Identification and Environmental Implications of Photo-Transformation Products of Trenbolone Acetate Metabolites

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*Supporting Information

ABSTRACT: Despite the widespread use of the anabolic androgen trenbolone acetate (TBA) in animal agriculture, evidence demonstrating the occurrence of TBA metabolites such as 17β-trenbolone (17β-TBOH), 17α-trenbolone (17α-TBOH), and trendione (TBO) is relatively scarce, potentially due to rapid transformation processes such as direct photolysis. Therefore, we investigated the phototransformation of TBA metabolites and associated ecological implications by characterizing the photoproducts arising from the direct photolysis of 17β-TBOH, 17α-TBOH, and TBO and their associated ecotoxicity. LC-HRMS/MS analysis identified a range of hydroxylated products that were no longer photoactive, with primary photoproducts consisting of monohydroxy species and presumptive diastereomers. Also observed were higher-order hydroxylated products probably formed via subsequent reaction of primary photoproducts. NMR analysis confirmed the formation of 12,17-dihydroxy-estra-5(10),9(11),dien-3-one (12-hydroxy-TBOH; 2.2 mg), 10,12,17-trihydroxy-estra-4,9(11),dien-3-one (10,12-dihydroxy-TBOH; 0.7 mg), and a ring-opened 11,12-dialdehyde oxidation product (TBOH-11,12-dialdehyde; 1.0 mg) after irradiation of ~14 mg of 17β-trenbolone. Though unconfirmed by NMR, our data suggest that the formation of additional isomeric products may occur, likely due to the reactivity of the unique 4,9,11 conjugated triene structure of trenbolone. In vivo exposure studies employing Japanese medaka (Oryzias latipes) indicate that low concentrations of 17α-TBOH photoproduct mixtures can alter ovarian follicular development, and photoproducts alter whole-body 17β-estradiol levels. Therefore, direct photolysis yields photoproducts with strong structural similarity to parent steroids, and these photoproducts still retain enough biological activity to elicit observable changes to endocrine function at trace concentrations. These data indicate that environmental transformation processes do not necessarily reduce steroid hormone ecotoxicity.

INTRODUCTION

In the United States and abroad, the androgenic steroid trenbolone acetate (TBA) is widely used as a growth promoter in beef cattle production.1−3 TBA is effective because it is 15−50 times more androgenically and anabolically potent than testosterone.4 We estimate that annual TBA production and use in the U.S. likely exceeds 5000 kg/year, and some studies report that 60−90% of U.S. beef cattle receive TBA implants.3−5 Most of these data suggest that TBA is one of the most heavily used synthetic steroids. Its use and the large-scale operations typical of animal agriculture also imply that TBA or its metabolites may be widespread in environments affected by animal agriculture. As many studies demonstrate endocrine
disruption and impaired reproduction in aquatic organisms resulting from exposure to potent endogenous and synthetic steroids,\textsuperscript{2,8–11} assessing the fate of TBA and related metabolites in aquatic environments is a priority.

After implant release, TBA is converted to the active androgen 17β-trenbolone (17β-TBOH), which is metabolized to 17α-trenbolone (17α-TBOH) and trendione (TBO).\textsuperscript{1} Together, these metabolites, of which 17α-TBOH composes 95\% of the known TBA metabolite mass excreted, account for \textasciitilde{}8\% of the total TBA implant mass, with the remainder composed of uncharacterized metabolites.\textsuperscript{1} Mass balance calculations suggest that up to 80 \(\mu\)g/day of 17α-TBOH is excreted from implanted cattle, resulting in concentrations potentially as high as several thousand ng/L in runoff from animal agriculture sites such as CAFOs.\textsuperscript{5,12} 17β-TBOH and TBO also are detected in runoff and soils near animal agriculture operations.\textsuperscript{12–16}

Low concentrations of TBA metabolites can affect morphology and reproduction in exposed aquatic organisms. Ankley et al. and Jensen et al. report fecundity reduction in fathead minnows at 11–27 ng/L for 17α-TBOH and 17β-TBOH, respectively.\textsuperscript{2,3,17} No-effects concentrations for these compounds are likely even lower, and are probably similar to the \textasciitilde{}1 ng/L no-effects concentrations frequently reported for estrogens.\textsuperscript{3,19} In addition, skewed sex ratios, physiological masculinization, inhibited embryonic development, and genotoxicity are observed in fish exposed to 17α-TBOH and 17β-TBOH.\textsuperscript{11–13,20} To date, no ecotoxicology studies have been reported for TBO.

Despite the widespread use of TBA in animal agriculture, these compounds are detected rather infrequently compared to endogenous steroids such as estrogens with similar chemical properties and often at lower concentrations than would be expected from TBA mass usage alone.\textsuperscript{12,15,21} For example, in our own experience, analysis of soils and water runoff from cattle CAFOs with extensive trenbolone use sometimes does not detect TBA metabolites even though we expected detection.\textsuperscript{16} However, a number of ecotoxicology studies have reported instances of endocrine disruption in aquatic organisms in close proximity to animal agriculture operations, although related chemical analyses, including known TBA metabolites, were unable to identify causative agents.\textsuperscript{9,11,13,22,23} These observations suggest the potential importance of transformation mechanisms (e.g., biotransformation, photolysis) and related product formation on TBA metabolite fate and ecosystem risk in aquatic environments.

