ES; (ref) van Lier et al17 α ethynyl-16 α [^{18}F]-ES; (ref) VanBrocklin et al16 β [^{18}F]-ES; (ref) Welch et al7 α substituted[^{18}F]-ES; (ref) van Lier et al11 β methoxy-16 α [^{18}F]-ES; (ref) Pomper et al**FIG. 1**

PRIOR ART

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**FIG. 2A****FIG. 2B**

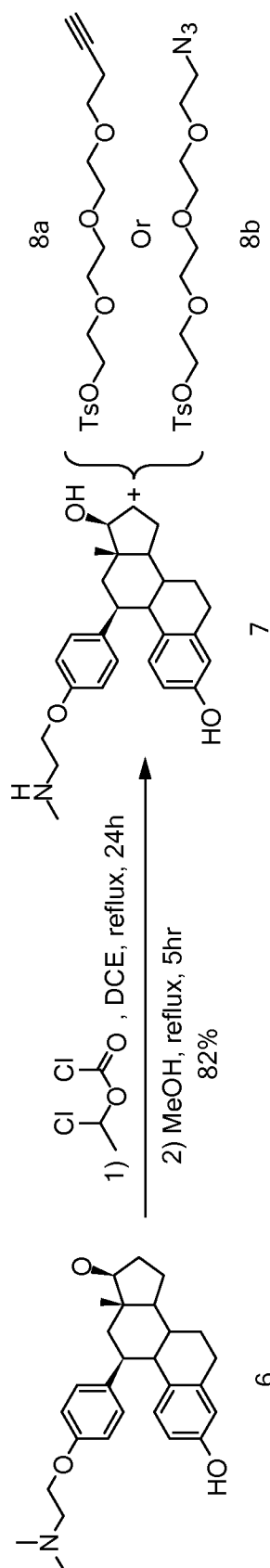


FIG. 2C

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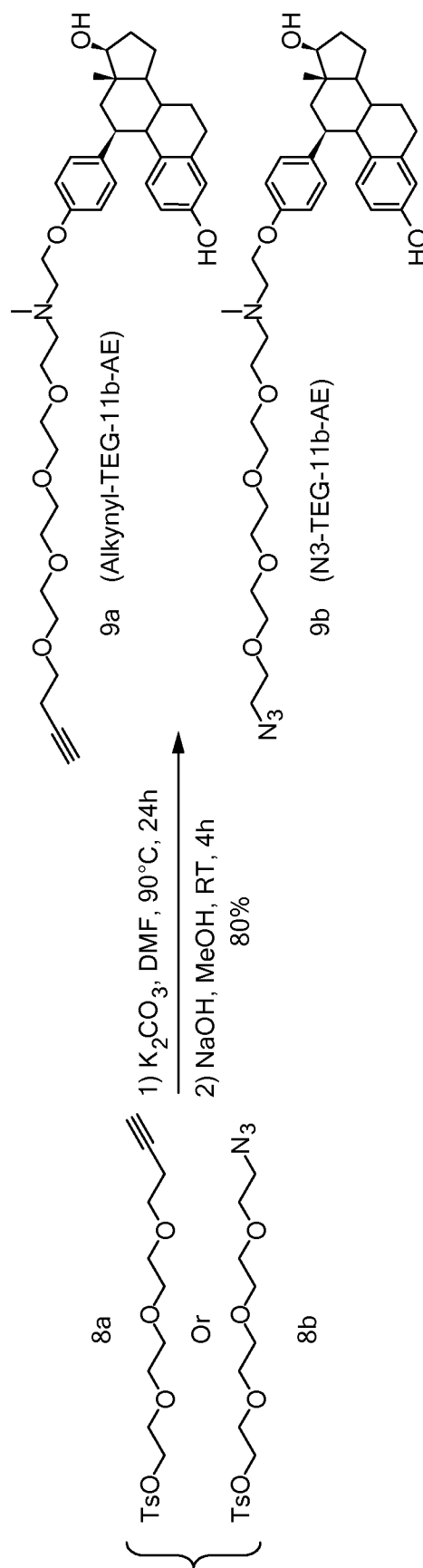
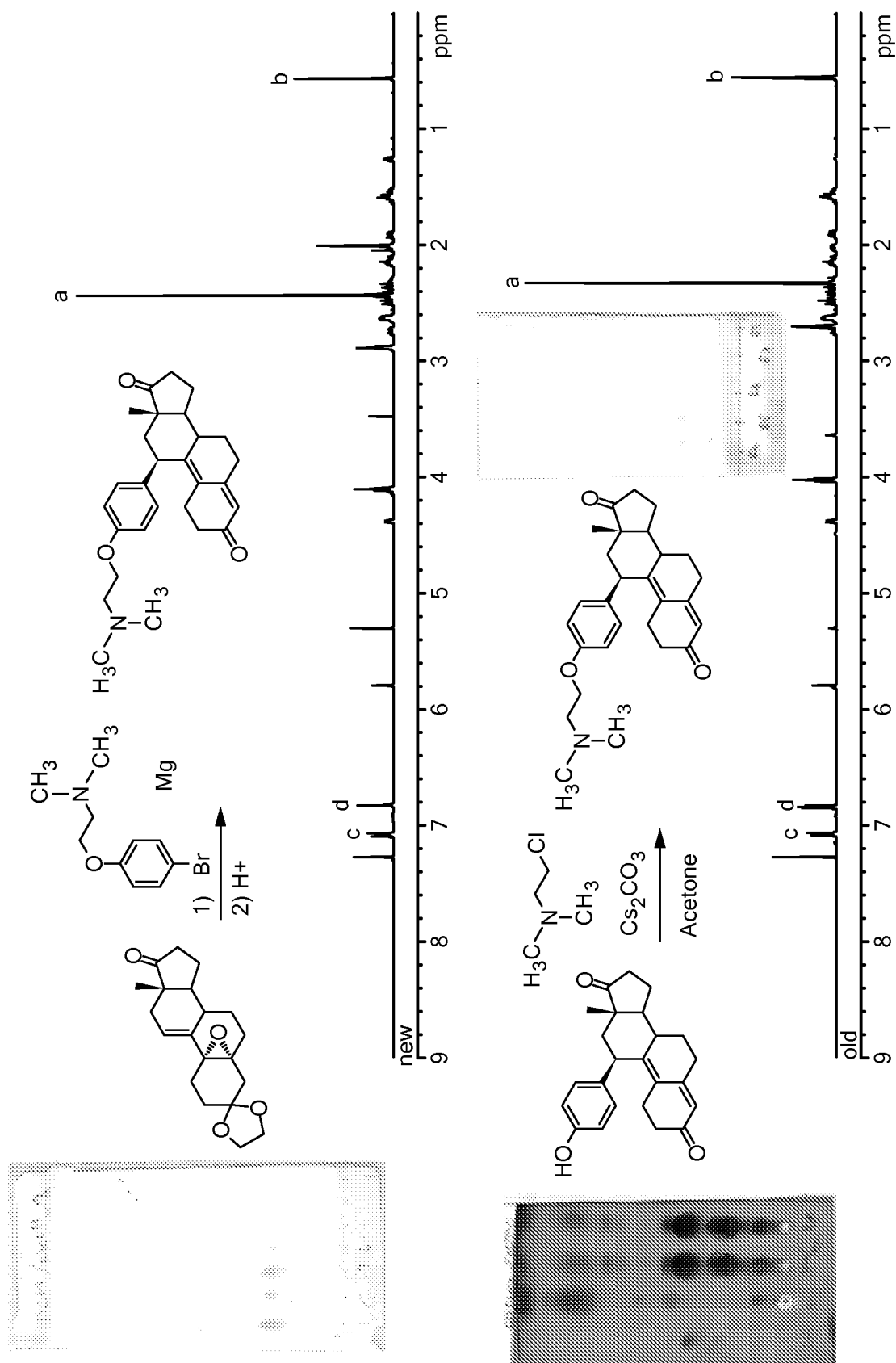
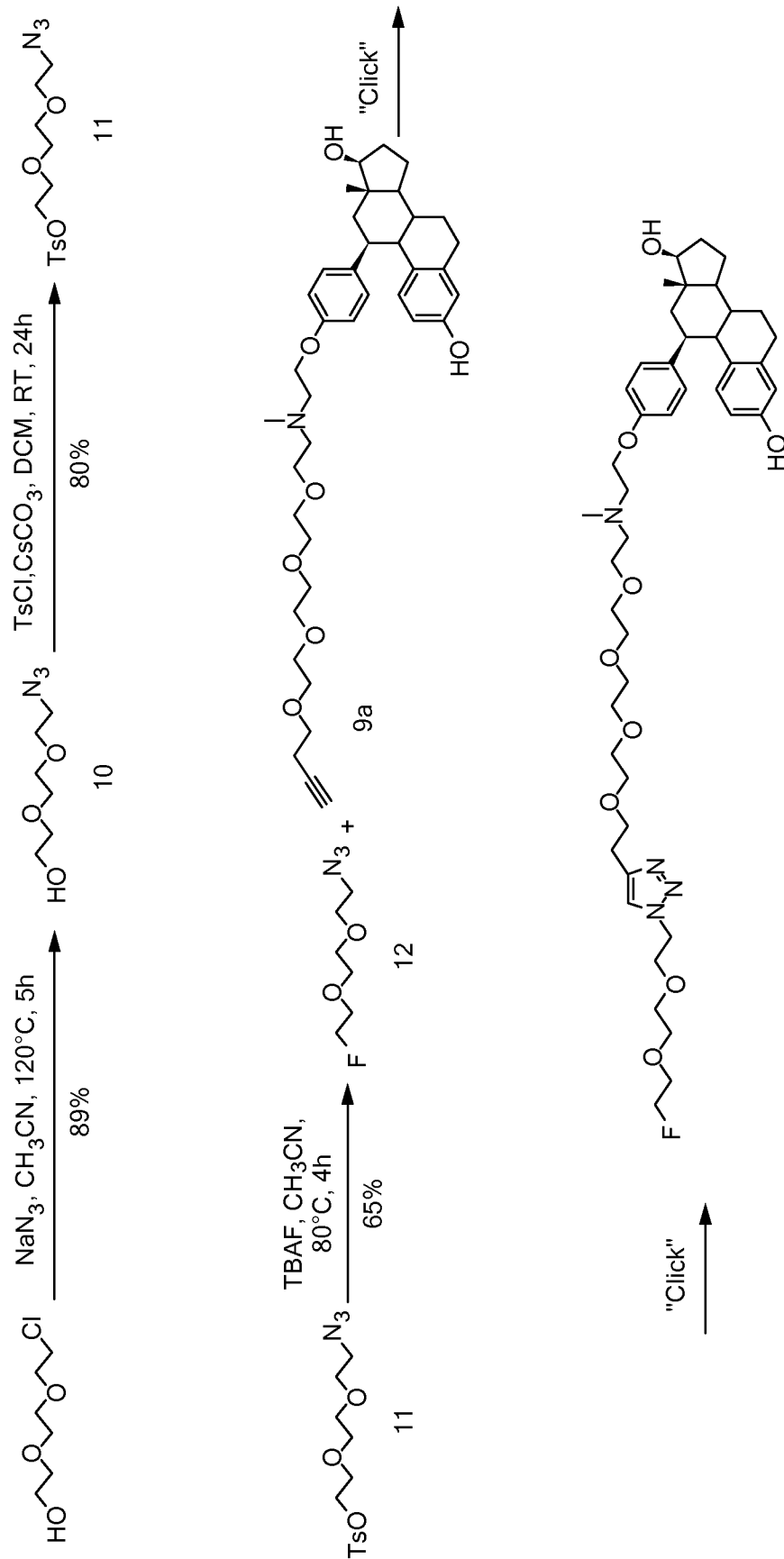


FIG. 2D

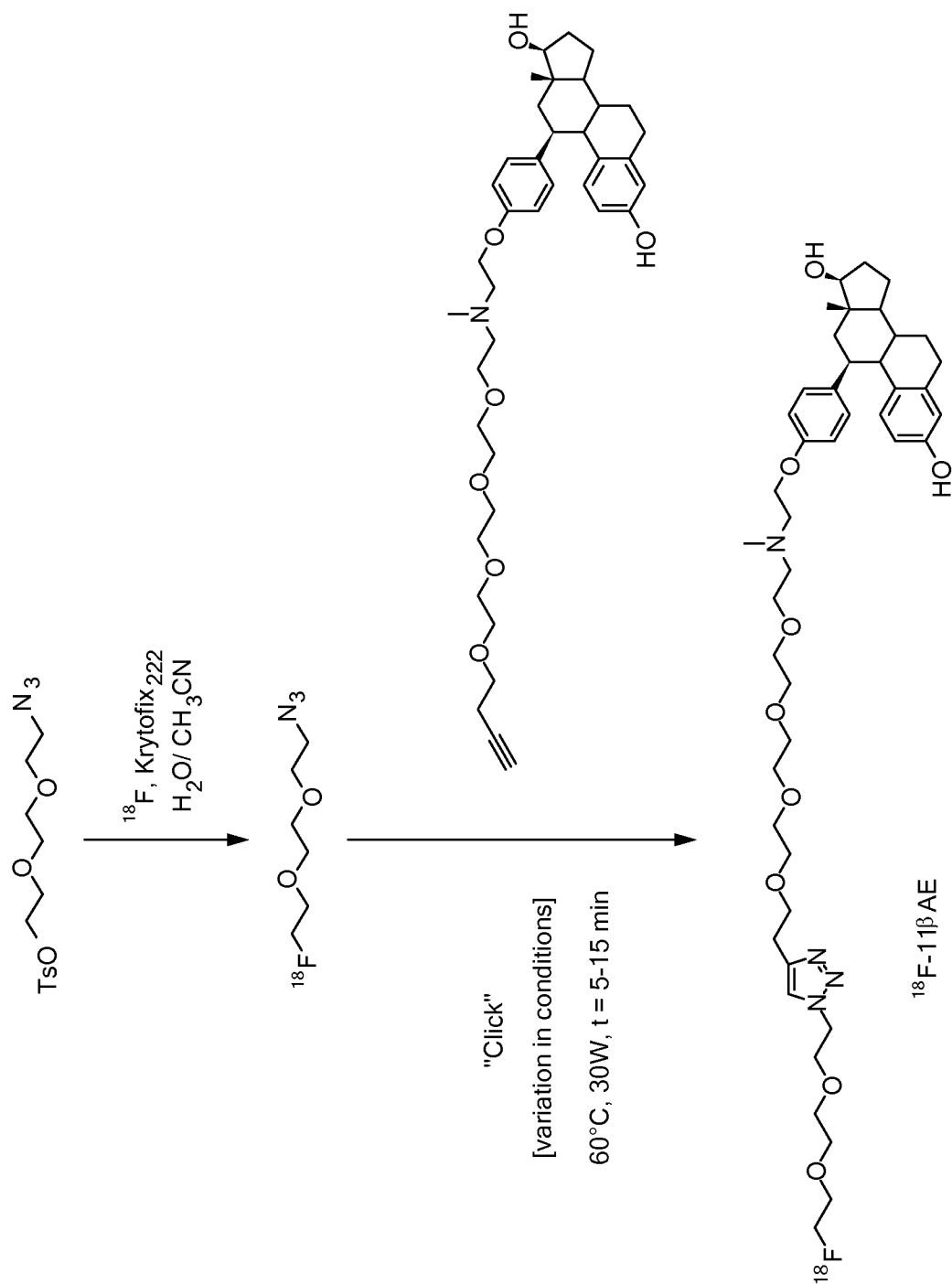
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**FIG. 3**

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**FIG. 4**

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**FIG. 5**

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Radiochromatography ^{18}F -11 β -AE

