Syntheses and structural confirmation of stereoisomers and various isotopologues of tetrahydromethyltestosterone

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1 INTRODUCTION

The following describes the syntheses of stereoisomers and isotopologues of 17ξ-methyl-5ξ-androstane-3ξ,17ξ-diol (tetrahydromethyltestosterone, THMT). Stereoisomers of THMT are known metabolites of inter alia methyltestosterone, metandienone, mestanolone and methandriol[1]. These products found application in our group as starting material in biotransformation experiments, as reference standards for mass spectrometric analysis, as target compounds and as model substances for fragmentation analysis in electron ionization-mass spectrometry. In a manner of transparent scientific work and open source the synthetic approaches and structural confirmation including analytical results are presented here.

2 EXPERIMENTAL

2.1 Reagents and chemicals

Ammonium iodide (≥99%), ethanethiol (97%), K-selectride solution (1 M in tetrahydrofuran), sulfur trioxide pyridine complex and palladium on carbon (10 wt. %) were purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany). Androsterone (3α-hydroxy-5α-androstan-17-one, 97%) and methylmagnesium bromide (3M in diethyl ether) were obtained from Acros Organics (Fair Lawn, New Jersey, USA). Sodium borohydride and Celite came from Merck KGaA (Darmstadt, Germany). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany) and N,O-bis(trimethyl-[$^2H_9$]-silyl)acetamide ([$^2H_{18}$]-BSA) from Abcr GmbH (Karlsruhe, Germany). Hydrogen gas was supplied by Air Liquide (Düsseldorf, Germany) and deuterium gas (99,8%) from Linde AG (Munich, Germany). 2,2,3,4,4-d_5-Androsterone has been obtained from LGC standards (Wesel, Germany). Further steroid starting material, epiandrosterone (3β-hydroxy-5α-androstan-17-one, >97%) and 17α-methyltestosterone (>98%), were obtained from TCI (Tokyo, Japan), etiocholanolone (3α-hydroxy-5β-androstan-17-one), epietiocholanolone (3β-hydroxy-5β-androstan-17-one) from Steraloids (Newport, RI, USA). 19,19,19-d_3-androst-4-ene-3,17-dione, 20,20,20-d_3-17α-methyltestosterone and 17α-methyl-5β-androstane-3α,17β-diol were purchased from TRC (Toronto, Canada).

2.2 Synthesis

The assignment of number, systematic name und chemical structure is described in Table 2 for products and in Table 3 for intermediates.

2.2.1 17ξ-Methyl-5ξ-androstane-3ξ,17ξ-diol

2.2.1.1 17α-Methyl-5α-androstane-3α,17β-diol (T1) and 17β-methyl-5α-androstane-3α,17α-diol (T5)

The reaction was performed under atmosphere of argon. A solution of methylmagnesium bromide in diethyl ether (3 M, 21 mL, 63 mmol) was boiled under reflux (80 °C) and a solution of androsterone (2 g, 6.9 mmol) in anhydrous tetrahydrofuran (THF, 9 mL) was added dropwise. The reaction was

quenched by adding ice after 4 h. After adding water (200 mL) the product was extracted three times using dichloromethane (DCM, 100 mL). Subsequently, the organic phase was washed with brine and dried over sodium sulfate. Purification was performed via gravity column chromatography (silica gel, hexane/ethyl acetate, 3:2, *v:v*) and subsequent semi-preparative HPLC-UV as described in 2.3.3 (reversed phase,mobile phase methanol/water, 80:20, *v:v*).

2.2.1.2 17α-Methyl-5α-androstane-3β,17β-diol (T 2) and 17β-methyl-5α-androstane-3β,17α-diol (T6)

The synthesis of 17α-methyl-5α-androstane-3β,17β-diol and 17β-methyl-5β-androstane-3α,17α-diol was performed likewise as described in 2.2.1.1 using epiandrosterone (2 g, 6.9 mmol) as starting material. The crude product was purified using gravity column chromatography (silica gel, hexane/ethyl acetate, 3:1, *v:v*) and semi-preparative HPLC- UV as described in 2.3.3 (reversed phase, mobile phase methanol/water, 75:25, *v:v*).

2.2.1.3 17α-Methyl-5β-androstane-3α,17β-diol (T3) and 17α-methyl-5β-androstane-3β,17β-diol (T4)

2.2.1.3.1 17β-Hydroxy-17α-methyl-5β-androstan-3-one (I1)

17α-Methyltestosterone (0.5 g, 1.7 mmol) was dissolved in 20 mL of a mixture of methanol/aqueous potassium hydroxide (KOH, 5 M, ratio 9:1, *v:v*) with additional palladium on charcoal (10% wt., 0.5 g). The mixture was stirred under hydrogen atmosphere at room temperature for 60 min. The reaction mixture was filtered through celite.

2.2.1.3.2 17α-Methyl-5β-androstane-3α,17β-diol (T3)

Unpurified intermediate **I1** was dissolved in 9 mL methanol and 1 mL water and stirred together with sodium borohydride (62 mg, 1.6 mmol) at room temperature for 60 min. The reaction was stopped by dropwise addition of hydrochloric acid and, subsequently, the solution was neutralized by KOH. Methanol was evaporated and water (20 mL) was added to finally extract three times with 50 mL of DCM each. The extracted product was purified using gravity column chromatography (silica gel, hexane/ethyl acetate, 4:1, *v:v*).

2.2.1.3.3 17α-Methyl-5β-androstane-3β,17β-diol (T4)

Unpurified intermediate **I1** was dissolved in 20 mL dry THF and stirred for 1 h after addition of K-selectride solution (1M in THF, 0.5 mL, 0.5 mmol). The reaction was quenched by dropwise addition of hydrochloric acid and, subsequently, the solution was neutralized by KOH. Methanol was evaporated and 20 mL of water was added to finally extract three times with 50 mL DCM each. Final purification was performed via gravity column chromatography (silica gel, hexane/ethyl acetate, 3:1, *v:v*) and subsequent semi-preparative HPLC-UV as described in 2.3.3 (reversed phase, mobile phase acetonitrile/water, 70:30, *v:v*).

2.2.1.4 17β-Methyl-5β-androstane-3α,17α-diol (T7) and 17β-methyl-5β-androstane-3β,17α-diol (T8)

2.2.1.4.1 17β-Methyltestosterone (I2)

17α-Methyltestosterone (2.5 g, 8.3 mmol) and sulfur trioxide pyridine complex (3 g, 18.8 mmol) were stirred at ambient temperature in dimethyl formamide (25 mL) for 2 h. After the precipitate by excess diethyl ether precipitation was filtered, dissolved in water and stirred overnight. Filtered newly precipitated product was dissolved in DCM and dried over sodium sulfate. The crude product was purified using gravity column chromatography (silica gel, hexane/ethyl acetate 3:1, *v:v*).

2.2.1.4.2 17α-Hydroxy17β-methyl-5β-androstane-3-one (I3)

Unpurified intermediate **I2** (130 mg) was dissolved in a mixture of methanol and potassium hydroxide solution (5 M) in a ratio 9:1 (*v:v*). Palladium on charcoal (10% wt., 80 mg) was added as catalyst to the solution. The reaction mixture was kept under hydrogen atmosphere for 1 h. The resulting mixture was purified via filtration through celite.

2.2.1.4.3 17β-Methyl-5β-androstane-3α,17α-diol (T7)

Unpurified intermediate **I3** was stirred in a mixture of methanol and water (9:1, *v:v*, 10 mL) at ambient temperature for 1 h after addition of sodium borohydride (45 mg, 1.2 mmol). After extraction with DCM final purification was achieved by gravity column chromatography (silica gel, hexane/ethyl acetate 3:1, *v:v*) and subsequent semi-preparative HPLC-UV as described in 2.3.3 (normal phase, mobile phase hexane/isopropyl alcohol, 88:12, *v:v*).

2.2.1.4.4 17β-Methyl-5β-androstane-3β,17α-diol (T8)

Unpurified intermediate **I3** was dissolved in THF (10 mL) and K-selectride solution (1 M in tetrahydrofuran, 0.25 mL) was added to the stirring solution. After 1 h excessive reagent was destroyed by adding hydrochloric acid (1 M). After neutralization and extraction with DCM a gravity column chromatography purification (silica gel, hexane/ethyl acetate 3:2, *v:v*) was performed followed by semi-preparative HPLC-UV purification as described in 2.3.3 (normal phase, mobile phase hexane/isopropyl alcohol, 92 : 8, *v:v*).

2.2.2 Stable isotopic labeled 17α-methyl-5ξ-androstane-3ξ,17β-diols

2.2.2.1 20,20,20-d_3-Derivatives of the 3β5α- (9) ,3α5β- (10) and 3β5β-isomers (11)

20,20,20-d_3-17α-Methyltestosterone (0.5 mg) and a tip of a micro spatula of palladium on charcoal were mixed in 250 µL of methanol. The reaction mixture was stirred under hydrogen atmosphere and was stirred for 19 h. The catalyst was removed by filtration through celite after flushing the system with air.

