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Synthesis and characterization of radiolabeled 17β-estradiol conjugates

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The use of radioactive tracers for environmental fate and transport studies of emerging contaminants, especially for those that are labile, offers convenience in tracking study compounds and their metabolites, and in calculating mass balances. The aim of this study was to synthesize radiolabeled glucuronide and sulfate conjugates of 17β-estradiol (17β-E2). The conjugates 17β-[4-^{14}C]estradiol-3-glucuronide ([^{14}C]17β-E2-3-G) and 17β-[4-^{14}C]estradiol-17-sulfate ([^{14}C]17β-E2-17-S) were synthesized utilizing immobilized enzyme and chemical syntheses, respectively. Microsomal proteins from the liver of a phenobarbital induced pig (*Sus scrofa domestica*) were harvested and used to glucuronidate [^{14}C]17β-E2. Synthesis of [^{14}C]17β-E2-17-S consisted of a three-step chemical process – introducing a blocking group at the C-3 position of [^{14}C]17β-E2, sulfation at C-17 position, and subsequent deblocking to yield the desired synthetic product. Successful syntheses of [^{14}C]17β-E2-3-G and [^{14}C]17β-E2-17-S were achieved as verified by liquid chromatography, radiochemical analyses, quadrupole-time-of-flight (QTOF) mass spectrometry, and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. Radiochemical yields of 84 and 44% were achieved for 17β-E2-3-G and 17β-E2-17-S, respectively. Synthetic products were purified using high-performance liquid chromatography and radiochemical purities of 98% or greater were obtained.

Keywords: 17β-estradiol; immobilized enzyme; conjugation; glucuronide; sulfate; steroid hormone

Introduction

Medical research has used radiolabeled estrogenic compounds to study breast and uterine cancers,[1] estrogenic receptors,[2] and as imaging agents in breast tumors.[3] More recently though, the radiolabeled hormonal compounds (e.g. [^{14}C]estradiol, [^{14}C]estrone, and [^{14}C] testosterone[4–8] and 6,7-^{3}H-estradiol[6]) have been used to study the fate and transport of steroids in the environment. Exposure to exogenous reproductive hormones has been associated with adverse effects in certain aquatic[9–11] and terrestrial[12–14] species. Human waste treatment and animal feeding operations (AFOs) are sources of estradiol (E2), estrone (E1), and estriol (E3) to the environment. Estradiol is the most potent of these natural estrogens.[15–18]

Laboratory studies suggest estrogens should have little to no mobility and should not persist in the environment because they bind rapidly and strongly to soil and degrade within hours.[4,8,19] Field studies, however, have indicated that estrogens are present in the environment at frequencies and concentrations that imply they are moderately mobile and persistent.[20,21] Estrogen conjugates, which have different water solubilities, sorption coefficients, and degradation rates relative to their 'free' estrogen counterparts, may offer insights into why steroidal estrogens are frequently detected in the environment.[20] Swine (*sus scrofa domesticus*), poultry (*gallus domesticus*), and cattle (*bos taurus*) excrete 96, 69, and 42%, respectively, of the estrogens as conjugates.[22] In fact, appreciable amounts of 17β-estradiol (17β-E2) in conjugated forms have been measured in swine manure slurry (liquid urine and feces) from AFO manure storage lagoons.[23] Conjugates form a major portion of total environmental estrogen loading from AFOs and might play a significant function in the detections of 'free' steroidal estrogens in the environment. Although, estrogen conjugates are biologically inactive,[24] they can potentially be cleaved by microbial enzymes to form the more potent parent compound.[24]

Conjugation reactions are a common vertebrate mechanism in which hormones, drugs, toxicants, and non-nutritive organic molecules are eliminated.[25] During conjugation a charged, polar moiety is attached to a hydrophobic compound (e.g. estrogen), which increases its water solubility and excretion in urine or bile. Estrogens are typically conjugated with glucuronic or sulfuric acid at the C-3 and/or the C-17 positions[24] (Schemes 1 and 2). Glucuronidation of estrogen is catalyzed by uridine 5′-diphospho-glucuronosyltransferase (UGT) enzymes in the endoplasmic reticulum and sulfation is catalyzed by cytosolic sulfotransferases (SULTs).[26]