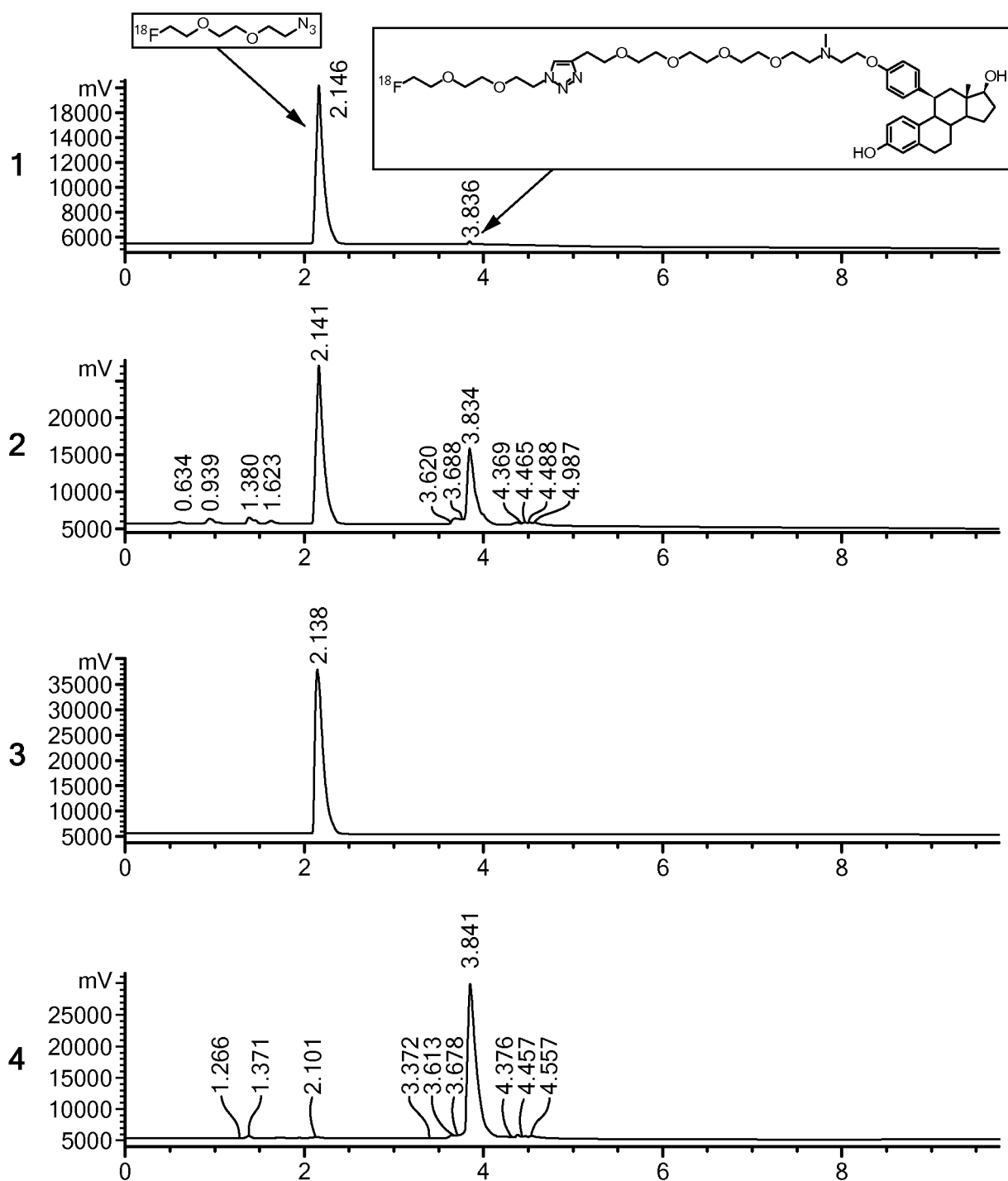
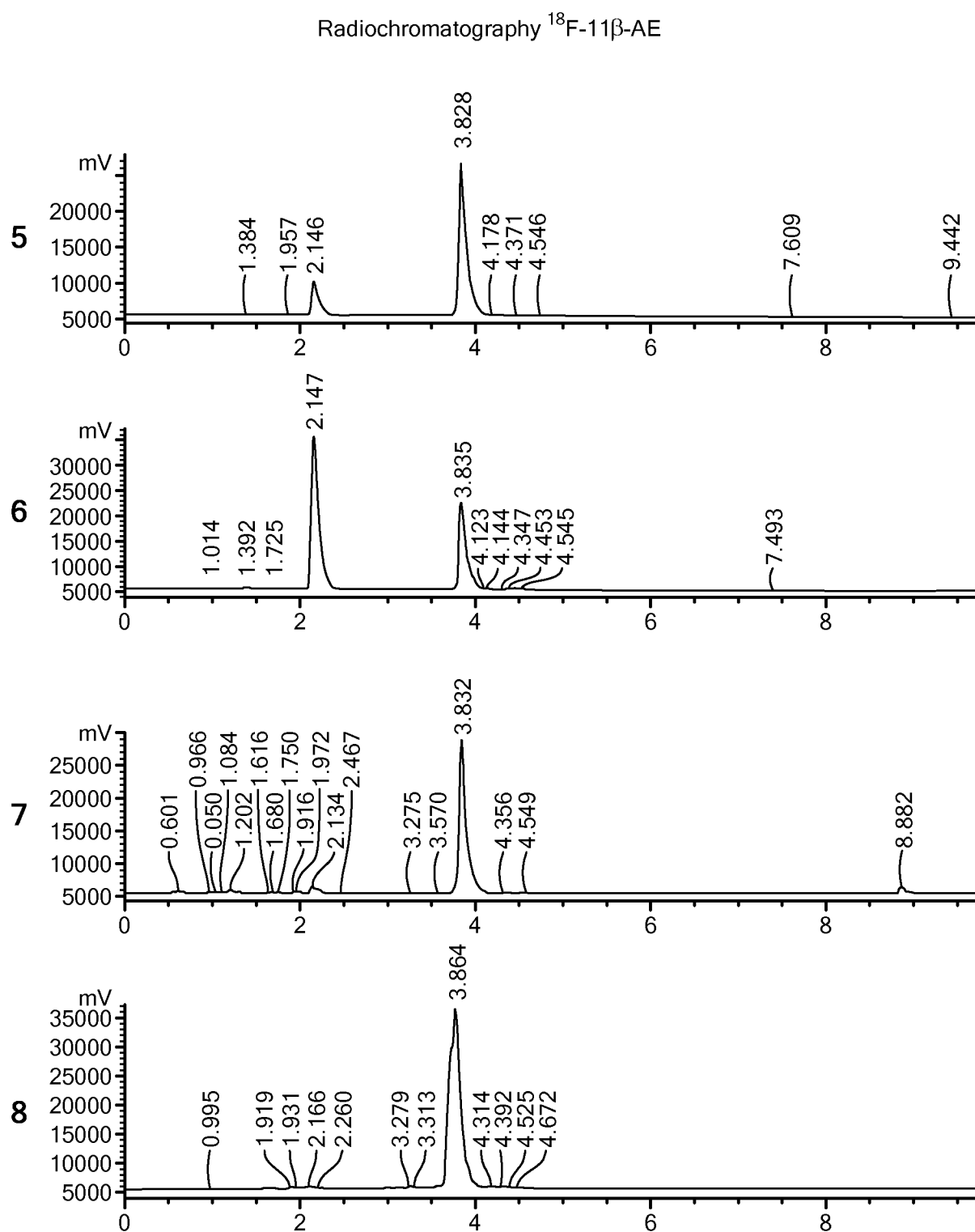


FIG. 6A

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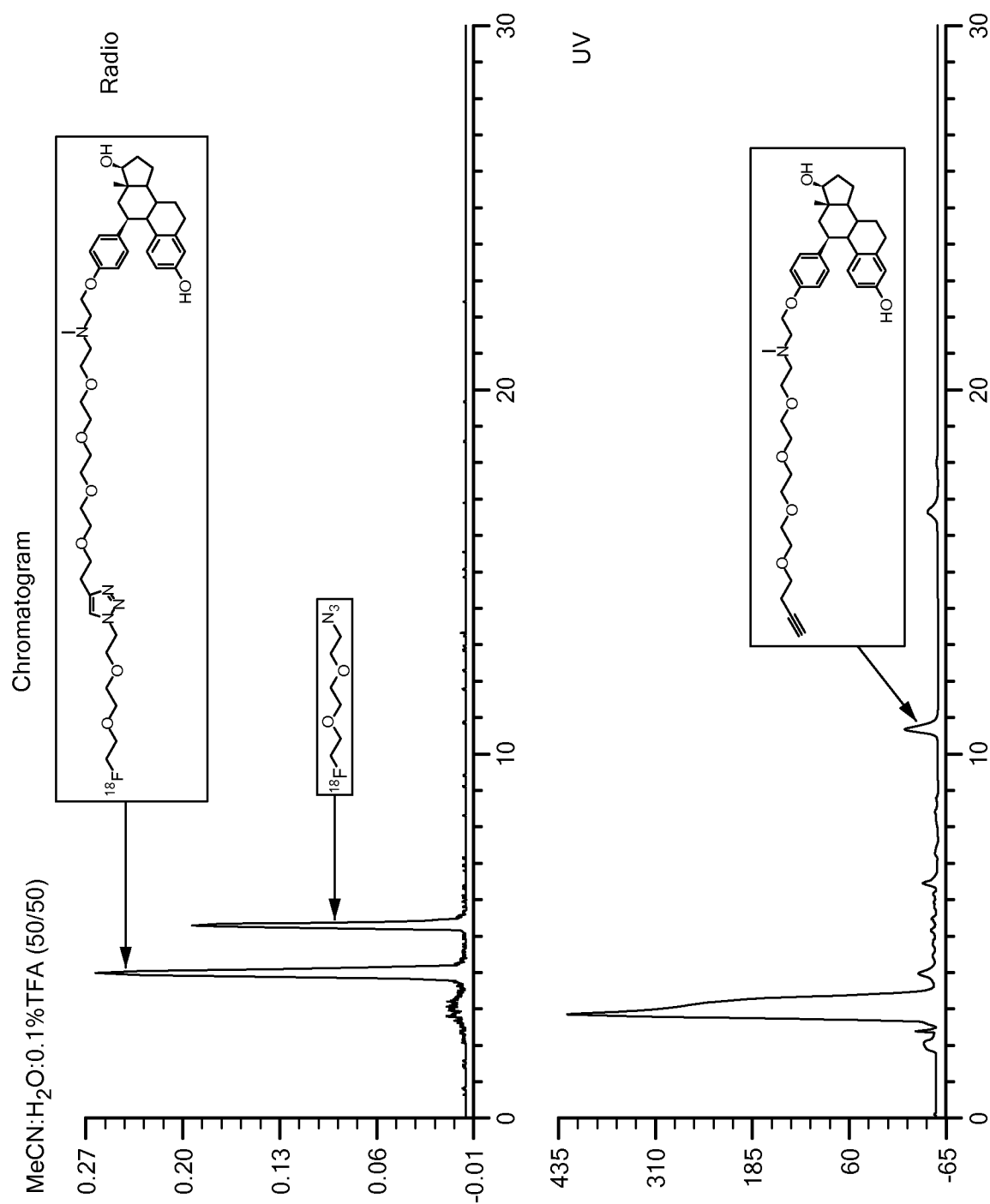
**FIG. 6B**

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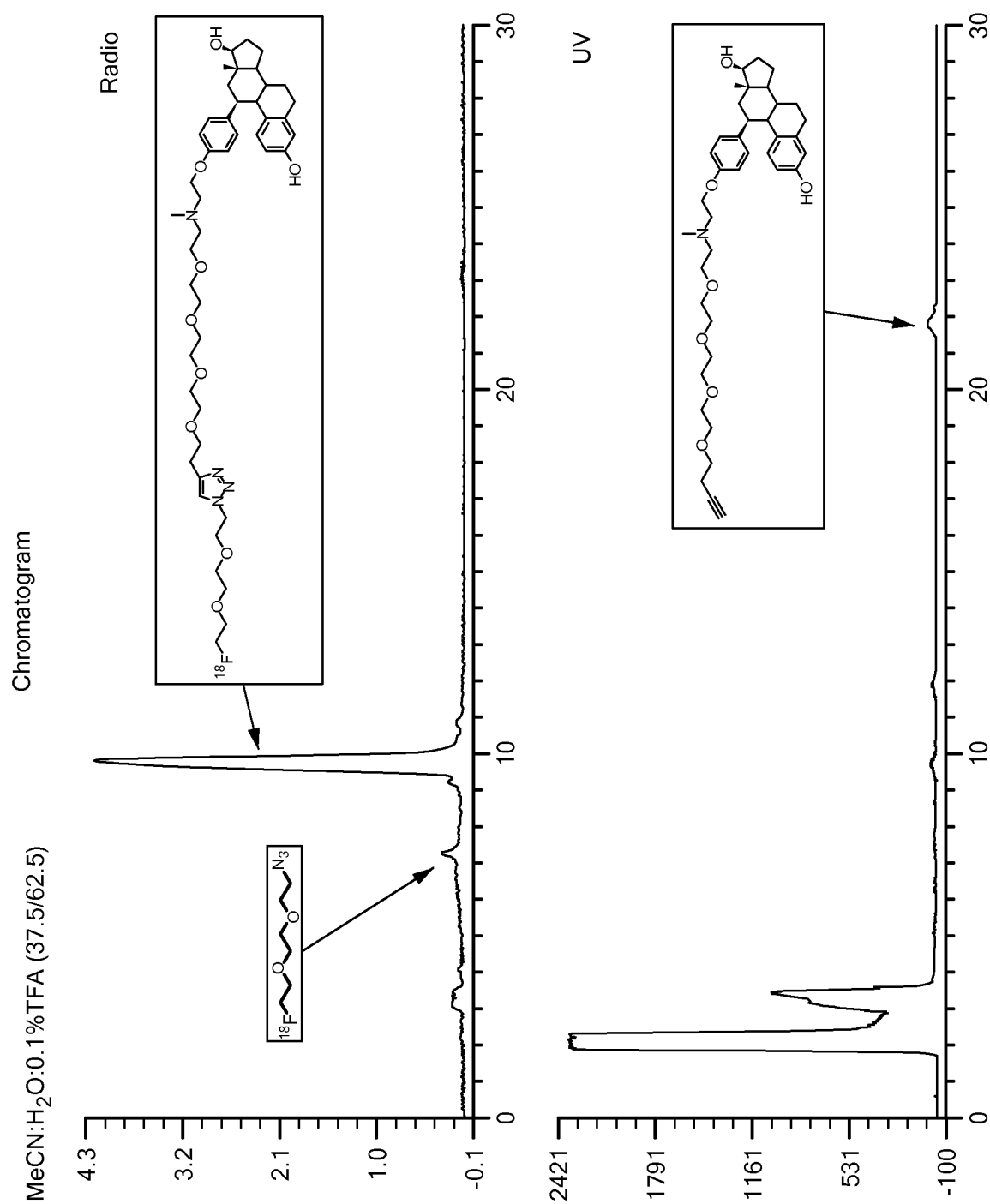
Condition	Cu ⁺² 80mM	ASC 200mM	BPDS 80mM	Cu ⁺ 80mM	11βAE 1mM	¹⁸ FN3	DMSO/ H ₂ O	Temp °C	Time min	Rxn vol (μl)	Yield %
1	10	30	20		20	5	100	60	5	185	0
2			20	20	20	10	50	60	5	120	30
3			20	20		10	50	60	5	120	30
4			40	40	40	10		60	5	130	95
5				40	40	10	DMF (μL) 40	60	5	130	80
6				60	40	10	20	60	5	130	36
7				60	40	10	20	60	10	130	94
8			40	40	40	100 9.73mCi		60	5	220	95