2.2.2.2 19,19,19-d_3-Derivatives of the 3β5α- (12) and 3β5β-isomers (13)

2.2.2.2.1 19,19,19-d_3-5ξ-Androstane-3ξ-ol-17-one (I4 and I5)

19,19,19-d_3-Androst-4-ene-3,17-dione (2 mg) and a tip of a micro spatula of palladium on charcoal were mixed in 0.5 mL of methanol. The reaction mixture was put under hydrogen atmosphere and was stirred for 20 h. The catalyst was removed by filtration through celite after flushing the system with air.

2.2.2.2.2 19,19,19-d_3-17α-Methyl-5ξ-androstane-3ξ,17β-diols

Unpurified intermediate **I4** or **I5** was dissolved in 1 mL of anhydrous THF and stirred in round bottom flask under atmosphere of argon at room temperature. A mixture of dry THF (225 µL) and methylmagnesium bromide in diethyl ether (3 M, 75 µL, 0.2mmol) was added dropwise. The reaction was ended after 3 h by adding 750 µL of water. THF was evaporated by a gentle stream of nitrogen and subsequently, the reaction mixture was extracted three times with 750 µL of DCM each.

2.2.2.3 16,16-d_2-Derivatives of the 3α5α- (14), 3β5α- (15) ,3α5β- (16) and 3β5β-isomers (17)

2.2.2.3.1 16,16-d_2-3ξ-Hydroxy-5ξ-androstane-17-ones (I6, I7, I8 and I9)

3 mg of Androsterone, Epiandrosterone, Etiocholanolone and Epietiocholanolone have been dissolved in d_1-ethanol (1 mL) separately with additional deuterium oxide (D_2O, 200 µL) and sodium deuteroxide in D_2O (40%, 40 µL). The mixtures were kept at 75°C for 3 h and subsequently extracted three times with 2 mL hexane each. This procedure was repeated four times.

2.2.2.3.2 16,16-d_2-17α-Methyl-5ξ-androstane-3ξ,17β-diols

Unpurified intermediate **I6**, **I7**, **I8** or **I9** was dissolved in anhydrous THF (1 mL) and kept under atmosphere of argon. Methylmagnesium bromide in diethyl ether (3 M, 150 µL, 0.5 mmol) was added dropwise. After 2.5 h the reaction was quenched by addition of 500 µL of water and extracted three times with 750 µL methyl tert-butyl ether each.

2.2.2.4 2,2,3,4,4-d_5-17α-Methyl-5α-androstane-3α,17β-diol (18)

2,2,3,4,4-d_5-androsterone (3 mg, 10µmol) was treated as described in 2.2.2.3.2.

2.2.2.5 5-d_1-Derivatives of the 3β5α- (**19**) ,3α5β- (**20**) and 3β5β-isomers (**21**)

2.2.2.5.1 4,5-d_2-17β-Hydroxy-17α-methyl-5ξ-androstane-3-one (**I10** and **I11**)

17α-methyltestosterone (20 mg, 66 µmol) and a tip of a spatula palladium on charcoal have been mixed in methanol and were kept under atmosphere of deuterium for 1 h. Reaction mixture was cleaned up by filtering through celite.

2.2.2.5.2 5-d_1-17β-Hydroxy-17α-methyl-5ξ-androstane-3-one (**I12** and **I13**)

Unpurified intermediate **I10** or **I11** was dissolved in methanol (2 mL), water (200 µL) and sodium hydroxide (40%, 80 µL) and incubated at 75 °C for 3 h. Thereafter, the reaction mixture was extracted three times with hexane (4 mL) and the combined organic phases were brought to dryness. This whole procedure was repeated three times.

2.2.2.5.3 5-d_1-17α-Methyl-5ξ-androstane-17β,3ξ-ol

Dried organic phase of 2.2.2.5.2 was dissolved in methanol (900 µL) and water (100 µL) and was stirred together with one granule of sodium borohydride at room temperature for 30 min. Reaction was ended by dropwise addition of hydrochloric acid and subsequently, the solution was neutralized by KOH. Methanol was evaporated and water (1 mL) was added to the reaction mixture, which was finally extract three times with 4 mL hexane.

2.3 Instrumentation

2.3.1 GC-MS

Low-resolution gas chromatography - electron ionization – mass spectrometry (GC-EI-MS) experiments were conducted on a 7890 gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA) hyphenated to a 5975C single quadrupole mass-selective detector (Agilent Technologies Inc). A HP-Ultra 1 column (17 m x 200 µm x 0.11 µm; Agilent Technologies Inc) was used with helium as carrier gas with constant flow rate of 1 mL/min. Inlet temperature was 300°C, injection volume 2 µL and split ratio of 25:1. The oven temperature started at 183°C, heating 3°C/min up to 232°C and then heated 40°C/min to 310°C with a hold time of 2 min. Ionization energy of 70 eV was applied using scan mode ranging from *m/z* 40 to 1000.

To improve chromatographic separation in the analysis of synthesis of 19,19,19-d_3-THMT structures an adjusted temperature program was used. It also started at 183°C and increased the temperature with 5°C/min up to 200°C. After a hold time of 10 min a ramp of 3°C/min up to 215°C was added and then heated with 40°C/min up to 310°C with a hold time of 2 min. Other conditions were kept unchanged.

2.3.2 GC-QTOF-MS

High-resolution data was acquired on an Agilent gas chromatography - electron ionization - quadrupole time-of-flight mass spectrometrer (GC-EI-QTOF-MS) 7890B/7250 (Agilent Technologies Inc., Milano, Italy) equipped with a HP-5MS capillary column (17 m x 200 µm x 0.11 µm, Agilent Technologies Inc.). Differences in the method described in 2.3.1 are the inlet temperature of 280°C, split ratio of 10:1, constant pressure of 18 psi and following oven parameters: initial setpoint 150°C, heating rate of 50°C/min up to 200°C, following heating of 3°C/min up to 230°C, heating rate of 50°C/min up to 320°C with a final hold time of 3 min. The coupled QToF was operated in full scan mode with an ionization energy of 15 eV and scan range from *m/z* 50 to 750. Mass calibration was performed twice at the beginning of every sequence and then repeated after every second injection of a sample.

2.3.3 HPLC purification

HPLC-purification was performed by using an Agilent 1260 Infinity Quaternary HPLC system detecting with an Agilent Infinity 1260 diode array detector (Agilent Technologies GmbH, Waldbronn, Germany).

This semi-preparative approach was either done as reversed phase chromatography using a Hypersil ODS C18 column (pore size: 120 Å, 250 mm length, 10 mm inner diameter, 5 µm particle size, Thermo Scientific, Schwerte, Germany) or as normal phase chromatography with an EC 250/4,6 Nucleosil 100-5 N(CH_3) column (pore size: 100 Å, 250 mm length, 4,6 mm inner diameter, 5 µm particle size, Macherey Nagel, Düren, Germany) as stationary phase. A constant flow was set at 2 mL/min and UV absorption was observed at 196 nm. Compositions of the mobile phases are mentioned under 2.2 for every purified compound.

2.3.4 Nuclear magnetic resonance

The nuclear magnetic resonance (NMR) analyses of **T1**, **T2**, **T4** and **T7** were performed at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) at 296 K on a Bruker (Rheinstetten, Germany) Avance III instrument equipped with a nitrogen-cooled 5 mm inverse TCI cryoprobe with actively shielded z-gradient coil. Chemical shifts are reported in δ values (ppm) relative to tetramethylsilane. Solutions of about 5 mg of each compound in deuterated chloroform ($CDCl_3$) were used for conducting ^{1}H; H,H COSY; ^{13}C; edited H,C HSQC; H,C HMBC, selective NOE and NOESY experiments. Two-dimensional experiments were recorded in non-uniform sampling (NUS) mode.

Furthermore, NMR analyses of **T5**, **T6** and **T8** were performed at 600 MHz (^{1}H NMR) and 150 MHz (^{13}C NMR) at 296 K on a Jeol (Freising, Germany) ECZ600 instrument. Chemical shifts are reported in δ values (ppm) relative to the solvent $CDCl_3$. Solutions of about 5 to 10 mg of each compound were used for conducting ^{1}H; H,H COSY; ^{13}C; H,C HSQC; H,C HMBC, selective NOE and NOESY experiments.

3 RESULTS AND DISCUSSION

3.1 Synthesis

3.1.1 17ξ-Methyl-5ξ-androstane-3ξ,17ξ-diols

The desired configuration in the A-ring of the steroid structures was achieved by selective reduction as reported by Schänzer and Donike [2]. Carbonyl groups in position 3 were either reduced by sodium borohydride or K-Selectride. Stereochemistry of the remaining molecule directs, if 3α- or 3β-configuration depicts the main or side product. Reduction of the 4(5) double bond was achieved using hydrogen and palladium on charcoal. The addition of aqueous potassium hydroxide to the reaction mixture led to an excess of 5β-configured products and addition of only water instead to a racemic mixture of 5α- and 5β-configuration.

To obtain 17β-methyl-5α-androstane-3ξ,17α-diols the well-established approach via Grignard addition of a methyl group at C17 was applied and modified [3]. From a mechanistic point of view Grignard reactions are not stereoselective in the attack at the planar carbon of a carbonyl. Nevertheless, in the case of androstanes this reaction yields in the 17α-methyl-17β-diol structure in high excess and the targeted epimer as minor side products [4]. This may be explained by the steric hindering by C18-CH_3 in β-configuration [5]. Therefore, harsh conditions especially elevated temperatures were needed to observe an increase of the 17β-methyl-17α-diol product which was be preparatively separated.