The environmental fate of estradiol conjugates has not been extensively studied, possibly because radiolabeled conjugates are not commercially available. The availability of radiolabeled conjugated hormones would enable studies to be conducted that would improve the understanding of the fate and transport of these labile compounds in the environment. The objective of

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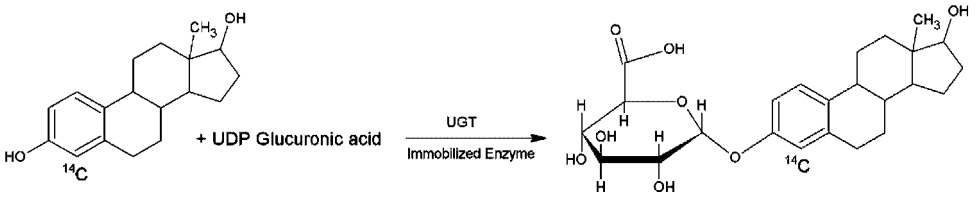
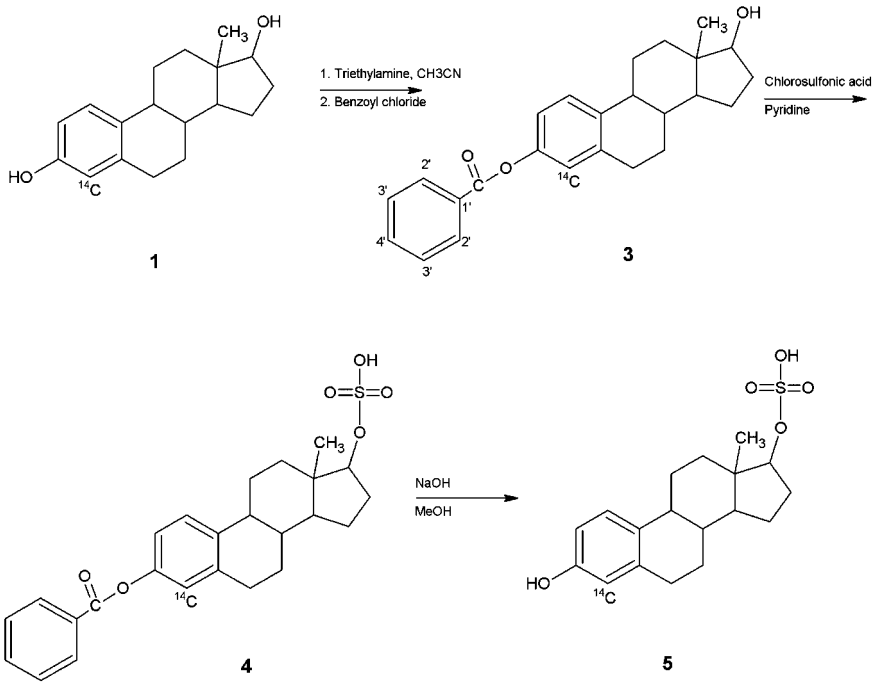


1 2

Scheme 1. Glucuronidation of the hydroxyl group at C-3 of 17-β-estradiol by uridine 5′-diphospho-glucuronosyltransferase (UGT).



1 3 4 5

Scheme 2. Chemical synthesis of [^{14}C]17β-estradiol-17-sulfate conjugate from [^{14}C]17β-estradiol.

this paper is to provide a method to synthesize carbon-14-labeled 17β-E2-3-G and 17β-E2-17-S.

Experimental

Materials

[^{14}C]17β-E2 (55 mCi/mmol) (**1**) was purchased from American Radiolabeled Chemicals (St Louis, MO). Unlabeled **1**, UDP glucuronic acid, magnesium chloride, ethanol, potassium phosphate monobasic, potassium phosphate dibasic, potassium hydroxide, hydrochloric acid, ethyl acetate, pyridine, sodium hydroxide, chlorosulfonic acid, and acetic acid were obtained from Sigma-Aldrich. Triethylamine (Fluka); benzoyl chloride (Bayer); trisodium phosphate (Mallinkrodt, Paris, KY) were obtained from other sources. Acetonitrile (ACN) was obtained from EMD Chemicals (Gibbstown, NJ). Scintillation fluid EcoLite™ was obtained from MP Biomedicals (Santa Ana, CA). SPE cartridges Bond Elut™ C18 (6 g, 20 mL) and Sep-Pak® Vac C18 were obtained from Varian (Harbor City, CA) and Waters (Milford, MA), respectively.

