STEROIDS 73 (2008) 760–764

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Synthesis of exemestane labelled with ^{13}C

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ARTICLE INFO

Article history:
Received 19 December 2007
Received in revised form
15 February 2008
Accepted 24 February 2008
Published on line 4 March 2008

Keywords:
Exemestane
Aromatase inhibitor
Labelling
^{13}C
Internal standard
LC–MS

ABSTRACT

The synthesis of exemestane (Aromasin®), an irreversible steroidal aromatase inhibitor, specifically labelled with ^{13}C is reported. The preparation of [$^{13}C_3$]exemestane was achieved according to an eight-step procedure starting from the commercially available testosterone.

1. Introduction

Exemestane (Aromasin®, 6-methylenandrosta-1,4-diene-3,17-dione; molecular weight 296.41) (Fig. 1) is an orally active irreversible aromatase inhibitor which is clinically in use for the treatment of postmenopausal women with early or advanced breast cancer [1–3]. The initial analytical methods developed for the determination of exemestane in biological fluids involved the use of high-performance liquid chromatography (HPLC) combined with ultraviolet detection or with thermospray mass spectrometry or followed by specific radioimmunoassay [4–6]. However, a higher samples throughput as well as lower limits of quantitation were needed to assay exemestane in human plasma. Therefore, the preparation of a stable labelled version of the compound was required to be used as analytical internal standard (IS) to develop and validate a sensitive, specific and reliable method for the quantitation of exemestane in biological fluids using liquid chromatography–mass spectrometry (LC–MS). In this paper, the approach followed to prepare a suitable stable labelled IS of exemestane is described.

2. Experimental

Chemicals and materials: All solvents and reagents were of analytical grade and were used without purification unless otherwise indicated. Testosterone (**1**) was purchased from Fluka, with a chemical purity >99%. [$^{13}C_2$]Acetyl chloride (99 atom % ^{13}C) and [^{13}C]formaldehyde (20% aqueous solution; 99 atom % ^{13}C) were obtained from Aldrich Chemical Co.

Instrumentation and equipment: Chemical purities were determined by HPLC using a series-200 pump (PerkinElmer) equipped with series-200 solvent degasser (PerkinElmer), series-200 autosampler (PerkinElmer) and a LC-235 UV diode array detector (PerkinElmer) connected with Totalchrom

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doi:10.1016/j.steroids.2008.02.010

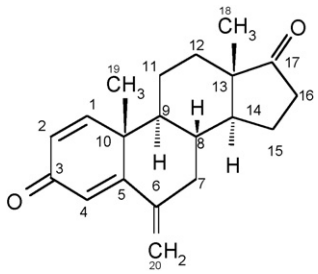

Fig. 1 – Structural formula of exemestane.

Client/Server (PENelson) as integrator *via* link 600 interface (PerkinElmer). Preparative-HPLC was performed at 25 °C using a PrepStar HPLC system (Varian). ^{1}H NMR data were recorded on Varian INOVA 400 spectrometer, equipped with a 5 mm 1H{15N-31P} *z*-axis pulse field gradient (PFG) indirect detection probe. ^{13}C NMR data were obtained by direct detection on a varian INOVA 500 spectrometer, equipped with a 3 mm broadband 15N-31P{1H-19F} probe optimized for 13C sensitivity and by heteronuclear correlation spectra (H-C) recorded on Varian Unity INOVA 500 spectrometer, equipped with triple resonance 1H{13C, 15N} z-axis pulse field gradient (PFG) indirect detection Cold-Probe. Chemical shifts were referenced with respect to the residual solvent signals (DMSO-d6: 2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C).

Analytical method: HPLC: X-Terra Waters RP18 column (mm 4.6 × 100, 5 μm) eluting with H_2O:CH_3CN:HCOOH 90:10:0.1 by volume (A) and H_2O:CH_3CN:HCOOH 10:90:0.1 by volume (B): from 100% A to 0% A in 10 min; 5 min at 0% A; from 100% B to 100% A in 1 min; 4 min at 100% A. Flow rate: 1 ml/min. Column temperature: 25 °C. Analytical wavelength: 215 nm.