On the other hand, the synthesis of 17β-methyl-5β-androstane-3ξ,17α-diols was achieved by synthetic epimerization of C17 adapted from Schänzer et al [6]. The targeted epimerization of C17 of 17α-methyltestosterone was achieved by sulfonation of the 17β-hydroxy group. The cleavage of the sulfate resulted in a tertiary carbenium ion and subsequently lead to a mixture of 17β- and 17α-methyltestosterone by an unselective attack of water.

3.1.2 Stable isotopic labeled 17α-methyl-5ξ-androstane-3ξ,17β-diols

In the case of **17**-**21** and **26** synthesis was performed as described in 3.1.1 but instead using with deuterium labelled starting material (positions: C19-CD_3, C20-CD_3 or 2,2,3,4,4-d_5).

Prior to the Grignard addition as described in 3.1.1 to obtain **22**-**25** starting material with a carbonyl function at C17 underwent proton-deuterium-exchange in position 16. Therefore, keto-enol tautomerism was triggered in a deuterated environment by alkaline conditions. This reaction bases on an establishment of equilibrium and needed to be repeated three times for a sufficient yield.

Deuterium labels at C5 of isotopologues **27**-**29** were established by reduction of 4(5) double bond with deuterium to obtain a 4,5-d_2 substructure and subsequent deuterium-proton-exchange in position 4.

3.2 Structural confirmation of synthetic products

After purification structural and stereochemical confirmation of synthesized products (**T1**-**T8**) was performed. The assignment was supported by gas chromatography high resolution mass spectrometry (GC/HRMS) experiments (2.3.2) by a low mass difference to the exact mass (<2.3 ppm) as depicted in Table 1. Final confirmation was achieved via NMR spectroscopy. All signals were assigned from shifts and correlations of 1D and 2D measurements. Assignment of stereochemistry at C5 was supported based on shielded or deshielded shifted C19. Blunt et al. have reported that $\delta_{C19} < 22$ ppm is typical for 5α-androstanes and $\delta_{C19} > 22$ ppm for 5β-androstanes [7]. Complete stereochemical assignment was performed by evaluation of NOESY experiments.

Products **9-21** were not finally confirmed by NMR experiments due to synthesis in analytical scale. Structural assignments of isotopologues **9-21** synthesized in 2.2.2 was achieved by comparison of mass spectra and retention time with d_0-isotopologues **T1-T4**, which were confirmed by NMR experiments.

*Table 1 Stereochemical assignment, accurate mass ($M^{+\bullet}$, EI, 70 eV), mass difference to exact mass (**1**-**8**: m_e = 450.3344, $C_{26}H_{50}O_2Si_2^{+\bullet}$, as per-TMS derivatives).*

number	accurate mass	Δ *m/z* [ppm]
T1	450.3351	1.55
T2	450.3354	2.22
T3	450.3351	1.55
T4	450.3344	0
T5	450.3353	2.00
T6	450.3350	1.33
T7	450.3349	1.11
T8	450.3350	1.33

3.2.1 NMR spectroscopy of tetrahydromethyltestosterone stereoisomers

3.2.1.1 17α-Methyl-5α-androstane-3α,17β-diol (T1)

^{1}H NMR (500 MHz, CDCl3) δ = 4.04 (p, *J*=2.7, 2.7, 2.6, 2.6, 1H, H-3β), 1.78 (m, 1H, H-16β), 1.73 (m, 1H, H-16α), 1.70 (m, 1H, H-2β), 1.68 (dd, *J*=4.5, 3.1, 1H, H-7β), 1.63 (dt, *J*=4.5, 2.6, 2.6, 1H, H-2α), 1.61 (td, *J*=4.7, 4.4, 2.3, 1H, H11α), 1.56 (m, 1H, H-15α), 1.54 (m, 1H, H-5α), 1.51 (d, *J*=2.7, 1H, H-4β), 1.49 (m, 1H, H-1β), 1.48 (m, 1H, H-12β), 1.43 (ddd, *J*=10.4, 4.0, 1.3, 1H, H-8β), 1.38 (dq, *J*=13.4, 2.7, 2.6, 2.6, 1H, H-4α), 1.31 (m, 1H, H-1α), 1.27 (m, 1H, H-12α), 1.24 (dd, *J*=5.9, 3.5, 1H, H-11β), 1.24 (m,1H, H-15β), 1.21 (s, 3H, CH3-20), 1.20 (m, 1H, H-6β), 1.19 (m, 1H, H-14α), 1.17 (m, 1H, H-6α), 0.90 (m, 1H, H-7α), 0.85 (s, 3H, CH3-18), 0.80 (s, 3H, CH3-19), 0.74 (m, 1H, H-9α).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 81.75 (s, C-17), 66.55 (s, C-3), 54.39 (s, C-9), 50.75 (s, C-14), 45.55 (s, C-13), 39.23 (s, C-5), 39.00 (s, C-16), 36.39 (s, C-8), 36.21 (s, C-10), 35.89 (s, C-4), 32.23 (s, C-1), 31.75 (s, C-7), 31.69 (s, C-12), 29.04 (s, C-2), 28.49 (s, C-6), 25.83 (s, C-20), 23.24 (s, C-15), 20.41 (s, C-11), 14.00 (s, C-18), 11.21 (s, C-19).

3.2.1.2 17α-Methyl-5α-androstane-3β,17β-diol (T2)

^{1}H NMR (600 MHz, $CDCl_3$) δ = 3.59 (ddd, *J*=15.9, 11.1, 4.8, 1H, H-3α), 1.80 (m, 1H, H-2β), 1.78 (m, 1H, H-16β), 1.74 (m, 1H, H-1β), 1.71 (m, 1H, H-16α), 1.68 (m, 1H, H-7β), 1.59 (m, 1H, H-11α), 1.57 (m, 1H, H-4α), 1.55 (m, 1H, H-15α), 1.47 (m, 1H, H-12α), 1.41 (m, 1H, H-8β), 1.40 (m, 1H, H-2α), 1.30 (m, 1H, H-11β), 1.29 (m, 1H, H-4β), z 1.27 (m, 1H, H-6β), 1.25 (m, 1H, H-12β), 1.24 (m, 1H, H-6α), 1.23 (m, 1H, H-15β), 1.21 (s, 3H, CH3-20), 1.16 (m, 1H, H-14), 1.10 (m, 1H, H-5α), 0.97 (td, *J*=13.5, 13.4, 3.8, 1H, H-1α), 0.87 (m, 1H, H-7α), 0.85 (s, 3H, CH3-18), 0.83 (s, 3H, CH3-19), 0.62 (ddd, *J*=11.9, 10.5, 4.1, 1H, H-9α).

^{13}C NMR (151 MHz, $CDCl_3$) δ = 81.74 (s, C-17), 71.32 (s, C-3), 54.42 (s, C-9), 50.70 (s, C-14), 45.55 (s, C-13), 44.98 (s, C-5), 39.01 (s, C-16), 38.19 (s, C-4), 37.06 (s, C-1), 36.40 (s, C-8), 35.60 (s, C-10), 31.81 (s, C-7), 31.68 (s, C-12), 31.53 (s, C-2), 28.64 (s, C-6), 25.80 (s, C-20), 23.27 (s, C-15), 20.89 (s, C-11), 13.99 (s, C-18), 12.36 (s, C-19).

3.2.1.3 17α-Methyl-5β-androstane-3α,17β-diol (T3)

The structure of **T3** has been confirmed by GC-MS comparison regarding retention time and fragmentation with authentic reference material supplied by the Laboratorio Antidoping FMSI, Rome (TRC, Toronto, Canada).

3.2.1.4 17α-Methyl-5β-androstane-3β,17β-diol (T4)

^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.12 (t, *J*=2.9, 2.9, 1H, H-3α), 1.99 (td, *J*=14.1, 14.0, 3.1, 1H, H-2α), 1.90 (tt, *J*=13.8, 13.8, 4.6, 4.6, 1H, H-6β), 1.80 (m, 1H, H-16β), 1.75 (m, 1H, H-5β), 1.72 (m, 1H, H-16α), 1.58 (m, 1H, H-4α), 1.55 (m, 1H, H-15α), 1.54 (m, 1H, H-1α), 1.51 (m, 1H, H-12α), 1.49 (m, 1H, H-4β), 1.48 (m, 1H, H-11α), 1.48 (m, 1H, H-8β), 1.43 (m, 1H, H-7β), 1.39 (m, 1H, H-1β), 1.34 (m, 1H, H-2β), 1.29 (d, *J*=2.3, 1H, H-9α), 1.27 (m, 1H, H-11β), 1.27 (m, 1H, H-12β), 1.26 (m, 1H, H-15β), 1.23 (m, 1H, H-14α), 1.22 (s, 3H, CH3-20), 1.17 (ddt, *J*=13.7, 4.2, 2.3, 2.3, 1H, H-6α), 1.02 (ddd, *J*=13.7, 4.2, 1.7, 1H, H-7α), 0.99 (s, 3H, CH3-19), 0.85 (s, 3H, CH3-18).