Uridine 5′-diphospho-glucuronosyltransferase (UGT)

A castrated, cross-bred hog weighing 24.4 kg was used as the source of the UGT enzymes, following USDA Animal Care and Use Committee guidelines. The hog was intramuscularly (2 d) then intraperitoneally dosed (2 d) with approximately 20 mg/kg phenobarbital for 4 consecutive days, after which, the hog was euthanized. The liver was homogenized and microsomes were isolated via differential centrifugation. Proteins were solubilized and immobilized onto Sepharose beads[27] and were stored in a 1:1 suspension with 0.1 M Tris buffer (pH 7.4) at 4°C until use.

Liquid scintillation counting

Radioactivity was quantitated with a Packard 1900 CA scintillation analyzer (Downers Grove, IL), and samples were dissolved in EcoLite™ scintillation cocktail.

High-performance liquid chromatography

Analytical high-performance liquid chromatography (HPLC) for **2** was performed using a Waters 600E System Controller and pump (Milford, MA), equipped with a Jasco FP 920 fluorescence detector (Jasco, Easton, MD) with the following conditions: Phenomenex-C18, 4.6 × 250 mm, 5 μm; A: 10% ACN in 50 mM ammonium acetate (pH 4.5), B: 90% ACN in 50 mM ammonium acetate (pH 4.5); gradient: 20–100% B, 29 min, 100% B, 3 min

hold, 1.0 mL/min, excitation and emission wavelengths of 280 and 312 nm, respectively. Prep-HPLC was performed on Jones Chromatography-C18, 10 × 250 mm, 5 μm; A: 5% ACN in 50 mM ammonium acetate (pH 4.5), B: 90% ACN in water; isocratic 85% solvent A, 15% solvent B; 4.7 mL/min.

For **5**, analytical HPLC was performed on a Gilson System 45NC Gradient Analytical instrument (Gilson Medical Electronics, Middleton, WI) equipped with a variable wavelength UV detector with the following conditions: Radial-Pak-C18, 8 × 100 mm (Waters Associates, Milford, MA); A: 10:90 methanol/water, B: 90:10 methanol/water; gradient: 20% B to 100% B, 28 min., 4 min hold; 1.0 mL/min 220 nm. HPLC for **3** was conducted using following conditions: Radial-Pak-C18, 8 × 100 mm; A: 10:90 methanol/water, B: 90:10 methanol/water; gradient: 20% B to 100% B, 30 min, 15 min hold; 1.0 mL/min, UV 220 nm.

Mass spectral analysis

Negative ion LC/MS was performed with a Waters Alliance 2695 HPLC (Symmetry-C18, 2.1 × 100 mm; A: 40% ACN in water, B: 60% ACN in water; gradient: 40–100% B, 10 min, 5 min hold, 0.2 mL/min), and a Waters Micromass QTOF (API-US in an ES-mode, MassLynx software, FWHM: 6500, source temperature 120°C, desolvation temperature 350°C, cone voltage 35 V, capillary voltage 2500 V, collision energy 5 eV for sulfate and 20 eV for glucuronide conjugates.

NMR spectra

A Bruker AM-400 spectrometer (Billerica, MA) operating at either 400.13 MHz or 100.61 MHz was used to record the ^{1}H and ^{13}C NMR spectra, respectively. ^{1}H NMR spectra were run in fully coupled mode with 128 scans and an acquisition time of 3.9713 s. ^{13}C NMR spectra were run in CPD mode, with 64K scans obtained with an acquisition time of 1.307 s. The chemical shifts for the NMR spectra for **1** were ^{13}C NMR (MeOH-d_4)δ: 155.84, 138.8, 132.32, 127.22, 116.05, 113.72, 82.49, 51.26, 45.34, 44.35, 40.5, 38.00, 30.72, 30.68, 28.83, 27.53, 24.03, 11.71; ^{1}H NMR (MeOH-d_4)δ(aromatic A-ring protons): 7.06 (d), 6.53 (d), 6.47 (s).

