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In vitro metabolism studies of desoxy-methyltestosterone (DMT) and its five analogues, and *in vivo* metabolism of desoxy-vinyltestosterone (DVT) in horses

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The positive findings of norbolethone in 2002 and tetrahydrogestrinone in 2003 in human athlete samples confirmed that designer steroids were indeed being abused in human sports. In 2005, an addition to the family of designer steroids called 'Madol' [also known as desoxy-methyltestosterone (DMT)] was seized by government officials at the US–Canadian border. Two years later, a positive finding of DMT was reported in a mixed martial arts athlete's sample. It is not uncommon that doping agents used in human sports would likewise be abused in equine sports. Designer steroids would, therefore, pose a similar threat to the horseracing and equestrian communities. This paper describes the *in vitro* metabolism studies of DMT and five of its structural analogues with different substituents at the 17α position (R=H, ethyl, vinyl, ethynyl and $^{2}H_{3}$-methyl). In addition, the *in vivo* metabolism of desoxy-vinyltestosterone (DVT) in horses will be presented.

The *in vitro* studies revealed that the metabolic pathways of DMT and its analogues occurred predominantly in the A-ring by way of a combination of enone formation, hydroxylation and reduction. Additional biotransformation involving hydroxylation of the 17α-alkyl group was also observed for DMT and some of its analogues. The oral administration experiment revealed that DVT was extensively metabolised and the parent drug was not detected in urine. Two *in vivo* metabolites, derived respectively from (1) hydroxylation of the A-ring and (2) di-hydroxylation together with A-ring double-bond reduction, could be detected in urine up to a maximum of 46 h after administration. Another *in vivo* metabolite, derived from hydroxylation of the A-ring with additional double-bond reduction and di-hydroxylation of the 17α-vinyl group, could be detected in urine up to a maximum of 70 h post-administration. All *in vivo* metabolites were excreted mainly as glucuronides and were also detected in the *in vitro* studies. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: designer steroids; doping control; equine urine; *in vitro* and *in vivo* metabolism

Introduction

Designer steroids can be derived from a simple chemical modification of known anabolic androgenic steroids. They have been synthesised by unscrupulous chemists for the purpose of performance enhancement and escaping detection. As long as these steroids remain unknown, it is difficult to detect their presence in biological samples by either target analysis or mass spectral library search, as both approaches would require first establishing the analytical characteristics with suitable reference materials. In recent years, abuses of designer steroids, such as norbolethone[1] and tetrahydrogestrinone,[2] have been reported in human sports. Strictly speaking, norbolethone is not a designer steroid because it is not novel but was originally synthesised in 1966 with intended pharmacological applications. It was, however, never marketed. As norbolethone was intended to be used in human sports to avoid detection, it can be considered to fall within the same category of designer steroids. 'Madol' (17α-methyl-5α-androst-2-ene-17β-ol), also known as desoxy-methyltestosterone (**DMT**),[3] was an addition to the family of designer steroids and was seized by government officials at the US–Canadian border in 2005. Two years later, **DMT** was detected in a mixed martial arts athlete's sample.[4] It is not uncommon that doping agents used in human sports would likewise be abused in equine sports. Therefore, designer steroids would pose a similar threat to the horseracing and equestrian communities.

The detection of unknown designer steroids has been proposed by Thevis *et al.*[5,6] and Nielen *et al.*[7] by using respectively precursor ion scans in triple quadrupole mass spectrometers and a combination of androgen bioactivity testing and quadrupole time-of-flight mass spectrometry. Pozo *et al.* later proposed a more comprehensive method to detect unknown anabolic steroids and their metabolites based on precursor ion scanning of three ions (*m/z* 105, *m/z* 91 and *m/z* 77) using liquid chromatography–tandem mass spectrometry.[8] The detection of unknown anabolic androgenic steroids in doping control was recently reviewed.[9] While these generic approaches to detect unknown steroids are valid and excellent concepts, there are limitations (such as uncertainty in detecting truly unknown substances), and these should only be used as

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complementary approaches. The identification of screening targets via *in vitro* and *in vivo* metabolism studies,[10–14] although requiring many resources, remains to be the most certain way to control the abuse of selected designer steroids. This paper describes a series of *in vitro* metabolism studies of **DMT** and five of its structural analogues with different substituents at the 17α position (R=H, ethyl, vinyl, ethynyl and $^{2}H_3$-methyl). In addition, the *in vivo* metabolism of one of the **DMT** analogues, desoxy-vinyltestosterone (**DVT**), in horses will be presented.