^{13}C NMR (126 MHz, $CDCl_3$) δ = 81.77 (s, C-17), 67.12 (s, C-3), 50.84 (s, C-14), 45.66 (s, C-13), 39.91 (s, C-9), 39.09 (s, C-16), 36.63 (s, C-5), 36.57 (s, C-8), 35.29 (s, C-10), 33.53 (s, C-2), 31.91 (s, C-12), 30.02 (s, C-1), 27.84 (s, C-4), 26.58 (s, C-6), 26.09 (s, C-7), 25.79 (s, C-20), 23.91 (s, C-19), 23.28 (s, C-15), 20.71 (s, C-11), 13.96 (s, C-18).

3.2.1.5 17β-Methyl-5α-androstane-3α,17α-diol (T5)

^{1}H NMR (600 MHz, $CDCl_3$) δ = 4.03 (p, *J*=2.8, 2.8, 2.6, 2.6, 1H, H-3β), 1.84 (ddd, *J*=14.5, 11.4, 2.8, 1H, H-16β), 1.69 (m, 1H, H-15α), 1.68 (s, 1H, H-7β), 1.67 (m, 1H, H-2β), 1.64 (m, 1H, H-11β), 1.63 (m, 1H, H-16α), 1.61 (m, 1H, H-2α), 1.61 (m, 1H, H-14α), 1.53 (m, 1H, H-5α), 1.51 (dd, *J*=13.1, 2.7, 1H, H-4β), 1.47 (m, 1H, H-1β), 1.41 (m, 1H, H-8β), 1.40 (m, 2H, H-12), 1.36 (m, 1H, H-4α), 1.31 (td, *J*=13.7, 13.6, 4.5, 1H, H-1α), 1.24 (m, 1H, H-11α), 1.18 (s, 3H, CH_3-20), 1.13 (qd, *J*=11.8, 11.5, 11.5, 5.5, 1H, H-15β), 1.00 (qd, *J*=12.6, 12.6, 12.4, 5.3, 1H, H-7α), 0.79 (s, 3H, CH_3-19), 0.78 (m, 1H, H-9α), 0.67 (s, 3H, CH_3-18).

^{13}C NMR (151 MHz, $CDCl_3$) δ = 82.52 (s, C-17), 66.90 (s, C-3), 54.52 (s, C-9), 50.30 (s, C-14), 47.03 (s, C-13), 39.54 (s, C-5), 38.63 (s, C-16), 36.53 (s, C-10), 36.32 (s, C-8), 36.25 (s, C-4), 32.58 (s, C-1), 32.49 (s, C-7), 30.28 (s, C-12), 29.36 (s, C-2), 28.92 (s, C-6), 24.24 (s, C-15), 22.99 (s, C-20), 20.64 (s, C-11), 16.31 (s, C-18), 11.55 (s, C-19).

3.2.1.6 17β-Methyl-5α-androstane-3β,17α-diol (T6)

^{1}H NMR (600 MHz, $CDCl_3$) δ = 3.52 (tt, *J*=11.1, 11.1, 4.8, 4.8, 1H, H-3α), 1.77 (m, 1H, H-4α), 1.72 (m, 1H, H-2a), 1,64 (m, 1H, H-1α), 1.63 (m, 1H, H-7β) , 1.61 (m, 1H, H-15α), 1.56 (m, 1H, H-16β), 1.54 (m, 1H, H-11β), 1.52 (m, 1H, H-14α), 1.48 (m, 1H, H-4β), 1.32 (m, 1H, H-2β), 1.31 (m, 2H, H-12), 1.30 (m, 1H, H-8β), 1.20 (m, 1H, H-16α), 1.19 (m, 2H, H-6), 1.10 (s, 3H, CH_3-20), 1.05 (m, 1H, H-15β), 1.02 (m, 1H,

H-5α), 0.91 (m, 1H, H-1β), 0.87 (m, 1H, H-7α), 0.74 (s, 3H, CH_3-19), 0.61 (m, 1H, H-9α), 0.59 (s, 3H, CH_3-18).

^{13}C NMR (151 MHz, , $CDCl_3$) δ = 82.34 (s, C-17), 71.44 (s, C-3), 54.35 (s, C-9), 50.06 (s, C-14), 46.83 (s, C-13), 45.06 (s, C-5), 38.45 (s, C-16), 38.33 (s, C-4), 37.20 (s. C-1), 36.11 (s, C-8), 35.70 (s, C-10), 32.35 (s, C-7), 31.64 (s, C-2), 30.07 (s, C-12), 28.85 (s, C-6), 24.06 (s, C-15), 22.81 (s, C-20), 20.92 (s, C-11), 16.10 (s, C-18), 12.49 (s, C-19).

3.2.1.7 17β-Methyl-5β-androstane-3α,17α-diol (T7)

^{1}H NMR (600 MHz, $CDCl_3$) δ = 3.62 (tt, *J*=11.1, 11.1, 4.7, 4.7, 1H, H-3β), 1.86 (m, 1H, H-6β), 1.85 (m, 1H, H-16β), 1.81 (m, 1H, H-1α), 1.76 (m, 1H, H-4α), 1.70 (m, 1H, H-15α), 1.68 (m, 1H, H-14α), 1.67 (m, 1H, H-2β), 1.65 (m, 1H, 16α), 1.52 (m, 1H, 11α), 1.51 (m, 1H, 4β), 1.46 (m, 1H, 12α), 1.45 (m, 1H, 7β), 1.44 (m, 1H, 9α), 1.43 (m, 1H, H-8β), 1.40 (m, 1H, H-5β), 1.39 (m, 1H, H-12β), 1.32 (dq, *J*=12.4, 3.2, 1.3, 1.3, 1H, H-2α), 1.27 (m, 1H, H-6α), 1.24 (m, 1H, H-11β), 1.19 (s, 3H, CH_3-20), 1.17 (m, 1H, H-7α), 1.13 (m, 1H, H-15β), 0.98 (td, *J*=14.2, 14.2, 3.4, 1H, H-1β), 0.94 (s, 3H, CH_3-19), 0.67 (s, 3H, CH_3-18).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 82.22 (s, C-17), 71.87 (s, C-3), 50.02 (s, C-14), 46.78 (s, C-13), 42.23 (s, C-5), 40.46 (s, C-9), 38.46 (s, C-16), 36.52 (s, C-4), 36.33 (s, C-8), 35.50 (s, C-1), 34.70 (s, C-10), 30.58 (s, C-2), 30.11 (s, C-12), 27.18 (s, C-6), 26.64 (s, C-7), 23.96 (s, C-15), 23.38 (s, C-19), 22.66 (s, C-20), 20.37 (s, C-11), 15.93 (s, C-18).

3.2.1.8 17β-Methyl-5β-androstane-3β,17α-diol (T8)

^{1}H NMR (600 MHz, $CDCl_3$) δ = 4.03 (p, *J*=2.8, 2.8, 2.6, 2.6, 1H, H-3β), 1.84 (ddd, *J*=14.5, 11.4, 2.8, 1H, H-16β), 1.69 (m, 1H, H-15α), 1.68 (s, 1H, H-7β), 1.67 (m, 1H, H-2β), 1.64 (m, 1H, H-11β), 1.63 (m, 1H, H-16α), 1.61 (m, 1H, H-2α), 1.61 (m, 1H, H-14α), 1.53 (m, 1H, H-5α), 1.51 (dd, *J*=13.1, 2.7, 1H, H-4β), 1.47 (m, 1H, H-1β), 1.41 (m, 1H, H-8β), 1.40 (m, 2H, H-12), 1.36 (m, 1H, H-4α), 1.31 (td, *J*=13.7, 13.6, 4.5, 1H, H-1α), 1.24 (m, 1H, H-11α), 1.18 (s, 3H, CH_3-20), 1.13 (qd, *J*=11.8, 11.5, 11.5, 5.5, 1H, H-15β), 1.00 (qd, *J*=12.6, 12.6, 12.4, 5.3, 1H, H-7α), 0.79 (s, 3H, CH_3-19), 0.78 (m, 1H, H-9α), 0.67 (s, 3H, CH_3-18).

^{13}C NMR (151 MHz, $CDCl_3$) δ = 82.52 (s, C-17), 66.90 (s, C-3), 54.52 (s, C-9), 50.30 (s, C-14), 47.03 (s, C-13), 39.54 (s, C-5), 38.63 (s, C-16), 36.53 (s, C-10), 36.32 (s, C-8), 36.25 (s, C-4), 32.58 (s, C-1), 32.49 (s, C-7), 30.28 (s, C-12), 29.36 (s, C-2), 28.92 (s, C-6), 24.24 (s, C-15), 22.99 (s, C-20), 20.64 (s, C-11), 16.31 (s, C-18), 11.55 (s, C-19).

3.2.2 Mass spectra of products 9-21

Mass spectra of GC-MS measurements of final isotopologue products of THMT (**9**-**21**) are depicted in Figure 1 to Figure 13.