FIG. 6C

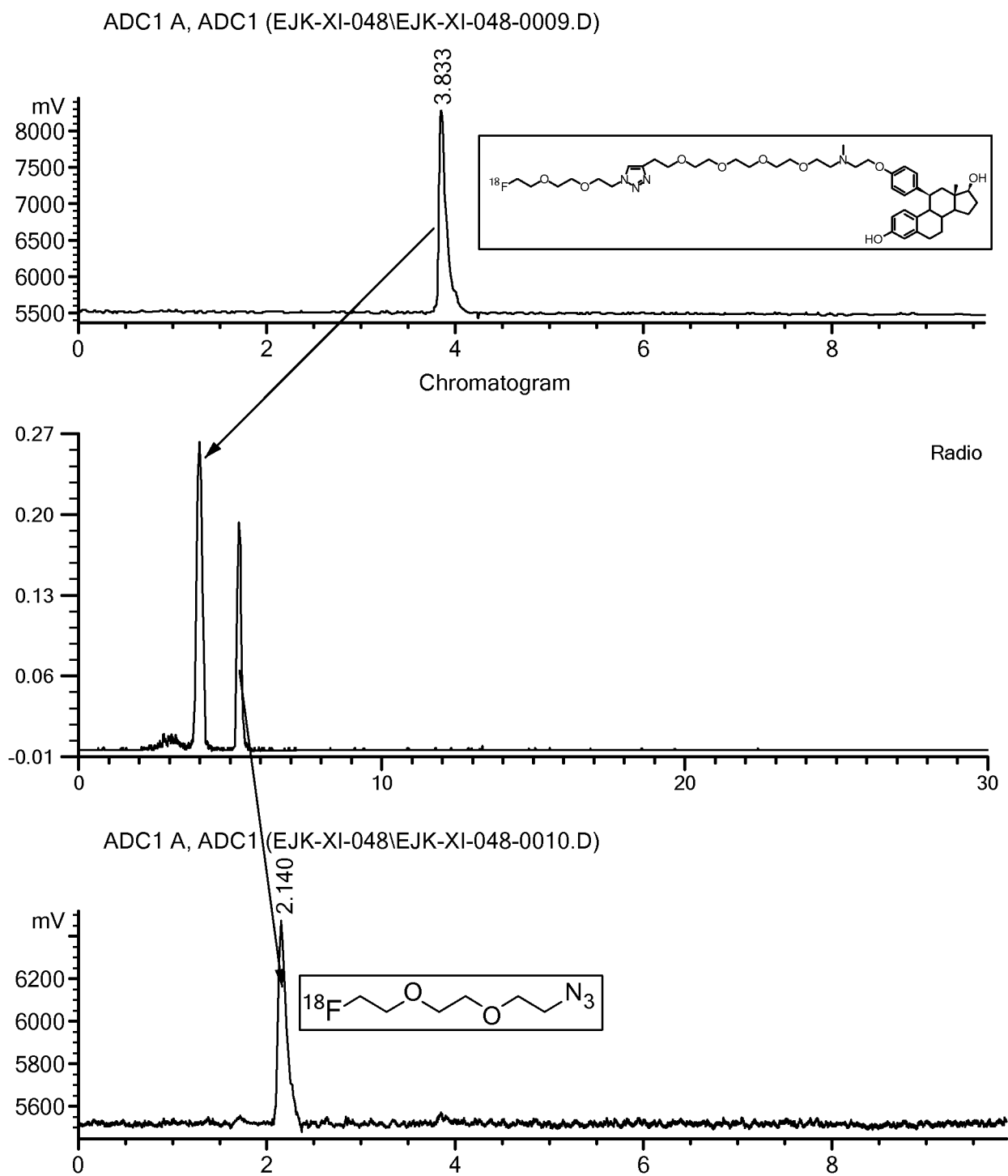
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**FIG. 7B-1**

The figure displays three HPLC chromatograms related to the synthesis and purification of a radiolabeled compound.

Top Chromatogram (HPLC analytical): The y-axis represents mV (5000 to 25000) and the x-axis represents time in minutes (0 to 10). The chromatogram shows several peaks, with the most prominent one at 3.838 minutes. Other labeled peaks are at 3.748, 5.050, 7.337, and 7.998 minutes. An inset shows the chemical structure of the compound, which is a complex molecule featuring a steroid-like core, a phenol group, and a long chain containing an azide group and a fluorine atom (^{18}F).

Middle Chromatogram (HPLC purification): The y-axis represents mV (-0.1 to 4.3) and the x-axis represents time in minutes (0 to 30). The chromatogram shows a major peak at 2.167 minutes and a minor peak at 10.0 minutes. The label "HPLC purification" is present.

Bottom Chromatogram (HPLC analytical): The y-axis represents mV (5000 to 25000) and the x-axis represents time in minutes (0 to 10). The chromatogram shows peaks at 2.167, 5.163, and 5.251 minutes. An inset shows the chemical structure of the compound, which is a simple molecule containing a fluorine atom (^{18}F) and an azide group (N_3).

FIG. 7B-2

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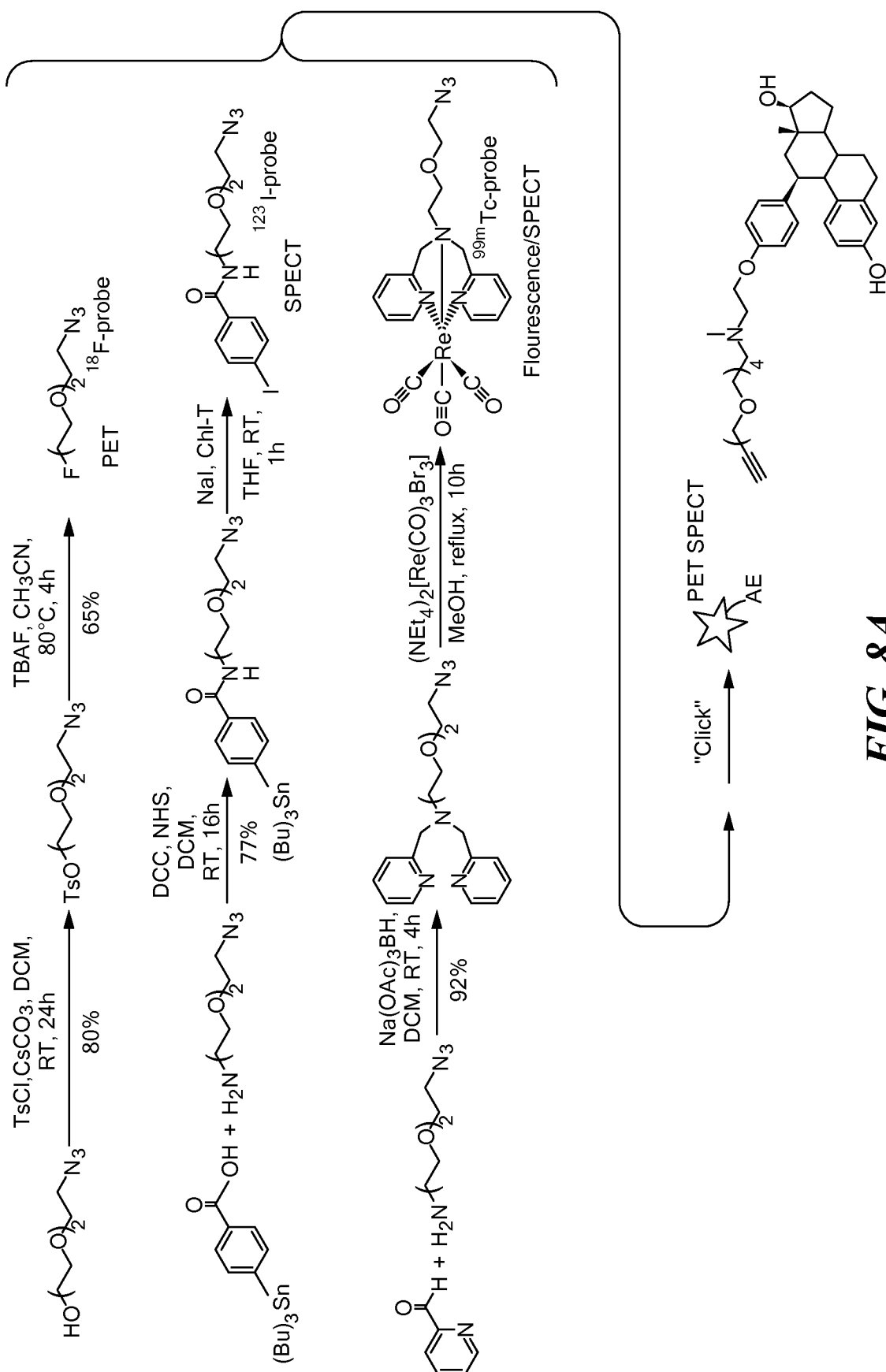
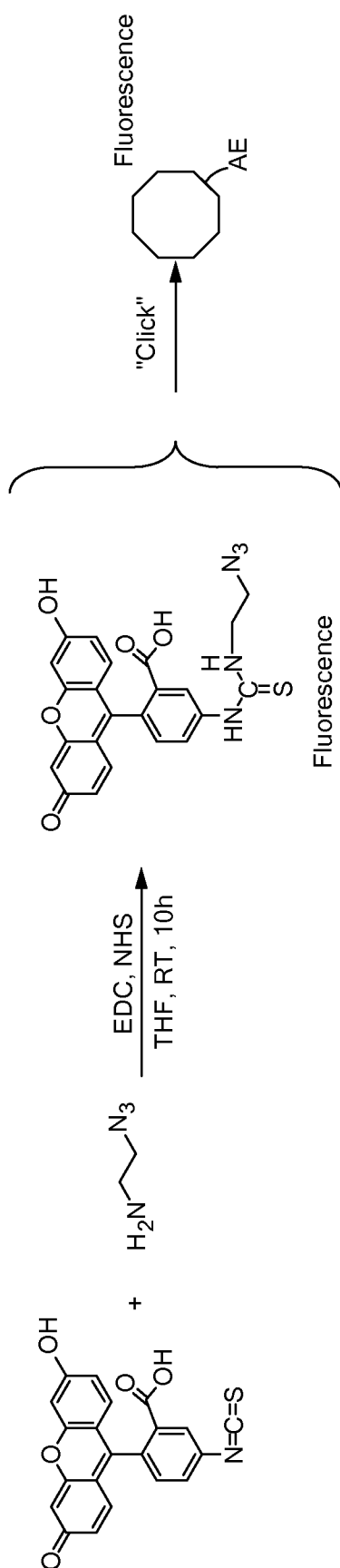


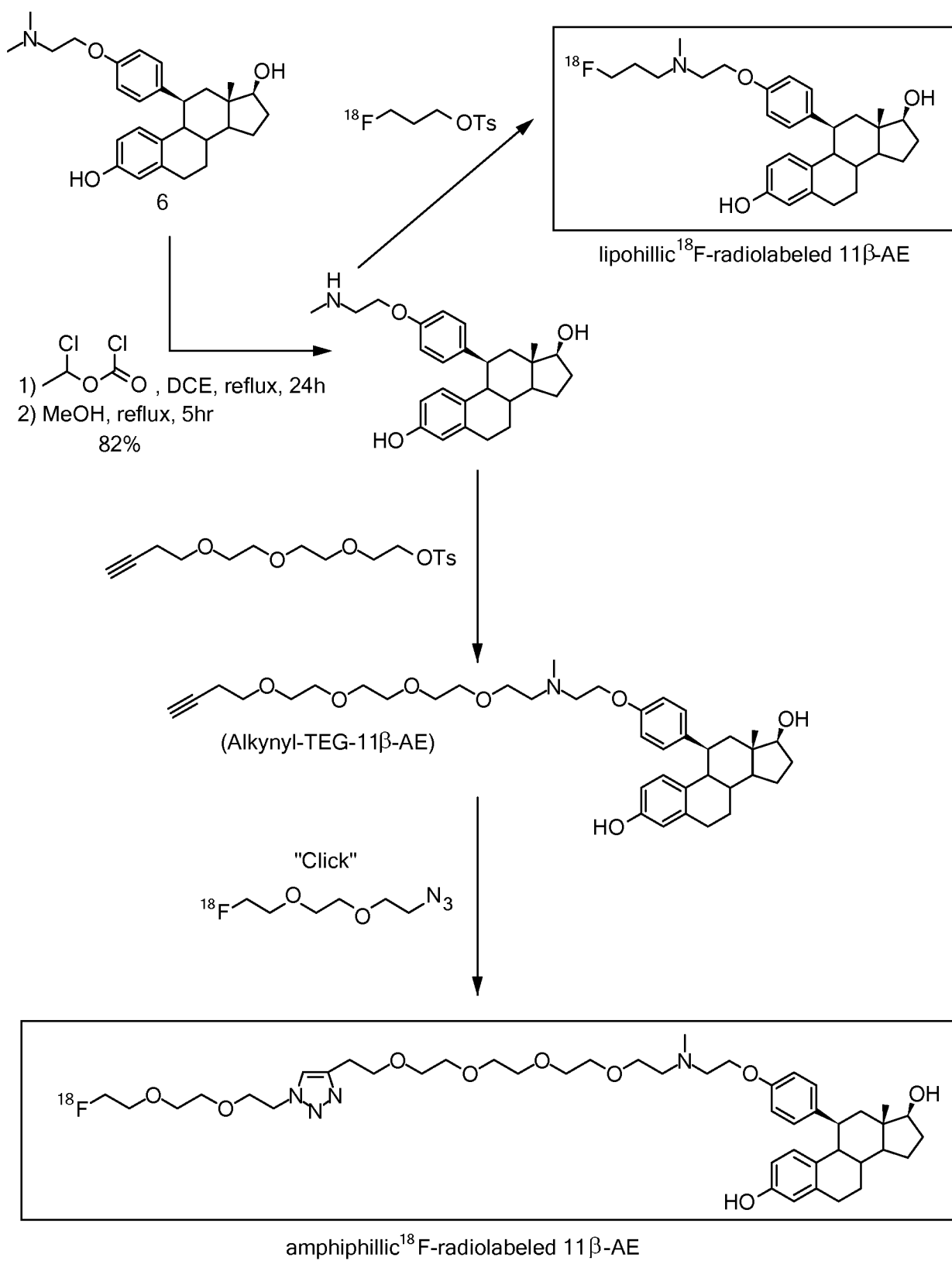
FIG. 8A



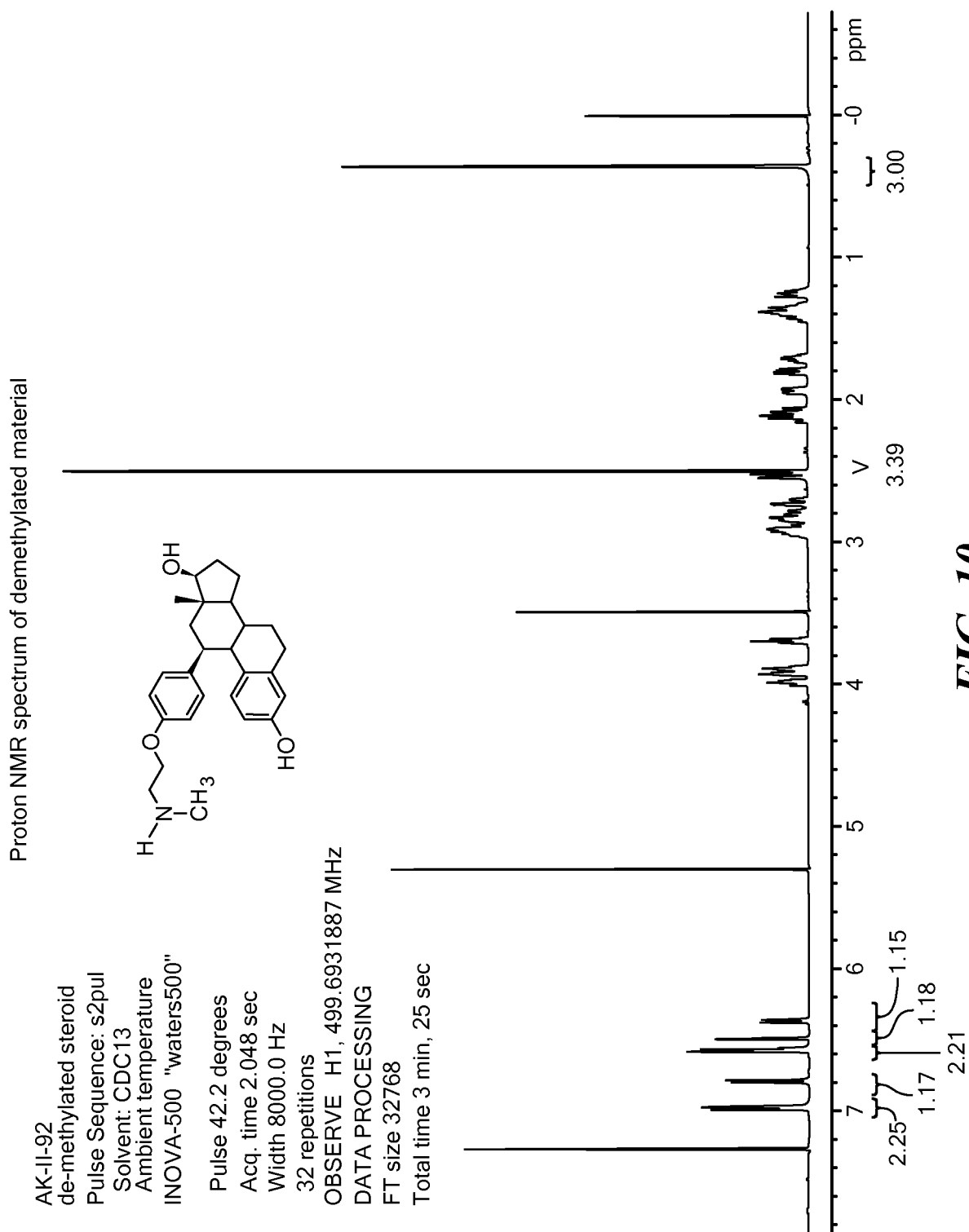
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**FIG. 8C**