Materials and methods

DMT, desoxy-testosterone (**DT**), desoxy-ethyltestosterone (**DET**), **DVT**, desoxy-ethynyltestosterone (**DENT**) and d_3-desoxy-methyltestosterone (***d_3*-DMT**) were custom synthesised by the Hong Kong Polytechnic University, and their identities were confirmed by ^{1}H NMR and ^{13}C NMR. 5α-Androst-1-ene-3α,17β-diol and 5α-androst-1-ene-3β,17β-diol were custom synthesised by The Chinese University of Hong Kong.[13] [16,16,17-d_3-5α-Androstane-3α,17β-diol (d_3-androstanediol) was synthesised in house according to the literature.[15] 17α-Methyl-1-testosterone and 4-androstene-3β,17β-diol were obtained from Steraloids (Rhode Island, USA). Epiandrosterone, testosterone, β-nicotine adenine dinucleotide (β-NAD), glucose 6-phosphate, glucose-6-phosphate dehydrogenase (G6PDH), magnesium chloride ($MgCl_2$), *N*-methyl-*N*-trimethylsilylfluoroacetamide (MSTFA), ammonium iodide (NH_4I), 1,4-dithioerythritol (DTE), protease (from bovine pancreases, type I, 6.9 U/mg solid) and β-glucuronidase (from *Patella vulgata*, lyophilised powder) were purchased from Sigma (St. Louis, MO, USA). *N*,*O*-Bis(trimethylsilyl)trifluroacetamide (BSTFA) with 5% trimethylchlorosilane (TMCS) was prepared by mixing BSTFA from Pierce (Illinois, USA) with TMCS from Aldrich (St. Louis, MO, USA). β-Glucuronidase (*Escherichia coli*) was from Roche (Indianapolis, USA). Diisopropyl ether (GR grade), ethyl acetate (GR grade), methanol (GR grade), sulfuric acid (Suprapur®, 96%), sodium chloride (GR grade) and sodium dihydrogen phosphate (GR grade) were obtained from Merck (Darmstadt, Germany). Sodium hydroxide (pro analysis grade) was from RDH (Seelze, Germany). Sodium sulfate was purchased from Farco Chemical Supplies (Beijing, China). ABS Elut NEXUS cartridge (60 mg, 3 ml) was obtained from Varian (Harbor City, CA, USA).

Preparation of horse liver microsomes

Horse liver microsomes were isolated in house from fresh horse liver, which was supplied by the Equine Hospital of The Hong Kong Jockey Club. Small pieces of horse liver were homogenised in Tris/KCl buffer (0.05 M, pH 7.4). The homogenate was centrifuged at 10 000 *g* for 25 min. Microsomes were isolated from the supernatant by centrifuging at 105 000 *g* for 1 h. The pellet of microsomes was then washed twice with Tris/KCl buffer (0.05 M, pH 7.4). All the preparation steps and reagent storage were carried out at 4 °C.

Microsomal incubation

For the *in vitro* metabolic studies, a mixture of freshly prepared horse liver microsomes (30 µl), β-NAD (1.5 mM), glucose-6-phosphate (7.5 mM), magnesium chloride (4.5 mM), EDTA (1 mM) and G6PDH (1 U/ml) in phosphate buffer (5 ml, 50 mM, pH 7.4) was incubated with the steroid under study (200 µg) at 37 °C overnight with shaking. The reaction was terminated by boiling at 100 °C for 10 min. The mixture was centrifuged at 2100 *g* for 10 min. The supernatant was extracted twice with ethyl acetate (5 ml), and the combined organic extract was evaporated to dryness. The residue was analysed by GC–MS as trimethylsilyl (TMS) derivatives. Control experiments in the absence of either (1) the steroid under study or (2) microsomes were performed in parallel.

Drug administration studies

Two thoroughbred geldings were each administered orally with a single dose of 500 mg of **DVT**. Urine samples were collected before administration and then at least twice daily for up to 8 days post-administration. Urine samples were extracted with and without prior hydrolysis in order to identify respectively conjugated and free **DVT** and its metabolites. The extracts were analysed by GC–MS after trimethylsilylation.

Extraction procedures for phase I metabolism study on DVT post-administration urine samples

Urine (3 ml) was fortified with d_3-androstanediol (30 ng) as the internal standard and diluted with potassium phosphate buffer (pH 6.0, 0.1 M, 1 ml). A solution of protease (5 mg/ml, 60 µl) and β-glucuronidase (*P. vulgata*, 18 000 U/ml, 360 µl) was added, and the sample incubated at 65 °C for 3.5 h. The enzyme-treated urine was then extracted twice with diisopropyl ether (5 ml). The combined extract was washed with base (2.0 ml containing 1 M sodium hydroxide and 0.15 M sodium chloride), passed through an anhydrous sodium sulfate drying tube and evaporated to dryness under nitrogen at 60 °C.

Extraction procedures for phase II metabolism studies

Unconjugated steroids

Urine (5 ml) was mixed with sodium sulfite solution (0.25 ml, 25% *w/v*), adjusted to pH 6.8 and extracted with diisopropyl ether (5 ml). The organic layer was base washed (4.0 ml containing 1 M sodium hydroxide and 0.15 M sodium chloride) and centrifuged (2100 *g* for 1 min). The supernatant was passed through an anhydrous sodium sulfate drying tube and evaporated to dryness under nitrogen at 60 °C.