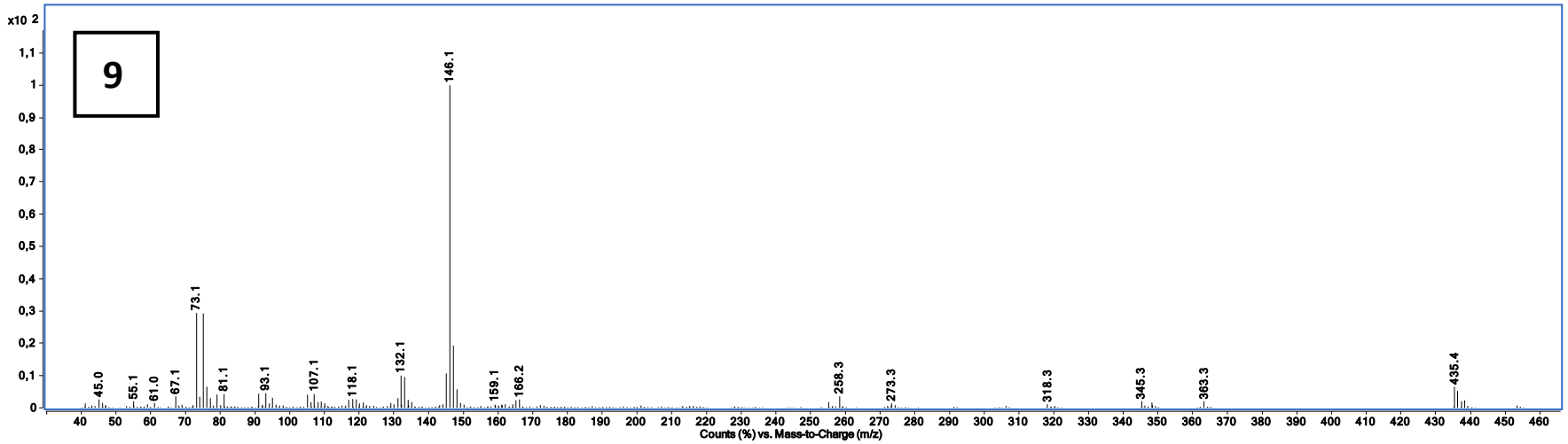

*Figure 1 GC-MS spectrum of **9**.*

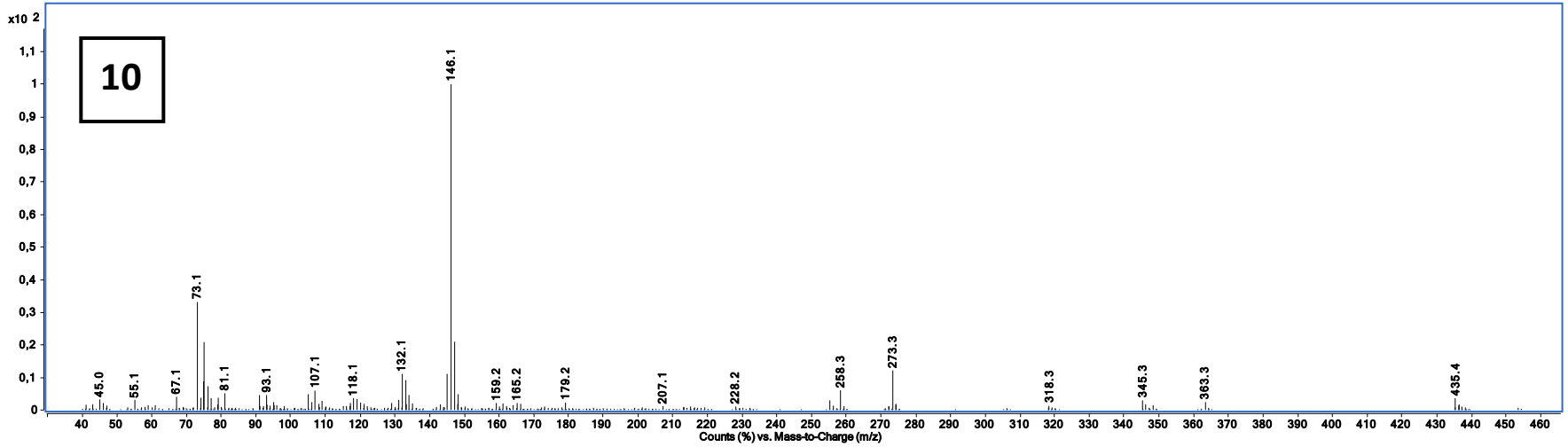

*Figure 2 GC-MS spectrum of **10**.*

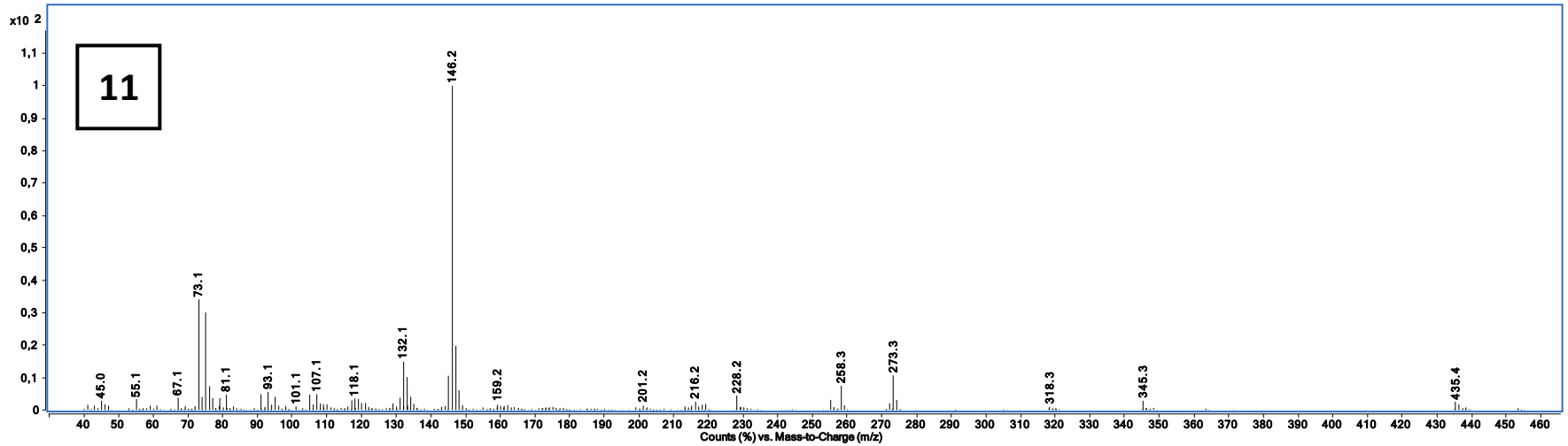

*Figure 4 GC-MS spectrum of **11**.*

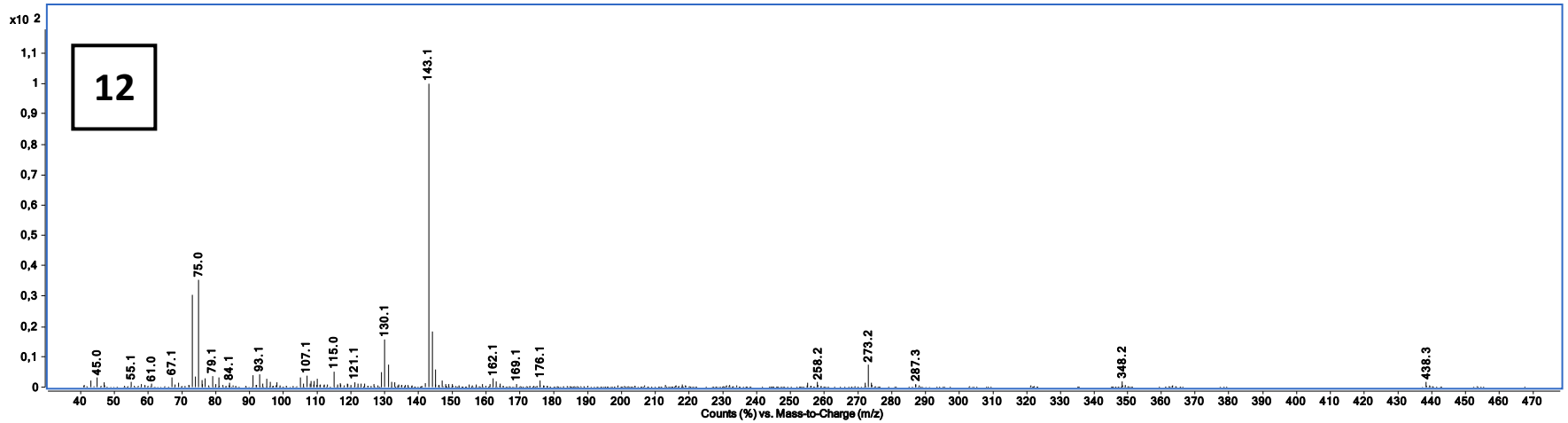

*Figure 3 GC-MS spectrum of **12**.*

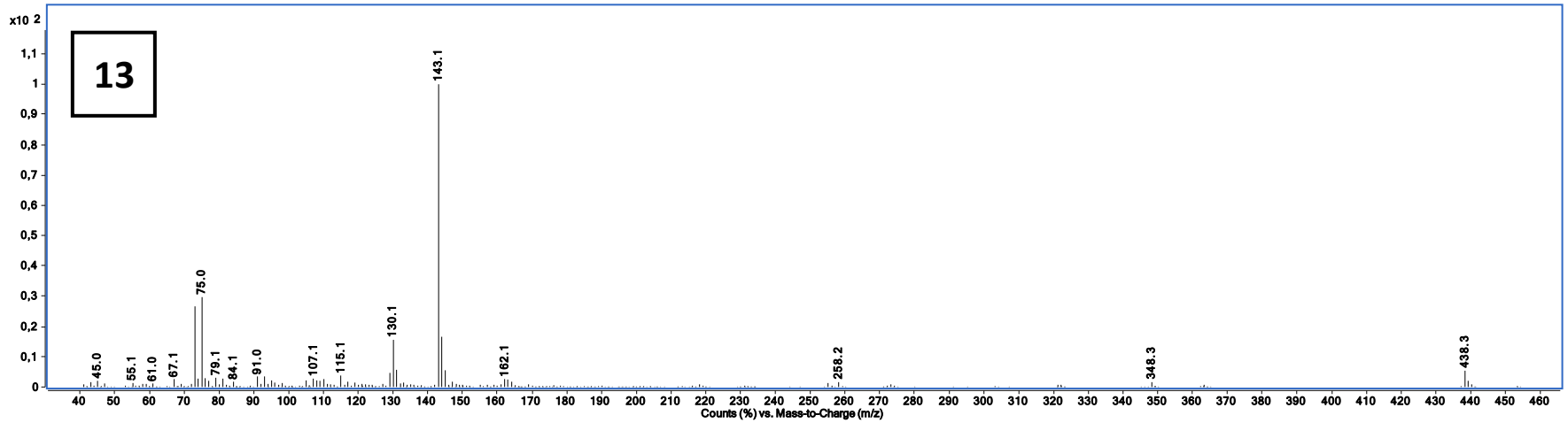

*Figure 5 GC-MS spectrum of **13**.*

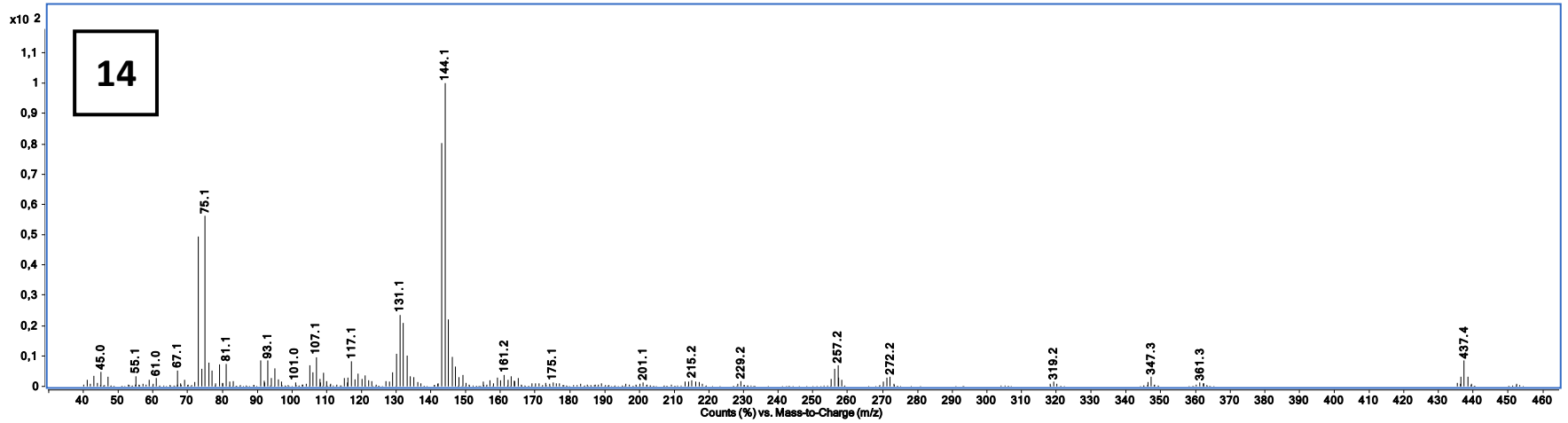

*Figure 6 GC-MS spectrum of **14**.*

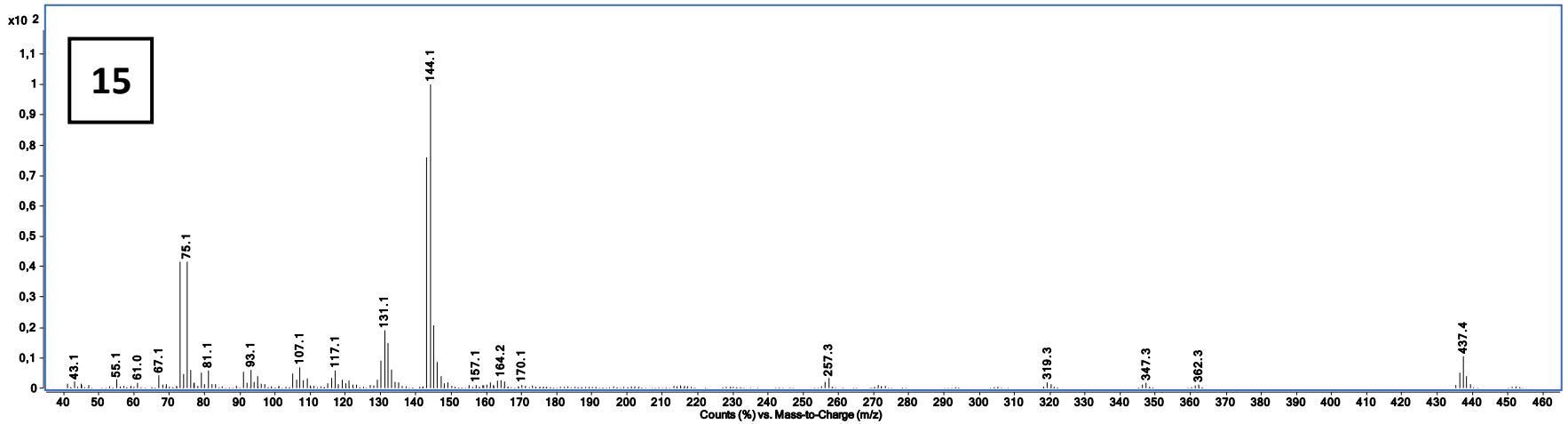

*Figure 8 GC-MS spectrum of **15**.*

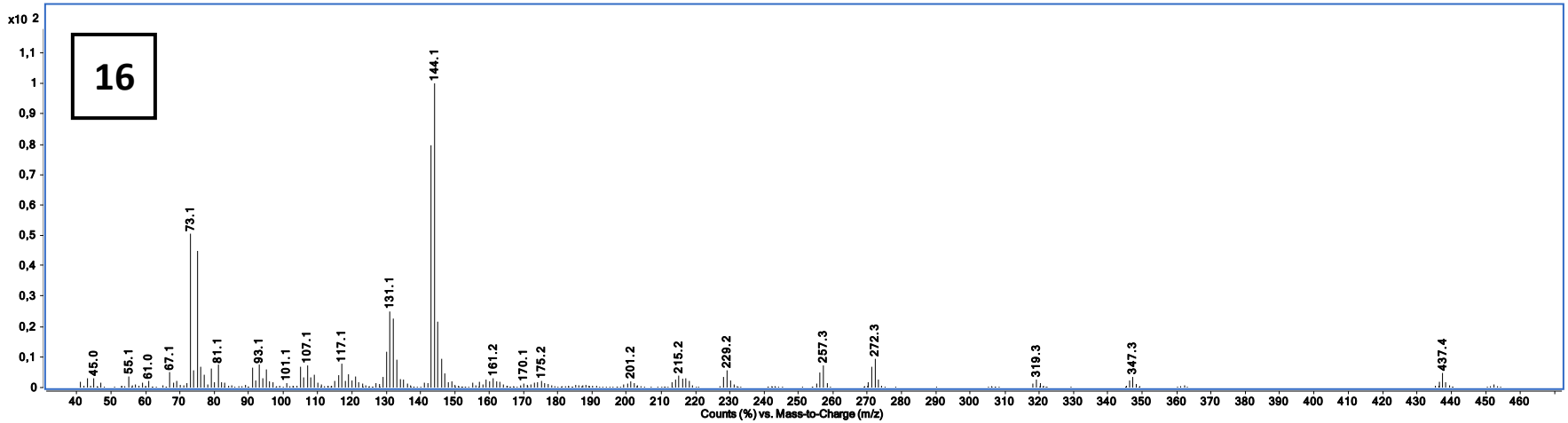

*Figure 7 GC-MS spectrum of **16**.*

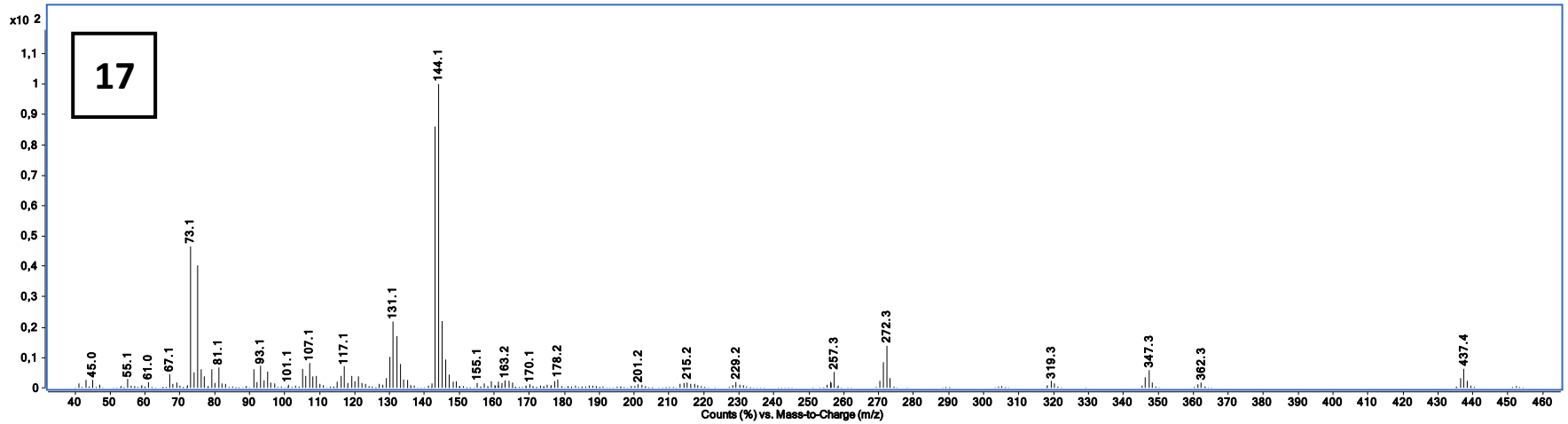

*Figure 9 GC-MS spectrum of **17**.*

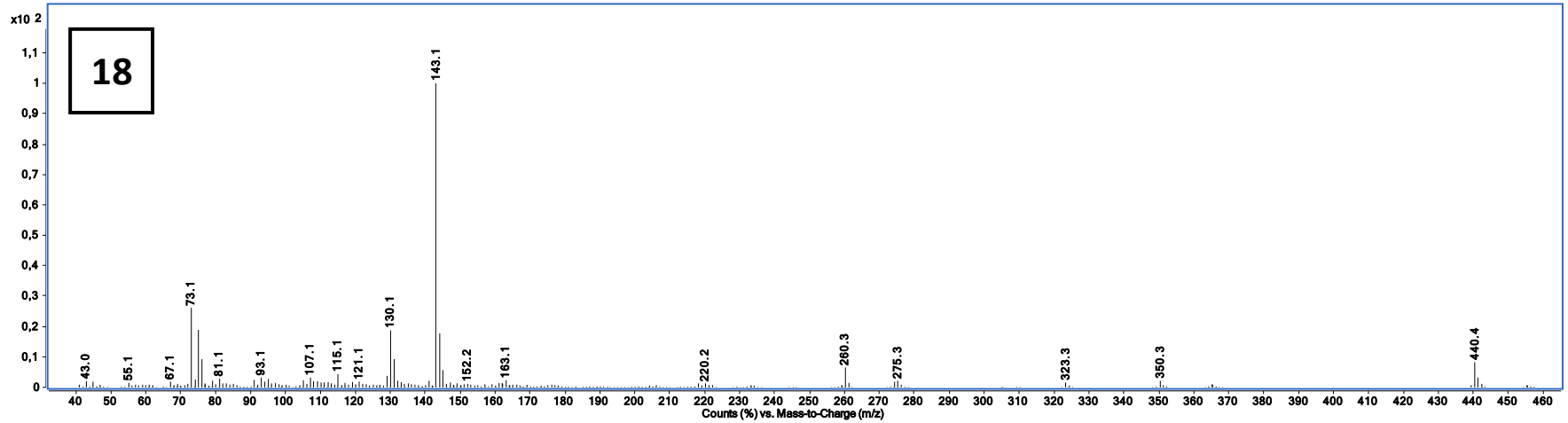

*Figure 10 GC-MS spectrum of **18**.*

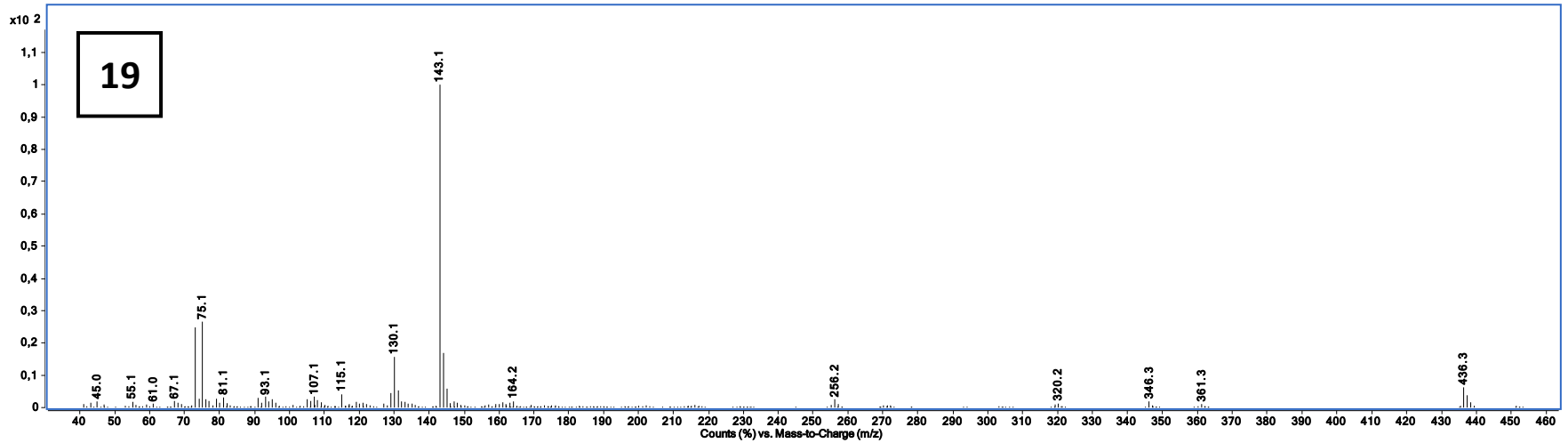

*Figure 12 GC-MS spectrum of **19**.*

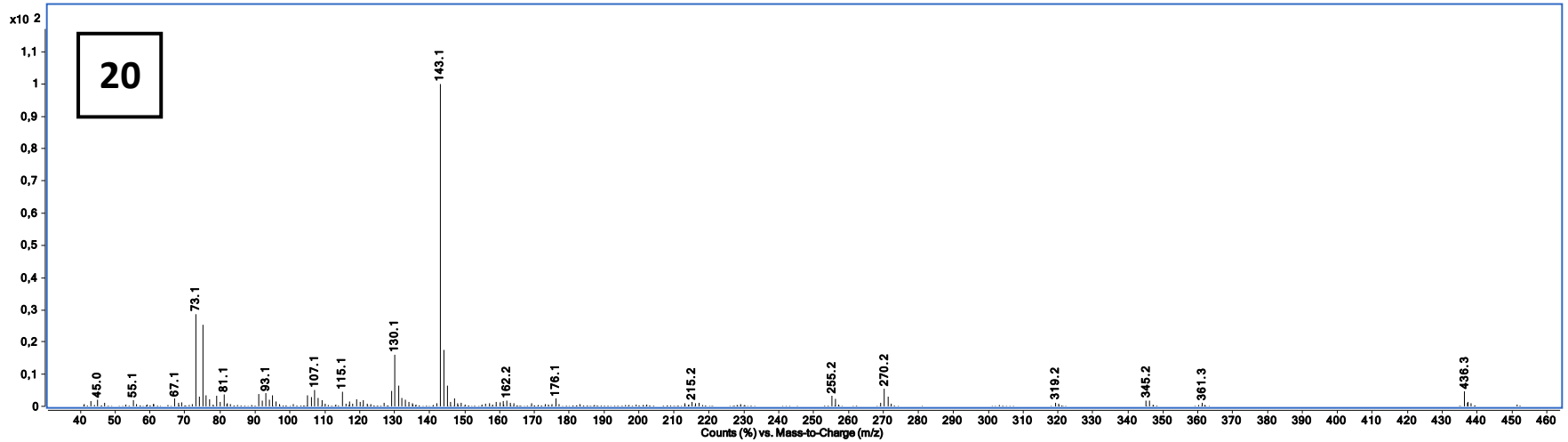

*Figure 11 GC-MS spectrum of **20**.*

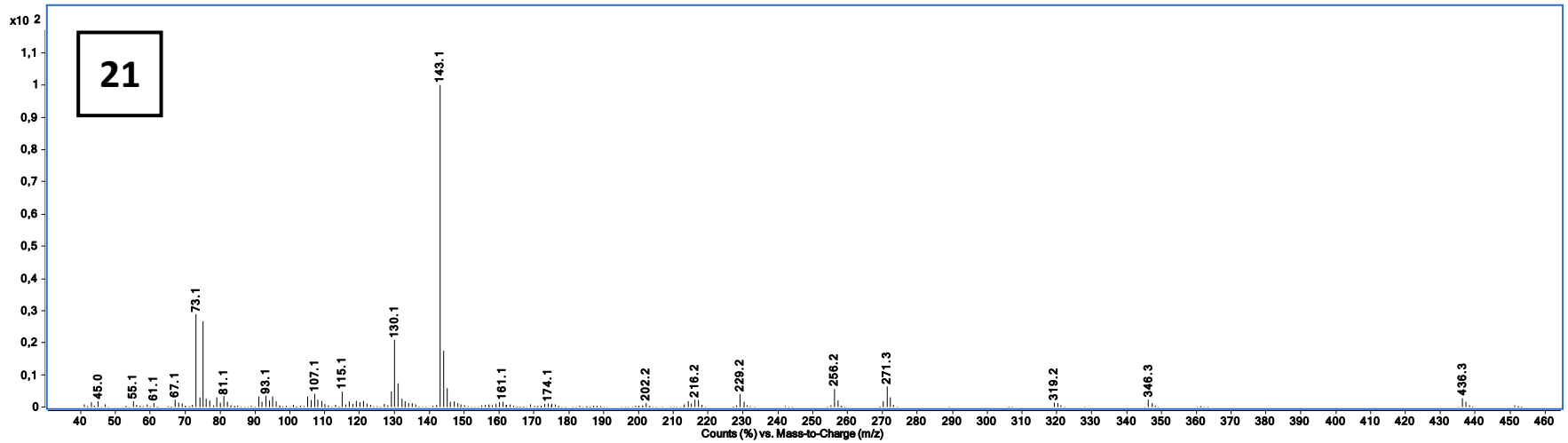

*Figure 13 GC-MS spectrum of **21**.*

4 ACKNOWLEDGEMENTS

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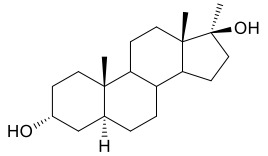
