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Biologically active equine estrogens and sulfate conjugates labelled with tritium at high specific activity

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HIGHLIGHTS

- This paper describes the synthesis of [^{3}H] equilin and a metabolite.
- Iodination—catalytic tritium dehalogenation was employed.
- This paper also describes the synthesis of several tritiated sulfate conjugates.

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ABSTRACT

Methods are presented to tritiate and characterize the steroid equilin as well as a structurally related 17-alpha alcohol metabolite and several 3-position sulfate conjugates.

1. Introduction

As a structural class, steroids are among the most ubiquitous and biologically active natural substances. Present in vertebrates, insects, plants as well as fungi, steroids display diverse biological influence including cardiovascular activity (Briet and Schiffrin, 2010), regulation of some neurotransmitter signaling pathways (Zheng, 2009), activation of certain cancers (Walsh et al., 2012) and a role in such diseases as diabetes (Morimoto et al., 2011). The family of estrogenic steroids and their interactions with both the alpha (Liu et al., 2011) and beta (Minutolo et al., 2011) nuclear estrogen receptors has been increasingly recognized as important to human health. In particular, the steroid equilin (**1**) and related components of equine estrogenic preparations are intriguing substances. In order to more fully investigate the biology of these compounds, we endeavored to label them with tritium at high specific activity and now report their synthesis and characterization.

2. Experimental

Evaporations were carried out on a Buchi rotary evaporator (Model RE 111) at bath temperatures less than 40 °C. Analytical TLC was performed on Analtech plates coated with silica gel (250 μm). Autoradiography was performed at 0 °C after spraying with PPO and exposing the plates to X-ray film. TLC plates were also scanned for radioactivity (~370 kBq) using a Vanguard Autoscanner. Analytical HPLC was performed on a PerkinElmer instrument with peak detection done simultaneously by UV (280 nm) and a IN/US Systems Beta RAM Model 3 radioactivity detector. Solution radioassays were conducted with a PerkinElmer Tri-Carb 3100T instrument. The NMR spectra were recorded on a Bruker 300 MHz instrument with chemical shifts being reported as parts per million (ppm) downfield from internal TMS. Mass spectra were recorded on a Kratos Model MS25 RF instrument. All chemicals used were reagent grade and authentic standards were obtained from Steraloids.

2.1. [2,4-^{3}H] equilin (**3**)

To a solution of 15 mg (0.03 mmol) of diiodo intermediate **2** (Rao and Somawardhana, 1987) in 4 ml of ethyl acetate with 100 mg of 5% palladium on alumina was introduced 2.22 TBq of tritium gas and the reaction was stirred for 2 h at ambient temperature. After this time, labile tritium was removed by several vacuum evaporations of methanol and the reaction was filtered free of catalyst, giving 46.4 GBq of crude product. A 22.2 GBq portion of this material was purified by preparative reverse phase HPLC employing methanol:water (65:35). The appropriate peaks were collected and pooled to afford 6.6 GBq (an extrapolated 28% radiochemical yield based on

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precursor **2**) of product **3** which was >98% radiochemically pure and completely co-chromatographed with authentic **1** (Steraloids catalog number E0600-000) on both reverse phase HPLC (same system as above) as well as TLC (benzene:ethanol (90:10)). The specific activity of product **3** was measured to be 1.61 TBq/mmol by radioassay with UV (ethanol) spectroscopy where $E_{281}=1{,}764$ for **1**. The distinctive UV (ethanol) spectum of **3** was also superimposable on that of **1**.

2.2. [2,4-^{3}H] equilin-3-sulfate (**4**)

A 6.3 GBq portion of product **3** was dissolved in 0.25 ml of dry pyridine and it was then added to a solution of 0.15 ml of chlorosulfonic acid in 0.4 ml of dry pyridine at 0 °C with stirring. The reaction was allowed to warm to ambient temperature and stirred overnight. After this time, 3 ml of water was added and the pH was adjusted to 9.0 with 6 M sodium hydroxide, causing a cloudy red solution. The solution was then diluted with 15 ml of ethanol, filtered through a silica gel sep-pak and washed with another 25 ml of ethanol, giving 4.74 GBq of crude product. A 2.2 GBq portion of this material was purified by preparative reverse phase HPLC eluted with 0.01 M aqueous potassium phosphate (pH 7.5):acetonitrile (75:25). The appropriate peaks were collected and pooled to afford 0.53 GBq (an extrapolated 18% radiochemical yield) of product **4** which was >98% radiochemically pure and completely co-chromatographed with authentic standard (Steraloids catalog number E0619-000) on both reverse phase HPLC (same system as above) as well as TLC (ethyl acetate:methanol:ammonium hydroxide (75:25:2)). The specific activity of product **4** was determined to be 1.61 TBq/mmol by mass spectrometry as seen in Fig. 1.