Previously, we reported that direct photolysis represents the dominant phototransformation mechanism for 17α-TBOH, 17β-TBOH, and TBO, and that the time scales of this process are quite short (half-lives \textasciitilde{} 1 h).\textsuperscript{24} Due to the likely dominance of this degradation pathway in surface waters,\textsuperscript{24} the objective of this study was to investigate the related direct photolysis products of known TBA metabolites (17α-TBOH, 17β-TBOH, and TBO) by utilizing liquid chromatography–high resolution tandem mass spectrometry (LC-HRMS/MS) analysis. After photolysis and fractionation, nuclear magnetic resonance (NMR) analysis was used to confirm the structure of the most abundant 17β-TBOH photoproducts. Finally, the endocrine disrupting capability of photoproduct mixtures was assessed using static in vivo and in vitro bioassays for estrogenic and androgenic end points.

### EXPERIMENTAL SECTION

**Photolysis Studies.** Most photolysis experiments were conducted in natural sunlight in clear mid-day conditions (temperatures averaged 15–23 °C) in Reno, NV (latitude 39.54, longitude –119.81). Cylindrical (12-mm diameter) borosilicate glass flasks were filled with 50 mL of deionized water spiked with \textasciitilde{}100 \(\mu\)L of concentrated steroid stock solution in methanol to a final 2–3 \(\mu\)M concentration. Dark controls consisted of identical flasks wrapped in foil, although analysis of these samples demonstrated no decay. After 0, 45, and 120 min of irradiation, 2-mL samples were collected and methanol was added (90\% water, 10\% methanol \(\nu/\nu\)) for immediate LC-HRMS/MS analysis.

A subset of experiments also was performed using simulated sunlight as described in our previous work.\textsuperscript{24} Using the same concentration and sampling intervals as for the natural sunlight experiments, 30-mL aqueous samples were extracted on preconditioned C-18 SPE cartridges, chilled, and shipped overnight from the University of Iowa to the University of Nevada, Reno for analysis. Upon arrival, SPE samples were extracted with 5-mL aliquots of methanol, concentrated to 200 \(\mu\)L and diluted with deionized water (90\% water, 10\% methanol \(\nu/\nu\)) for LC-HRMS/MS analysis. In most cases, initial evaluation of photoproducts, parent transformation, and photoproduct stability were performed using standard high-pressure liquid chromatography–diode array (HPLC-DAD) or low-resolution LC-MS/MS analysis.\textsuperscript{24}

**LC-HRMS/MS Analysis.** LC-HRMS/MS separation protocols are described in the Supporting Information (SI). Photoproducts were analyzed using an LTQ-Orbitrap XL (ThermoElectron) detector equipped with an Ion Max source using Xcalibur v 2.0.7 software for data processing. The MS was operated in data-dependent mode switching between Orbitrap-MS for high-resolution full scan data and LTQ-MS/MS for low-resolution MS/MS fragmentation data. Full scan spectra (\(m/z\) 100–350) were acquired in the positive ion mode with resolution of 100,000 in profile mode. The three most intense data-dependent peaks at each analysis interval were subjected to MS/MS using collision-induced dissociation with a minimum signal of 5000, isolation width of 3.0 amu, and normalized collision energy of 35.0\%. Ions already selected were dynamically excluded for 30 s after a repeat count of 2 with a repeat duration of 10 s. Also, during each scan, all \(m/z\) 271.17 and 269.15 ions, representing 17α- or 17β-TBOH parents and TBO, respectively, were subjected to MS/MS analysis using collision-induced dissociation with a normalized collision energy of 35.0\% and an isolation width of 3.0.

Because pure standards of potential products are not available, identification of prospective products in irradiated samples by LC-HRMS/MS first employed the time-dependent growth of chromatographic peaks which were not present in the standard or control solutions as the primary screening criteria. Concurrent HRMS full scan (all) and MS/MS spectra (most) then were collected for the most intense peaks in the chromatogram over a 120–350 amu range (Figure 2). All peaks with a minimum integrated area unit of 10,000 or greater in single mass chromatograms over the 250–350 amu range were examined. Peaks were eliminated as prospective products if their observed mass was not within 0.03 amu of the predicted product structure exact mass. For observed peaks with accurate masses close enough to the predicted exact mass to imply a structural relationship, MS/MS fragment comparisons were
performed, with photoproducts typically exhibiting at least 3–4, and occasionally as many as 8, of the same MS/MS fragments as parents. Fragments at m/z 159 and 133 were particularly diagnostic of trenbolone; these fragments are conserved across all parents and present in nearly all of the identified photoproducts, especially those with masses closest to parent mass. At larger masses (e.g., trihydroxy species), fragment conservation diminished as these structures fragmented by increasingly unique mechanisms.

**NMR Analysis.** Structure elucidation of the most stable 17β-TBOH photoproducts was accomplished by NMR analysis, including results from 1H NMR, COSY (correlation spectroscopy), HSQC (heteronuclear single quantum coherence), and HMBC (heteronuclear multiple bond correlation) experiments (see SI for isolation protocol, NMR spectra, and signal assignments). Two-dimensional (COSY, HSQC, and HMBC) experiments were performed on a 600-MHz Bruker AVANCE-III equipped with a 1.7-mm triple-resonance (1H, 13C, 15N) inverse probe. 1H NMR data were acquired on a Bruker AVANCE 500. Due to the high cost of 17α-TBOH and TBO standards, NMR analysis of these compounds was not performed, though we presume that 17β-TBOH photoproducts analysis also applies to 17α-TBOH and TBO.

**Theoretical Methods.** To assist in their identification, UV/vis spectra for proposed product structures were calculated using computational methods. Details are provided in the SI.