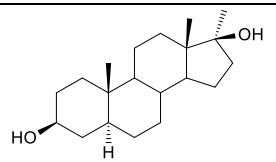
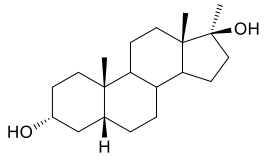
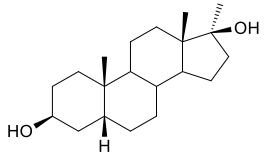
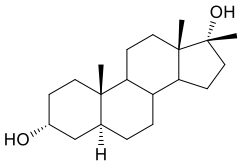
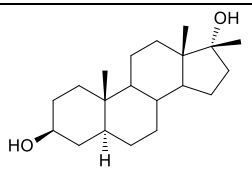
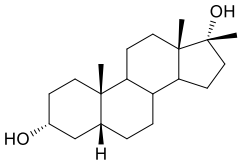
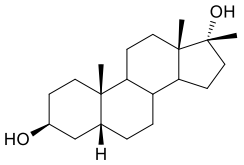
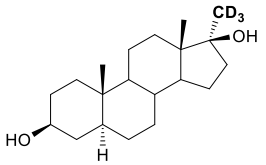
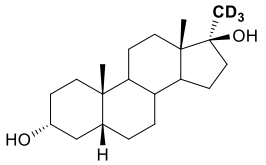
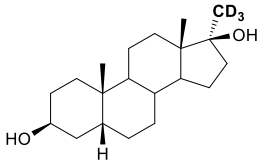
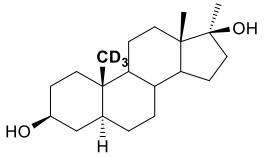
5 APPENDIX

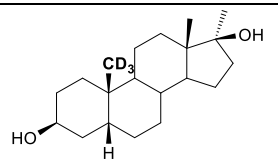
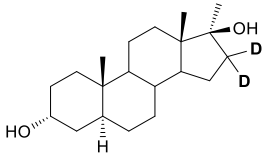
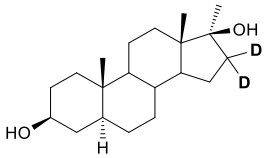
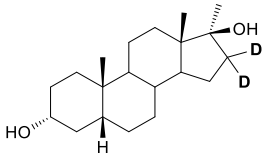
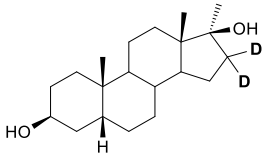
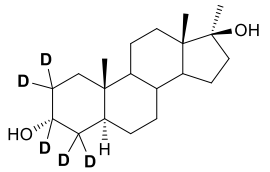
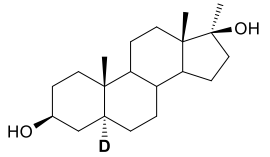
Data availability

Further Data will be made available on request.

Table 2 List of number, systematic name and molecule structure of products.

number	***systematic name***	***molecule structure***
T1	17α-Methyl-5α-androstane-3α,17β-diol	
T2	17α-Methyl-5α-androstane-3β,17β-diol	
T3	17α-Methyl-5β-androstane-3α,17β-diol	
T4	17α-Methyl-5β-androstane-3β,17β-diol	