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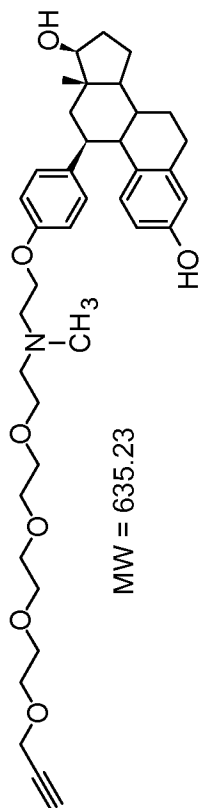
**FIG. 9**

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Proton NMR spectrum of material with
propargyl tetraethylene glycol linker



NU STANDARD 1H OBSERVE

Pulse Sequence: s2pul

Solvent: CDC13

Ambient temperature

Mercury-400BB "nmr400"

Relax. delay 1.000 sec

Pulse 60.0 degrees

Acq. time 1.365 sec

Width 6002.4 Hz

54 repetitions

OBSERVE H1, 399.1216301 MHz

DATA PROCESSING

Line broadening 0.2 Hz

FT size 16384

Total time 4 min, 59 sec

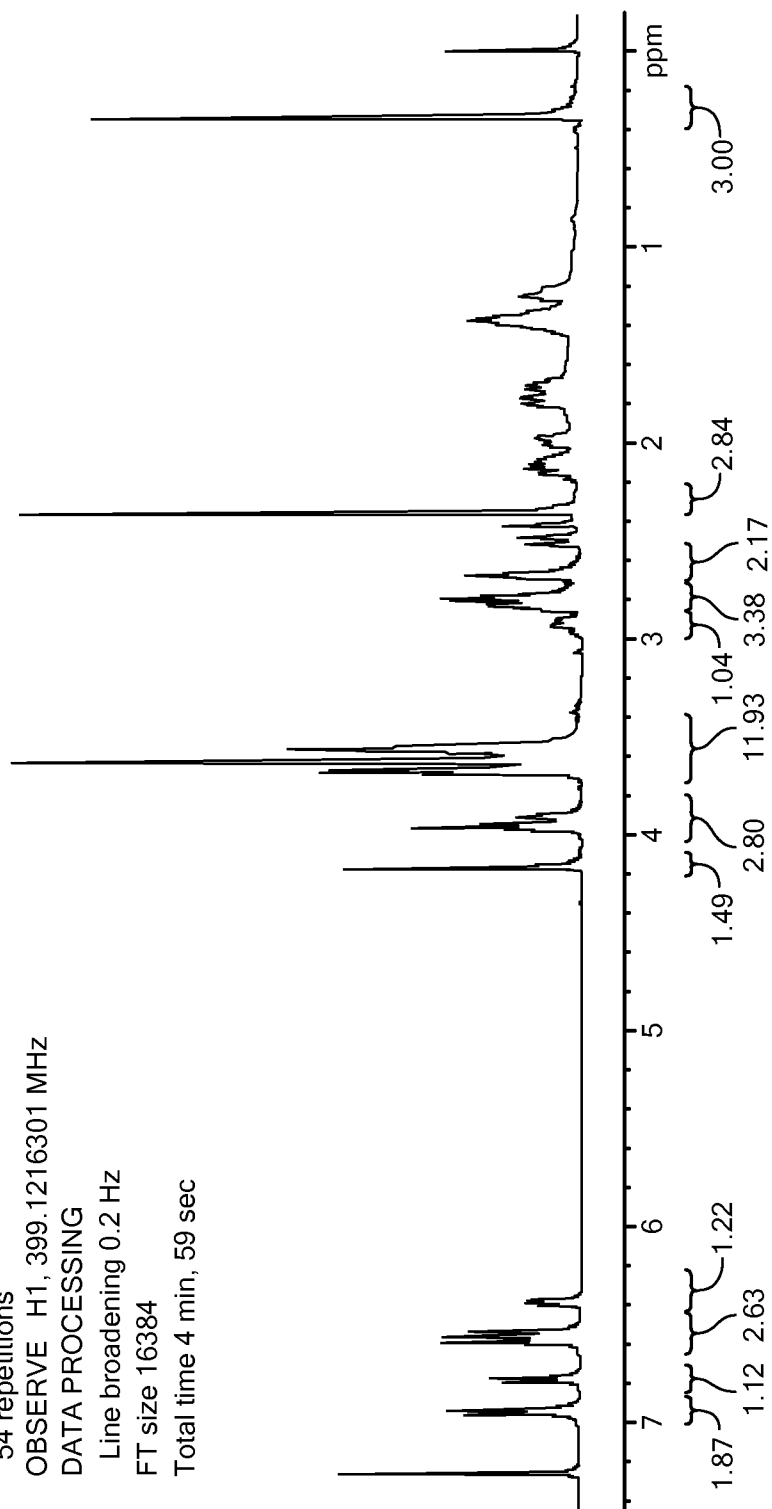


FIG. 11

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Carbon-13 NMR spectrum of material with
propargyl tetraethylene glycol linker

NU 13C OBSERVE

Pulse Sequence: s2pul

Solvent: CDC13

Ambient temperature

Mercury-400BB "nmr400"

Pulse 37.5 degrees

Acq. time 1.311 sec

Width 25000.0 Hz

22540 repetitions

OBSERVE C13, 100.3591898 MHz

DECOUPLE H1, 399.1231403 MHz

Power 39 dB

continuously on

WALTZ-16 modulated

DATA PROCESSING

Line broadening 1.0 Hz

FT size 65535

Total time 939 hr, 19 min, 25 sec

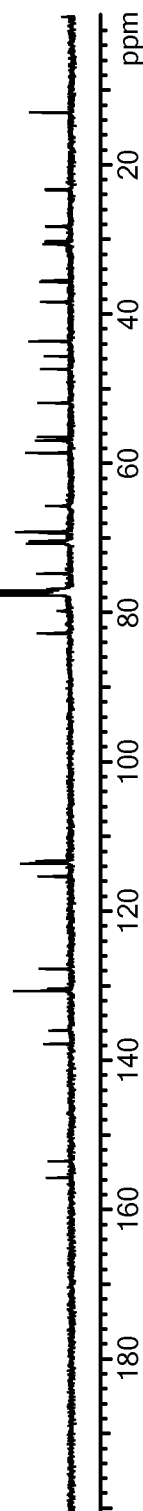
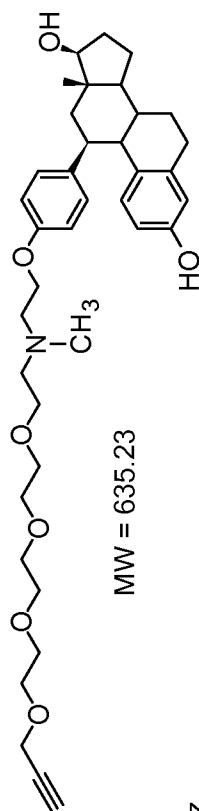
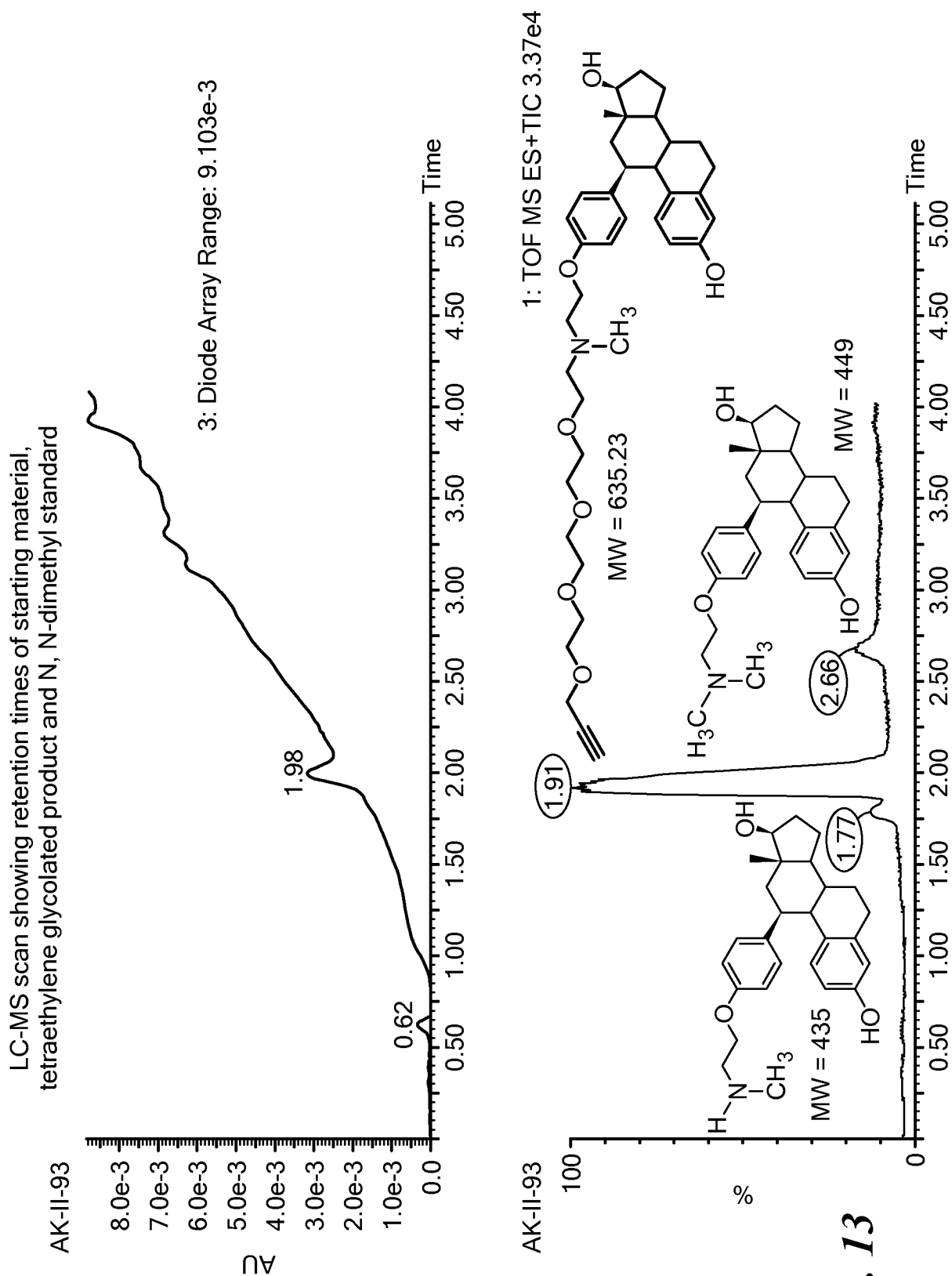
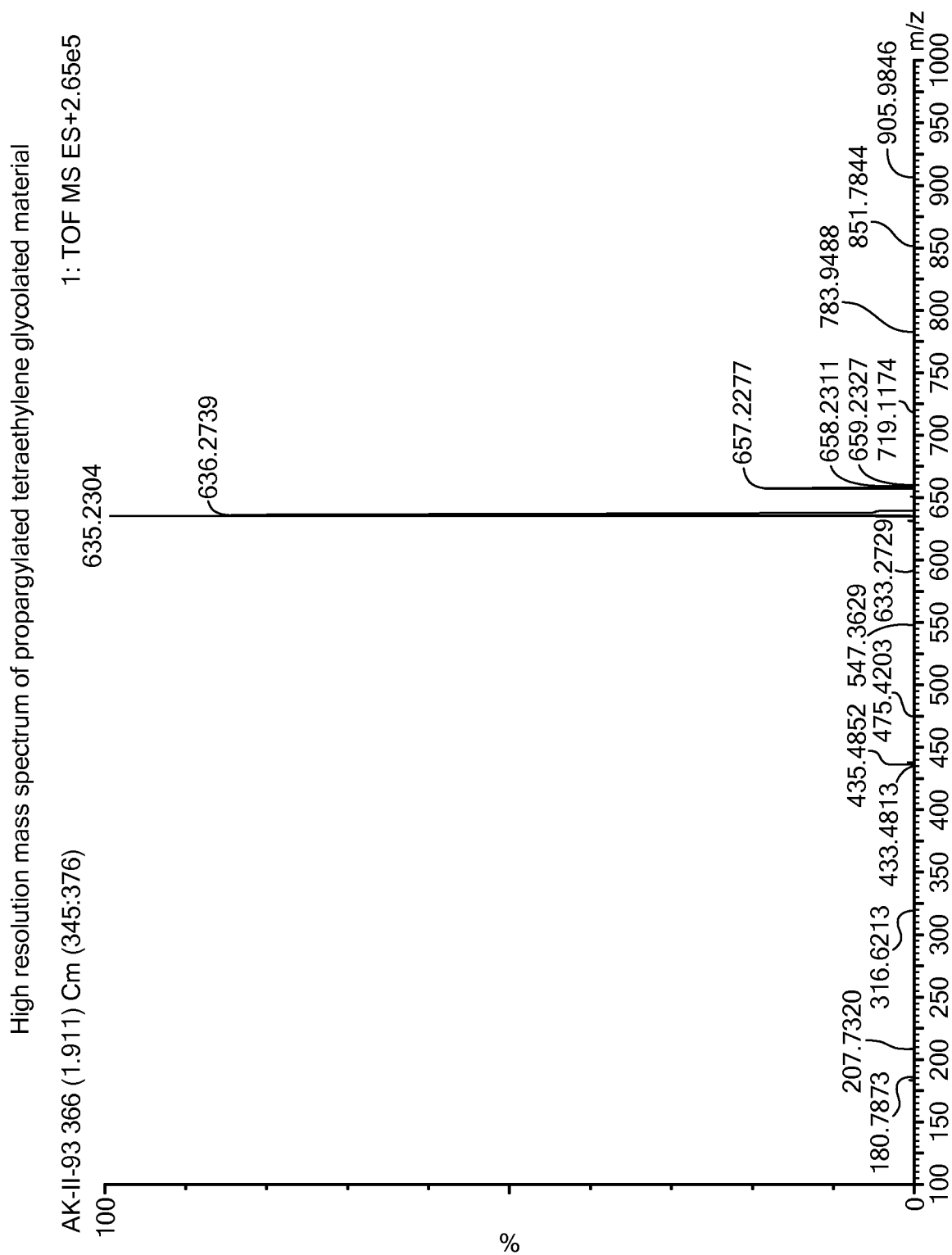