2.1. 17β–Benzoyloxy-testosterone (2)

A solution of benzoyl chloride (BzCl; 49 ml, 42 mmol) in dry toluene (60 ml) was slowly dripped into a stirred and cooled (0 °C) solution of testosterone (**1**) (10.08 g, 34.9 mmol) in dry toluene (100 ml). After addition of dry pyridine (14 ml, 173 mmol), the reaction mixture was stirred at reflux for 20 h then cooled to room temperature. An aqueous solution of 0.5 N HCl (60 ml) was added and the mixture was stirred for 1 h. After separation, the organic layer was treated with 0.5 N HCl (4 ml × 100 ml), 8% $NaHCO_3$ (3 ml × 100 ml), brine (2 ml × 100 ml) and dried over Na_2SO_4. The solvent was evaporated to dryness giving the intermediate (**2**) (14.03 g, 35.7 mmol), with a chemical purity of 91% (determined by HPLC; see analytical method; R_t = 14.0 min).

2.2. 17β-Benzoyloxy-5-oxo-3,5-seco-4-norandrostan-3-oic acid (3)

An aqueous solution of 2M K_2CO_3 (32.6 ml) was added to a suspension of (**2**) (13.00 g, 33.2 mmol) in *tert*-butanol (325 ml). The reaction mixture was heated at 40 °C under stirring and two separate solutions of 0.13 M $KMnO_4$ (42.25 ml) and 0.75 M KIO_4 (419 ml) were contemporaneously introduced into the reaction flask. At the end of the addition (about 2 h) the mixture was stirred for 2 h at 40 °C then cooled to room temperature and evaporated to dryness. The obtained residue was dissolved with water (500 ml), transferred into a separating funnel and extracted with ethyl acetate (EtOAc 2 ml × 200 ml). After separation, the combined organic layers were back extracted with 8% $NaHCO_3$ (3 ml × 100 ml). The basic aqueous phases were combined with the aqueous phase previously obtained, then 6 N HCl was added up to pH 2. After extraction with EtOAc (4 ml × 250 ml) the obtained organic phases were combined, extracted with brine (2 ml × 300 ml) and dried over Na_2SO_4. The solvent was removed by evaporation, and the obtained intermediate (**3**) (11.09 g, 28.9 mmol) was used without further purification in the next step.

2.3. 3-Oxo-4-oxa-5-androsten-17β-yl benzoate (4)

The intermediate (**3**) (11.09 g, 28.9 mmol) was dissolved in dry EtOAc (480 ml) under nitrogen then a freshly prepared solution of acetic anhydride (94 ml) and 65% $HClO_4$ (0.24 ml) in dry EtOAc (480 ml) was introduced into the reaction flask. After stirring at room temperature for exactly 10 min, 8% $NaHCO_3$ (740 ml) was added, the mixture was stirred for 30 min then transferred into a separating funnel. After separation of the aqueous layer, the organic phase was extracted with 8% $NaHCO_3$ (3 ml × 300 ml), water (2 ml × 300 ml), brine (2 ml × 300 ml) and dried over Na_2SO_4. After solvent evaporation, the oily yellow residue was submitted to 3 crystallizations from mixtures of CH_3OH:H_2O 3:1 by volume. After drying under high vacuum at 40 °C, the intermediate (**4**) was recovered as a white powder (5.79 g, 14.7 mmol) with a chemical purity of 90% (determined by HPLC; see analytical method; R_t = 11.7 min). MS (ESI-MS): *m*/*z* 395 ($[MH]^+$).