T5	17β-Methyl-5α-androstane-3α,17α-diol	

T6	17β-Methyl-5α-androstane-3β,17α-diol	
T7	17β-Methyl-5β-androstane-3α,17α-diol	
T8	17β-Methyl-5β-androstane-3β,17α-diol	
9	20,20,20-d_3-17α-Methyl-5α-androstane-3β,17β-diol	
10	20,20,20-d_3-17α-Methyl-5β-androstane-3α,17β-diol	
11	20,20,20-d_3-17α-Methyl-5β-androstane-3β,17β-diol	
12	19,19,19-d_3-17α-Methyl-5α-androstane-3β,17β-diol	

13	19,19,19-d_3-17α-Methyl-5β-androstane-3β,17β-diol	
14	16,16-d_2-17α-Methyl-5α-androstane-3α,17β-diol	
15	16,16-d_2-17α-Methyl-5α-androstane-3β,17β-diol	
16	16,16-d_2-17α-Methyl-5β-androstane-3α,17β-diol	
17	16,16-d_2-17α-Methyl-5β-androstane-3β,17β-diol	
18	2,2,3,4,4-d_5-17α-Methyl-5α-androstane-3α,17β-diol	
19	5-d_1-17α-Methyl-5α-androstane-3β,17β-diol	

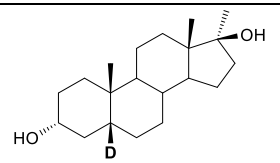
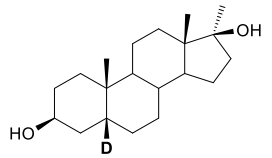
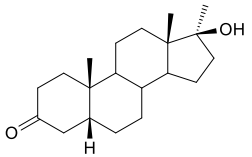
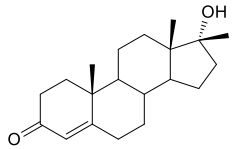
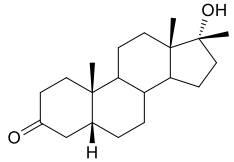
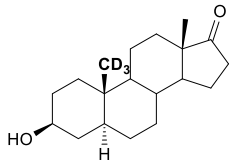
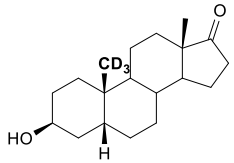
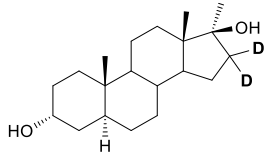
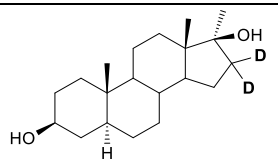