FIG. 12

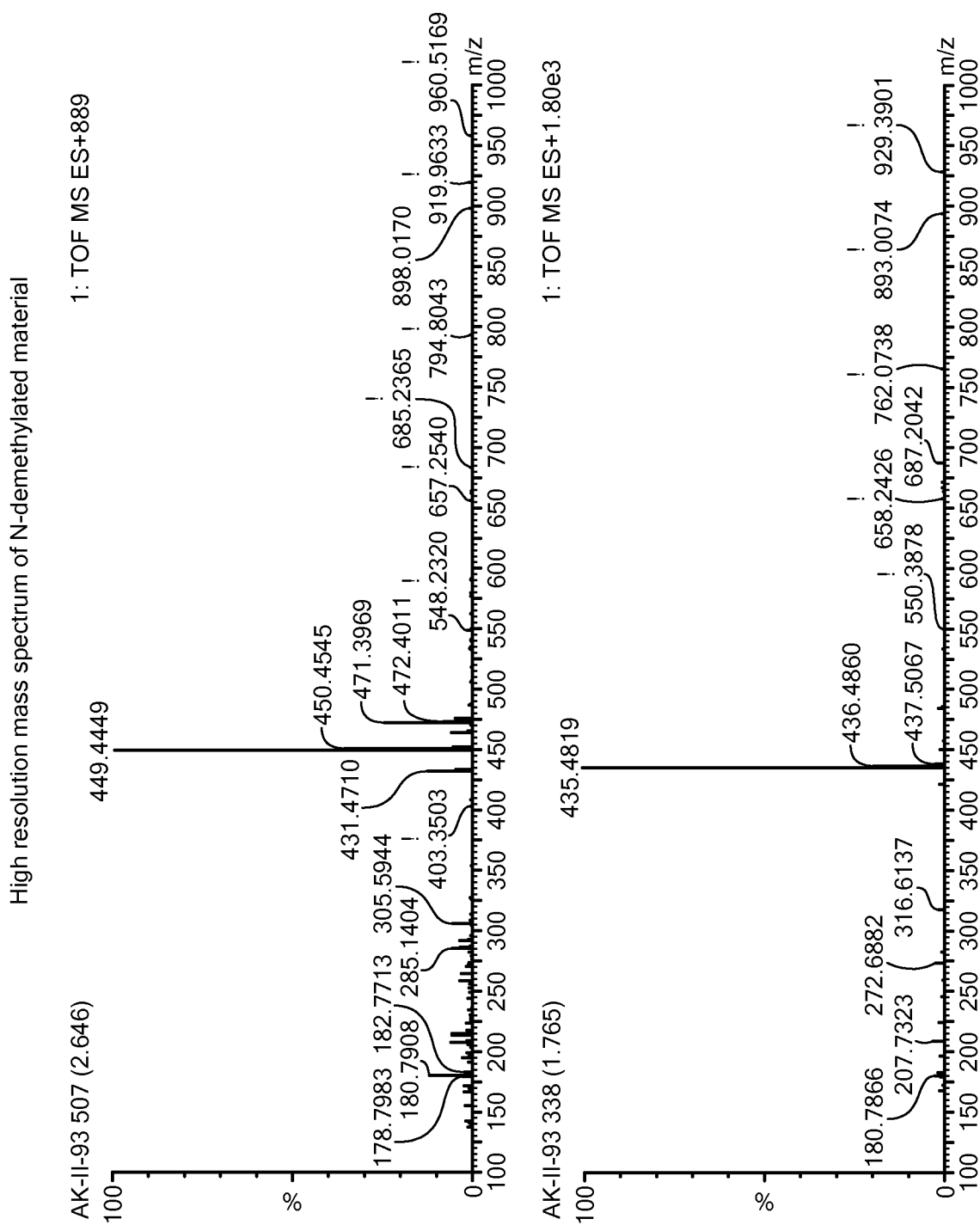
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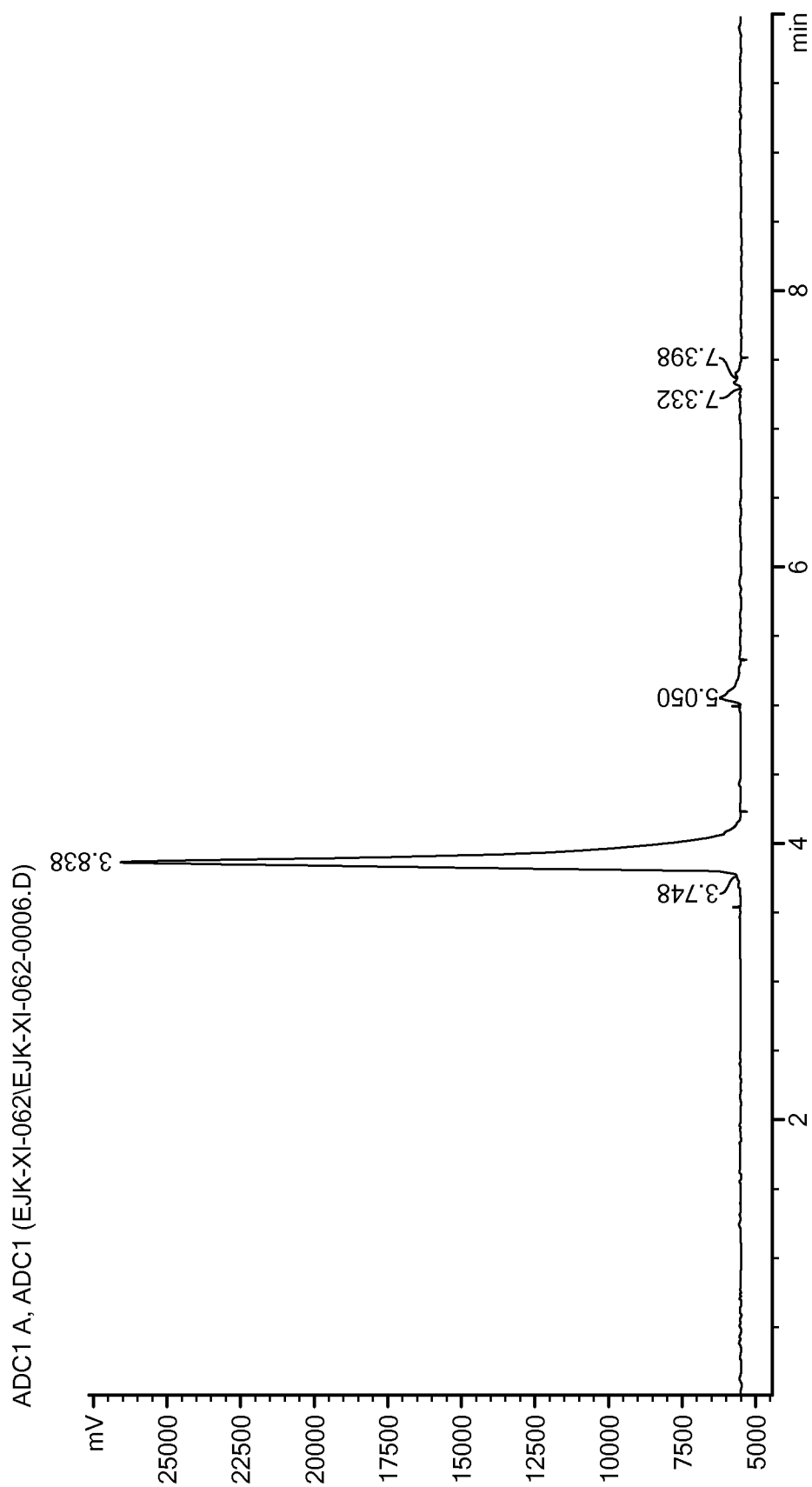
22/42

**FIG. 14**

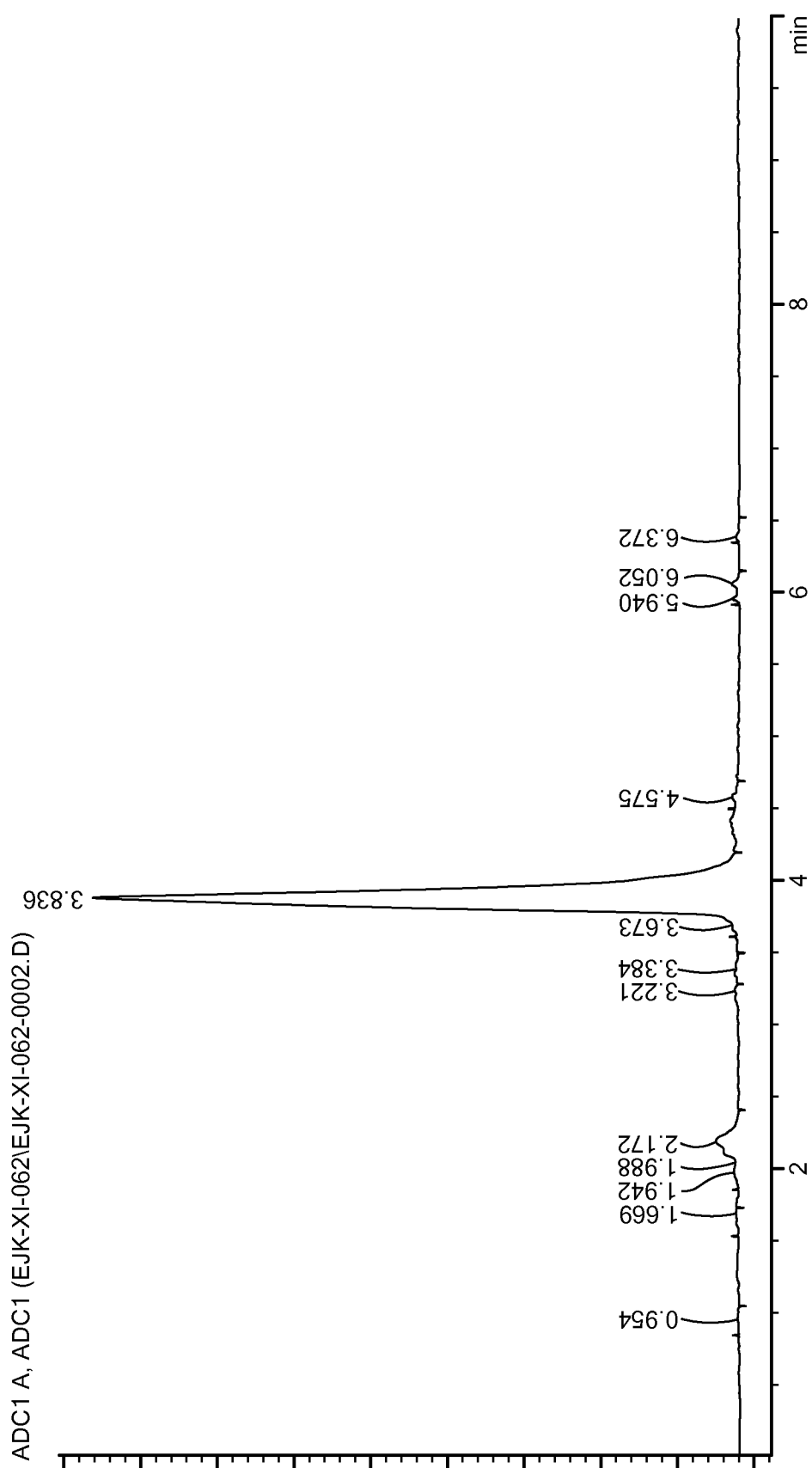
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**FIG. 15**

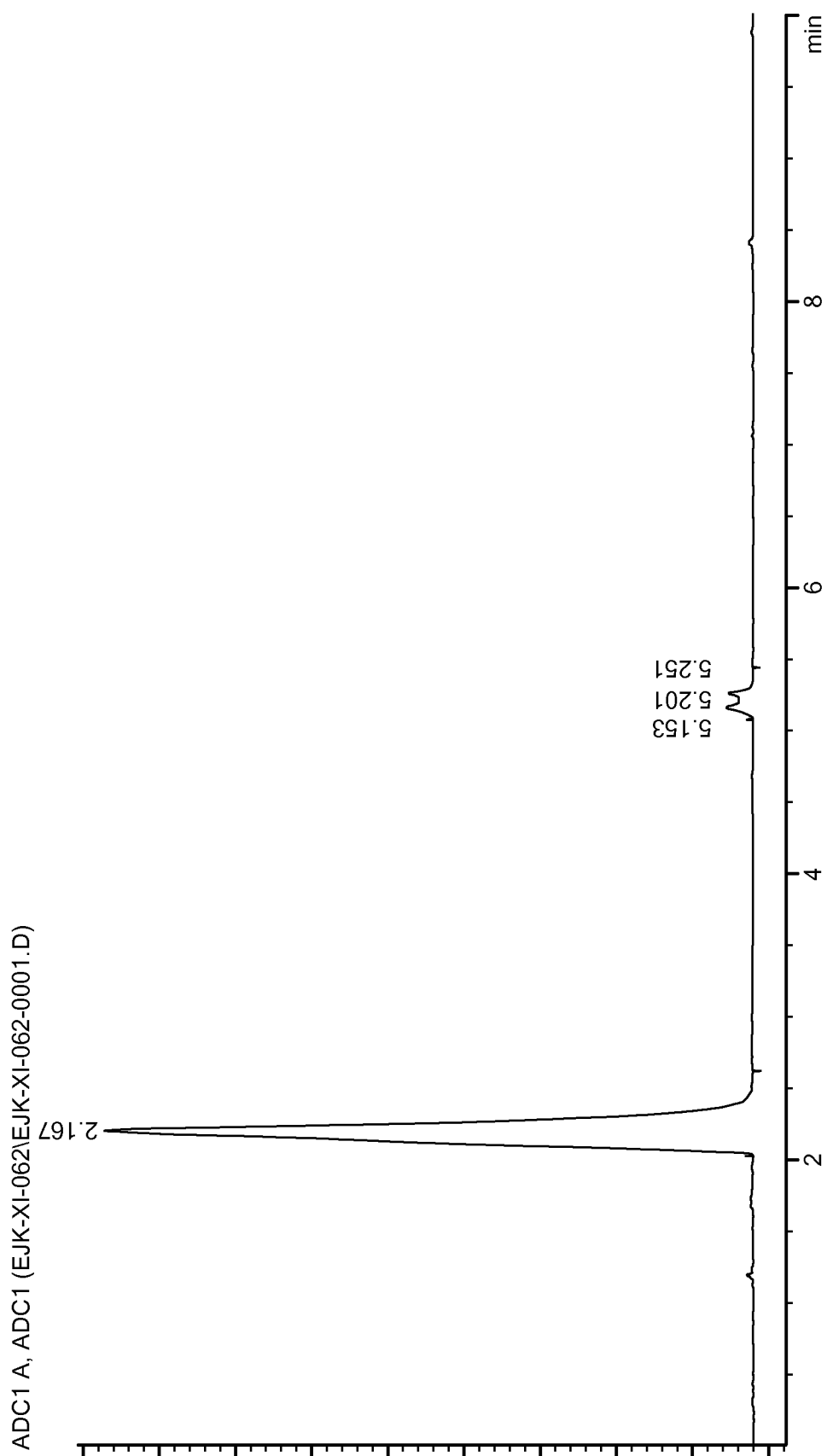
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**FIG. 16**

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**FIG. 17**

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**FIG. 18**

Proton NMR spectrum of N-flouropropyl derivative

STANDARD PROTON PARAMETERS

LD7-38: frac 36

F-propyl-11b-AE

Nov 29, 2012

nmr500/dao

Pulse Sequence: s2pul
Solvent: CDC13
Ambient temperature
INOVA-500 "waters500"

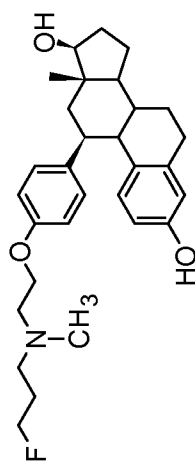
Pulse 42.2 degrees
Acq. time 2.048 sec
Width 8000.0 Hz
258 repetitions