**Photoproduct Ecotoxicology.** Both in vivo and in vitro bioassays were used to assess biological effects of photoproduct mixtures generated from 17β-TBOH, 17α-TBOH, and TBO phototransformation. Static in vivo studies (n = 3 replicates with 10 fish per replicate, for each TBA metabolite) employed female Japanese medaka (*Oryzias latipes*) as test organisms, and both histological and whole-body steroid level analyses were performed after exposure to photoproduct mixtures. Photoproduct exposure concentrations (10, 100, or 1000 ng/L) represent the concentrations of parent TBA metabolites present in photoreactors prior to phototransformation. Redosing of photoproduct mixtures was accomplished by

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*Figure 1.* Representative time courses and corresponding UV/vis absorbance scans for photoproducts detected during direct photolysis experiments with (a,b) 17α-TBOH, (c,d) 17β-TBOH, and (e,f) TBO. All experiments were conducted at pH 7 with ~10 μM initial concentration.
Table 1. Observed Photoproducts of 17α-TBOH, 17β-TBOH, and TBO

<table>
<thead>
<tr>
<th>proposed product</th>
<th>retention time (min)</th>
<th>product ion</th>
<th>mass</th>
<th>comment</th>
</tr>
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<tbody>
<tr>
<td>17α-TBOH Parent</td>
<td>8.31</td>
<td>[M + H]⁺</td>
<td>271.1685</td>
<td>proposed structure by analogy to 17β-TBOH products</td>
</tr>
<tr>
<td>10-hydroxy-17α-TBOH</td>
<td>4.91</td>
<td>[M + H−H₂O]⁺</td>
<td>271.1684</td>
<td>proposed structure by analogy to confirmed 17β-TBOH products</td>
</tr>
<tr>
<td>12-hydroxy-17α-TBOH</td>
<td>6.20</td>
<td>[M + H−H₂O]⁺</td>
<td>271.1685</td>
<td>proposed structure by analogy to confirmed 17β-TBOH products</td>
</tr>
<tr>
<td>unknown</td>
<td>7.42</td>
<td>likely [M + H]⁺</td>
<td>271.1684</td>
<td>potential stereoisomer or structural analog of 17α-TBOH</td>
</tr>
<tr>
<td>unknown</td>
<td>10.25</td>
<td>likely [M + H]⁺</td>
<td>271.1685</td>
<td>likely stereoisomer or structural analog of 17α-TBOH</td>
</tr>
<tr>
<td>unknown</td>
<td>3.34, 3.74, 3.98</td>
<td>[M + H]⁺</td>
<td>303.1581</td>
<td>poorly resolved group of potential dialdehyde or dihydroxy products of unresolved structure</td>
</tr>
<tr>
<td>trihydroxy-17α-TBOH</td>
<td>3.71</td>
<td>[M + H]⁺</td>
<td>321.1687</td>
<td>potential 10,11,12 trihydroxy product, but other hydroxylation sites also possible</td>
</tr>
<tr>
<td>trihydroxy-17α-TBOH</td>
<td>4.75</td>
<td>[M + H]⁺</td>
<td>321.1687</td>
<td>dominant trihydroxy product</td>
</tr>
<tr>
<td>17β-TBOH Parent</td>
<td>8.07</td>
<td>[M + H]⁺</td>
<td>271.1685</td>
<td></td>
</tr>
<tr>
<td>10-hydroxy-17β-TBOH</td>
<td>4.93</td>
<td>[M + H−H₂O]⁺</td>
<td>271.1685</td>
<td>proposed structure; less stable, decays to secondary products quickly</td>
</tr>
<tr>
<td>12-hydroxy-17β-TBOH</td>
<td>5.55</td>
<td>[M + H−H₂O]⁺</td>
<td>271.1685</td>
<td>NMR confirmed; dominant product</td>
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<td>hydroxy-17β-TBOH?</td>
<td>7.73</td>
<td>[M + H−H₂O]⁺</td>
<td>271.1685</td>
<td>potential hydroxy-trenbolone product or structural analog</td>
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<tr>
<td>presumed dialdehyde or dihydroxy products</td>
<td>3.03, 3.36, 3.70, etc</td>
<td>[M + H]⁺</td>
<td>303.1583</td>
<td>poorly resolved dialdehyde or dihydroxy products; NMR confirmed 11,12 dialdehyde present</td>
</tr>
<tr>
<td>10,12,12-dihydroxy-17β-TBOH</td>
<td>2.90</td>
<td>[M + H]⁺</td>
<td>305.1739</td>
<td>NMR confirmed 10,12 dihydroxy structure</td>
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<td>tridydroxy-17β-TBOH</td>
<td>3.57</td>
<td>[M + H]⁺</td>
<td>321.1688</td>
<td>dominant trihydroxy product</td>
</tr>
<tr>
<td>trihydroxy-17β-TBOH</td>
<td>4.01</td>
<td>[M + H]⁺</td>
<td>321.1688</td>
<td>trihydroxy product of unresolved structure</td>
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<tr>
<td>TBO Parent</td>
<td>9.54</td>
<td>[M + H]⁺</td>
<td>269.1528</td>
<td></td>
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<tr>
<td>10-hydroxy-TBO</td>
<td>6.10</td>
<td>[M + H−H₂O]⁺</td>
<td>269.1528</td>
<td>proposed structure by analogy to confirmed 17β-TBOH products</td>
</tr>
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<td>12-hydroxy-TBO</td>
<td>6.47</td>
<td>[M+H−H₂O]⁺</td>
<td>269.1528</td>
<td>dominant product; proposed structure by analogy to confirmed 17β-TBOH products</td>
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<tr>
<td>dialdehyde or dihydroxy</td>
<td>3.29</td>
<td>[M + H]⁺</td>
<td>303.1582</td>
<td>observed product</td>
</tr>
<tr>
<td>trihydroxy-TBO</td>
<td>3.34</td>
<td>[M + H]⁺</td>
<td>319.1531</td>
<td>potential 10,11,12 trihydroxy product, but other hydroxylation sites also possible</td>
</tr>
<tr>
<td>trihydroxy-TBO</td>
<td>4.04</td>
<td>[M + H]⁺</td>
<td>319.1532</td>
<td>potential 10,11,12 trihydroxy product, but other hydroxylation sites also possible; dominant trihydroxy product</td>
</tr>
</tbody>
</table>

