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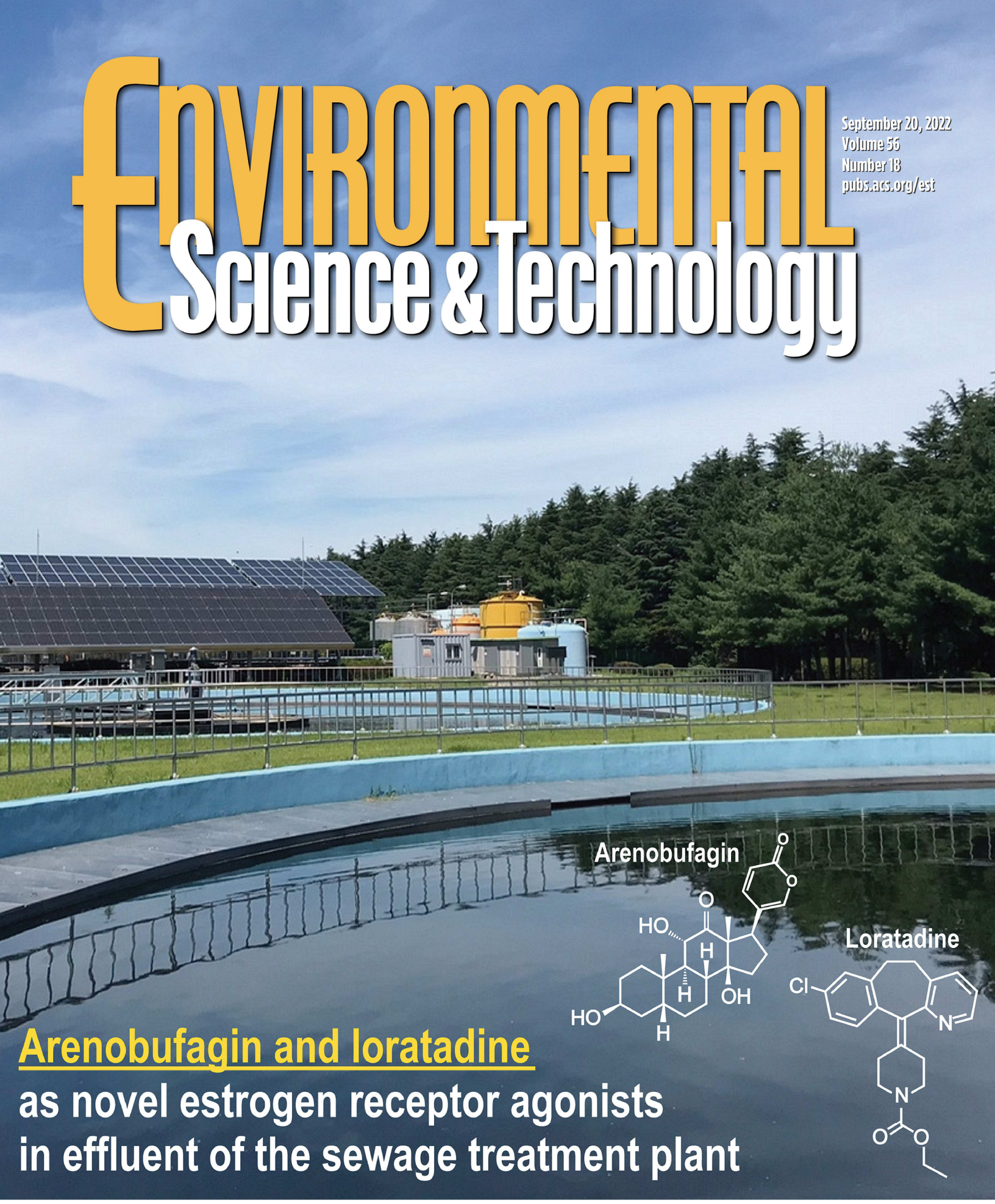

Arenobufagin and loratadine as novel estrogen receptor agonists in effluent of the sewage treatment plant

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Article

Molecular Characterization of Estrogen Receptor Agonists during Sewage Treatment Processes Using Effect-Directed Analysis Combined with High-Resolution Full-Scan Screening

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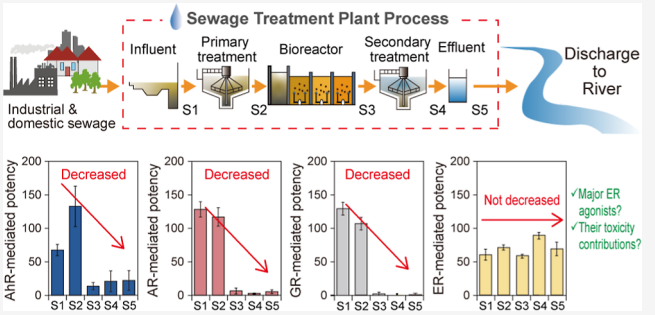
ABSTRACT: Endocrine-disrupting potential was evaluated during the sewage treatment process using in vitro bioassays. Aryl hydrocarbon receptor (AhR)-, androgen receptor (AR)-, glucocorticoid receptor (GR)-, and estrogen receptor (ER)-mediated activities were assessed over five steps of the treatment process. Bioassays of organic extracts showed that AhR, AR, and GR potencies tended to decrease through the sewage treatment process, whereas ER potencies did not significantly decrease. Bioassays on reverse-phase high-performance liquid chromatography fractions showed that F5 (log K_{OW} 2.5−3.0) had great ER potencies. Full-scan screening of these fractions detected two novel ER agonists, arenobufagin and loratadine, which are used pharmaceuticals. These compounds accounted for 3.3−25% of the total ER potencies and 4% of the ER potencies in the final effluent. The well-known ER agonists, estrone and 17β-estradiol, accounted for 60 and 17% of the ER potencies in F5 of the influent and primary treatment, respectively. Fourier transform ion cyclotron resonance mass spectrometry analysis showed that various molecules were generated during the treatment process, especially CHO and CHOS (C: carbon, H: hydrogen, O: oxygen, and S: sulfur). This study documented that widely used pharmaceuticals are introduced into the aquatic environments without being removed during the sewage treatment process.



KEYWORDS: *endocrine-disrupting potentials, estrogen receptor-mediated activity, full-scan screening, sewage treatment process water, in vitro bioassays*

INTRODUCTION

Endocrine-disrupting chemicals (EDCs), including natural and synthetic steroids, bind to receptors in cells and interfere with normal functioning of the endocrine system.[1] Various EDCs are introduced directly to the aquatic environment or via sewage and wastewater treatment processes.[2] However, some EDCs are not sufficiently eliminated during the sewage treatment process and remain in the final effluent.[3] Transformation products of EDCs generated in the sewage treatment process are also present in the effluent.[4] Thus, sewage treatment plant (STP) outfalls are considered a significant source of EDCs in the aquatic environment.[3] Even when present in concentrations below the limit of detection, EDCs adversely affect the endocrine hormonal systems of aquatic organisms by mixture effects.[5,6]

Several studies have used effect-directed analysis (EDA) on treated water and the final effluent of STPs[7−9] to identify the major toxic substances in samples for various endpoints of endocrine-disrupting effects. EDA is used to evaluate endocrine-disrupting activity in samples using bioassays, which reduces the complexity of samples through fractionation and allows key toxicants to be identified through instrumental analysis in highly potent fractions.[10] Among bioassays used in EDA, an in vitro bioassay is a valuable screening tool to evaluate the potential risk of EDCs in raw influents, treated water, and effluents of STPs and aquatic ecosystems.[11−17] Aryl hydrocarbon receptor (AhR)-, androgen receptor (AR)-, glucocorticoid receptor (GR)-, and estrogen receptor (ER)-mediated potencies in samples from STPs are frequently evaluated using in vitro bioassays.[11−17] Although the EDA used in vitro bioassays are useful for identifying EDCs, target compounds often cannot explain total induced toxicity because many EDCs exist in STP samples.[18] Thus, the identification of

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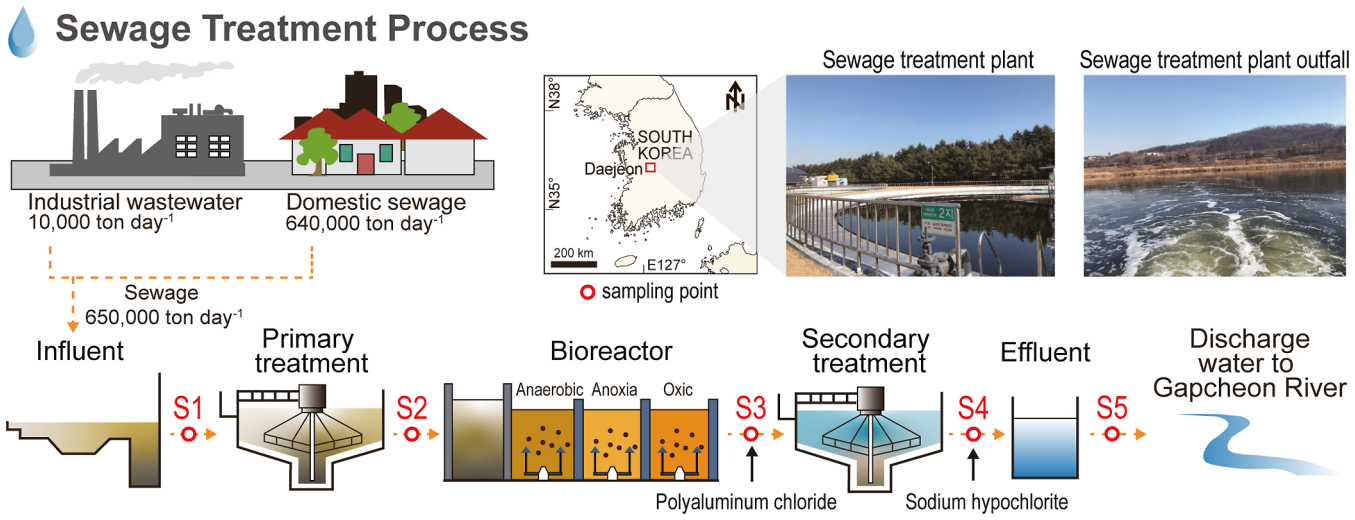

Figure 1. Processes in the Daejeon STP (South Korea) and sampling points (S1: influent, S2: primary treatment, S3: bioreactor, S4: secondary treatment, and S5: effluent).

unknown or unmonitored toxic substances is very challenging in EDA.[19]

Over the last 10 years, EDA combined with full-scan screening analysis (FSA) has been applied to identify unmonitored toxicants in environmental samples through more sophisticated mass detection using high-resolution mass spectrometry (MS), such as quadrupole time-of-flight MS (QTOFMS).[20] In parallel, the development of library-matching software makes it easy to search for information on the identification, appearance, and relevance of candidate compounds based on the molecular mass by matching compounds with chromatograms from the instrumental analysis.[21] Previous studies successfully identified unmonitored toxicants in environmental samples using FSA.[10,22] For example, ER agonists have been identified in wastewater, including benzophenone, diethyl phthalate, and diisobutyl phthalate.[22]

Fourier transform ion cyclotron resonance MS (FT-ICRMS) is a powerful technique that has a high resolution and high mass accuracy.[23] Its high mass resolution enables the detection of thousands of mass spectra in complex environmental samples. Recently, this approach has been used to confirm the composition of compounds in complex environmental samples, including the effluent in STPs, seawater, freshwater, and soil.[24−27] Using Van Krevelen diagrams, the compounds in samples are distinguished into CHO, CHON, CHOS, and CHONS molecules (C: carbon, H: hydrogen, O: oxygen, N: nitrogen, and S: sulfur).[28] By applying FT-ICRMS to nontarget analysis in EDA, information on the molecular compositions in highly toxic fractions can be obtained. To the best of our knowledge, FT-ICRMS analysis has not been applied to EDA studies.