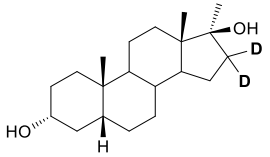
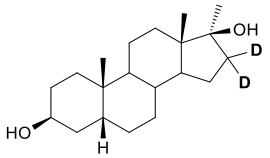
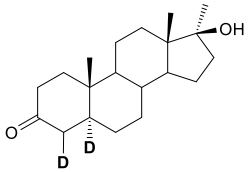
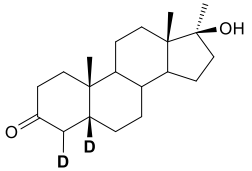
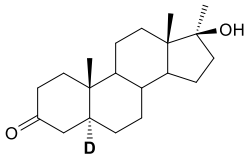
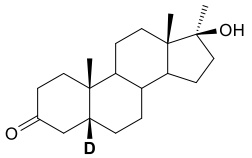
20	5-d_1-17α-Methyl-5β-androstane-3α,17β-ol	
21	5-d_1-17α-Methyl-5β-androstane-3β,17β-ol	

Table 3 List of number, systematic name and molecule structure of intermediates.

number	***systematic name***	***molecule structure***
I1	17β-Hydroxy-17α-methyl-5β-androstan-3-one	
I2	17β-Methyltestosterone	
I3	17α-Hydroxy-17β-methyl-5β-androstane-3-one	
I4	19,19,19-d_3-3β-Hydroxy-5α-androstane-17-one	
I5	19,19,19-d_3-3β-Hydroxy-5β-androstane-17-one	
I6	16,16-d_2-3α-Hydroxy-5α-androstane-17-one	

I7	16,16-d_2-3β-Hydroxy 5α-androstane-17-one	
I8	16,16-d_2-3α-Hydroxy-5β-androstane-17-one	
I9	16,16-d_2-5β-Androstane-3α-ol-17-one	
I10	4,5-d_2-17β-Hydroxy-17α-methyl-5α-androstane-3-one	
I11	4,5-d_2-17β-Hydroxy-17α-methyl-5β-androstane-3-one	
I12	5-d_1-17β-Hydroxy-17α-methyl-5α-androstane-3-one	
I13	5-d_1-17β-Hydroxy-17α-methyl-5β-androstane-3-one	

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