2.3. 2,4-Diiodo-17-alpha-acetoxydihydroequilin (**6**)

To a solution of iodine (402 mg, 1.58 mmol) in 29 ml of benzene with 0.4 ml of morpholine was added 162 mg (0.52 mmol) of acetate **5** (Arnold et al., 1977) and the reaction was stirred for 2 h at ambient temperature. After this time, 30 ml of a 5% aqueous hydrochloric acid solution was added and the organic layer was removed by rotary evaporation. The aqueous layer was extracted with three 10 ml portions of chloroform which was then dried over sodium sulfate and filtered. The filtrate was evaporated and recrystallized from acetone–water to afford 258 mg (88% yield) of a yellow solid which was essentially homogeneous on TLC (benzene:ethanol (90:10)) and well separated (Rf=0.9) from starting material **5** (Rf=0.53) in this system. A proton NMR ($CDCl_3$) of 6 was especially informative in the aromatic region where the three proton multiplet pattern between 6.5 and 7.2 ppm for starting material **5** was replaced by a single proton (A ring 1-position) noncoupled resonance at 7.6 ppm as seen in Figs. 2 and 3.

2.4. [2,4-^{3}H]-17-alpha-acetoxydihydroequilin (**7**)

To a solution of 19 mg (0.034 mmol) of diiodo intermediate **6** in 4 ml of ethyl acetate with 100 mg of 5% palladium on alumina was introduced 2.22 TBq of tritium gas and the reaction was stirred for 2 h at ambient temperature. After this time, labile tritium was removed by several vacuum evaporations of methanol and the reaction was filtered free of catalyst, giving 45.3 GBq of crude product. An 11.1 GBq portion of this material was purified by preparative TLC on two 500 μm plates developed with chloroform:-methanol (95:5). The plates were visualized by UV and the most prominent bands were scraped and eluted with three 20 ml portions of ethanol. Pooling the ethanol aliquots afforded 5.4 GBq (an extrapolated 35.7% radiochemical yield based on precursor **6**) of product **7** which was >95% radiochemically pure and completely co-chromatographed with authentic **5** on TLC (chloroform:methanol (95:5)). The specific activity of product **7** was measured to be 1.80 TBq/mmol by radioassay with UV (ethanol) spectroscopy where $E_{281}=1{,}951$ for **5**. The distinctive UV (ethanol) spectrum of **7** was also superimposable on that of **5**.

2.5. [2,4-^{3}H] 17-alpha-17-dihydroequilin-3-sulfate (**8**)

Product **7** from the above synthesis was dissolved in 0.25 ml of dry pyridine and then added to a solution of 0.15 ml of chlorosulfonic acid in 0.4 ml of dry pyridine at 0 °C with stirring. The reaction was allowed to warm to ambient temperature and stirred overnight.

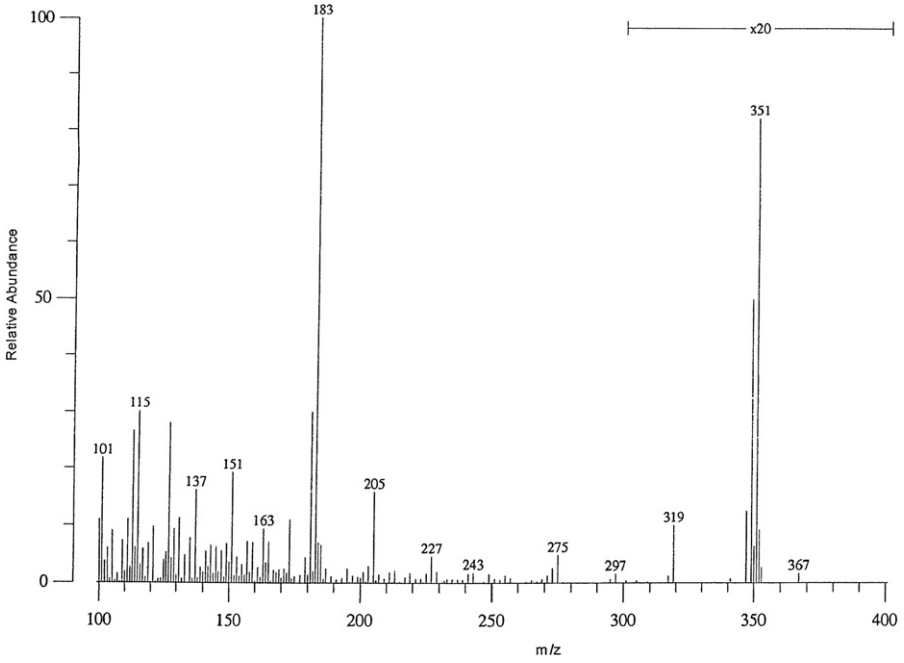

Fig. 1. Mass Spectrum of **4**.