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Synthesis of three hydroxyraloxifenes as reference compounds in doping analytics

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Introduction

Raloxifene (Evista®, **4**) is a selective estrogen receptor modulator that has estrogenic action on the estrogen receptors in bone and liver and anti-estrogenic effect on uterus and breasts. Raloxifene is used for the treatment and prevention of osteoporosis in postmenopausal women and for reduction in risk of invasive breast cancer[1]. Raloxifene can be abused in sports because it counteracts the adverse effects of extensive use of anabolic androgenic steroids (gynaecomastia). It also increases testosterone concentration by stimulation of testosterone biosynthesis[2].

Raloxifene is included in the list of prohibited substances and methods in sports of the World Anti-Doping Agency (WADA). According to WADA's international standard for laboratories and the ISO 17025 standard, well-characterized reference compounds should be used for the identification of a prohibited substance, if available.

As a part of this ongoing WADA-funded research project we synthesized three hydroxylated phase one raloxifene metabolites (Fig. 1) for doping analytical purposes. The synthesized metabolites were purified and fully characterized by chromatographic and spectroscopic methods.

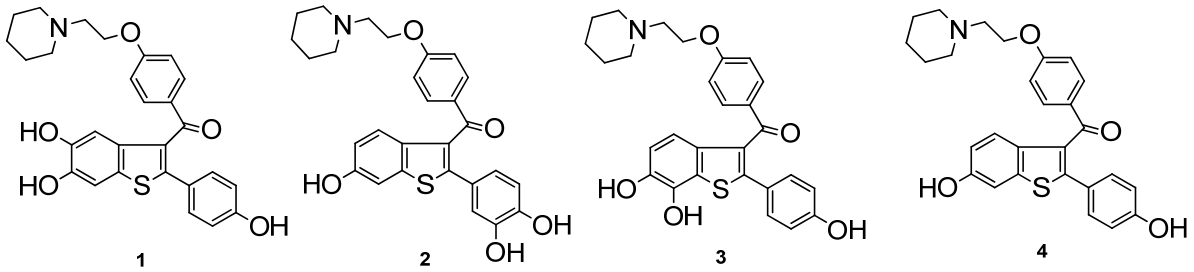


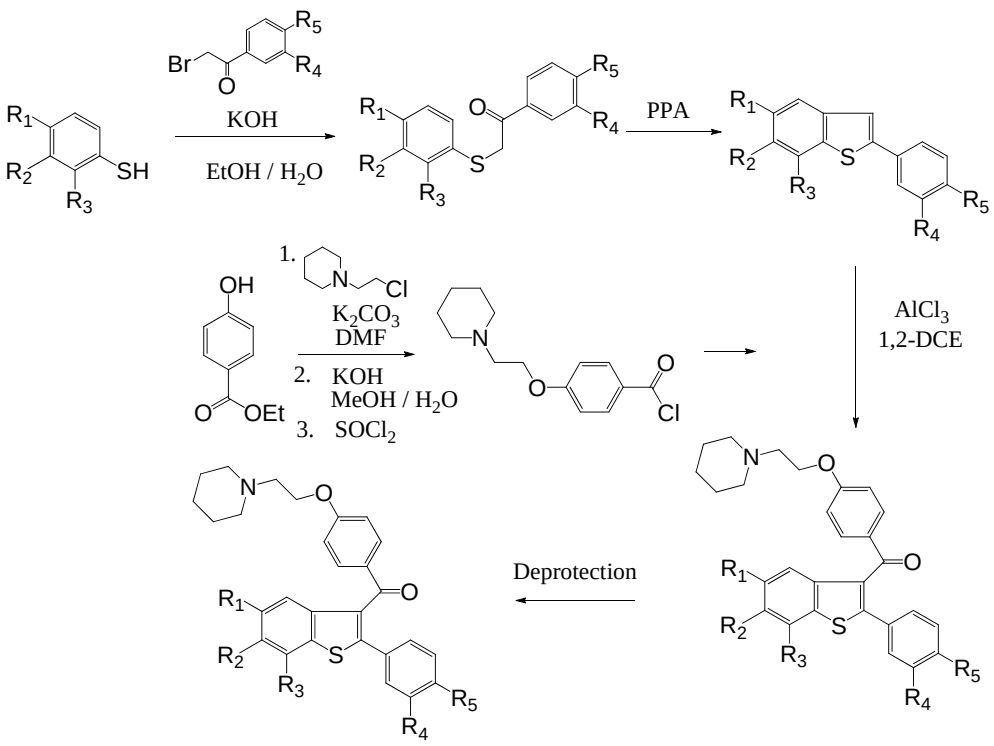
Figure 1. Hydroxyraloxifenes (**1**-**3**) and raloxifene (**4**)

Chemical synthesis

Metabolite (**1**) can be prepared using the synthetic route shown in Scheme 1[3]. Methoxythiophenol is first alkylated with 2-bromomethoxyacetophenone in the presence of potassium hydroxide. Methoxybenzo[*b*]thiophene is then obtained by polyphosphoric acid (PPA)-mediated cyclization-rearrangement reaction.