complete water changes every 48 h. As histological evaluations indicated estrogenic activity, in vitro assays also were employed using rainbow trout (Oncorhynchus mykiss) hepatocytes to quantify vitellogenin induction following exposure to photoproducts.22,23 Full details are provided in the SI.

RESULTS AND DISCUSSION

Photoproduct Analysis. As described in our prior work,24 TBA metabolites rapidly decay to residual levels upon irradiation, with corresponding increases in primary photoproduct concentrations (Figure 1). Direct photolysis half-lives for 17α-TBOH, 17β-TBOH, and TBO were 31, 25, and 23 min, respectively.24 We observed all major TBA metabolite photoproducts (i.e., Figure 1) to be photostable, consistent with their absorption maxima decreasing from 350 nm for parent TBA metabolites (a value characteristic of the 4,9,11 triene system) to 240–260 nm for major products (Figure 1, S1).24 This suggests that disruption of the conjugated 4,9,11 π-system has occurred, shifting product absorption essentially out of the solar spectrum, reducing the potential for further phototransformation, and increasing photoproduct persistence.24 We note that minor decay observed for 17β-TBOH and TBO photoproducts is attributable to nonphotochemical processes (e.g., hydrolysis), as the same decay rate is observed in the dark. Another observation from initial HPLC-DAD analysis is the striking similarity in the TBA metabolite photoproduct absorbance spectra, evidence that these major photoproducts are likely all similar in structure (Figure S1). However, there are some apparent differences in the character of the photoproducts. For instance, each metabolite has a unique ratio of major to minor product (based on HPLC-DAD peak areas, the corresponding ratios for 17α-TBOH, 17β-TBOH, and TBO are 5:1, 2:1, and approximately 1.5:1, respectively). We also note increased polarity of 17β-TBOH photoproducts relative to those of 17α-TBOH and TBO based on their relative elution times.

LC-HRMS/MS analysis was used to characterize these TBA metabolite photoproducts. Observed accurate masses and MS/MS data for 17α-TBOH, 17β-TBOH, and TBO photoproducts are found in Table 1 and SI Table S1. Two substantial analytical challenges were encountered with this approach. First, photoproducts exhibited decreased ionization efficiency in positive ESI mode compared to the parent TBA metabolites that contain an intact 3-keto, 4-ene structure.26 Alteration of the 3-keto, 4-ene structure in the steroid A-ring can significantly reduce ionization efficiency in positive ion ESI, implying reduced ionization for products exhibiting disruption of 4,9,11 triene resonance.26 For example, the structural analog 9,11-delta-estradiol has been proposed as a potential biotransformation product of 17β-TBOH.27 Using positive ESI, the peak area for a 3 μM standard of 9,11-delta-estradiol was far less than 1% of the observed peak areas for 3 μM TBA metabolites. Additionally, summarizing the peak areas of parents and photoproducts was not conservative, implying substantial variation in peak area responses between parents and photoproducts. This variable product ionization in positive ESI precluded estimation
of product yields using LC-HRMS/MS analysis, and is characteristic of many steroids such as estrogens that often are analyzed with alternative ionizations. Given the potential difficulty in resolving minor from major products due to ionization efficiency differences, we included all products related to TBA metabolites in this analysis.

Second, under the conditions encountered in the Orbitrap detector, we determined that \([M + H]^+\) molecular ions (expected \(m/z\) 289.1805) were not observed for monohydroxy species subsequently identified by NMR (discussed below). Instead, \([M + H - H_2O]^+\) fragments (observed \(m/z\) 271.1685) were observed for monohydroxy products, initially confounding their correct mass assignments. However, ancillary LC-MS/MS analysis yielded the expected \(m/z\) 289 mass for two 17\(\alpha\)-TBOH monohydroxy product \([M + H]^+\) molecular ions, although at low abundance (<5%) relative to the \([M + H - H_2O]^+\) fragment (Figure S2). For these \(m/z\) 271 photoproducts, the characteristic decrease in LC retention time (e.g., Figure S4, S9, or S16) due to their increased polarity is considered to be a valuable diagnostic for their presence. This approach is complicated by the fact that these \([M + H - H_2O]^+\) species cannot be readily differentiated from potential structural analogs and stereoisomers with \(m/z\) 271.1685 \([M + H]^+\) ions, although we expect that any structural analogs and stereoisomeric products should exhibit relatively longer chromatographic retention times that are closer to parent retention times.