In the present study, various endocrine-disrupting potentials (AhR-, AR-, GR-, and ER-mediated potencies) in five steps of the sewage treatment process were screened using in vitro bioassays, and the degree of potential toxicity reduction was evaluated. Among them, we focused on the point that ER-mediated potencies were not significantly reduced during the sewage treatment process. This study aimed to identify major ER agonists in samples from STPs using EDA combined with FSA. Fractionation, targeted analysis, and nontargeted analysis were performed, and the contributions of known ER agonists and newly identified ER agonists in samples of STPs were evaluated. Chemical and toxicological information was then compared to quantitative structure−activity relationship (QSAR) modeling for candidates of ER agonists. Finally, the molecular composition of more potent fractions was examined using FT-ICRMS analysis.

MATERIALS AND METHODS

Sampling and Pretreatment. The STP located in Daejeon city in South Korea receives 10,000 tons of industrial wastewater and 6,40,000 tons of domestic sewage per day (Figure 1). The effluent is discharged into the Gapcheon River (area: 648 km^2; length: 73.7 km; mean annual flow: 22.9 m^3 s^{-1}; available online at the Water Resources Management Information System, https://wamis.go.kr). Water samples were collected at each step in the STP: influent (S1), primary treatment (S2), bioreactor (after the treatment of anaerobic, anoxic, and oxic processes) (S3), secondary treatment (S4), and effluent (S5) (Figure 1). The primary treatment separates and treats the debris mixed in the dirty water, the bioreactor uses microorganisms to remove contaminants, and the secondary treatment separates the clear water layer and the debris layer. Polyaluminum chloride was added after S3, and sodium hypochlorite was added after S4 in the STP. Thus, some residual chlorines may be present in samples S4 and S5. However, preservatives were not added to the samples because ascorbic acid would potentially influence the endocrine-disrupting activity.[29] In previous studies, no preservatives were added to stabilize the chemicals in samples of the STPs.[9,30]

Ten liters of water was collected from each of the five treatment processes (S1−S5) using a precleaned bottle in July 2020. Samples were filtered through Whatman GF/F (0.7 μm nominal pore size, Merck KGaA, Darmstadt, Germany) to remove suspended particles, extracted using solid-phase extraction (SPE) cartridges, and stored at −20 °C until analysis. For bioassays, the S1−S5 samples were extracted using SPE using an Oasis HLB cartridge (500 mg, 6 cc, Waters, Milford, MA). The cartridge was conditioned with 10 mL of methanol (MeOH)/ethyl acetate (1:1, v/v) and 30 mL of deionized water. One liter of filtered water was loaded into the cartridge. After loading, cartridges were dried under air for 30

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min, and extracts were eluted using 10 mL of MeOH/ethyl acetate (1:1, v/v). The eluent was concentrated to 1 mL using a nitrogen gas concentrator (concentration factor = 1000). Of the 1 mL raw extracts (REs), 0.2, 0.5, and 0.3 mL were used for bioassays, reverse-phase high-performance liquid chromatography (RP-HPLC) fractionation, and chemical analysis, respectively. The RE of each sample was separated into 20 fractions using RP-HPLC. Details on the RP-HPLC fractionation conditions are provided in Table S1 of the Supporting Information. Experimental blank samples were analyzed using the same procedures as all pretreatments, including SPE and RP-HPLC fractionation, to evaluate the contamination during the experiment.

In Vitro Bioassays. AhR-, AR-, GR-, and ER-mediated potencies of REs and RP-HPLC fractions were measured using H4IIE-*luc*,[31,32] MDA-kb2,[33,34] and T47D-kb*luc*[11,30] bioassays following the methods of previous studies. The cell culture and experimental conditions are shown in Table S2. Both AR- and GR-mediated potencies were measured using MDA-kb2 bioassays. To distinguish between AR- and GR-mediated potencies, the anti-GR agonist mifepristone was added before sample dosing when measuring AR-mediated potency and the anti-AR agonist flutamide when measuring GR-mediated potency.[35,36] Luciferase luminescence was measured using a Victor X3 multilabel plate reader (PerkinElmer, Waltham, MA). Responses of bioassays were converted to percentages of maximum responses to benzo[*a*]pyrene (BaP) for AhR, dihydrotestosterone (DHT) for AR, dexamethasone (Dexa) for GR, and 17β-estradiol (E2) for ER. In this study, the H4IIE-*luc* bioassay with a shorter exposure time (4 h) was mainly focused on polar fractions, and thus, BaP was used as a reference material.[32] Potency-based E2-equivalent concentrations (EQs) (ng E2-EQ L^{-1}) were calculated from the dose−response curve for six concentrations. All bioassays were conducted in triplicate to check reproducibility.

Targeted Analysis. Steroidal estrogens, including estrone (E1), E2, estriol (E3), and 17α-ethinylestradiol (EE2), were quantitated using a 1290 Infinity II high-performance liquid chromatograph coupled with an Agilent 6470 triple quadrupole mass spectrometer MS/MS system (Agilent Technologies, Santa Clara, CA). E1, E2, E3, and EE2 were separated using a Waters X-Bridge C18 column (3.0 mm × 150 mm, 5.0 μm). The mobile phase consisted of (A) 0.075% ammonium hydroxide in water (pH 9.0 adjusted with NH_4OH) and (B) MeOH/acetonitrile (1:1, v/v). The injection volume was 5 μL, and the flow rate of the mobile phase was set to 0.4 mL min^{-1}. E1, E2, E3, and EE2 were detected in the negative ion mode (details in Table S3). Details on multiple reaction monitoring (MRM) transitions are provided in Table S4. The method detection limits of E1, E2, E3, and EE2 were 1.3, 0.8, 0.7, and 0.2 ng L^{-1}, respectively. The recovery during the RP-HPLC fractionations of steroidal estrogens was estimated by comparing the concentrations in samples (S1−S5) before and after fractionations. The average recovery rates of E1, E2, E3, and EE2 were 98, 74, 87, and 87%, respectively.

Six alkylphenols (APs) in REs, such as 4-*t*-octylphenol (OP), 4-*t*-octylphenol monoethoxylate (OP1EO), 4-*t*-octylphenol diethoxylate (OP2EO), nonylphenols (NPs, isomer mix), nonylphenol monoethoxylates (NP1EOs, isomer mix), and nonylphenol diethoxylates (NP2EOs, isomer mix), were quantified using an Agilent 7900B gas chromatograph equipped with a 5977A mass-selective detector (MSD) (Agilent Technologies, Santa Clara, CA).[37] Details of the instrumental conditions of gas chromatography (GC)-MSD are shown in Table S5. The method detection limits of *t*-OP, *t*-OP1EO, *t*-OP2EO, NPs, NP1EOs, and NP2EOs were 0.12, 0.61, 0.08, 3.7, 0.45, and 1.5 ng L^{-1}, respectively. An isotopically labeled surrogate standard (bisphenol A-d16) was added in aliquots of extracts, and the average recovery rate of surrogate standard was 97%.

FSA Using LC-QTOFMS. FSA was performed using LC-QTOFMS on RP-HPLC fractions that showed high ER-mediated potencies in sewage samples (Table S6). Details of the instrumental conditions of LC-QTOFMS are shown in Table S6. Potential ER agonists were selected via a five-step process. In the first step, all chromatographic peaks detected in the sewage sample were identified. In the second step, tandem mass spectrometry (MS/MS) data were collected for chromatogram peaks. In the third step, the m/z data of the compounds were matched with known compounds in Traditional Chinese Medicine (TCM) library software (AB Sciex, Framingham, MA). In the fourth step, compounds that had a matching score ≥70 were selected. It was assumed that compounds commonly detected in each step of the sewage treatment process (S1−S5 samples) were the tentative ER agonists. This is because the ER-mediated potency was not significantly reduced by the treatment process. By using this data processing as the fifth step, candidates for ER agonists were selected. Seven compounds, such as loratadine, arenobufagin, etofenprox, corticosterone, resibufogenin, omethoate, and 6-beta naltrexol, were selected as tentative ER agonists. Corticosterone is generally known as a GR agonist.[36] Since compounds can have various toxicological endpoints, corticosterone was selected as a tentative ER agonist. Arenobufagin and omethoate were purchased for chemical and toxicological confirmation from Cayman Chemical (Ann Arbor, MI). Loratadine, etofenprox, corticosterone, and resibufogenin were purchased from Sigma-Aldrich (St. Louis, MO). 6-Beta-naltrexol could not be confirmed toxicologically or chemically because the standard material was unavailable.

Analysis of Newly Identified ER Agonists. Arenobufagin and loratadine were quantitated using a 1290 Infinity II high-performance liquid chromatograph coupled with a QTRAP 6500 tandem mass spectrometer (AB Sciex). ER agonists were isolated using the ZORBAX Eclipse XDB-C18 (150 mm × 2.1 mm i.d. × 5 μm film). The mobile phase consisted of 0.1% formic acid and 10 mM ammonium formate in water (A) and 0.1% formic acid in acetonitrile (B). The injection volume was 3 μL, and the flow rate of the mobile phase was set to 0.4 mL min^{-1}. Arenobufagin and loratadine were detected in the positive ion mode. Details of the instrumental conditions are shown in Table S7.

Relative Potency Values of ER Agonists and Potency Balance Analysis. The relative potency (RePs) values of the ER-mediated potencies of steroidal estrogens (E1, E2, E3, and EE2) and ER agonist candidates were determined using the T47D-kb*luc* bioassay with effective concentrations (ECs) at 50% of the maximum level achieved by E2 (EC_{50}). EC_{50} values of each compound were calculated from dose−response curves at seven concentrations for steroidal estrogens (0.5, 0.2, 0.1, 0.02, 0.01, 0.002, and 0.001 ng L^{-1}) (Figure S1) and nine concentrations (1000, 333, 111, 37, 12, 4, 1, 0.5, and 0.2 μg L^{-1}) for ER agonist candidates. Potency balance analysis was performed between instrument-derived E2 EQs (EEQs) and bioassay-derived E2-EQs to confirm the contribution of each ER agonist to the total induced ER-mediated potency.