2.4. 2-[1′,2′-$^{13}C_2$]Acetyl-3-oxo-4-oxa-5-androsten-17β-yl benzoate (5)

A solution of the intermediate (**4**) (1.51 g, 3.83 mmol) in dry tetrahydrofuran (THF; 34.4 ml) was slowly added under nitrogen to a cooled (−78 °C) and stirred 1 M solution of lithium bis(trimethylsilyl)amide (7.66 ml; 7.66 mmol) in dry THF. After stirring under nitrogen at −78 °C for 1 h, a solution of [$^{13}C_2$]acetyl chloride (308 mg, 3.83 mmol) in dry THF (2.7 ml) was added. The mixture of reaction was stirred at −78 °C for 90 min, then 1 N HCl (4.8 ml) and water (4.8 ml) were added and the mixture was allowed to reach room temperature then diluted with EtOAc (50 ml). After transferring into a separating funnel and separation, the organic layer was washed with brine (4 ml × 200 ml), dried over Na_2SO_4 and evaporated to dryness. The obtained crude intermediate (**5**) was purified by flash chromatography on silica gel using a mixture *n*-hexane:EtOAc 2:1 by volume as the eluting solvent system. The collected fractions were combined as appropriate and after solvent evaporation to dryness, the intermediate (**5**) (446 mg; 1.02 mmol) was recovered as a white solid with a chemical purity >95% (determined by HPLC; see analytical method; R_t = 13.8 min) and an isotopic purity of 99 atom % ^{13}C. MS (ESI-MS): *m*/*z* 439 ($[MH]^+$).

2.5. [3,4-$^{13}C_2$]Testosterone (6)

The intermediate (**5**) (446 mg; 1.02 mmol) was dissolved in a solution of KOH (500 mg) in a mixture of CH_3OH:H_2O 75:25 by volume (50 ml). The mixture of reaction was refluxed for about 20 h. After cooling to room temperature, 1 N HCl was added up to pH 4 and methanol was removed by evaporation. The aqueous residue was transferred into a separating funnel and extracted with EtOAc (3 ml × 25 ml). The combined organic phases were washed with 1 N HCl (3 ml × 20 ml), 4% $NaHCO_3$ (4 ml × 20 ml), brine (2 ml × 20 ml) and dried over Na_2SO_4. After solvent evaporation to dryness, the intermediate (**6**) was recovered as a yellow solid (270 mg, 0.93 mmol) with a chemical purity >77% (determined by HPLC; see analytical method; R_t = 6.2 min) and an isotopic purity of 99 atom % ^{13}C. MS (ESI-MS): *m*/z 291 ($[MH]^+$).

2.6. [3,4-$^{13}C_2$]Androstenedione (7)

Pyridinium chlorochromate (PCC; 599.7 mg, 2.78 mmol) was added under nitrogen to a stirred solution of the intermediate (**6**) (270 mg, 0.93 mmol) in dry dichloromethane (10 ml). After 4 h stirring at room temperature, the conversion was completed (determined by TLC on silica gel; *n*-hexane:EtOAc 1:1 by volume) and diethyl ether (18 ml), silica gel and celite were introduced into the reaction flask. After stirring at room temperature for about 1 h, the suspention was filtered and washed with diethyl ether (20 ml). The filtrate was evaporated to dryness giving the intermediate (**7**) as a yellowish solid (235 mg, 0.82 mmol) with a chemical purity >90% (determined by HPLC; see analytical method; R_t = 9.1 min) and an isotopic purity of 99 atom % ^{13}C. MS (ESI-MS): *m*/z 289 ($[MH]^+$).

2.7. 6-[^{13}C]Methylenandrost-[3,4-$^{13}C_2$]4-ene-3,17-dione (8)