Under these chromatographic conditions, 17\(\alpha\)-TBOH, 17\(\beta\)-TBOH, and TBO standards eluted at 8.06, 8.31, and 9.55 min, respectively (Figures 2, S3, S8, and S15). Transformation products were primarily monohydroxy, dihydroxy, and trihydroxy species, often detected as groups of closely eluting peaks in chromatograms (Table 1). Notably, hydroxyl addition is a common phototransformation pathway and subsequent photolysis or hydrolysis reactions can form secondary products.\(^{28,29}\) HPLC-DAD analysis observed 2−4 primary products upon direct photolysis of 17\(\alpha\)-TBOH and 17\(\beta\)-TBOH (Figure 1). In the case of 17\(\beta\)-TBOH, 2 product peaks were observed by HPLC-DAD (Figure 1c, 1d) and confirmed by LC-HRMS/MS (Figures 2 and S4, retention times of 4.93 min for the minor product and 5.55 min for the dominant product) as hydroxy-trenbolone products with \([M + H - H_2O]^+\) ions at \(m/z\) 271.1685. Several stable 17\(\beta\)-TBOH photoproducts produced in reasonably high yields were isolated by HPLC and identified by NMR (Figure 3, Tables S2−S5, and Figures S21−S25). The three most abundant products were
identified as 10,12,17-trihydroxy-estra-4,9(11),dien-3-one (2.90 min), a ring-opened species containing aldehyde groups at C-11 and C-12 (3−4 min), and 12,17-dihydroxy-estra-5(10),9(11),dien-3-one (5.55 min). For simplicity, we abbreviate these compounds as 10,12-dihydroxy-trenbolone, TBOH-11,12-dialdehyde, and 12-hydroxy-trenbolone, respectively.

UV/vis spectra calculated for these 17β-TBOH photoproducts also are consistent with those observed via HPLC-DAD (compare Figures 1, S1, and S20).

NMR signal assignments were established for 17β-TBOH (Table S2) to aid in recognition of structural changes in the products encountered. The 1H NMR signals for the olefinic protons of the A- and C-rings at δH 5.75 (H-4), δH 6.49 (H-11), and δH 6.54 (H-12) resolved well and were particularly useful in recognizing structural differences and changes among the photoproducts. The methyl shifts (H3-18) and those of the oxygenated CHs also proved to be diagnostic when comparing photoproducts to each other and to 17β-TBOH.

The 10,12-dihydroxy product was identified by analysis of 1H NMR, HSQC, HMBC, and COSY data. The addition of two hydroxy groups created two new stereocenters, but their configurations were not determined. Key features in the 1H NMR spectrum include the conservation of the olefinic CH α to the ketone at δH 5.78, the appearance of a new sp3 oxygenated CH signal as a doublet at δ3.98, the absence of the signals for the isolated CH=CH unit in the C-ring of the starting material, and the presence of a different C=CH signal at δH 6.02. COSY data were used to verify the presence of the same C-4 to C-17 spin system found in 17β-TBOH. The methyl singlet (H3-18) showed an HMBC correlation to the new oxygenated CH (δH 3.84, δC 69.6; C-12), whose proton, in turn, showed a correlation to C-9. The C-CH signal at δH 6.02 showed HMBC correlations to C-8, C-10, and C-13. These results indicated a change in oxygenation in the C-ring. The location of the second hydroxy group was determined based on the shift of C-10 (δC 70.8) and HMBC correlations from both H-4 and H-11 to that carbon.

The dialdehyde product was similarly identified. Key features include the loss of both signals for the isolated CH=CH unit in the C-ring and the appearance of two new aldehyde singlets at δH 9.13 and δH 9.99. The locations of the new aldehyde units were assigned by analysis of HMBC correlations. The aldehyde signal at δH 9.13 (H-12) showed correlations to C-13 and C-18, while the signal at δH 9.99 (H-11) correlated to C-8 and C-9. This compound degraded further over time to form another product containing only one aldehyde unit (the C-12 aldehyde group remained intact), but the process did not proceed to completion and other minor products were also formed. A similar dialdehyde has been reported as an oxidation product derived from a trenbolone analog.

The 12-hydroxy product was the major product in the mixture based on both HPLC peak intensity and on the amount isolated. Evidence for a similar change in C-ring oxidation was observed in the 1H NMR spectrum of this product, but the A-ring olefin CH signal was absent. The 13C NMR spectrum indicated that the molecule contained only two oxygenated carbons (δC 70.3 and δC 73.9), ruling out dihydroxylation. The signal at δC 73.9 was determined to be

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Figure 3. Proposed 17β-TBOH phototransformation pathway. Although the stereochemical configurations of the products were not definitely determined, most products have one or more possible diastereomeric or epimeric forms. Structures marked with an asterisk are confirmed by NMR. We propose analogous transformation pathways for 17α-TBOH and TBO photoproducts.
C-17 based on HMBC correlations from H$_3$-18 and H$_2$-16; correlations that also were observed for 17$\beta$-TBOH. The location of hydroxylation at C-12 ($\delta_2$ 70.3) was established by HMBC correlations from the corresponding CH proton signal ($\delta_1$ 3.89) to carbons 9, 11, 13, 14, and 18, similar to those observed for 17$\beta$-TBOH. $^1$H NMR analysis over time revealed that 12-hydroxy-trenbolone gradually degrades to form the dialdehyde, among other products, upon storage in methanol. A final product was isolated in sufficient quantity for identification and was found to be a corresponding 12-methoxy analog, presumably an artifact arising from storage in methanol.