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Environ. Sci. Technol. 2022, 56, 13085−13095

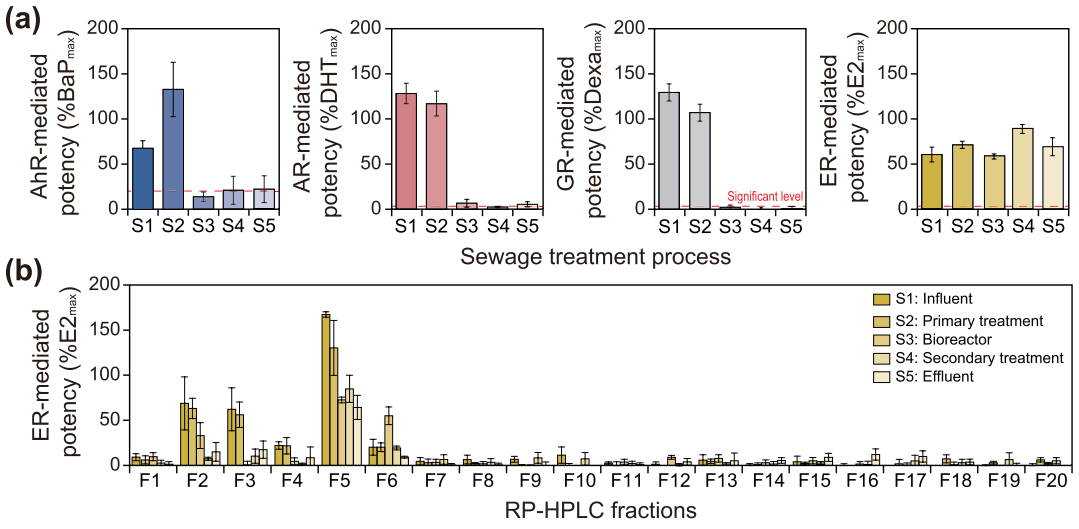

Figure 2. (a) Endocrine-disrupting potential (AhR-, AR-, GR-, and ER-mediated potencies) of REs and (b) ER-mediated potency in RP-HPLC fractions of S1−S5 (error bar: mean ± SD; n = 3).

Instrument-derived EEQs were used to calculate the sum of the products of measured concentrations for individual compounds in the water samples of STPs multiplied by their RePs.

In Silico Modeling Using VituralToxLab and VEGA QSAR. ER-binding affinities of seven tentative ER agonists were also predicted using VirtualToxLab.[38] VirtualToxLab was used to measure the toxic potential of compounds using four-dimensional (4D) Boltzmann scoring through standardized individual binding affinities for a set of protein models known to cause adverse effects. Seven tentative ER agonists identified by FSA were screened to predict mutagenicity, carcinogenicity, developmental toxicity, and ER activity using VEGA QSAR.[38,39] The VEGA platform is an in silico program that contains dozens of QSAR models for various endpoints. In silico techniques are used to predict various toxicological endpoints of chemicals based on their physicochemical properties and structures.[40] Additionally, EPA ToxCast in the US EPA CompTox dashboard, which contains actual bioactivity data of various chemicals, was used to evaluate AR, GR, and ER activity for seven tentative ER agonists (available online at https://comptox.epa.gov/dashboard).

Molecular Composition Analysis Using FT-ICRMS. The composition ratio of the molecular class (CHO, CHON, CHOS, and CHONS) in F2, F3, and F5 (highly potent fractions) and F7 (blank) of water samples of the STP (S1−S5) was determined using a 15-T Fourier transform ion cyclotron resonance mass spectrometer interfaced with an Apollo II electrospray ionization source (Bruker Daltonik, Bremen, Germany) in the Korea Basic Science Institute (Ochang, South Korea). The instrumental conditions included running the device in the negative ion mode, with a flow rate of 4 L min^{-1}, a voltage of −45 V, and a mass range of 150−1200 m/z. Details of the instrumental conditions are shown in Table S8. The FT-ICRMS dataset was assigned formulas using the composer program (Sierra Analytics, Modesto, CA). The matching tolerance was <0.5 ppm. C, H, O, N, and S were analyzed at the corresponding ranges of 0−100, 0−200, 0−60, 0−4, and 0−2, respectively.

RESULTS AND DISCUSSION

Endocrine-Disrupting Potentials in Water Samples of the STP. AhR-, AR-, GR-, and ER-mediated potencies were evaluated in the water sample REs of the STP (S1−S5) (Figure 2a and Table S9). The AhR-mediated potency of S2 was relatively greater compared to that of S1, which seems to be because the chemical composition of the influent sample was too complicated to interfere with AhR binding.[41,42] AhR-, AR-, and GR-mediated potencies tended to decrease sharply as the sewage treatment process progressed, in general. This phenomenon was attributed to 90% of endocrine-disrupting activity being eliminated in the bioreactor of the STP.[5] However, ER-mediated potency showed no significant decrease during the sewage treatment process and had high ER-mediated potency in the RE of the final effluent (S5, >50% E2$_{max}$). Thus, the removal of ER-active substances in sewage and wastewater was not sufficient in the STP. This issue reflects the limitation of the anaerobic−anoxic−aerobic method (A2O method) used in the STP at Daejeon (South Korea). A similar result was also obtained in a previous study.[30] ER-mediated potencies were noticeably reduced in the STP due to the use of the ozone treatment method.[43] The EDCs that were removed differed depending on the treatment methods of the bioreactor in the STP. Thus, additional treatment is likely necessary to eliminate ER-active substances.

RP-HPLC fractionation (F1−F20) was performed to search for major ER-active substances in the REs of S1−S5. The ER-mediated potencies in each fraction were evaluated (Figure 2b and Table S10). Among the 20 fractions, high ER-mediated potency was found in F5 (log K_{OW} 2.5−3.0), followed by F2 (log K_{OW} 1.0−1.5) and F3 (log K_{OW} 1.5−2.0). This trend was commonly found in S1−S5. Thus, ER-active substances in the water samples of the STP are likely less hydrophobic, with a log K_{OW} of 3 or less. F5 of S1−S5 had a higher ER-mediated potency compared to that of REs. Various chemicals are present in complex mixtures in STP samples and might alter endocrine-disrupting effects.[44] Antagonistic effects arose due to the complexity of the samples in REs.[7,41]

The EC$_{50}$ values for ER-mediated potencies in the highly potent fraction (F5) were calculated from the dose−response curves (Figure S2). Potency-based E2-EQs of F5 for S1, S2, S3,

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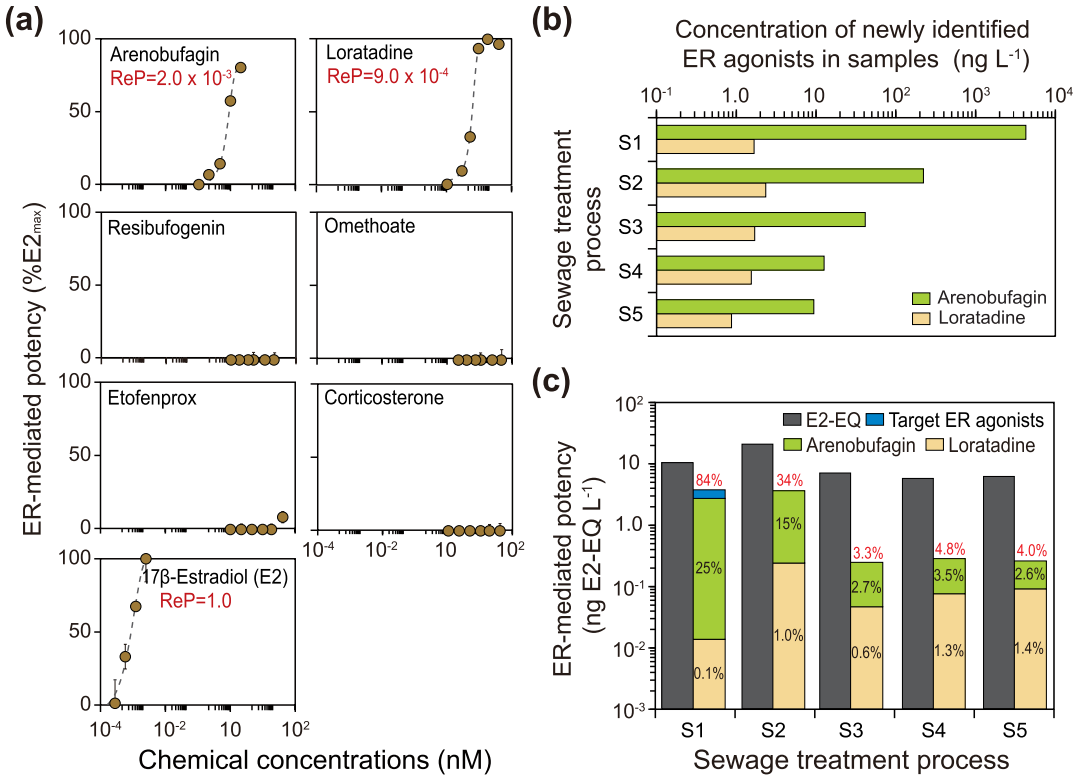

Figure 3. (a) Dose−response curves for ER-mediated potencies of six candidates of ER agonists and 17β-estradiol in the T47D-kb*luc* bioassay (error bar: mean ± SD, n = 3). (b) Concentrations of novel ER agonists (arenobufagin and loratadine) in F5 of S1−S5 and (c) contribution of steroidal estrogens and novel ER agonists to E2-EQs (potency-based).

S4, and S5 were 11, 22, 7, 5, and 6 ng L^{-1}, respectively, which were calculated based on the EC_{50} values. The calculated E2-EQs were used for the potency balance analysis. The E2-EQ (6 ng E2-EQ L^{-1}) of the F5 in the final effluent exceeded the safe concentration of estrogenic equivalent in municipal wastewater treatment plant effluents (0.1−0.4 ng E2-EQ L^{-1} for long-term exposure) suggested in the previous study.[44] In this study, the dilution factor could not be calculated due to the lack of river flow data, but it was found that at least 60-fold dilution was required in order not to affect the aquatic ecosystem adversely.

Target ER Agonists. Steroidal estrogens were detected in all samples of the STP. The concentrations of steroid estrogens (E1 + E2 + E3 + EE2) in REs were 334, 363, 109, 22.2, and 11.9 ng L^{-1} in the S1−S5 samples, respectively, indicating that most steroid estrogens were removed during the sewage treatment process (Table S10). In the result for RP-HPLC fractions, E1 and E2 were mainly detected in F5, and E3 and EE2 were mainly found in F2 and F3. Concentrations of E1 and E2 in F5 were relatively great in S1 and S2 but significantly decreased through the bioreactor, and the final effluent showed a very low concentration (E1: 1.2 ng L^{-1}; E2: < DL). Assay-specific ReP values for ER-mediated potency were 1.5×10^{-2}, 4.9×10^{-3}, and 2.3×10^{-3} in E1, E3, and EE2, respectively, compared to that of E2 (1.0) (Figure S1). Potency balance analysis showed that in F5 of S1 and S2, 60 and 17% of ER-mediated potency, respectively, was explained by E1 and E2. However, a small portion of ER-mediated potency was accounted for in S3−S5 samples due to the drastic decrease in E1 and E2 concentrations (Figure S3). Steroidal estrogens are strong ER-active substances and are known to be commonly detected in samples of STPs.[41,45] During the sewage treatment process, steroidal estrogens were adsorbed to the sludge and removed or biodegraded;[46] thus, the concentrations in the effluent seemed to have decreased. However, ER-mediated potency was still relatively great in the effluent, suggesting that unmonitored ER agonists were present.