Piperidine-containing moiety is synthesized in three reaction steps starting with alkylation of ethylparaben with *N*-(2-chloroethyl)piperidine. The resulting ethyl ester is hydrolyzed to the corresponding carboxylic acid, which is subsequently converted to acyl chloride using thionyl chloride. Methoxybenzo[*b*]thiophene is reacted with the acyl chloride using aluminium chloride-catalyzed Friedel-Crafts (F-C) reaction to give methoxyraloxifene. Metabolites of raloxifene are obtained after deprotection of the hydroxy groups with aluminium chloride-ethanethiol system (**1** and **3**)[4] or with lithium diisopropylamide (**2**, LDA)[5].

This general route applies to metabolites (**2**) and (**3**) with few exceptions. For (**2**) protecting groups need to be changed from methyl to mesyl to facilitate a cleaner F-C reaction. This is done after polyphosphoric acid-catalyzed (PPA) cyclization reaction using boron tribromide to cleave the methyl groups and mesyl chloride to mesylate the free phenols. For (**3**) the starting material for the first reaction step has to be prepared by diazotizing 2,3-dimethoxyaniline and then replacing the diazo group with ethyl xanthate which is hydrolyzed to give the corresponding thiol[6].



Scheme 1. Synthesis of hydroxyraloxifenes (**1-3**)

Characterization results

So far, the metabolite (**1**) has been synthesized. Its structure has been confirmed using ^{1}H-NMR, LC-MS/TOFMS and its purity has been determined by LC-UV measurements.

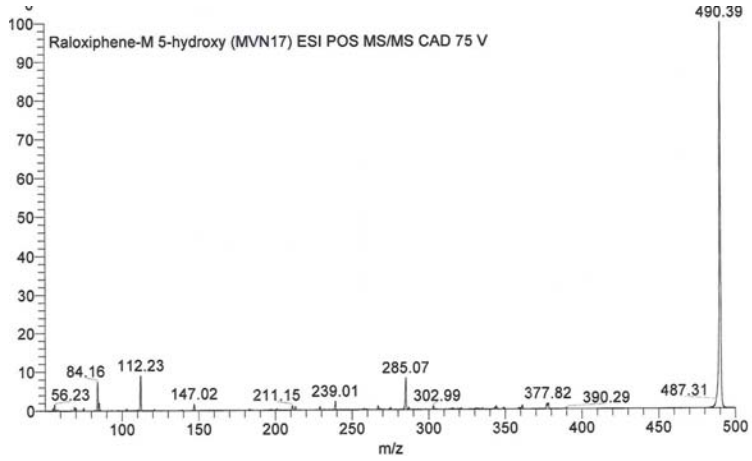
LC:	Column: LiChroCART 125-3, Purospher RP-18e (5 µm)
Mobile phase:	A: 2.5 mM ammonium acetate with 0.1% acetic acid, pH 4 and B: methanol. Gradient from 25% B to 95% B in 7 min, 95% B for 3 min
Flow Rate:	0.8 mL/min
Detector	UV/VIS
Retention time:	5.41 min
UV-VIS spectrum peaks:	230 nm, 285 nm
Chromatographic purity:	99.3%

ESI-MS: Instrument: TSQuantum (Thermo Finnigan)

Operation: Direct infusion MS/MS: precursor ion *m/z* 490

Ionization: ESI, positive mode, spray voltage 4000 V

Product ion spectrum peaks: *m/z* 490, 378, 285, 147, 112



ESI-TOFMS: Instrument: microTOF (Bruker Daltonics)

Operation: Direct infusion

Ionization: ESI, positive mode, spray voltage 4500 V

Accurate mass [$M+H^+$]: *m/z* 490.168270 (mass error -2.32 mDa)

^{1}H NMR: Instrument: Varian Mercury Plus 300 MHz

Spectral data: δ ^{1}H NMR (CD_3OD) 7.68 (d, *J* = 8.9 Hz, 2H), 7.23 (s, H), 7.15 (d, *J* = 8.6 Hz, 2H), 7.02 (s, 1H), 6.81 (d, *J* = 8.9 Hz, 2H), 6.60 (d, *J* = 8.6 Hz, 2H), 4.11 (t, *J* = 5.5 Hz, 2H), 2.81 (t, *J* = 5.5 Hz, 2H), 2.60 (m, 4H), 1.63 (m, 4H), 1.48 (m, 2H).

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