Anhydrous triethyl orthoformate (0.24 ml, 1.44 mmol) and *p*-toluenesulfonic acid monohydrate (*p*-TsOH; 9.1 mg, 0.047 mmol) were added under nitrogen to a solution of the intermediate (**7**) (235 mg, 0.82 mmol) in dry THF (2.8 ml) and absolute ethanol (0.25 ml) and the reaction mixture was stirred at 40 °C for about 1 h. After cooling to room temperature, *N*-methylaniline (97 μl, 0.9 mmol) and a 20% [^{13}C]formaldehyde aqueous solution (0.225 ml, 1.47 mmol) were introduced into the reaction flask and the mixture was stirred at 40 °C for about 2 h. As soon as the conversion was completed (determined by TLC on silica gel; *n*-hexane:EtOAc 1:1 by volume), the mixture was cooled to room temperature, 37% HCl was added (0.65 ml) and the stirring was continued for further 4 h. The obtained brown solution was transferred into a separating funnel with water (40 ml) and the mixture was extracted with EtOAc (3 ml × 30 ml). The combined organic phases were washed with 0.1 N HCl (3 ml × 20 ml), brine (2 ml × 20 ml) and dried over Na_2SO_4. After solvent evaporation, the oily crude intermediate (**8**) was purified by flash chromatography on silica gel using a mixture *n*-hexane:EtOAc 4:1 by volume as the eluting solvent system. The collected fractions were combined as appropriate and after solvent evaporation to dryness, the intermediate (**8**) (101 mg, 0.335 mmol) was recovered as a white solid with a chemical purity >89% (determined by HPLC; see analytical method; R_t = 9.8 min) and an isotopic purity of 99 atom % ^{13}C. MS (ESI-MS): *m*/z 302 ($[MH]^+$).

2.8. [$^{13}C_3$]Exemestane

The intermediate (**8**) (0.101 mg, 0.335 mmol) was dissolved under nitrogen in dry 1,4-dioxane (7 ml) then *p*-TsOH (128.7 mg, 0.674 mmol) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ; 133.6 mg, 0.588 mmol) were added. The reaction mixture was stirred under reflux for about 1 h then cooled to room temperature. After solvent evaporation, the residue was dissolved in EtOAc (25 ml), transferred into a separating funnel and washed with water (3 ml × 25 ml), 4% $NaHCO_3$ (3 ml × 25 ml), brine (2 ml × 25 ml) and dried over Na_2SO_4. The crude [$^{13}C_3$]exemestane was purified on a silica gel column eluting with a mixture *n*-hexane:EtOAc 1:1 by volume. The obtained fractions were combined as appropriate and, after solvent evaporation to dryness, [$^{13}C_3$]exemestane (74.5 mg; 0.25 mmol) was recovered 84% chemically pure (determined by HPLC; see analytical method). The obtained [$^{13}C_3$]exemestane was submitted to a further purification by preparative HPLC (XTerra RP18 column, mm 100 × 50 I.D., 5 μm, eluting with H_2O (A) and CH_3CN (B): from 90%A to 20%A in 20 min; from 20%A to 0%A in 3 min; from 0% A to 90% A in 5 min; Flow rate: 120 ml/min. Column temperature: ambient; Analytical wavelength: 215 nm]. After solvent evaporation to dryness, [$^{13}C_3$]exemestane (52 mg; 0.038 mmol) was recovered as a white solid with a chemical purity >98% (determined by HPLC; see analytical method; R_t = 7.9 min) and an isotopic purity of 99 atom % ^{13}C. MS (ESI-MS): *m*/z 300 ($[MH]^+$). ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 0.86 (s, 3 H, CH_3-18) 1.12 (s, 3 H, CH_3-19). 1.17–1.33 (m, 2H, H12 ax, H9), 1.39 (m, 1H, H14), 1.51–1.73 (m, 3H, H11 ax, H12 eq, H15 β), 1.80–1.94 (m, 4H, H11 eq, H7 ax, H8, H15 α), 2.02 (ddd, J = 19.08, 9.21, 9.02 Hz, 1 H, H16 α) 2.42 (dd, J = 19.14, 8.41 Hz, 1 H, H16 β) 2.62 (dd, J = 9.08, 5.18 Hz, 1 H, H7 eq) 5.04 (d, J_{H-13C} = 159.1 Hz, 2 H, CH_2-20) 5.99 (d, J_{H-13C} = 162.1 Hz, 1 H, H4) 6.16 (m, 1 H, H2) 7.25 (t, J = 10.06 Hz, J_{H-13C} = 10.2 Hz 1 H, H1). ^{13}C NMR (125 MHz, DMSO-d6): δ ppm 12.8 (C18), 19.1 (C19), 20.7 (C15), 21.3 (C11), 30.5 (C12), 34.2 (C8), 34.8 (C16), 37.9 (C7), 43.9 (C10), 47.5 (C13), 48.7 (C9), 49.6 (C14), 112.1 (d, J = 3.6) (C20), 121.7 (dt, J = 53.3, 3.6) (C4), 127.4 (C2), 145.9 (C6), 155.3 (C1), 167.6 (C5), 185.1 (d, J = 53.3) (C3), 219.4 (C17).