OBSERVE H1, 499.6931877 MHz

DATA PROCESSING

FT size 32768

Total time 34 min, 16 sec



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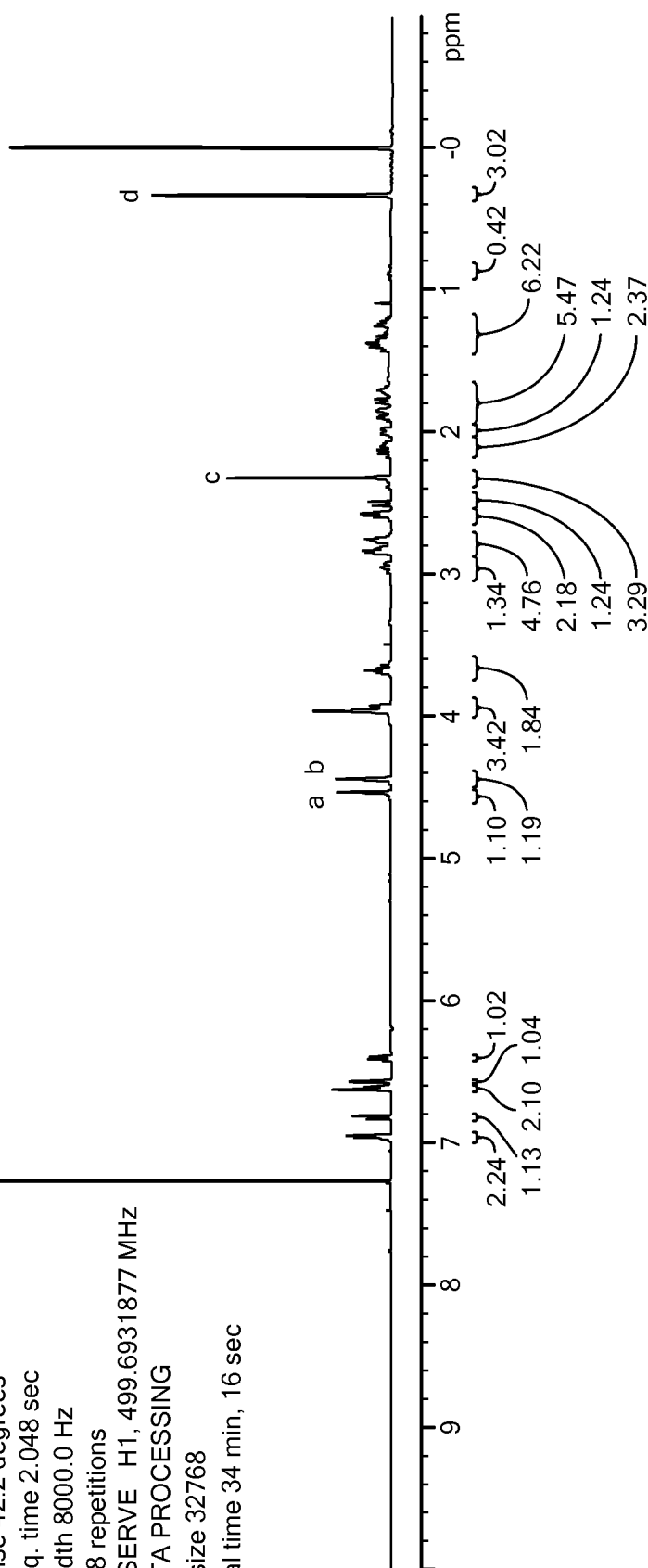


FIG. 19

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STANDARD PROTON PARAMETERS

LD7-38: frac 36

F-propyl-11b-AE

Nov 29, 2012

nmr500/dao

Pulse Sequence: s2pul

Solvent: CDC13

Ambient temperature

INOVA-500 "waters500"

Pulse 42.2 degrees

Acq. time 2.048 sec

Width 8000.0 Hz

292 repetitions

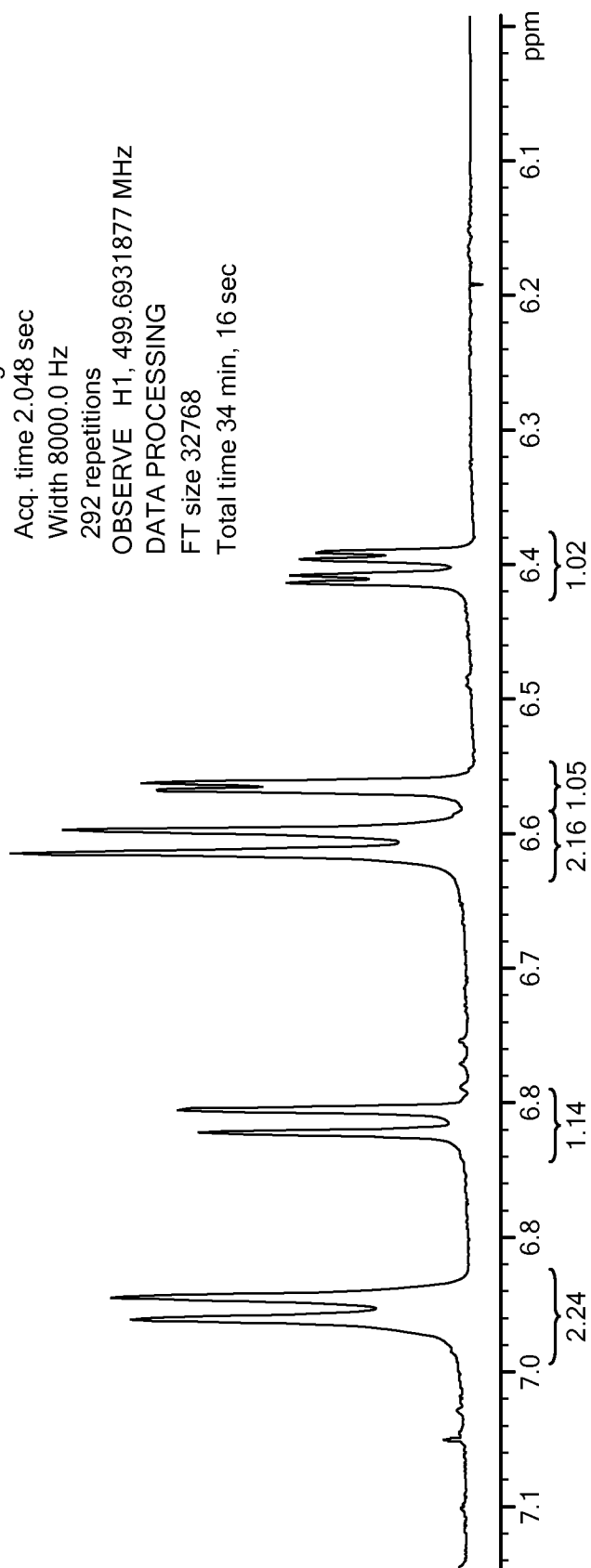
OBSERVE H1, 499.6931877 MHz

DATA PROCESSING

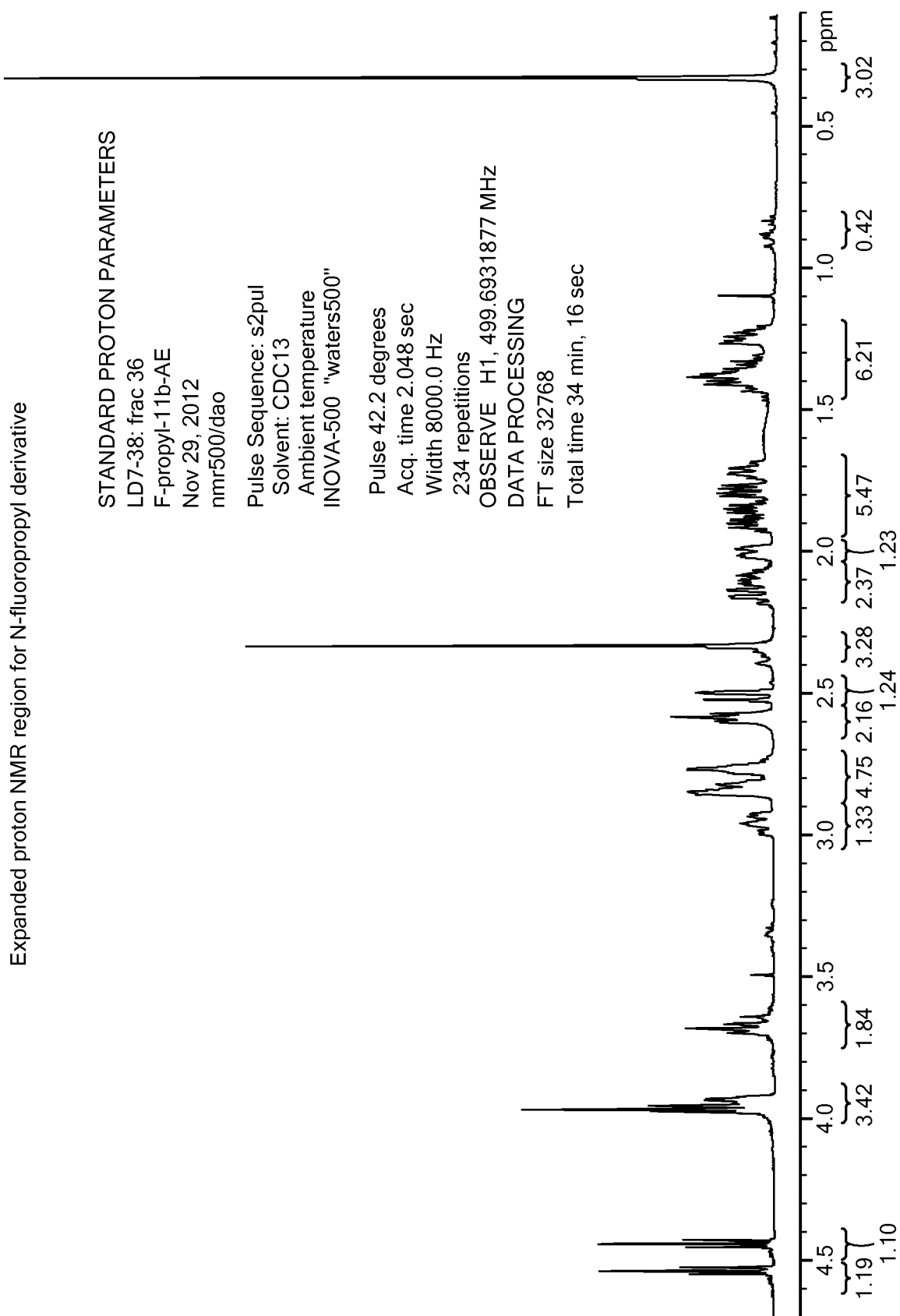
FT size 32768

Total time 34 min, 16 sec

Expanded proton NMR region for N-fluoropropyl derivative

**FIG. 20**

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**FIG. 21**

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Carbon-13 NMR spectrum for N-fluoropropyl derivative

LD7-38

F-propyl-11b-ae

nov 29, 2012

nmr500/dao

Pulse Sequence: s2pul

Solvent: CDC13

Ambient temperature

User: 1-14-87

INOVA-500 "waters500"

Pulse 27.7 degrees

Acq. time 0.964 sec

Width 33999.2 Hz

41800 repetitions

OBSERVE C13, 125.6479222 MHz

DECOUPLE H1, 499.6961872 MHz

Power 34 dB

on during acquisition

off during delay

GARP-1 modulated

DATA PROCESSING

FT size 65536

Total time 27019 hr, 14 min, 40 sec

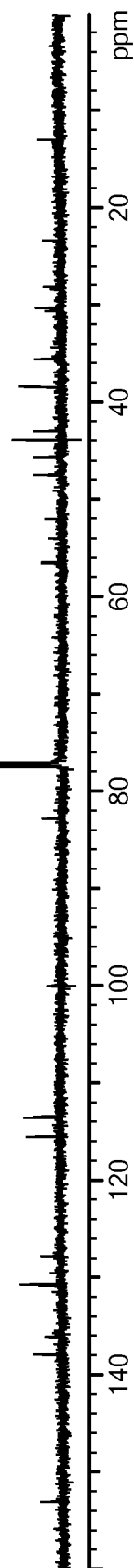
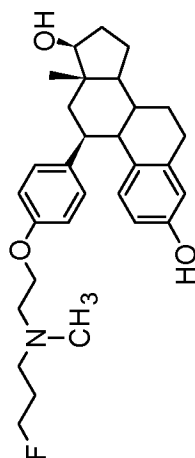
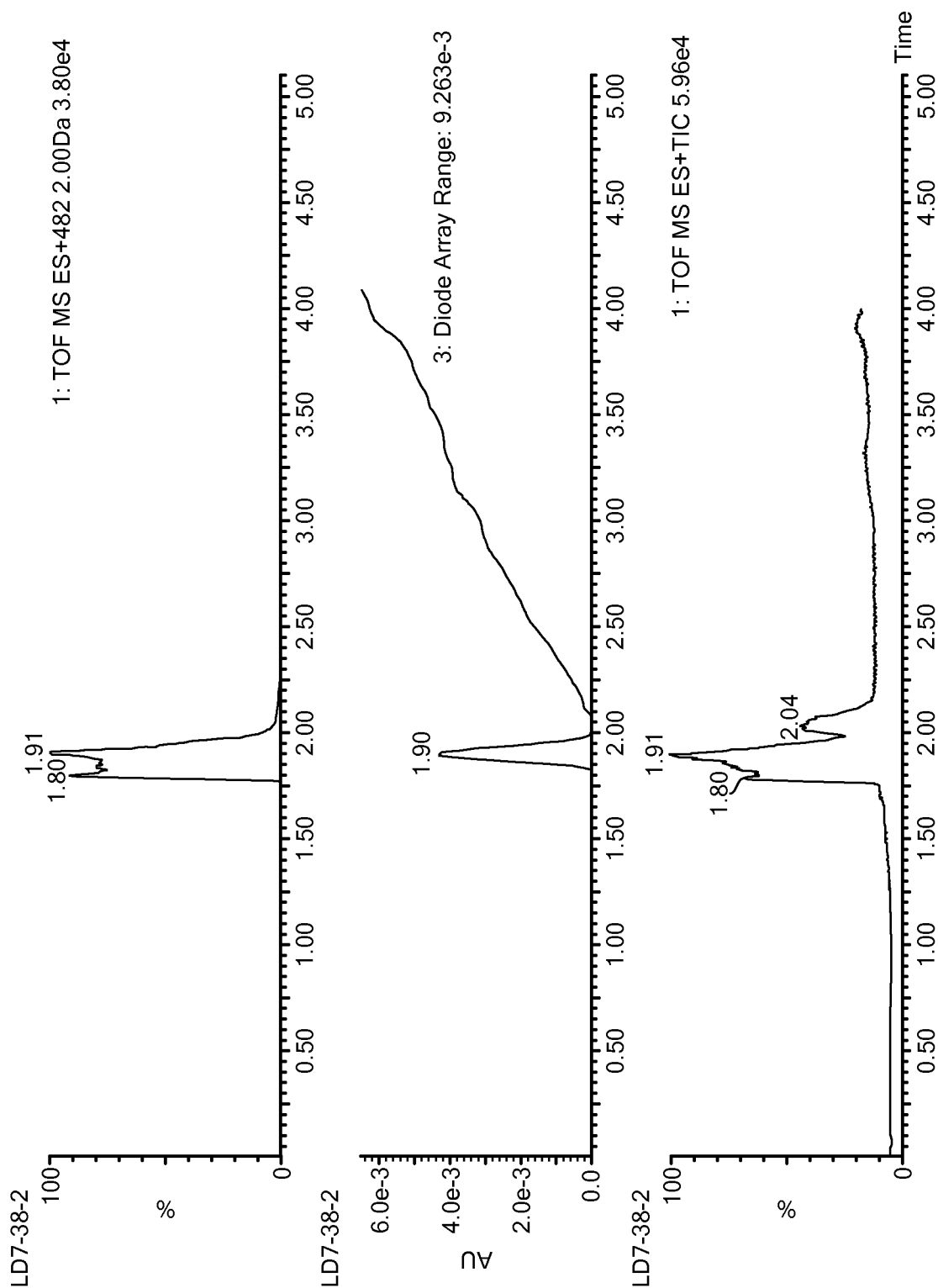