Synthesis of 17β-[4-^{14}C]estradiol-3-glucuronide (2)

Five millilitres of 0.1 M phosphate buffer (pH 7.4) was added to 20 mL of pre-rinsed microsomal proteins immobilized on Sepharose beads. Forty microlitres of 2.63 M magnesium chloride, 63 mg of UDP glucuronic acid (5 mM final concentration), and 164.7 μg of [^{14}C] labeled **1** (0.60 μmol; 33 μCi; dissolved in 567 μL ethanol) and 6477 μg of unlabeled **1** (23.78 μmol, dissolved in 540 μL ethanol) were added to the reaction flask. The reaction flask was slowly stirred on a Roto-Vap (Büchi, Flawil, Switzerland) without vacuum at 37°C for 24 h determined *a priori*. The aqueous fraction was collected by filtration. **2** was partially purified on a Bond Elut™ C18 SPE cartridge preconditioned with ACN and nanopure water by eluting with 20:80 ACN-water. The final radiochemical purity was 99% obtained after preparative HPLC. ^{13}C NMR (MeOH-d_4)δ: 176.52, 156.99, 135.66, 127.20, 117.96, 115.41, 102.65, 82.47, 77.71, 76.68, 74.74, 73.59, 51.26, 45.41, 44.32, 40.34, 38.97, 37.97, 30.69, 30.04, 28.40, 27.51, 24.00, 11.67. ^{1}H NMR (MeOH-d_4)δ(aromatic A-ring protons): 7.18 (d), 6.87 (d), 6.81 (s). LC/MS-QTOF: M-H = 447.21, *m/z* 271.17, 175.03, 113.02.

Synthesis of 17β-[4-^{14}C]estradiol-17-sulfate (5)

*[^{14}C]17β-estradiol-3-benzoate (**3**)*

Radiolabeled **1** (259.5 μg, 0.95 μmol, 47.7 μCi) was mixed with unlabeled **1** (11.43 mg, 42 μmol) in ethanol and the solvent was evaporated.[28] The residue was re-dissolved in 2 mL of acetonitrile, and 13 μL triethylamine and 11 μL benzoyl chloride (13.3 mg, 94.7 μmol) were added; the reaction mixture was stirred at room temperature for 2 h and subsequently dried under a stream of nitrogen. To the white residue, 4 mL of 0.1 M trisodium phosphate solution was added and the mixture was sonicated for 30 min resulting in a light yellow suspension. The suspension was extracted with ethyl acetate (3 mL × 3), and the organic solvent was evaporated under a stream of nitrogen. The residue (**3**) was dissolved in ethyl acetate (3 mL) and water (1 mL) for further purification using HPLC. The yield of **3** was 59.3% and radiochemical purity was 98%. ^{13}C NMR (MeOH-d_4)δ: 165.94, 150.13, 139.49, 139.38, 134.86, 130.99, 130.99, 129.83, 129.83, 127.47, 122.63, 119.79, 82.45, 51.33, 45.55, 44.34, 40.14, 37.99, 30.69, 30.56, 28.27, 27.48, 24.04, 11.68. ^{1}H NMR (MeOH-d_4) δ(aromatic A-ring protons): 7.44 (d), 6.94 (d), 6.89 (d); δ(benzoate protons): 8.14 (d), 7.66 (dd), 7.54 (dd). LC/MS-QTOF: M-H = 375.21, *m/z* 361.21, 356.85, 334.82, 332.82.

*[^{14}C]17β-estradiol-3-benzoate-17-sulfate (**4**)*

Sulfur trioxide-pyridine complex was synthesized in-house[29] by adding chlorosulfonic acid (138 μL, 2.07 μmol) with stirring to dry pyridine (1.66 mL) at 0°C. The solution was allowed to warm to room temperature, followed by dilution with dry pyridine (623 μL). **3** was dissolved into 1.1 mL of pyridine, and the solution was heated to 50°C, to which sulfur trioxide-pyridine complex, also heated to 50°C, was added. The mixture was stirred for 30 min at 50°C followed by solvent evaporation under nitrogen, addition of water (4 mL), and adjusting to pH 8 (1 M NaOH). The mixture was partially purified with a Sep-Pak® Vac C18 cartridge and **4** eluted with methanol. ^{13}C NMR (MeOH-d_4)δ: 166.95, 150.11, 139.42, 139.27, 134.95, 130.85, 130.85, 129.89, 129.89, 127.52, 122.64, 119.84, 88.22, 50.78, 45.41, 44.23, 40.29, 37.98, 30.53, 29.22, 28.18, 27.47, 24.11, 12.24. ^{1}H NMR (MeOH-d_4)δ(aromatic A-ring protons): 7.30 (d), 6.90 (d), 6.86 (s); δ(benzoate protons): 8.13 (d), 7.66 (dd), 7.52 (dd). LC/MS-QTOF: M-H = 455.10, *m/z* 351.12.