Based on the LC-HRMS/MS and NMR data, and the observed 0.61 min separation between the two peaks, we propose that an additional minor 17$\beta$-TBOH photoproduct (4.93 min) likely represents 10-hydroxy-trenbolone, which further reacts along with the 12-hydroxy species to form the 10,12-dihydroxy-trenbolone and 11,12-dialdehyde species. However, these two peaks also may represent well resolved diastereomers of 12-hydroxy-trenbolone, although we were unable to confirm this. The data suggest an initial photolytic transformation to the monohydroxy species, with subsequent higher order hydroxylated products forming via hydrolysis because the absorption maxima of the monohydroxy products no longer fall within the solar spectrum (Figures 1, S1, and S20). A third, minor m/z 271.1685 peak also was observed at 7.73 min (Figure 2), although we cannot determine whether this is another monohydroxy species or a closely eluting stereoisomer or structural analog. Because four m/z 271.1685 photoproducts were observed for 17$\alpha$-TBOH, we consider this 7.73-min product to likely correspond to the analogous 17$\alpha$-TBOH product, though at minor yield for 17$\beta$-TBOH. Based on these observations, a coupled photolysis and hydrolysis transformation pathway for 17$\beta$-TBOH is proposed (Figure 3). We believe analogous pathways apply to 17$\alpha$-TBOH and TBO.

In addition to the monohydroxy products, rapidly eluting 17$\beta$-TBOH photoproducts observed by LC-HRMS/MS included m/z 303, 305, and 321 species, which likely arise from subsequent hydroxylation of the primary photoproducts. Though these polar fractions were collected and analyzed, the fractions did not contain sufficient material for NMR characterization. Several poorly resolved m/z 303.1583 products eluted between 3 and 4 min (Figure S5), and likely include the 11,12-dialdehyde observed by NMR, but also may represent a family of dihydroxy species or diketone products of unknown structure. Additionally, the m/z 305.1739 product observed at 2.90 min (Figure S6) likely represents the 10,12-dihydroxy-trenbolone product observed by NMR. Although the 11,12-dialdehyde product observed by NMR strongly suggests the presence of a corresponding 11,12-dihydroxy-trenbolone secondary product, other m/z 305 dihydroxy species were not observed. Also, unlike the m/z 305 dihydroxy product, the m/z 303 products presumably include a third $\pi$-bond elsewhere in the molecule, although additional studies are needed to elucidate these structural differences. Finally, two trihydroxy peaks at m/z 321.1688, one dominant peak at 3.57 min, and a much smaller peak at 4.01 min were observed (Figure S7). Based upon the NMR product characterization, a C10, C11, C12-trihydroxylation product seems to be the most likely candidate structure, but hydroxylation at C5 and/or C9 is also possible.

As expected from similar UV/vis absorbances, LC-HRMS/MS analysis of 17$\alpha$-TBOH and TBO photoproducts suggested many similarities to 17$\beta$-TBOH transformation mechanisms, with a couple of notable exceptions. For example, instead of the three, more polar, m/z 271.1685 products observed for 17$\beta$-TBOH (10- and 12-hydroxy-trenbolone [M + H – H$_2$O$^+$] ions and a third hydroxy species), four m/z 271.1685 products were observed for 17$\alpha$-TBOH, including one product which eluted at 10.25 min, a full 1.94 min later than the 17$\alpha$-TBOH parent (Figures S9–S12). The three early (i.e., more polar) m/z 271 product peaks of 17$\alpha$-TBOH elute at 4.91, 6.20, and 7.42 min. Due to the close similarity in relative retention times, we propose that the first two of these peaks (observed as [M + H – H$_2$O$^+$] ions) correspond to the 10-hydroxy and 12-hydroxy-trenbolone products observed for 17$\beta$-TBOH. For the third 17$\alpha$-TBOH product (7.42 min), no m/z 289 parent peak was apparent in the ancillary LC-MS/MS data (Figure S2), although this product might correspond to the 7.73-min peak only weakly observed for 17$\beta$-TBOH.

However, the close proximity to the 8.31-min retention time of the parent 17$\alpha$-TBOH also might suggest a [M + H$^+$] ion of a photolytically generated stereoisomer or structural analog of 17$\alpha$-TBOH. Similarly, the 10.25-min product peak almost certainly suggests formation of a much less polar stereoisomer or structural analog of 17$\alpha$-TBOH, as hydroxylation products are unlikely to exhibit increased retention times. Possible structural attributes that could account for this increased retention time might include stereochemical inversion or photochemically induced rearrangement of the C13 methyl group, as reported for lumi-estrone. Additional isolation and NMR characterization would be required to resolve this issue, although the high cost of 17$\alpha$-TBOH limits additional assessment for us.

Similar to 17$\beta$-TBOH photoproducts, several poorly resolved m/z 303.1581 peaks at 3–4 min are observed for 17$\alpha$-TBOH which likely represent dialdehyde or dihydroxy products (Figure S13). Two m/z 321.1687 peaks were observed (Figure S14): a dominant peak (4.75 min) and a smaller peak (3.71 min) that represent trihydroxy products. Unlike 17$\beta$-TBOH products, no m/z 305 dihydroxy products were observed for 17$\alpha$-TBOH, which probably indicates that these products are less stable compared to the dihydroxy 17$\beta$-TBOH product. For TBO, an analogous set of products were observed, although apparently more limited in range. Two [M + H – H$_2$O$^+$] ions at m/z 269.1528 are observed upon TBO irradiation (presumably analogous 10-hydroxy and 12-hydroxy species), along with a single m/z 303 dihydroxy product and a single trihydroxy-trendione species at m/z 319.1532 (Figures S16–S19). Unlike the primary 17$\alpha$- and 17$\beta$-TBOH products, peak areas for the TBO hydroxy species are substantially larger, which might indicate increased yields, decreased reactivity to secondary products, or more effective ionization of the TBO photoproducts due to the C17 ketone. No other TBO products were observed.