3. Results and discussion

Standard requirements of an acceptable IS usually include a molecular weight at least three mass unit higher than that of the non-labelled material and stability of the labels during sample preparation and analyses. In case of exemestane, deuterium and ^{18}O were discarded due to possible exchange reactions during sample preparation as well as possible formation of ions interfering with the quantitation of the analyte during the ionization processes in the mass spectrometer. Therefore, the introduction of three ^{13}C was considered. In the literature several preparations of

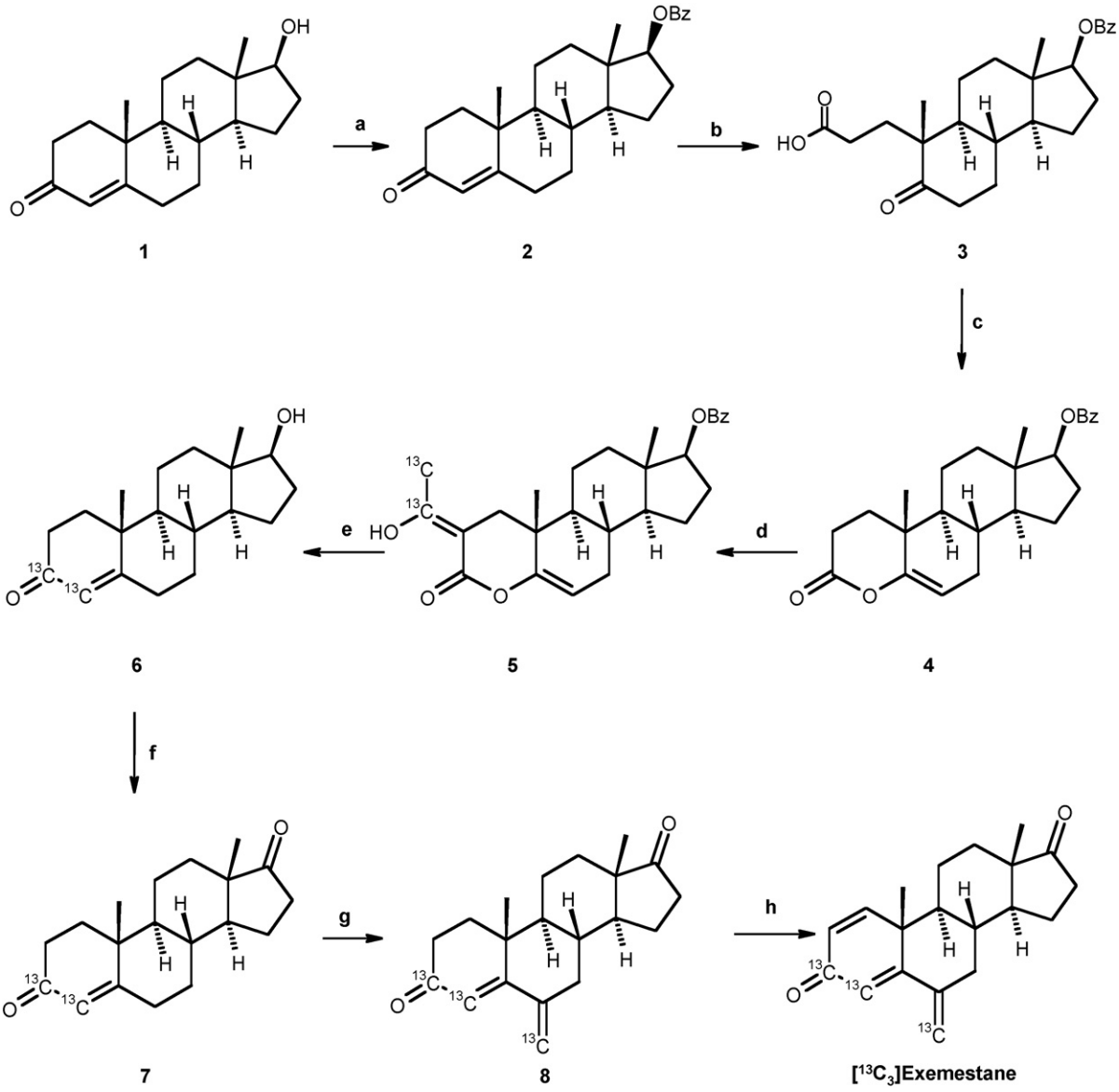

Fig. 2 – Synthesis of carbon-13 labelled exemestane. Reagents and conditions: (a) BzCl, Pyridine, toluene, reflux, 20 h; (b) $KMnO_4$, KIO_4, K_2CO_3, tBuOH, 40 °C, 5 h; (c) $HClO_4$, Ac_2O, EtOAc, rt, 1 h; (d) $^{13}CH_3{}^{13}COCl$ $((CH_3)_3Si)_2N$-Li, THF, −78 °C; (e) KOH, MeOH, H_2O, reflux, 20 h; (f) PCC, CH_2Cl_2, rt, 5 h; (g) 1. Triethylorthoformate, THF, EtOH, *p*-TsOH, 40 °C 1 h 2. N-methylaniline, $H^{13}CHO$, 40 °C, 2 h; (h) DDQ, *p*-TsOH, dioxane, reflux, 1 h.

carbon-13 labelled testosterone [7–10] are reported involving the partial degradation of the ring A of the non-labelled testosterone followed by reconstruction of the ring using suitable carbon-13 labelled synthons. According to these procedures up to five carbon-13 atoms can be introduced in the ring A of testosterone. However, when considering the preparation of [$^{13}C_3$]exemestane, the commercial availability at reasonable prices of both [$^{13}C_2$]acetyl chloride and [^{13}C]formaldehyde and the simplicity of synthetic methods indicated the synthesis of [$^{13}C_2$]testosterone followed by the introduction of the required third carbon-13 in the 6-methylene group as the most convenient