*17β-[4-^{14}C]estradiol-17-sulfate (**5**)*

Hydrolysis[30] of **4** was accomplished with 5% NaOH in methanol (5 mL) added to **4,** stirring for 1 h at room temperature, then neutralization with 10% acetic acid, and evaporation under nitrogen. After purification by HPLC, 21 μCi (18.9 μmol; 7.1 mg; 98% pure) of **5** was obtained (overall yield: 44%). ^{13}C NMR (MeOH-d_4)δ: 155.89, 138.76, 132.54, 127.26, 116.04, 113.76, 88.19, 50.78, 45.3, 44.24, 40.34, 38.00, 30.71, 29.22, 28.48, 27.48, 24.10, 12.19. ^{1}H NMR (MeOH-d_4)δ(aromatic A-ring protons): 7.06 (d), 6.53 (d), 6.47 (s). LC/MS-QTOF: M-H = 351.07, *m/z* 96.96.

Results and discussion

Synthesis of 17β-[4-^{14}C]estradiol-3-glucuronide (2)

A one-step enzymatic synthesis of **2** is described that permitted regioselective attachment of a glucuronide acid moiety to **1**.

As the reaction occurred in a buffered solution, reaction progress (Scheme 1) could be readily followed by reversed-phase HPLC. The radiolabeled parent peak (**1**) at 27.57 min dropped steadily in intensity, while the increase in peak intensity at 5.55 min occurred for the desired product (**2**) (Figure 1). The reaction was essentially complete by 24 h. C-18 SPE purification yielded a radiochemical purity of 95%; semipreparative HPLC improved radiochemical purity to 99%. LC/MS-QTOF analysis of **2** showed ions at *m/z* 447.21, 271.17, 175.03, and 113.02, representing the molecular ion of **2** and ions of **1**, glucuronic acid, and a glucuronide fragment, respectively.

To determine the site of conjugation, ^{13}C nuclear magnetic resonance (NMR) spectra of **1** and **2** were compared with each other and with literature values of **1**[31,32] and bisphenol A glucuronide.[33] Glucuronidation was indicated by the presence of an additional six carbons in the ^{13}C NMR spectrum of **2**; and the site of conjugation was indicated by the downfield shift of C-3 from 132.32 to 135.66 ppm in the spectrum of **2**. Chemical shifts in the ^{1}H NMR spectrum of **2** were also consistent with glucuronidation at C-3. For example, protons ortho and meta to C-3 were shifted downfield 6.53–6.87, 7.06–7.18, and 6.47–6.81 ppm for **1** and **2**, respectively. In addition, as one of the most diagnostic components of a sugar conjugated spectrum, an anomeric singlet at 4.30 ppm also confirms the formation of **2**. Chemical shift assignments for the C-17 remained invariant for **1** and **2**.

Diglucuronide conjugation was theoretically possible due to two hydroxyl groups in **1**, one a phenolic in the A-ring, and the other an aliphatic on the D-ring. However, only one site of conjugation was expected because enzyme-catalyzed reactions are usually regiospecific and stereospecific.[34] UGT enzymes are divided into two distinct subfamilies, UGT1 and UGT2.[35,36] Phenobarbital treatment of hepatoma cell lines is known to induce hepatic bilirubin UGTs,[37] which show a strong selectivity for phenolics.[38]