APs are also well-known ER-active substances[47] and were detected in the REs of S1−S5, ranging from 1860 to 3310 ng L^{-1} (mean 2390 ng L^{-1}) (Figure S4 and Table S11). Relatively high concentrations of APs in samples (S1 and S2) were detected. However, AP concentrations tended to decrease as the sewage treatment process progressed, particularly in S3 (bioreactor), whereas the ER-mediated potencies did not. Meanwhile, NPs were less removed in the STP and showed similar concentration levels in the influent (S1) and effluent (S5) (Figure S4). The concentrations of APs were slightly increased in S4 and S5 compared to those in S3, which is likely due to slight concentration and/or dilution occurring during the sewage treatment process. For example, after adding polyaluminum chloride in S3, it is diluted with water to neutralize the chemicals. Meanwhile, APs were not detected in F5, where ER-mediated potency was greatest. Thus, our results suggest that APs were not the major ER agonists in the S1−S5 samples. A previous study also showed that APs were not the major ER agonists in the STP effluent due to their low toxic potencies.[48]

Full-Scan Screening Analysis. FSA using LC-QTOFMS was conducted on the F5 fractions of S1−S5, which exhibited greater ER-mediated potencies. Candidate ER agonists were selected through a five-step process (Figure S5). The numbers of chromatographic peaks detected in F5 of S1−S5 were 207, 80, 188, 100, and 114, respectively. In the second step, 128, 49, 109, 55, and 79 compounds in F5 of S1−S5, respectively, were

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selected based on the MS/MS data. In the third step, the compounds matched with the TCM library software in F5 of S1−S5 were 83, 39, 73, 30, and 55, respectively. In the fourth step, the compounds with a matching score ≥70 in F5 of S1−S5 were 25, 14, 24, 12, and 15, respectively.[49] These compounds were selected as tentative ER agonist candidates in F5 of S1−S5 (Table S12). Finally, as the sewage treatment step progressed, the remaining compounds were selected as candidates for ER agonists. Seven compounds were selected as tentative ER agonists, loratadine, arenobufagin, etofenprox, corticosterone, resibufogenin, omethoate, and 6-beta-naltrexol. Of the seven candidate compounds, except for 6-beta-naltrexol for which a standard material was not available, ER-mediated activities were assessed for six compounds. In addition, the concentrations in samples were measured for substances showing significant ER activity.

Toxicological and Chemical Confirmation. Six candidates of ER agonists were evaluated for their ER-binding activation using T47D-kb*luc* bioassays (Figure 3a). Among the six candidates, arenobufagin and loratadine exhibited significant ER-mediated potencies. ReP values of arenobufagin and loratadine were 2.0×10^{-3} and 9.0×10^{-6}, respectively, compared to those of the reference standard E2. Arenobufagin and loratadine were confirmed to have lower RePs than steroidal estrogens. Arenobufagin and loratadine exhibited relatively greater ER-binding activation compared to NP1EO (ReP = 1.3×10^{-5}) and bisphenol A (ReP = 2.4×10^{-5}).[50,51] Quantification conditions of arenobufagin and loratadine, which were newly identified ER agonists, were optimized using HPLC-MS/MS (Figure S6 and Table S13). Parent ions of arenobufagin and loratadine were 417.22 and 383.15 m/z, respectively. Transition ions of arenobufagin were 399.20, 147.20, and 105.20 m/z and those of loratadine were 337.20, 267.20, and 259.20 m/z.

Concentrations of Newly Identified ER Agonists. Arenobufagin is derived from herbal medicines and has been used as a drug to treat liver cancer.[52,53] The present study is the first to demonstrate the ER-mediated activity of arenobufagin and its presence in samples of the STP. Loratadine is an antihistamine and is used to treat allergies.[54] Concentrations of arenobufagin and loratadine in F5 of S1−S5 ranged from 100 to 2100 ng L^{-1} and from 22 to 400 ng L^{-1}, respectively (Figure 3b). Concentrations of arenobufagin tended to decrease through treatment of sewage (from S1 to S5) and were 105 ng L^{-1} in the final effluent. In contrast, concentrations of loratadine were similar in S1−S5. Thus, arenobufagin was partially removed during the sewage treatment process, whereas loratadine was not eliminated from the STP. Overall, removal of these novel ER-active substances was not sufficient in the STP at Daejeon and appeared to flow into the Gapcheon River. In the current study, concentrations of loratadine in the final effluent of the STP (150 ng L^{-1}) exceeded the previously reported predicted no-effect concentration (21 ng L^{-1}).[55] Loratadine is widely used as a drug worldwide and has been continuously detected in the STP effluent in Colombia, Spain, and China.[55−57] Thus, concerns about the risk to the aquatic ecosystem due to the ER-mediated activity of loratadine are not just regional, and the potential risk worldwide requires evaluation.

Contribution of Novel ER Agonists to the Total ER-Mediated Potencies. Contributions of novel ER agonists ranged from 3.3 to 25% of the total ER-mediated potencies in F5 of S1−S5 (Figure 3c). Arenobufagin and loratadine explained 2.6−25 and 0.12−1.4% of the ER-mediated potencies, respectively. As arenobufagin concentrations decreased from S1 to S5, the contribution also decreased (from 25 to 2.6%). Since loratadine concentrations were constant from S1 to S5, the contributions were similar but were highest in S5 (1.4%). Potency balance analysis showed that novel ER agonists explained only a small portion of the E2-EQ. This result was attributed to samples containing high concentrations of arenobufagin but lower ReP values than E2. Loratadine also had low concentrations with a small ReP value. Although the contributions of arenobufagin and loratadine in the final effluent were relatively low (4%), they were greater than those of steroidal estrogens, and they were not easily removed in the sewage treatment process. It is necessary to perform follow-up studies for the identification of recalcitrant pharmaceuticals in the effluent of the STP.

Future studies should investigate ginkgolide B, hydramethylnon, sclareol, diphenoxylate, clonidine, cinnamic acid, amygdalin, and flavin mononucleotide which are expected to exist in the effluent, but ER activity has not been confirmed in the present study (Table S12). These compounds were detected in the final effluent of the STP and were directly introduced to the Gapcheon River. Diphenoxylate, clonidine, cinnamic acid, and amygdalin are pharmaceutical compounds, while hydramethylon is used in insecticides. Hydramethylon and cinnamic acid were previously identified as ER agonists.[58,59] Diphenoxylate is a metabolite of difenoxin[60] and is considered to be formed during the sewage treatment process.

Additional Toxicity Prediction Using QSARs. Arenobufagin may cause cardiotoxicity to aquatic organisms in high concentrations in the environment.[61] In previous studies, loratadine was found to have acute and chronic toxicity upon exposure to aquatic organisms, particularly in algae and crustaceans.[62] Loratadine can be degraded to desloratadine in the environment and can be as chronically toxic to aquatic organisms as the parent compound.

The seven tentative ER agonists, loratadine, arenobufagin, etofenprox, corticosterone, resibufogenin, omethoate, and 6-beta-naltrexol, were evaluated for AhR-, AR-, GR-, TR-, and ER-binding potencies using VirtualToxLab (Table 1). Arenobufagin, etofenprox, corticosterone, and resibufogenin were predicted to have AhR-, AR-, GR-, and TR-binding affinities. Etofenprox, corticosterone, and resibufogenin were predicted to have ER-binding affinity, but this was not confirmed within the concentration ranges tested in the present study. VirtualToxLab only uses thermodynamics to evaluate the potential binding affinity between compounds and receptors.[63] Thus, VirtualToxLab might not always match the results of the toxicity test (Table S14).

VEGA QSAR was used to evaluate seven tentative ER agonists for potential toxicities, including AR activity, ER activity, mutagenicity, carcinogenicity, and developmental toxicity (Table 1). The model predicted that only arenobufagin, loratadine, and 6-beta-naltrexol could bind to the ER. ER-mediated activity of arenobufagin and loratadine was consistent with that from VEGA QSAR (Table S14). ER-mediated activity of arenobufagin and loratadine was consistent with one model for VEGA QSAR (Table S14). Because VEGA QSAR is based on compound structures, some models may produce different results. Thus, an integrated evaluation that carefully considers the necessary information and quantifies the mechanisms of toxic expression is

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Environ. Sci. Technol. 2022, 56, 13085−13095

Table 1. Predicted Potential Toxic Effects of Newly Identified ER Agonists in Water Samples Collected during the Sewage Treatment Process

compound	VEGA QSAR					VirtualToxLab (nM)				ER binding	EPA ToxCas		
	ER activity[a]	AR activity[b]	mutagenicity[c]	carcinogenicity[d]	developmental toxicity[e]	AR	AhR	ER	GR	TR	T47D-kb*luc* bioassay	AR	GR	ER
etofenprox	–[f] –[g]	–	+ + – + +	+ + + +	+ +	540	40	3100	4300	1800	–	+	n.a	n.a
arenobufagin	– +	–	– – – – –	– – + +	+ –	119	66200	n.a[h]	47.5	3300	+	n.a	n.a	n.a
loratadine	+ –	–	+ + + – +	+ + – +	– –	n.a	n.a	n.a	n.a	n.a	+	+	n.a	n.a
omethoate	– –	–	+ + + – +	+ + + +	+ –	n.a	n.a	n.a	n.a	n.a	–	n.a	n.a	n.a
6-beta-naltrexol	+ +	–	– – – – –	– – + +	+ +	n.a	n.a	n.a	n.a	n.a	–	n.a	n.a	n.a
resibufogenin	– –	–	+ + – – +	+ + + +	+ –	30.6	13800	106	7.44	614	–	+	+	+
corticosterone	– –	+	– + – – –	– + + +	+ +	290	4.0	5.3	10.2	180	–	+	n.a	+

[a]Predicted using IRFMN and IRFMN/CERAPP models (ER activity). [b]Predicted using the IRFMN/COMPARA model (AR activity). [c]Predicted using CAESAR, ISS, CONSENSUS, KNN/Read-Across, and SarPY/IRFMN models (mutagenicity). [d]Predicted using CAESAR, ISS, IRFMN/Antares, and IRFMN/ISSCAN-CGX models (carcinogenicity). [e]Predicted using CAESAR and PG models (developmental toxicity). [f]+: active. [g]–: nonactive. [h]n.a: not available.

required.[64] From the result of investigating the additional toxicity of seven tentative ER agonists through EPA ToxCast, some compounds were confirmed to have AR, GR, and ER activities (Table 1). Overall, the newly identified ER agonists were confirmed to have various potential toxicities, such as mutagenicity, carcinogenicity, and developmental toxicity. The final effluent of the STP containing these toxic substances has the potential to affect aquatic ecosystems adversely.