approach. According to this procedure, the preparation of [$^{13}C_3$]exemestane was accomplished in eight steps starting from testosterone (**1**). The synthetic pathway is shown in Fig. 2. After protection of the 17-hydroxyl group of testosterone (**1**) by reaction with benzoylchloride, the A-ring of (**2**) was opened by oxidation with $KMnO_4/KIO_4$. The obtained ketoacid (**3**) was treated with $HClO_4$ giving the lactone (**4**). The reaction of (**4**) with lithium bis(trimethylsilyl)amide at −78 °C followed by treatment with [$^{13}C_2$]acetyl chloride, afforded the intermediate (**5**). The rearrangement of (**5**) to the desired [$^{13}C_2$]testosterone (**6**) was obtained at reflux in methanol/water in the presence of KOH. The conversion of (**6**) to the labelled androstenedione (**7**) was achieved by treatment with pyridinium chlorochromate in methylene chloride. The reaction of (**7**) with the commercially available [^{13}C]formaldehyde in the presence of *N*-methylaniline afforded the intermediate (**8**). The treatment of (**8**) with 2,6-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in dioxane in the presence of *p*-toluenesulfonic acid gave

the crude [$^{13}C_3$]exemestane. After purification by preparative HPLC, [$^{13}C_3$]exemestane was obtained >98% chemically pure and with an isotopic purity of 99 atom % ^{13}C. The overall chemical yield was approximately 10% from (**1**). The compound obtained was suitable for use as internal standard to develop a convenient LC–MS assay [11], which was then used to measure exemestane levels in a number of clinical studies [12–15].

Acknowledgement

The authors wish to thank Dr. Nicoletta Colombo, Analytical Chemistry Unit at Nerviano Medical Sciences (Nerviano, Italy), for NMR spectroscopy.

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