FIG. 22

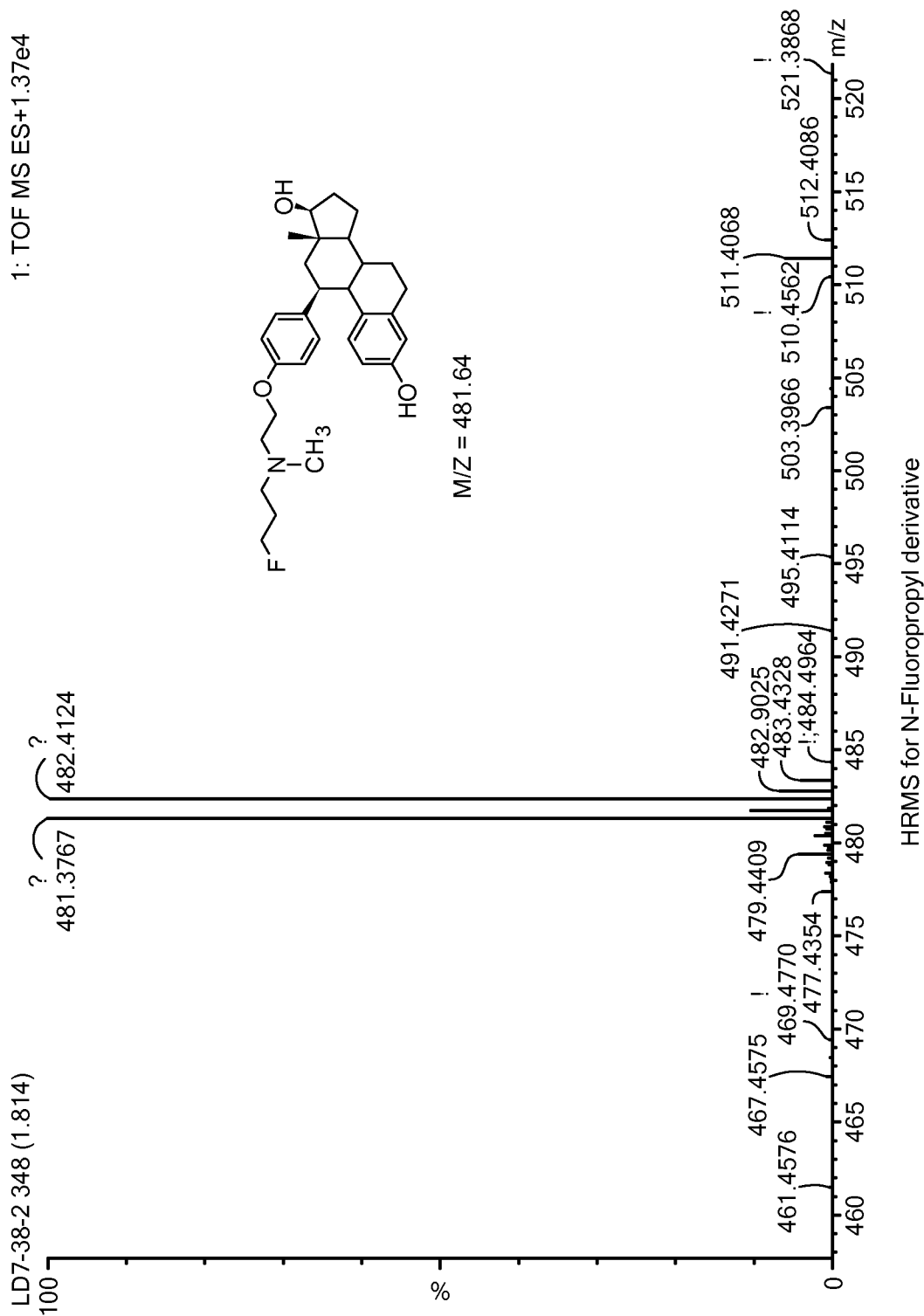
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LC-MS traces for N-fluoropropyl derivative

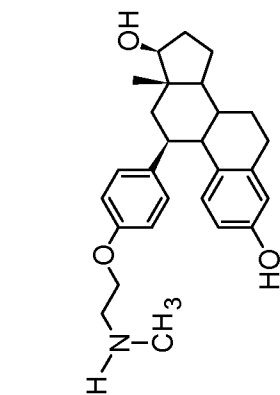
FIG. 23

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**FIG. 24**

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Proton NMR spectrum of N-demethylated antiestrogen



AK-II-92
de-methylated steroid
Pulse Sequence: s2pul
Solvent: CDC13
Ambient temperature
INOV A-500 "waters500"
Pulse 42.2 degrees
Acq. time 2.048 sec
Width 8000.0 Hz
32 repetitions
OBSERVE H1, 499.6931887 MHz
DATA PROCESSING
FT size 32768
Total time 3 min, 25 sec

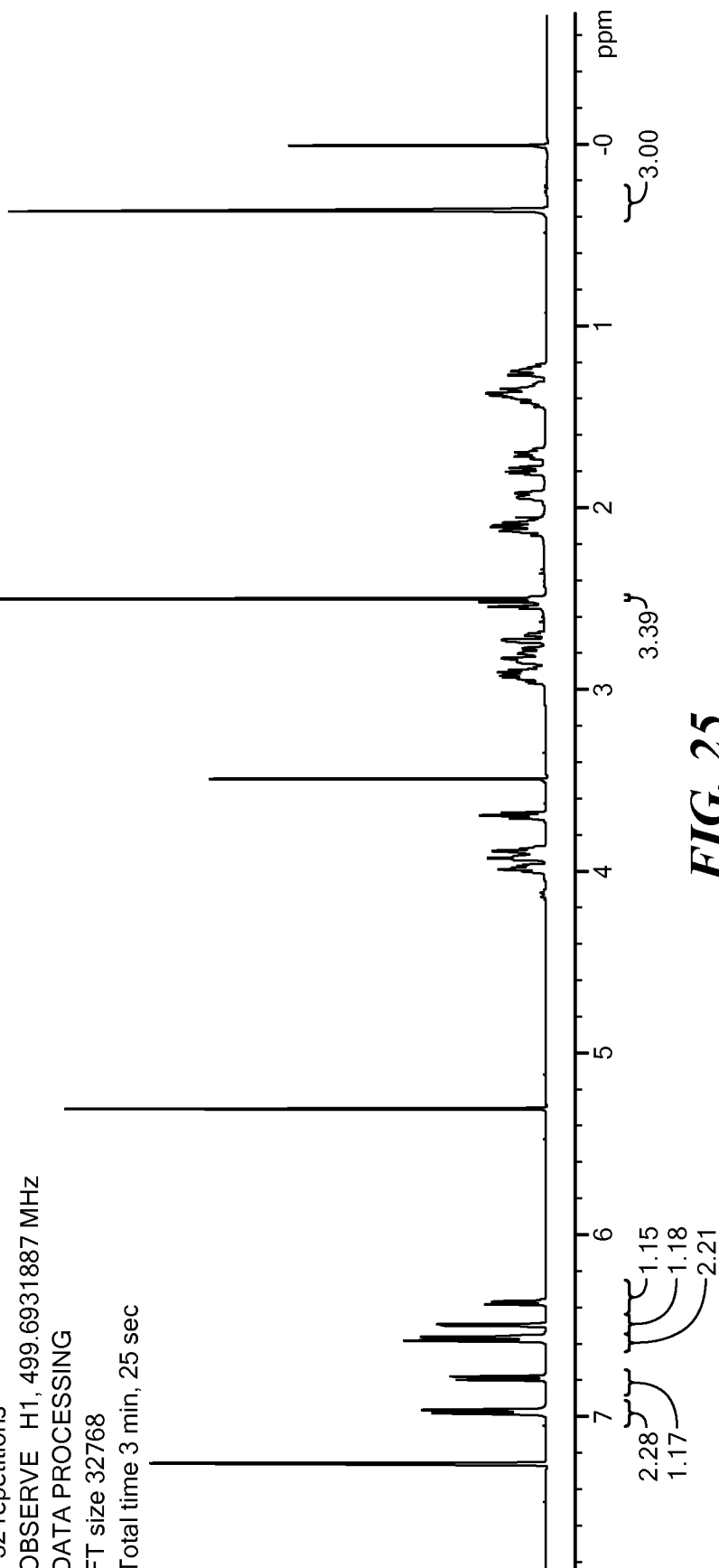
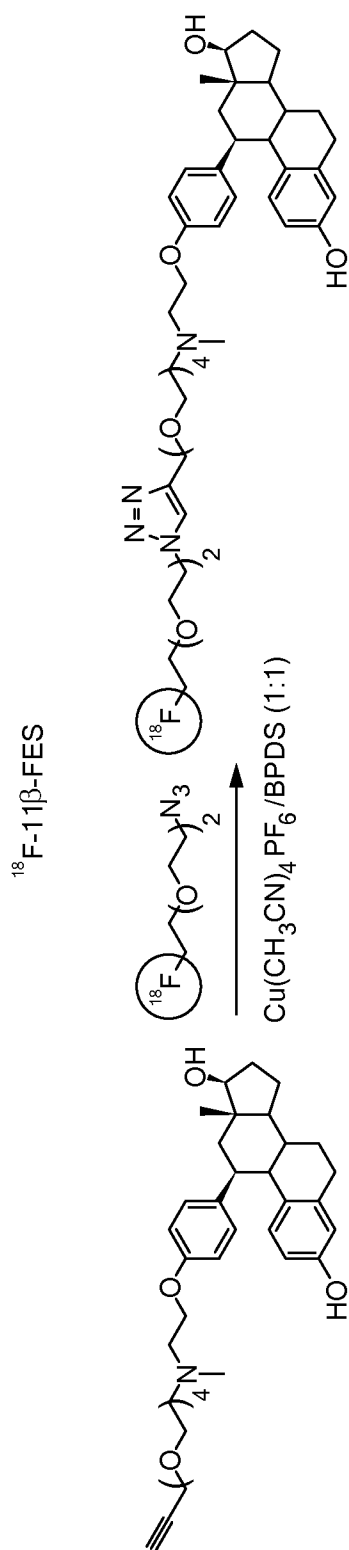
**FIG. 25**



FIG. 26



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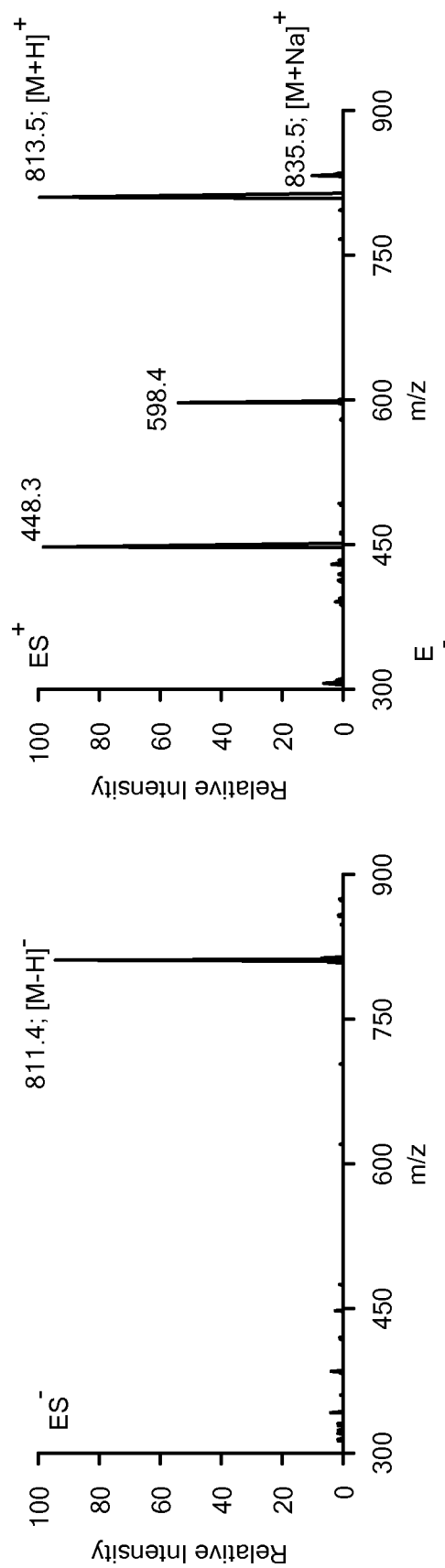
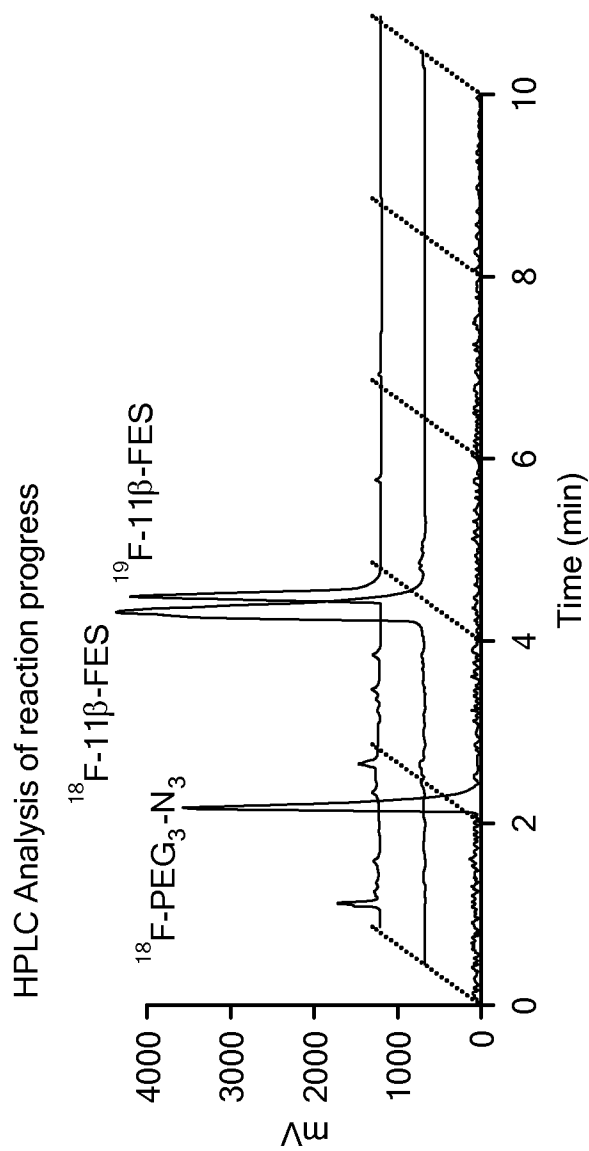
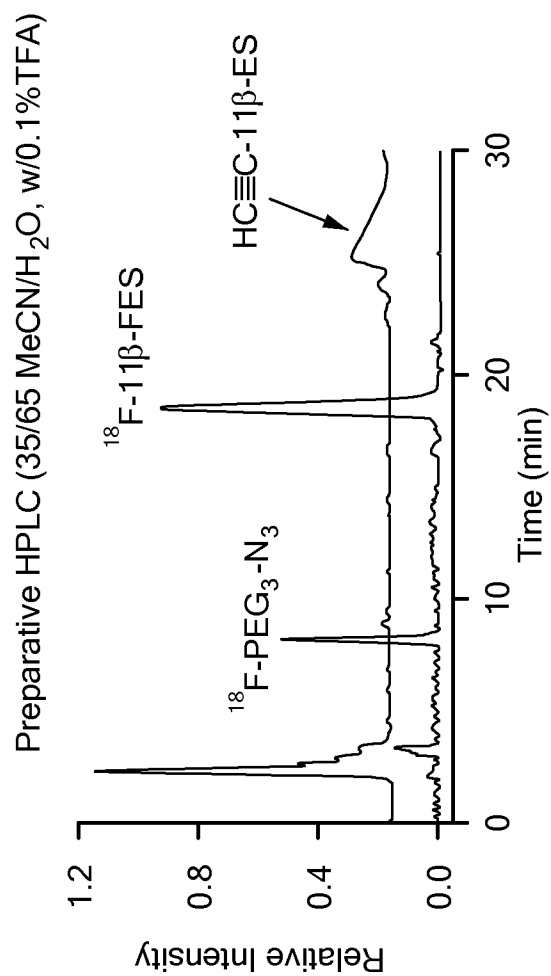
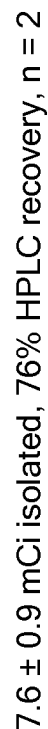