Molecular Characterization of Highly Potent Fractions Using FT-ICRMS. FT-ICRMS analysis was performed to investigate the molecular composition of potential ER agonists in F2, F3, F5, and F7 of S1−S5. It was assumed that ER agonists would have significant ER-mediated potency in F2, F3, and F5 and that no ER agonists would be present in F7, which did not exhibit ER-mediated potency. Thus, it was expected that ER agonists would be isolated if the compounds in F7 were removed from the compounds in F2, F3, and F5. Molecules in the fractions (F2−F7, F3−F7, and F5−F7) were distributed in 0.0−0.4 O/C and 1.0−2.0 H/C based on the Van Krevelen diagram (Figure 4), which showed that CHO molecules were dominant in F2, F3, and F5. Molecular compositions of the fractions showed that more CHO molecules were present in S5 compared to those in S1. This trend was attributed to the denitrification of CHON and CHONS molecules by microorganisms in anaerobic, anoxic, and oxygen tanks in the bioreactor.[27] Unexpectedly, the number of molecules in F5 with the greatest ER activity was less compared to those in F2 and F3. Thus, strong ER-active substances might be present in F5 despite not being fully identified in the present study.

Additional data analysis was performed to identify ER-active substances in F5. Compounds that were only detected in F5 were selected, excluding compounds detected in F2, F3, and F7. CHO, CHON, CHOS, and CHONS molecules were classified according to the sewage treatment steps (S1−S5) as follows: (1) generated compounds, (2) recalcitrant compounds, and (3) removed compounds (Figure 5). Consequently, more compounds were generated in F5 during the sewage treatment process compared to removed and recalcitrant compounds. CHO molecules accounted for 61% of molecules generated in S2, while CHOS molecules accounted for 57% of molecules in S5. CHOS was previously reported to be the most abundant molecule in effluents,[65,66] which is consistent with the results of the study presented here. CHOS molecules in the effluent might include sulfonates, sulfonamides, and their metabolites.[66] Sulfonamides are ER agonists.[67] Thus, these CHO and CHOS molecules might be associated with ER-active substances in F5, which were not investigated in this study and require additional confirmation.

Environmental Implications. This study evaluated various endocrine-disrupting activities in STP water samples. AhR-, AR-, and GR-mediated potencies decreased during sewage treatment processes, whereas ER-mediated potencies did not decrease. Arenobufagin and loratadine, which are novel ER agonists present in STP water samples, were successfully identified using EDA combined with FSA. These compounds are used as pharmaceuticals and are not easily removed in the sewage treatment process. QSAR predictions indicated that these compounds have mutagenicity, carcinogenicity, and developmental toxicity in addition to ER-mediated activity. We suggest that more studies on identifying unknown toxic substances are needed. Transformation products of compounds during sewage treatment and their ER potency changes

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Environ. Sci. Technol. 2022, 56, 13085−13095

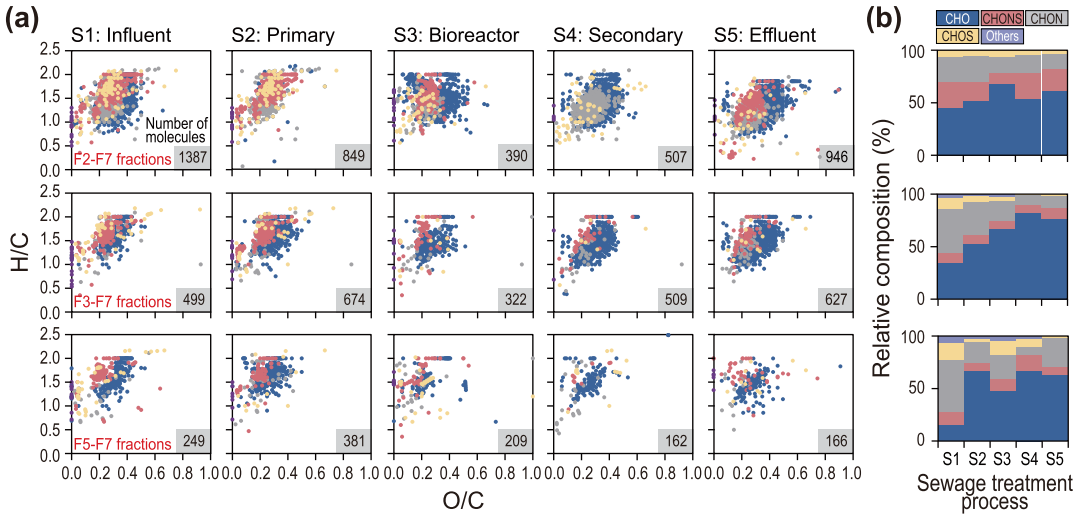

Figure 4. (a) Van Krevelen diagrams and (b) relative composition ratio of molecules (CHO, CHON, CHOS, CHONS, and other compounds) detected in the fractions (F2, F3, and F5) measured using FT-ICRMS analysis. Compounds detected in each fraction were subtracted from compounds detected in F7.

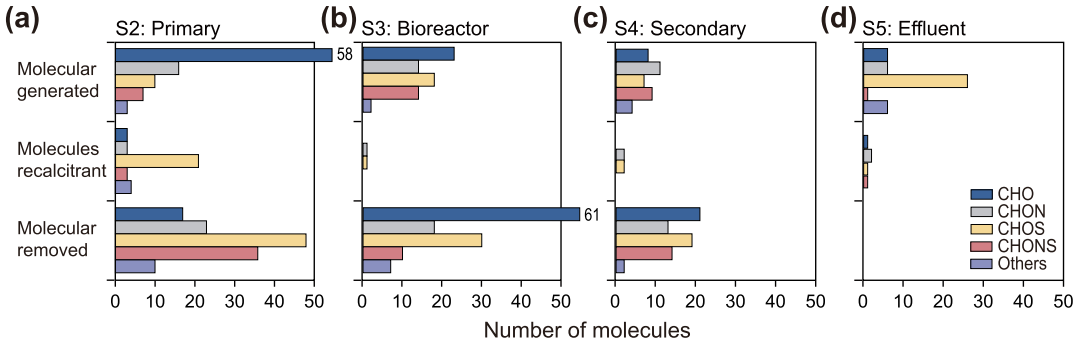

Figure 5. Molecules generated, recalcitrant, and removed during the sewage treatment process (a) influent to primary, (b) primary to bioreactor, (c) bioreactor to secondary, and (d) secondary to final effluent measured using FT-ICRMS analysis. Molecules from F5 were subtracted from compounds detected in F2, F3, and F7.

are also considered to be important research topics.[30] In addition, studies on mixture toxic effects (synergistic or antagonistic effects) associated with various substances present in samples of the STP are needed. Overall, this study is expected to be a good case study in terms of identification procedures for unknown toxic substances in samples of STPs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c03428.

RP-HPLC conditions for fractionation; experimental conditions for in vitro bioassays; HPLC-MS/MS conditions for steroidal estrogens analysis; optimization of compound-specific parameters in a MS/MS system for the analysis of E1, E2, E3, and EE2; instrumental conditions of GC-MSD, LC-QTOFMS, HPLC-MS/MS, and FT-ICRMS for targeted and nontargeted analyses; bioassay-derived EQs in REs; concentrations of steroids and AP; list of candidates of ER agonists; MS/MS parameters for quantifying ER agonists; comparison of T47D-kb*luc* bioassay results with VEGA QSAR and VirtualToxLab results; dose−response curves of steroidal estrogens and F5 of S1−S5; concentrations and contribution of steroidal estrogens to E2-EQs; concentrations of APs; candidates selection criteria for ER agonists; and extracted ion chromatograms and Q1/Q3 masses of arenobufagin and loratadine (PDF)

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Supporting Information for

Molecular characterization of estrogen receptor agonists during sewage treatment processes using effect-directed analysis combined with high-resolution full-scan screening

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This PDF file includes:

Number of pages: 22

Number of Supplementary Tables: 14, Tables S1 to S14

Number of Supplementary Figures: 6, Figures S1 to S6

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Supplementary Tables

Table S1. Reverse phase (RP)-HPLC conditions for fractionation of organic extracts.[1]

Instrument	Agilent 1260 HPLC system (Preparative scale) 1260 Multiple wavelength detector
Column	PrepHT XDB-C18 column (250 mm × 21.2 mm × 7 μm)
Mobile phase	A: Water, B: Methanol
Flow rate	10 mL min^{-1}
Injection volume	1 mL
Mobile phase gradient	40% A (0 min) → 40–0% A (0–40 min) → 0% A (40–60 min) → 0–40% A (60–62 min) → 40% A (62–70 min) 60% B (0 min) → 60–100% B (0–40 min) → 100% B (40–60 min) → 100–60% B (60–62 min) → 60% B (62–70 min)
Test standards	34 polychlorinated biphenyls 16 polycyclic aromatic hydrocarbons 7 alkylphenols 5 phthalates
Fractions collected times	

RP-HPLC Sub-fraction	Starting –End sampling time (min.)	Volume (mL)	Log K_{OW}
1	3.11 – 6.35	38	< 1
2	6.35 – 9.59	65	1 – 1.5
3	9.59–12.83	65	1.5– 2.0
4	12.83–16.08	65	2.0–2.5
5	16.08–19.32	65	2.5–3.0
6	19.32–22.58	65	3.0–3.5
7	22.58–25.83	65	3.5–4.0
8	25.83–29.06	65	4.0–4.5
9	29.06–32.29	65	4.5–5.0
10	32.29–35.54	65	5.0–5.5
11	35.54–38.78	65	5.5–6.0
12	38.78–42.02	65	6.0–6.5
13	42.02–45.26	65	6.5–7.0
14	45.26–48.48	65	7.0–7.5
15	48.48–51.70	65	7.5–8.0
16	51.70–54.97	65	8.0–8.5
17	54.97–58.20	65	8.5–9.0
18	58.20–61.48	65	9.0–9.5
19	61.48–64.72	65	9.5–10
20	64.72–70.00	65	>10

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Table S2. Experimental conditions for in vitro bioassays for AhR-, AR-, GR-, and ER-mediated potencies in samples.