**Photoproduct Ecotoxicology.** In vivo ecotoxicology studies with medaka suggest that photoproduct mixtures retain biological activity and also indicate potential mechanisms of action. Additional details from these studies are found in the SI. Observed effects were most pronounced for 17$\alpha$-TBOH photoproducts. For example, female medaka exposed to 17$\alpha$-TBOH product mixtures (100 and 1000 ng/L nominal concentrations) for 14 days resulted in alteration of the ovarian follicle composition at different developmental stages (Figure 4). Exposure to concentrations of 100 ng/L 17$\alpha$-TBOH photoproducts significantly increased the percentage of vitellogenic follicles from 6.3 ± 5.2% (controls) to 30.4 ± 2.9% (Figure 4B). Although the percentage of primary stage
ovarian follicles did not significantly change at this exposure, there is a trend for a decrease in primary stage follicles compared to control fish. At higher exposures (1000 ng/L 17α-TBOH photoproducts), the percentage of primary stage ovarian follicles was significantly reduced from 77.4 ± 10.6% (controls) to 40.3 ± 4.6%, while vitellogenic follicles increased from 6.3% (controls) to 31.6 ± 12.9%. No similar effects were observed for 17α-TBOH exposures at equivalent concentrations (p = 0.1724). Interestingly, 17α-TBOH photoproduct mixtures had the highest estrogenicity (4.25 ± 0.06 ng E2/L), but whole body 17β-estradiol (pg/mg) levels were reduced and androgen levels did not change after exposure to 17α-TBOH photoproducts (Figures 5, S26). These results suggest that 17α-TBOH photoproducts either directly act upon the ovary advancing the ovarian follicles into vitellogenic growth or indirectly by acting upon the liver to induce vitellogenin production to be incorporated into ovarian follicles. In contrast, 17β-TBOH photoproduct mixtures did not alter the composition of medaka ovaries after exposure, but did apparently elevate whole body E2 (pg/mg) levels significantly at the highest exposure. TBO photoproducts increased vitellogenin mRNA levels in hepatocytes indicating that these products also are estrogenic (3.43 ± 0.04 ng/L, Figure S26), although no significant difference in estrogenicity (p = 0.0634) was observed between TBO and TBO photoproducts. Most surprisingly, the observed biological effects of 17β-TBOH and TBO photoproduct mixture exposures generally exhibited inverse trends when compared to 17α-TBOH photoprodut exposures (Figure 5). These results indicate that although 17β-TBOH and TBO photolysis products are estrogenic and elevate whole body E2 levels, they apparently do not act upon the ovary by stimulating ovarian follicle development.

**Implications.** Understanding the fate of TBA metabolites and related products in environmental systems is important because both 17α-TBOH and 17β-TBOH are potent steroid structures that can alter endocrine function in aquatic organisms. However, despite the large mass production and widespread use of TBA, the TBA metabolites 17α-TBOH, 17β-TBOH, and TBO are infrequently detected in receiving waters or runoff. Observations of 0−350 ng/L are typically reported, often at detection frequencies of only a few percent of samples and with low median concentrations. Photo-transformation is likely a major degradation pathway for these compounds in some receiving waters. These data may suggest

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Figure 4. Percentage of ovarian follicle stages (primary, secondary, and vitellogenic stage follicles) of Japanese medaka after 14-day exposure to TBA metabolite photoproduction mixtures at different concentrations: (A) 10 ng/L (p > 0.05), (B) 100 ng/L (p = 0.008), and (C) 1000 ng/L (p = 0.0018). Data are shown as mean ± SEM; different letters indicate significant differences.

Figure 5. Whole-body sex steroid levels of Japanese medaka exposed to TBA metabolite photoproduction mixtures for 14 days: (A) 17β-estradiol (E2; p < 0.0001), (B) testosterone (T; p = 0.4920), and (C) 11-ketotestosterone (11-KT; p = 0.1755). Data are shown as mean ± SEM; different letters indicate significant differences.
that a considerable mass of uncharacterized TBA-related transformation products exists in environments affected by animal agriculture. More interestingly, observations of effects consistent with endocrine disruption in receiving waters affected by animal agriculture are commonly reported, along with variations in the biological effects of 17β-TBOH on aquatic organisms that cannot be explained by concurrent chemical analysis. Therefore, characterization of novel products arising from environmental steroid transformations may improve the ecological risk assessment of TBA metabolites. This process is particularly important when transformation products are closely related in structure to parent molecules, as the potential for steroid receptor binding and related biological effects is strongly dependent on structural similarity to parent molecules.

Although we cannot address these possibilities in full with this study, we observe several characteristics of TBA metabolites that may suggest potential explanations for some of these data. Most of these characteristics arise from the 4,9,11 triene structure of TBA, unique among steroids, and it is reasonable to expect similarly unique reactivity and fate of TBA metabolites in the aquatic environment. First, TBA metabolite photoproducts, at least initially, strongly resemble parent compounds and retain many key structural attributes related to steroid function. Conservation of the basic steroid ring structure is critical for conserving steroid receptor binding, and the initial phototransformations certainly do not alter the steroid ring structure. Photoproducts also exhibit shifts in absorption maxima to wavelengths out of the solar spectrum which imply increased environmental persistence relative to parents. Increasing photoproduct polarity also implies reduced partitioning to environmental surfaces and increased transport potential in affected waters.