FIG. 27B

FIG. 27A

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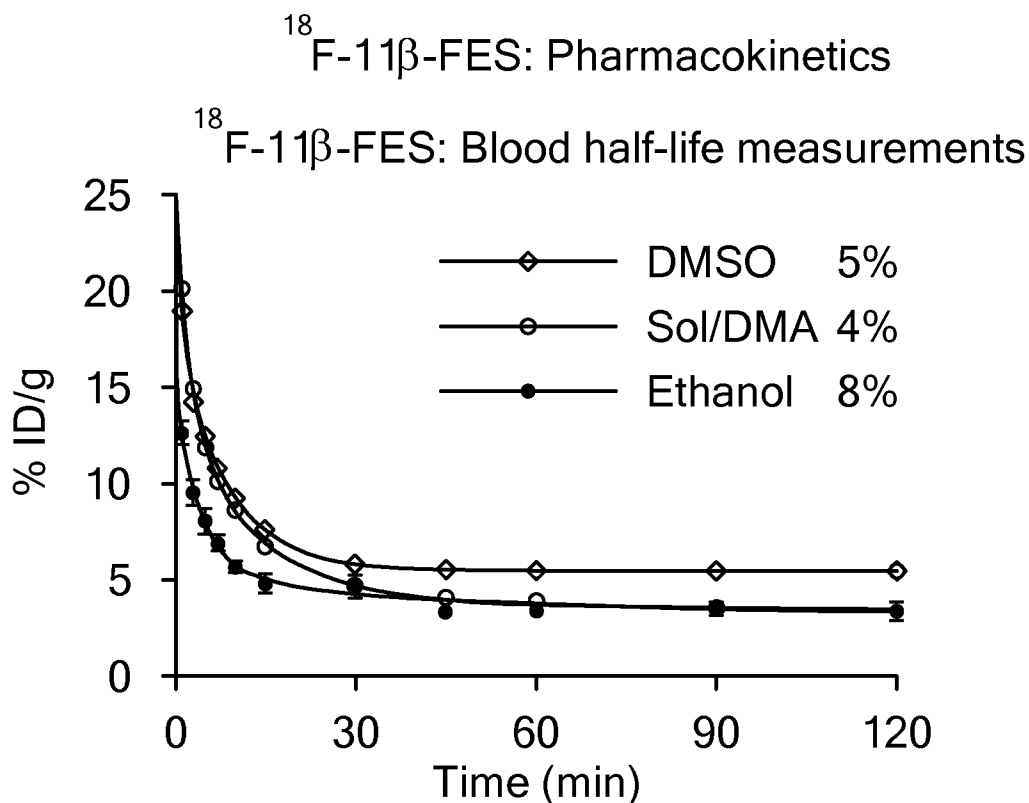
**FIG. 27C****FIG. 27D**



Average Conversion = $89.3 \pm 5.0\%$, $n = 9$

FIG. 28

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**FIG. 29A**

¹⁸F-11β-FES: Pharmacokinetics

Co-solvent in Saline (0.9% NaCl)	Half-life (min)		Percent Fast (%)	Half-life Weighted (min)	Terminal % at 2 h
	Slow	Fast			
DMSO (5%)	5.9	0.4	49.6	3.2	5.3
1:1 Solutol:DMA (4%)	10.1	1.9	54.6	5.6	3.4
Ethanol (8%)	25.1	2.8	79.3	7.4	3.5

n = 2 C57B1/6 mice for each formulation; 36% reduction in terminal concentration using ethanol as co-solvent

FIG. 29B

¹⁸F-11β-FES: Biodistribution

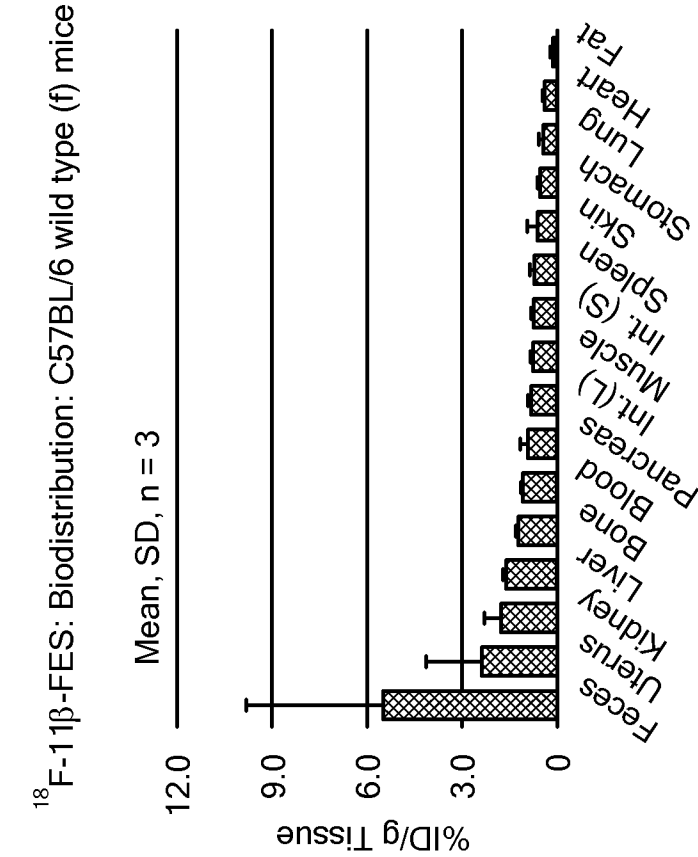


FIG. 29C

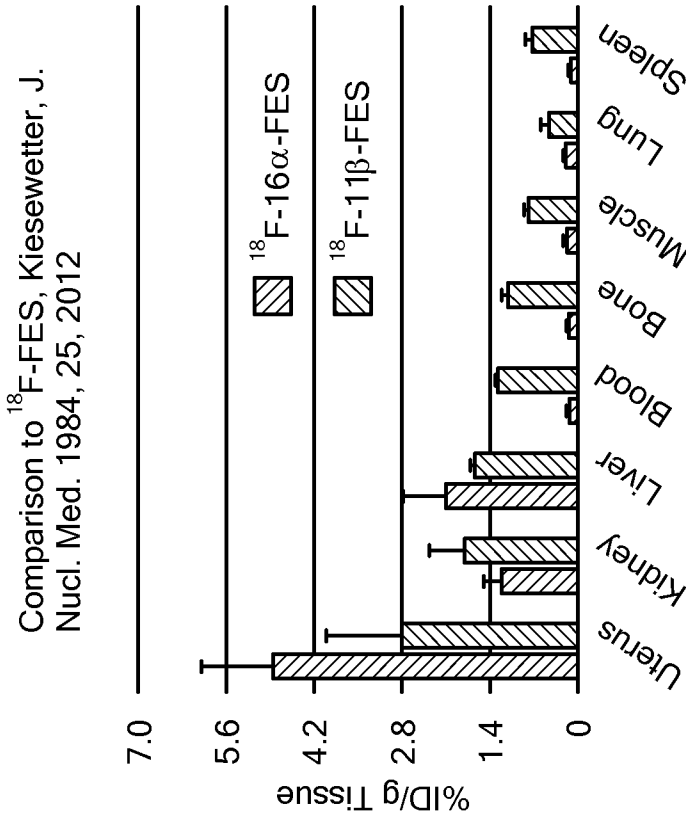


FIG. 29D

¹⁸F-11β-FES: Breast Cancer Model

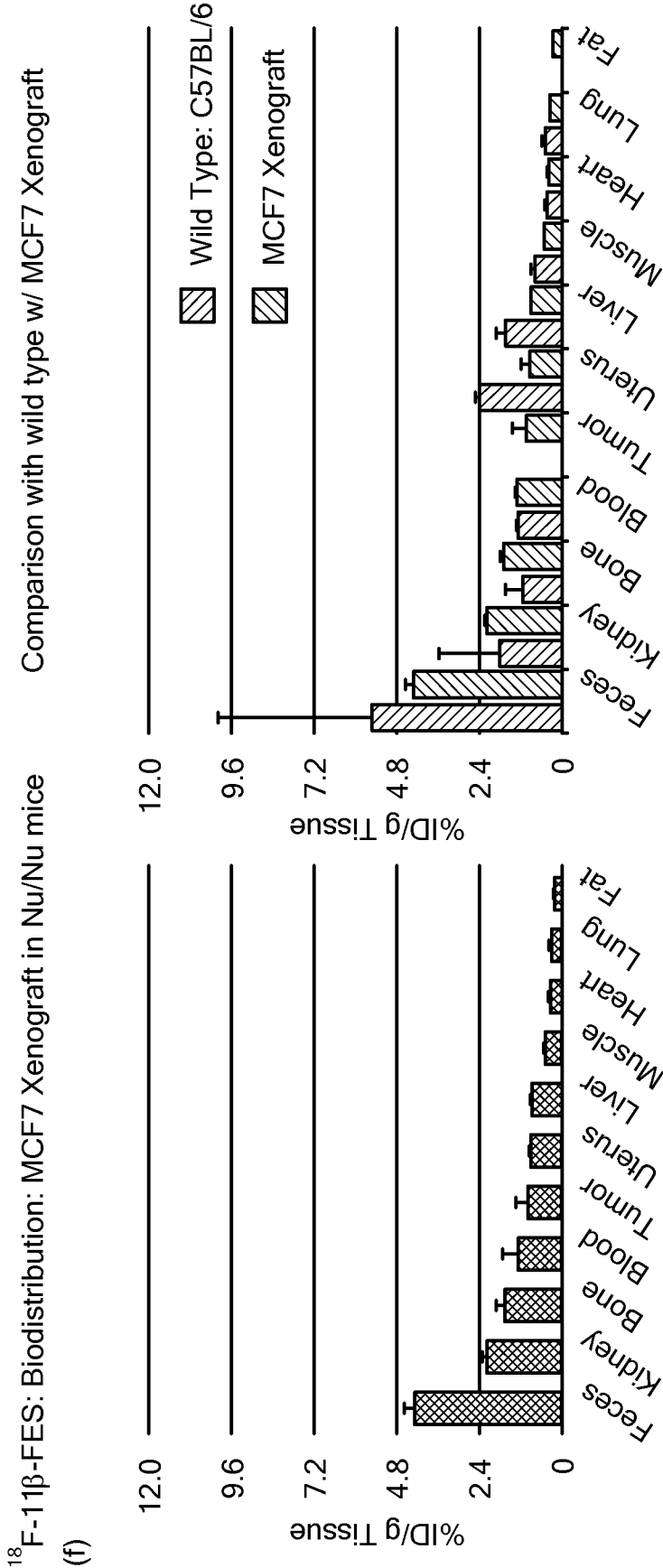


FIG. 30A

FIG. 30B

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¹⁸F-11β-FES: Biodistribution

Organ	Wild Type: C57BL/6		MCF7 Xenograft	
	%ID/g Tissue	St Dev	%ID/g Tissue	St Dev
Feces	5.549	4.492	4.303	0.324
Uterus	2.43	1.813	0.952	0.086
Kidney	1.807	0.590	2.229	0.115
Liver	1.669	0.097	0.924	0.045
Blood	1.280	0.050	1.313	0.489
Bone	1.136	0.118	1.710	0.317
Pancreas	0.977	0.284		
Int. (L)	0.877	0.12		
Muscle	0.810	0.092	0.509	0.035
Int. (S)	0.795	0.117		
Spleen	0.764	0.138		
Skin	0.658	0.344		
Stomach	0.585	0.040		
Lung	0.485	0.161	0.345	0.113
Heart	0.449	0.027	0.395	0.031
Fat	0.178	0.018	0.243	0.083
Tumor			1.036	0.426

FIG. 30C

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^{18}F -11 β -FES: 2 h dynamic PET scan, C57BL/6 mouse

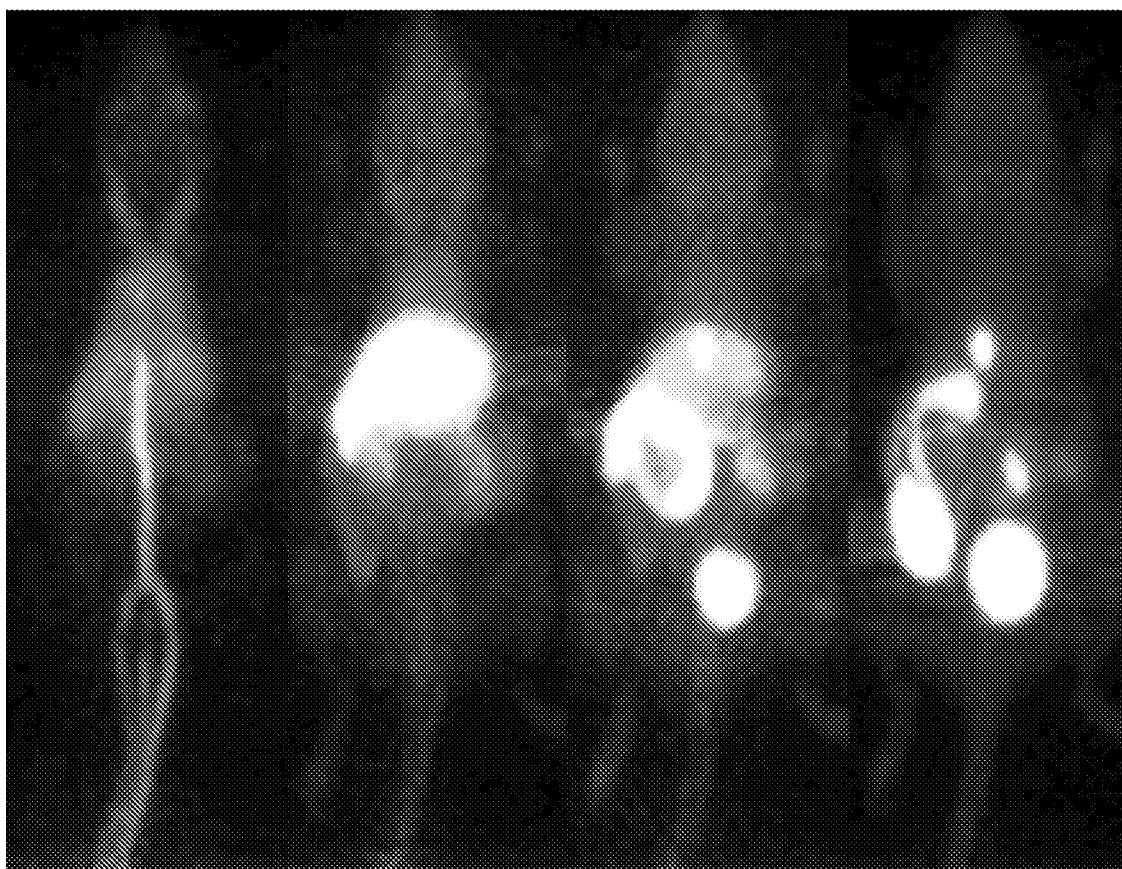


FIG. 31

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/074183

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07J 41/00 (2014.01)

USPC - 552/626

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C07J 41/00 (2014.01)

USPC - 552/625, 626

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C07J 41/00, 41/0083 (2014.02)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, Orbit, STN, PubChem, Google Scholar

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DAO et al. Design, Synthesis, and Initial Biological Evaluation of a Steroidal Anti-estrogen-Doxorubicin Bioconjugate for Targeting Estrogen Receptor-Positive Breast Cancer Cells. Bioconjug. Chem. 23(4):785-795, 18 April 2012. [retrieved on 18 March 2014]. Retrieved from the Internet. <URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3335367/pdf/nihms368734.pdf > entire document	1-3, 5, 7, 9-14,16, 18-32
A	US 2004/0229853 A1 (PRAT et al) 18 November 2004 (18.11.2004) entire document	1-3, 5, 7, 9-14,16, 18-32
A	WO 2010/085747 A1 (HANSON) 29 July 2010 (29.07.2010) entire document	1-3, 5, 7, 9-14,16, 18-32
A	US 2004/0171595 A1 (BENEDETTI et al) 02 September 2004 (02.09.2004) entire document	1-3, 5, 7, 9-14,16, 18-32

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 March 2014

Date of mailing of the international search report

15 APR 2014

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/074183

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4, 6, 8, 15, 17, 33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.