Bioassay		**In vitro bioassays**			
		H4IIE-*luc*	**MDA-kb2**		**T47D-kb*luc***
Endpoint		AhR-mediated potencies	AR-mediated potencies	GR-mediated potencies	ER-mediated potencies
Test samples		SPE extracts	SPE extracts	SPE extracts	SPE extracts & RP-HPLC fractions
Experimental conditions	Test chamber	96-well plate	96-well plate	96-well plate	96-well plate
	Solvent carrier	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO
	Temperature (°C)	37	37	37	37
	Test duration	4 h	24 h	24 h	24 h
	Seeding density (cells mL^{-1})	7.0×10^4	8.0×10^4	8.0×10^4	10×10^4
	Antagonists		Mifepristone	Flutamide	
Replicates		3	3	3	3
Positive control		Benzo[*a*]pyrene	Dihydrotestosterone	Dexamethasone	17β-Estradiol
		50, 17, 6.0, 2.0, 0.62, and 0.21 nM	4.0, 1.3, 0.44, 0.15, 0.049, and 0.016 nM	10, 3.3, 1.1, 0.4, 0.1, and 0.041 nM	0.0038, 0.0012, 0.00042, 0.00014, and 0.000047 nM

Table S3. Instrumental conditions for quantifying estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethinylestradiol (EE2) in water samples of STP using HPLC-MS/MS.

Instrument	HPLC: Agilent Infinity 1290 II MS/MS: Agilent 6470 triple quadrupole mass spectrometer
Samples	F1–F20 fractions from influent, primary treatment, bioreactor, secondary treatment, effluent of STP
Analytical column	Waters X-Bridge C18 column (3.0 mm × 150 mm, 5.0 μm)
Column temperature	40 ℃
Injection volume	5 μL
Flow rate	0.4 mL min^{-1}
Mobile phase	A: 0.075% ammonium hydroxide in water (pH 9.0 adjusted with NH_4OH) B: MeOH/acetonitrile (1:1 v/v)
Mobile phase gradient	

Time (min)	Solvent	
	A	B
0.0	65	35
1.5	65	35
6.5	25	75
6.6	5	95
8.0	5	95
8.1	65	35
10	65	35

Ionization mode	Electrospray ionization (ESI) Negative mode
TOF masses (Da)	100-2000 Da
Nebulizer gas	N_2 (35 psi)
Sheath gas flow	N_2 (11 L min^{-1})
Sheath gas temperature	350 °C
Gas temperature	350 °C
Ion source	AJS ESI
Ion spray voltage	Negative: 4,000 V

Table S4. Optimization of compound-specific parameters in a tandem mass spectrometer for the analysis of E1, E2, E3, and EE2.

Compounds	MRM transition Parent ion (*m/z*)	Daughter ion (*m/z*)	Fragementor	Collision Energy (volts)	Cell Accelerator (volts)	Ion monitoring	Method detection limit (ng L^{-1})
E1	269.15	183.1	150	30	5	Negative	1.3
	269.15	159.2	150	40	5	Negative	
	269.15	145.3	150	40	5	Negative	
E2	271.17	237.3	160	35	5	Negative	0.8
	271.17	183.3	160	45	5	Negative	
	271.17	145.2	160	50	5	Negative	
E3	287.16	211.3	160	40	5	Negative	0.7
	287.16	171.1	160	36	5	Negative	
	287.16	145.4	160	40	5	Negative	
EE2	295.17	189.1	150	15	5	Negative	0.2
	295.17	159.1	150	40	5	Negative	

Table S5. Abbreviations, GC-MSD target ions, and method detection limits for alkylphenols.

Compounds	Abbreviation	Target ions		Method detection limit (ng L^{-1})
		Quantification ion	Confirmation ion	
Alkylphenol (APs)				
4-tert-Octylphenol	t-OP	207	221, 193	0.12
4-tert-Octylphenol monoethoxylate	t-OP1EO	251	265, 135	0.61
4-tert-Octylphenol diethoxylate	t-OP2EO	295	309, 351	0.08
Nonylphenols	NPs	207	221, 193	3.7
Nonylphenol monoethoxylates	NP1EOs	251	265, 207	0.45
Nonylphenol diethoxylates	NP2EOs	295	309	1.5
Internal standard				
2-Fluorobiphenyl	IS	172	171, 170	

Table S6. Instrumental conditions of LC-QTOFMS for full-scan screening analysis**.**

Instrument	LC: 1290 infinity II (Agilent Technologies, Santa Clara, CA) QTOFMS: Triple time-of-flight (TripleTOF®) 5600+ mass spectrometer (AB Sciex, Framingham, MA)
Samples	F5 RP-HPLC fractions from influent, primary treatment, bioreactor, secondary treatment, and effluent
Analytical column	ZORBAX Eclipse XDB-C18 (150 mm × 2.1 mm i.d. × 5 μm film)
Column temperature	40 °C
Injection volume	3 μL
Flow rate	0.4 mL min^{-1}
Mobile phase	A: 0.1% Formic acid and 10mM ammonium formate in water, B: 0.1% Formic acid in acetonitrile
Mobile phase gradient	Time (min) / Solvent A / Solvent B 0 / 90 / 10 1 / 90 / 10 15 / 0 / 100 24 / 0 / 100 25 / 90 / 10 30 / 90 / 10
Ionization mode	Electrospray ionization (ESI) Positive and Negative mode
Mass scan type	Full scan and Information Dependent Acquisition (IDA) Scanning
TOF masses (Da)	100–2000 Da
Ion source gas 1	50 psi
Ion source gas 2	50 psi
Curtain gas	30 psi
Temperature	500 °C
Ion source	DuoSpray Ion Source
Ion spray voltage	Positive: 5,500 V, Negative -4,500 V
Software	All-in-One_HRMS/MS TCM library 1.0 metabolite software

Table S7. Instrumental conditions for arenobufagin and loratadine in water samples of STP using HPLC-MS/MS.

Instrument	HPLC: Agilent Infinity 1290 II MS/MS: Agilent 6470 triple quadrupole mass spectrometer
Samples	F5 fraction from influent, primary treatment, bioreactor, secondary treatment, effluent
Analytical column	ZORBAX Eclipse XDB-C18 (150 mm × 2.1 mm i.d. × 5 μm film)
Column temperature	40 ℃
Injection volume	3 μL
Flow rate	0.4 mL min^{-1}
Mobile phase	A: 0.1% Formic acid and 10 mM ammonium formate in water B: 0.1% Formic acid in acetonitrile
Mobile phase gradient	

Time (min)	Solvent	
	A	B
0.0	70	30
0.5	70	30
3.0	45	55
5.0	0	100
6.0	0	100
6.5	70	30
7.0	70	30

Ionization mode	Electrospray ionization (ESI) Positive mode
TOF masses (Da)	100–2000 Da
Nebulizer gas	N_2 (35 psi)
Sheath gas flow	N_2 (11 L min^{-1})
Sheath gas temperature	350 °C
Gas temperature	350 °C
Ion source	AJS ESI
Ion spray voltage	Positive: 4,000 V

Table S8. Instrumental conditions and formula assignment parameters for analyzing fractions of water samples from STP using FT-ICRMS.

FT-ICRMS condition : parameter settings	
Instrument	FT-ICRMS: Apollo II electrospray ionization source
Samples	F2, F3, F5, and F7 fractions from water samples of STP
Source	ESI^-
Mass range	150–1200 *m/z*
Scan number	100
Capillary voltage	3,500 V
Nebulizer	0.5 Bar
Dry gas flow rate	4 L min^{-1}
Dry gas temperature	180 ℃
Skimmer voltage	-45 V
Mass resolving power	> 440,000 at *m/z* 400
External calibration solution	100 ppm NaTFA calibration solution (Error ppm: 0.091)
Formula assignment parameter	
Processing software	Composer (Sierra Analytics)
S/N ratio	> 10
Element condition	$C_{0–100}H_{0–200}O_{0–60}N_{0–4}S_{0–2}$
Matching tolerance	< 0.5 ppm

Table S9. Bioassay-derived equivalent concentrations in raw organic extracts of S1–S5.

Endpoint	Samples	Bioassay-derived equivalent concentrations (EQs)	
		Magnitude-based EQ	**Magnitude-based EQ (%)**
		Magnitude-based BaP_{max}-EQ[a] (ng BaP-EQ L^{-1})	**Magnitude-based BaP_{max}-EQ (%)**
AhR-mediated potency	S1	2.0	68
	S2	49	130
	S3	0.1	14
	S4	ns[b]	ns
	S5	ns	ns
		Magnitude-based DHT_{max}-EQ (ng DHT-EQ L^{-1})	**Magnitude-based DHT_{max}-EQ (%)**
AR-mediated potency	S1	8.2	130
	S2	2.2	110
	S3	ns	ns
	S4	ns	ns
	S5	ns	ns
		Magnitude-based $Dexa_{max}$-EQ (ng Dexa-EQ L^{-1})	**Magnitude-based $Dexa_{max}$-EQ (%)**
GR-mediated potency	S1	3.0	130
	S2	2.0	37
	S3	9.6×10^{-3}	6.8
	S4	ns	ns
	S5	ns	ns

[a] Magnitude-based EQ concentrations were calculated from the percentage of the maximum response observed for a 50 nM BaP, 4.0 nM DHT, and 10 nM Dexa elicited by raw extracts.

[b] ns: Not significant.

Table S10. Concentrations of steroidal estrogens in RP-HPLC fractions of organic extracts of water samples from STP (S1–S5).

Sites	Compounds	Raw extracts (ng L^{-1})	RP-HPLC fractions (ng L^{-1})										Sum of compounds in RP-HPLC fractions (ng L^{-1})	Recoveries (%)[a]
			F1	F2	F3	F4	F5	F6	F7	F8	F9	F10–F20		
S1	E1	150	< DL[b]	< DL	< DL	< DL	140	4.0	< DL	< DL	< DL	< DL	144	96
	E2	4	< DL	< DL	< DL	< DL	3.0	< DL	< DL	< DL	< DL	< DL	3	75
	E3	140	1.1	130	5.6	< DL	< DL	< DL	< DL	< DL	< DL	< DL	137	98
	EE2	40	5.1	15	1.0	2.8	2.0	< DL	< DL	< DL	< DL	< DL	26	65
S2	E1	246	< DL	< DL	< DL	14	200	< DL	< DL	< DL	< DL	< DL	214	87
	E2	10	< DL	< DL	< DL	< DL	8.0	< DL	< DL	< DL	< DL	< DL	8	80
	E3	86	< DL	85	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	85	99
	EE2	21	0.48	15	0.32	0.2	0.24	< DL	< DL	< DL	< DL	< DL	16	77
S3	E1	45	< DL	< DL	< DL	< DL	40	8.2	< DL	< DL	< DL	< DL	48	107
	E2	12	< DL	< DL	< DL	< DL	8.1	< DL	< DL	< DL	< DL	< DL	8	68
	E3	28	1.0	14	1.6	< DL	< DL	< DL	< DL	< DL	< DL	< DL	17	60
	EE2	24	4.9	1.7	3.1	0.65	0.39	< DL	< DL	< DL	< DL	< DL	11	45
S4	E1	6.1	< DL	< DL	< DL	< DL	5.2	< DL	< DL	< DL	< DL	< DL	5	85
	E2	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	-	-
	E3	3.1	< DL	2.8	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	3	90
	EE2	13	< DL	11	1.0	< DL	< DL	< DL	< DL	< DL	< DL	< DL	12	92
S5	E1	4.8	< DL	< DL	< DL	< DL	1.2	4.4	< DL	< DL	< DL	< DL	6	117
	E2	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	-	-
	E3	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	-	-
	EE2	7.1	< DL	5.2	1.0	< DL	< DL	< DL	< DL	< DL	< DL	< DL	6	87
Experimental	E1	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	-	-
Blank	E2	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	-	-
	E3	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	-	-
	EE2	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	-	-

[a] Recoveries were calculated from the percentage divided by the concentrations of the compounds in RP-HPLC fractions and those in raw extracts.