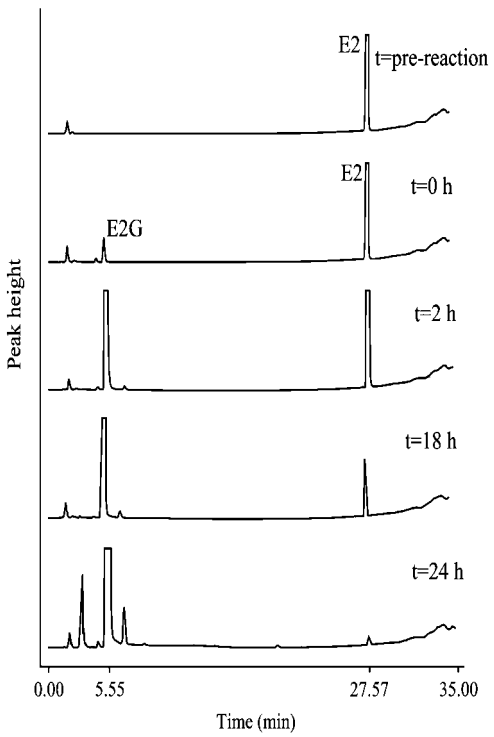


Figure 1. Progress of enzymatic synthesis of 17β-estradiol-3-glucuronide with time and the concurrent consumption of 17β-estradiol.

Product yield of **2** was 84%, and was attributed to the induction of UGT's by phenobarbital.[39] The same microsomal proteins also were active at glucuronidating hydroxylated polybrominated diphenyl ether metabolites, triclosan, and ractopamine hydrochloride.

Synthesis of 17β-[4-^{14}C]estradiol-17-sulfate (5)

The synthesis of **5**, presented in Scheme 2, was initiated by blocking the more reactive C-3 hydroxyl in **1**, which was accomplished with a 59.3% product yield to form the intermediate **3**.

The negative ion LC/MS analysis of **3** resulted in a molecular ion at 375.21, a methyl loss fragment at 361.21, and a water loss at 356.85. Losses of propanyl and propenyl groups were consistent with fragments at *m/z* 332.82 and 334.82, respectively. ^{1}H NMR analysis of **1** and **3** indicated shifts in protons ortho- and meta- to C-3 occurred in **3** relative to **1** (6.53–6.94; 7.06–7.44; 6.47–6.89 ppm). Benzoate protons were present at 8.14, 7.54, and 7.66 ppm of **3**. The ^{13}C NMR spectrum confirmed that the blocking had occurred at C-3 because carbons ortho- and meta- to C-3 of **3** were shifted downfield relative to their chemical shift position in **1** (113.72–119.79; 132.32–134.86; 116.05–122.63 ppm).

The formation of **4** was confirmed by a molecular ion at 455.09 in the LC/MS spectrum, and was accompanied by a prominent fragment at *m/z* 351.12, which was consistent with a benzoate fragment loss. Sulfation at C-17 was suggested by significant downfield chemical shifts for the C-17 proton (3.67–4.31 ppm) and carbon (82.45–88.22 ppm) in the ^{1}H and ^{13}C NMR spectra of **3** and **4**, respectively. A radiochemical purity of 95% was achieved and was considered satisfactory for the next step.

The purification of the final product (**5**) yielded 21 μCi (18.9 μmol; 7.1 mg) of 98% radiochemical purity. The formation of **5** was confirmed by a molecular ion at 351.07 and the sulfate moiety ion at *m/z* 96.96 in the LC/MS spectrum of **5**. ^{13}C NMR analyses of **5** indicated a significant upfield chemical shift of C-3 relative to **4** (132.54 from 134.95 ppm), as well as for carbons ortho to C-3 (116.04 from 122.64; 113.76 from 119.84 ppm). Upfield shifts of the aromatic protons between **5** and **4** were observed (6.53 from 6.90; 7.06 from 7.30; 6.47 from 6.86 ppm), but no chemical shift difference was observed for the C-17 proton. Collectively the physical data provide convincing evidence that sulfation of **1** had occurred at C-17.

The overall yield of **5** was 44%, which possibly could have increased if the reaction conditions had been optimized; however, optimization was not an immediate objective. The radiochemical yield was satisfactory for the immediate needs of the research program.

Conclusion

[^{14}C]Radiolabeled 17β-E2-3-G and 17β-E2-17-S were successfully synthesized using enzymatic and chemical approaches, respectively, which permitted their use for laboratory scale fate and transport experiments in soil–water systems. Though our objective was measuring and modeling the movement of endocrine disrupting compounds in the environment, these studies are but a small portion of the potential studies in which

radiolabeled conjugates could be used. As glucuronidation and sulfation are the major conjugation pathways in vertebrates for not only steroid hormones, but other xenobiotics,[40] we hypothesize that radiolabeled glucuronides and sulfates of other emerging contaminants can also be synthesized following the approaches provided in this paper, or with appropriate modifications of them.

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