Second, although we have not been able to confirm such structures with NMR, there are indications that phototransformation processes lead to the formation of structural analogs or stereoisomers of the TBA metabolites. Although they are very likely minor products, the 7,73- and 7,42-min photoproducts of 17β-TBOH and 17α-TBOH, respectively, are eluting after the observed hydroxy-trenbolone species (e.g., 50 min retention time) and only ~0.6 min before the parent compounds. The proximity in retention time to parent TBA metabolites may suggest that as-yet uncharacterized structural analogs or stereoisomeric products are formed. Similarly, the observed product eluting at 10.25 min after 17α-TBOH may also offer evidence for transformation to such a structural analog. We speculate that if generated, such structural analogs or stereoisomers will exhibit chemical characteristics similar to those of the parents and have important implications for environmental fate and ecotoxicology if stable in environmental systems. For example, we expect that structural analogs with retention times substantially different from TBA metabolites would yield a “no detect” under typical analytical conditions although a closely related compound is indeed present (e.g., 10.25-min 17α-TBOH product). Conversely, structural analogs could elute at nearly identical times to parents, yielding a case of mistaken identity and potential false positive at that retention time for a structure with different chemical properties and biological activity. For example, our LC-HRMS/MS analysis of 9,11-delta estradiol (the only commercially available structural analog), demonstrated that 9,11-delta estradiol essentially coeluted with 17α- and 17β-TBOH while yielding many TBA metabolite MS/MS fragments, including the diagnostic m/z 133 and 159 fragments. Though its ionization proved relatively inefficient, for some analyses the co-occurrence of these compounds may have been difficult to resolve under conditions typically employed for environmental analyses.

Third, the ecotoxicology data, although not extensive, indicate that photoproduct mixtures retain biological activity even after near complete phototransformation of parents. TBA metabolites themselves can alter circulating steroid levels and ovarian composition with reduced primary ovarian follicles and increases in vitellogenic follicles in several species of fish. Key differences in this study include the use of static exposures to photoproducts mixtures which may increase the importance of transformation processes to toxicology assessment as compared to parent exposures alone in flow-through systems. Flow-through systems likely remove transformation products and may explain the contrasting estrogenic effects in the current study relative to androgenic and defeminization effects previously reported. The in vitro responses likely resulted from transformation of the metabolites and/or photoproducts to estrogen receptor ligands within the hepatocytes which possess significant biotransformation capacity. In the current study, only female medaka exposed to 17α-TBOH photoproducts experienced reproductive dysfunction with altered ovarian follicle composition and reduced 17β-estradiol levels. We believe these effects likely are due to estrogenic properties of the 17α-TBOH photoproduct mixture. Interestingly, using the same measure of estrogenicity, 17α-TBOH had the lowest relative estrogenic activity, with 17β-TBOH having greater activity and TBO the greatest activity of the three. These observations indicate that TBA metabolites themselves have very different effects on the adult teleost ovary compared to photoproducts mixtures, though both are bioactive at similar concentrations.

A key characteristic of estrogenic activity in steroids is the presence of an aromatic A-ring. Although no such products were detected in this analysis, we speculate that the conjugated 4,9,11 triene system of trenbolone may be structurally poised to develop an aromatic A-ring upon simple shifts in the conjugated π-system during transformations, potentially providing a mechanism for conversion of androgenic TBA parents to estrogenic products. This process might be promoted by abiotic processes such as photoenolization, which would be expected to yield at least two π-bonds in the TBA metabolite product A-ring. Also, hydroxylation is a common transformation pathway for steroids, and we speculate that subsequent dehydration reactions also may provide a potential initiating mechanism for A-ring aromatization. Steroids are often prone to acid-catalyzed transformations that give rise to unsaturated derivatives. This behavior is well documented for steroids with an allylic hydroxyl group at the C3 position, for which acid-catalyzed elimination followed by rearrangement can yield 3,5-diene structures. Most relevant to our photoproducts, allylic 10-hydroxy steroids also undergo acid-catalyzed dehydration where rearrangement produces an aromatic A-ring structure. Interestingly, we also have observed unexpectedly high concentrations of parent 17α-TBOH in samples analyzed after direct photolytic mechanisms should have eliminated nearly all parent mass, suggesting potential reversibility of phototransformation pathways. Acidifying TBA photoproduct solutions results in the immediate formation of a secondary product that coelutes with the parent TBA metabolites, also suggesting the occurrence of product dehydration. While follow-up studies and data supporting this observation will be
presented elsewhere, it is clear to us that abiotic regeneration of parent TBA metabolites or similar structures from hydroxylated photoproducts does occur under certain conditions. Further studies to evaluate the mechanisms and implications of potential product-to-parent reversion observations are ongoing.

Observations of closely related photoproducts, including potential structural analogs or stereoisomers, and bioactive attributes of products suggest the presence of novel transformations and unique reactivities of TBA metabolites. Although our existing data are limited, and often well beyond the original scope of our studies, effectively understanding the environmental fate and ecological implications of TBA metabolites, widely used in animal agriculture, should consider these transformation mechanisms. A key element of all of these studies is a focus upon the stability of the parent TBA metabolites and any structurally similar products because their potential ecological significance is strongly dependent on their environmental persistence.

**ASSOCIATED CONTENT**

 Supporting Information

Additional information related to the LC-HRMS/MS, NMR, and ecotoxicology analyses. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

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