[b] < DL: Below detection limit.

Table S11. Concentrations of alkylphenols in water samples of STP (S1–S5).

Compounds	Abb.[a]	Sewage treatment process water (ng L^{-1})				
		S1	S2	S3	S4	S5
4-tert-Octylphenol	t-OP	75.6	139	55.1	55.3	58.0
4-tert-Octylphenol monoethoxylate	t-OP1EO	127	169	51.6	57.7	67.5
4-tert-Octylphenol diethoxylate	t-OP2EO	1,050	162	43.5	55.8	62.9
Nonylphenols	NPs	1,000	1,030	978	984	983
Nonylphenol monoethoxylates	NP1EOs	358	467	170	217	194
Nonylphenol diethoxylates	NP2EOs	698	936	560	627	518

Table S12. List of candidates of ER agonists in the fraction samples (F5) from STP water samples (S1–S5).

Influent	Primary treatment	Bioreactor	Secondary treatment	Effluent	Molecular formula	Molecular weight	CAS number
Etofenprox	**Etofenprox**	**Etofenprox**	**Etofenprox**	**Etofenprox**	**$C_{25}H_{28}O_3$**	**376.50**	**80844-07-1**
Arenobufagin	**Arenobufagin**	**Arenobufagin**	**Arenobufagin**	**Arenobufagin**	**$C_{24}H_{32}O_6$**	**416.51**	**464-74-4**
Loratadine	**Loratadine**	**Loratadine**	**Loratadine**	**Loratadine**	**$C_{22}H_{23}ClN_2O_2$**	**382.89**	**79794-75-5**
Omethoate	**Omethoate**	**Omethoate**	**Omethoate**	**Omethoate**	**$C_5H_{12}NO_4PS$**	**213.19**	**1113-02-6**
6-Beta-Naltrexol	**6-Beta-Naltrexol**	**6-Beta-Naltrexol**	**6-Beta-Naltrexol**	**6-Beta-Naltrexol**	**$C_{20}H_{25}NO_4$**	**343.42**	**49625-89-0**
Resibufogenin	**Resibufogenin**	**Resibufogenin**	**Resibufogenin**	**Resibufogenin**	**$C_{24}H_{32}O_4$**	**384.51**	**465-39-4**
Corticosterone		**Corticosterone**		**Corticosterone**	**$C_{21}H_{30}O_4$**	**346.47**	**50-22-6**
Naltrexone	Naltrexone	Naltrexone			$C_{20}H_{23}NO_4$	341.40	16590-41-3
Sesamoside	Sesamoside				$C_{17}H_{24}O_{12}$	420.37	117479-87-5
Doxycycline	Doxycycline				$C_{22}H_{24}N_2O_8$	444.40	564-25-0
3-Methyladenine	3-Methyladenine				$C_6H_7N_5$	149.15	5142-23-4
Rocuronium	Rocuronium				$C_{32}H_{53}N_2O_4^+$	609.69	143558-00-3
Enrofloxacin-D5		Enrofloxacin-D5			$C_{19}H_{22}FN_3O_3$	359.40	1173021-92-5
Ziprasidone		Ziprasidone			$C_{21}H_{21}ClN_4OS$	412.94	146939-27-7
Cortisol					$C_{21}H_{30}O_5$	362.46	50-23-7
Norbuprenorphine					$C_{25}H_{35}NO_4$	413.55	78715-23-8
Diniconazole					$C_{15}H_{17}Cl_2N_3O$	326.22	83657-24-3
Triptonide					$C_{20}H_{22}$	358.39	38647-11-9
Deacetyl asperulosidic acid methyl ester					$C_{17}H_{24}O_{11}$	404.40	52613-28-2
Norbuprenorphine					$C_{25}H_{35}NO_4$	413.55	78715-23-8
Glycyrrhetinic acid					$C_{30}H_{46}O_4$	470.68	471-53-4
Buturon					$C_{12}H_{13}ClN_2O$	236.70	3766-60-7
Benzoylmesaconine					$C_{31}H_{43}NO_{10}$	589.67	63238-67-5
Ranitidine					$C_{13}H_{22}N_4O_3S$	314.40	66357-35-5
5-hydroxy metabolite					$C_{13}H_{11}N_3O_5$	289.24	460741-57-5
	Ginkgolide B			Ginkgolide B	$C_{20}H_{24}O_{10}$	424.14	15291-77-7
	Hydramethylnon			Hydramethylnon	$C_{25}H_{24}F_6N_4$	494.48	67485-29-4
		Sclareol	Sclareol	Sclareol	$C_{20}H_{36}O_2$	308.49	515-03-7
		Betulonicacid	Betulonicacid				
		Diphenoxylate		Diphenoxylate	$C_{30}H_{32}N_2O_2$	452.59	915-30-0

	Clonidine		Clonidine	$C_9H_9Cl_2N_3$	230.09	4205-90-7
	Propiconazole			$C_{15}H_{17}C_{l2}N_3O_2$	342.22	60207-90-1
	Triphenylphosphate			$C_{18}H_{15}O_4P$	326.28	115-86-6
	Phenazepam			$C_{15}N_2H_{10}OBrCl$	349.61	51753-57-2
	Linthospermic Acid			$C_{27}H_{22}O_{12}$	538.46	28831-65-4
	Phendimetrazine			$C_{12}H_{17}NO$	191.27	634-03-7
	Naringenin			$C_{15}H_{12}O_5$	272.257	480-41-1
	Dibutyl phthalate			$C_{16}H_{22}O_4$	278.34	
	Triamterene			$C_{12}H_{11}N_7$	253.263	396-01-0
	Lithocholic acid			$C_{24}H_{40}O_3$	376.5726	
	Isorhamnetin			$C_{16}H_{12}O_7$	316.26	
		Fenhexamid		$C_{14}H_{17}C_{l2}NO_2$	302.196	126833-17-8
		Tuberostemonine		$C_{22}H_{33}NO_4$	375.502	6879-01-2
		Flunixin		$C_{14}H_{11}F_3N_2O_2$	296.245	38677-85-9
			Cinnamic acid	$C_9H_8O_2$	148.16	140-10-3
			Amygdalin	$C_{20}H_{27}NO_{11}$	457.431	29883-15-6
			Flavin Mononucleotide	$C_{17}H_{21}N_4O_9P$	456.344	146-17-8

Table S13. Conditions of HPLC-MS/MS for quantifying ER agonists in water samples of STP.

Compounds	MRM transition Parent ion (*m/z*)	Daughter ion (*m/z*)	Fragementor	Collision Energy (volts)	Cell Accelerator (volts)	Polarity	Method detection limit (ng L^{-1})
Arenobufagin	417.22	399.20	175	30	5	Positive	0.15
	417.22	147.20	175	45	5	Positive	
	417.22	105.20	175	60	5	Positive	
Loratadine	383.15	337.20	145	30	5	Positive	0.42
	383.15	267.20	145	40	5	Positive	
	383.15	259.20	145	35	5	Positive	

Table S14. Comparison of T47D-kb*luc* bioassay results with VEGA QSAR and VirtualToxLab results.

Compounds	Bioassay (using T47D-kb*luc*)	VirtualToxLab	VEGA QSAR	
			IRFMN Model	IRFMN/CERAPP model
Etofenprox	–	+	–	–
Arenobufagin	+	–	–	+
Loratadine	+	–	+	–
Omethoate	–	–	–	–
6-Beta-Naltrexol	n.a.	–	+	+
Resibufogenin	–	+	–	–
Corticosterone	–	+	–	–

[a] +: active.
[b] n.a: not available.

Supplementary Figures

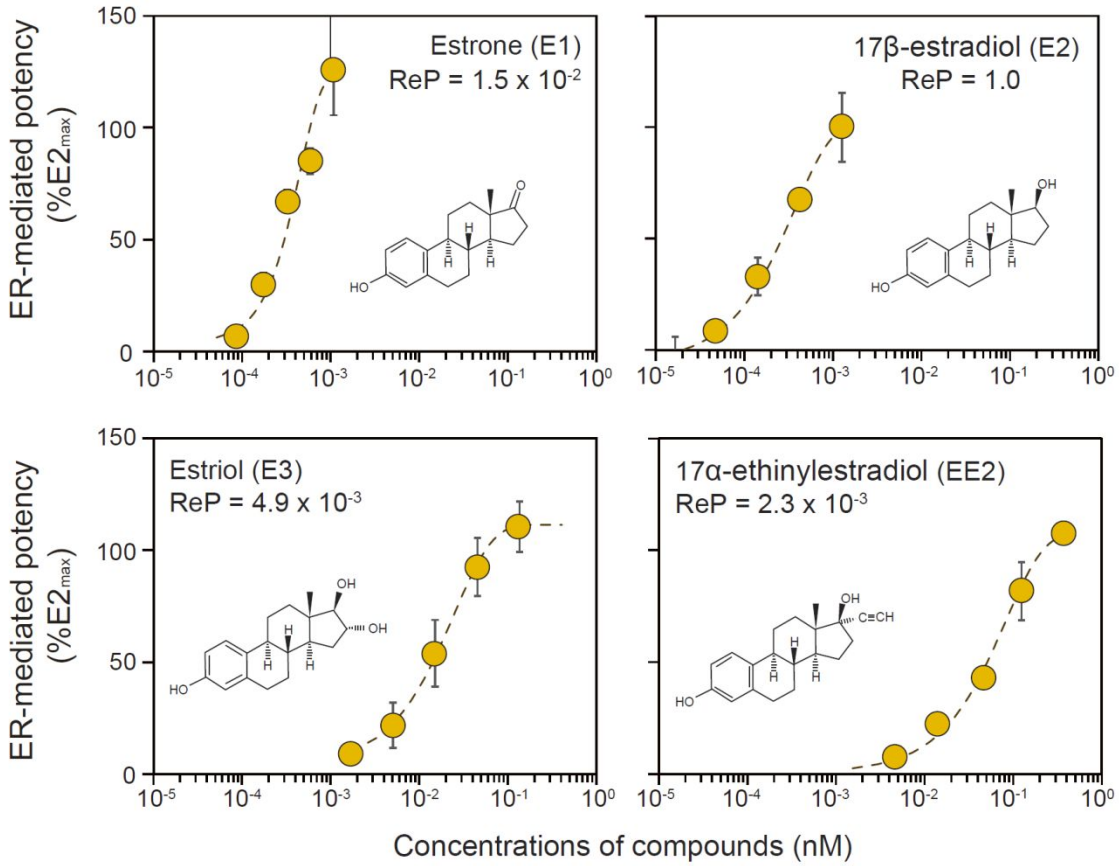

Figure S1. Dose-response curves for ER-mediated potencies of steroidal estrogens (E1, E2, E3, and EE2) in the T47D-Kb*luc* bioassay (error bar: mean ± SD, n = 3).

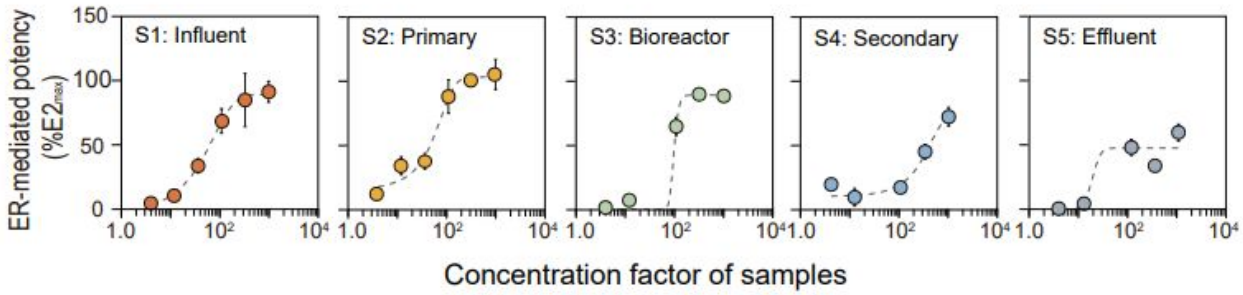

Figure S2. Dose-response curves for ER-mediated potencies of selected RP-HPLC fractions (F5 of S1–S5) (error bar: mean ± SD, n = 3).

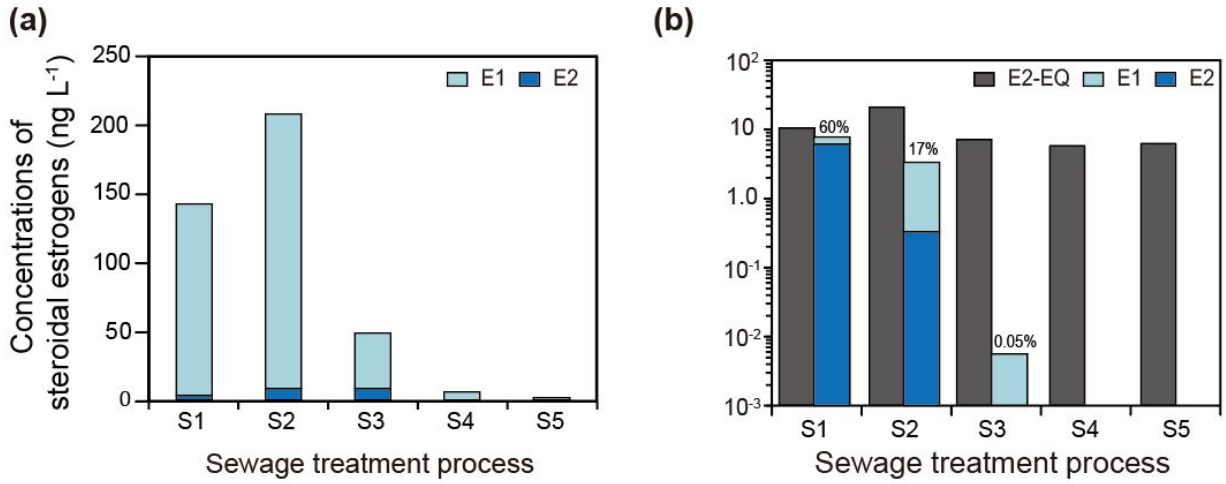

Figure S3. (a) Concentrations of steroidal estrogens (E1 and E2) in F5 of S1–S5 and **(b)** contribution of steroidal estrogens to E2-EQs (potency-based).

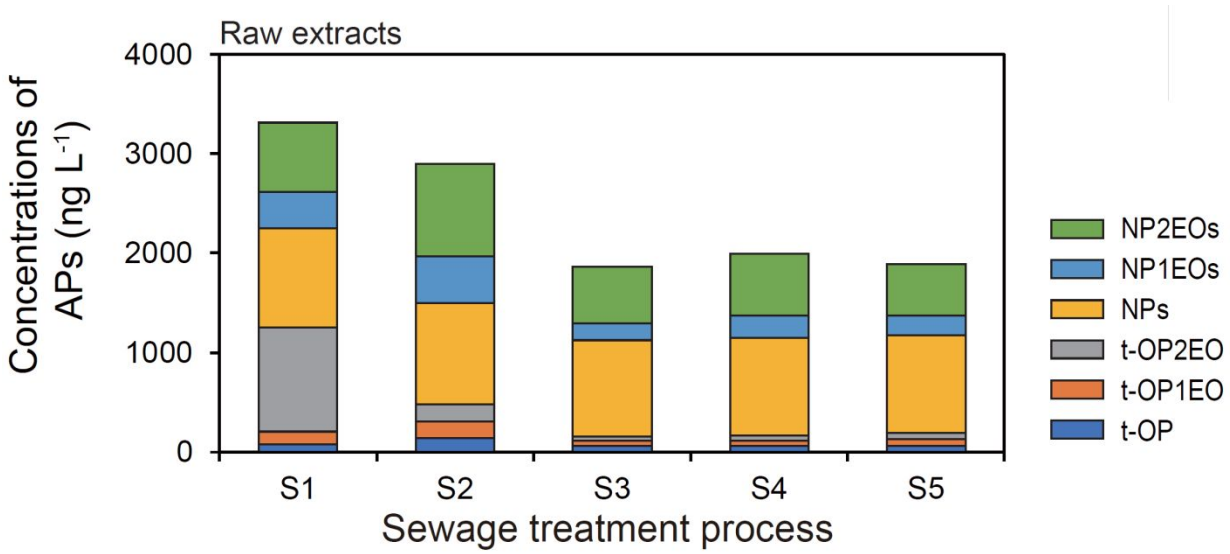

Figure S4. Concentrations of APs in water samples of STP (S1–S5).

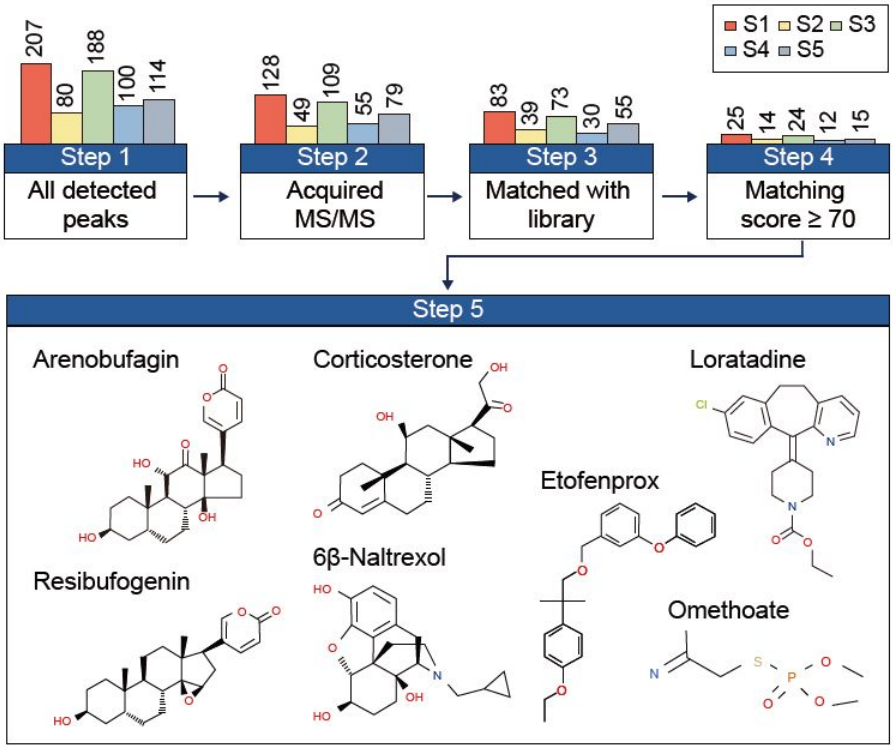

Figure S5. LC-QTOFMS data analysis for selecting candidates for ER agonists (Five steps).

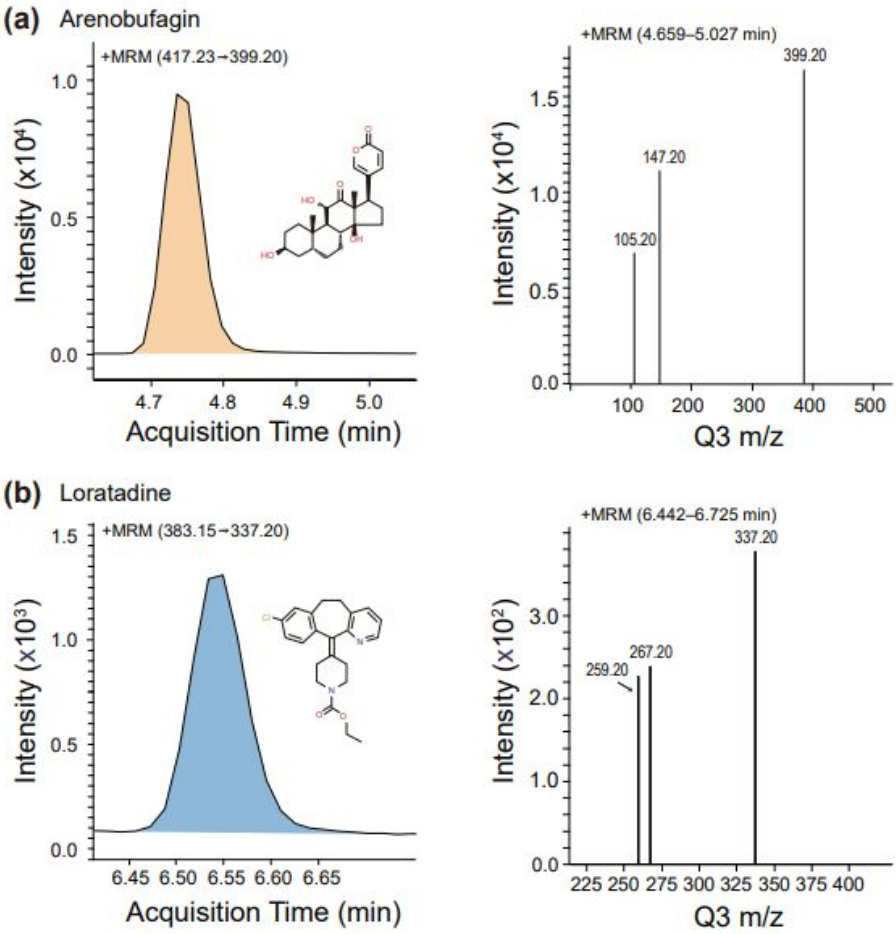

Figure S6. Extracted ion chromatograms and Q1/Q3 masses of (a) arenobufagin and (b) loratadine.