Glucuronide-conjugated steroids

The remaining aqueous layer was then adjusted to pH 6.4 and incubated at 45 °C for 1 h with β-glucuronidase from *E. coli* (100 µl). The enzyme-treated steroids were extracted from the mixture according to the aforementioned procedures for unconjugated steroids.

Sulfate and sulfate glucuronide-conjugated steroids

The sulfate and sulfate-glucuronide conjugates remaining in the aqueous layer after enzyme hydrolysis were extracted using solid-phase extraction (SPE) (ABS Elut Nexus cartridge). The loaded SPE cartridge was washed with deionised water (3 ml) and eluted with a solvolysis reagent (3 ml; ethyl acetate: methanol: conc. H_2SO_4, 100:20:0.2, *v/v/v*). The eluate was incubated at 55 °C for 2 h. NaOH/NaCl (1 M/0.15 M, 1.2 ml) was then added. The mixture was vortexed for 0.5 min. The separated organic layer was evaporated to dryness under nitrogen at 60 °C. The cooled residue was re-dissolved in diisopropyl ether (3 ml) and washed with deionised water (1 ml). The organic layer was passed through an anhydrous sodium sulfate drying tube and evaporated to dryness under nitrogen at 60 °C.

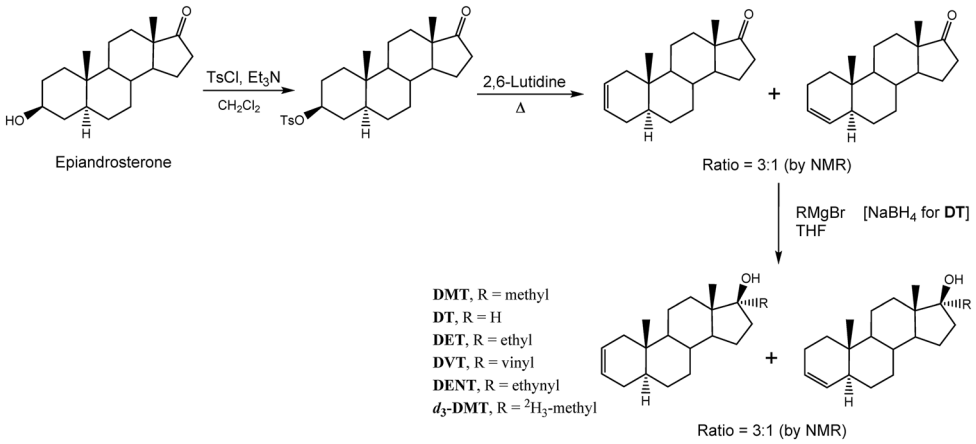
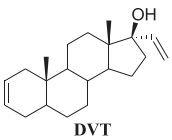
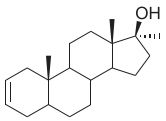
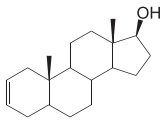
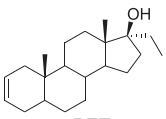
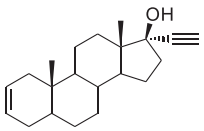
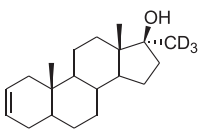


Figure 1. Synthetic route for **DMT** and its five analogues.

Table 1. Structures of **DVT**, **DMT**, **DT**, **DET**, **DENT** and ***d*$_3$-DMT**, and *major* metabolites identified after incubation with horse liver microsomes

Structure	*In vitro* metabolites			
	Enone formation in the A-ring	Mono-hydroxylation in the A-ring	Reduction + di-hydroxylation in the A-ring	Additional metabolites
 **DVT**	DVT-M1a DVT-M1b DVT-M1c DVT-M1d	DVT-M2a DVT-M2b DVT-M2c DVT-M2d	DVT-M3a DVT-M3b DVT-M3c DVT-M3d	DVT-M4a DVT-M4b DVT-M5a DVT-M5b DVT-M6 DVT-M7a DVT-M7b DVT-M8
 **DMT**	DMT-M1a DMT-M1b*	DMT-M2a DMT-M2b	DMT-M3a DMT-M3b	DMT-M4a DMT-M4b DMT-M5
 **DT**	DT-M1a DT-M1b*	DT-M2a DT-M2b* DT-M2c DT-M2d DT-M2e* DT-M2f*	DT-M3	Not detected
 **DET**	DET-M1a DET-M1b	Not detected	DET-M3a DET-M3b DET-M3c	DET-M4 DET-M5
 **DENT**	DENT-M1a DENT-M1b	DENT-M2a DENT-M2b	DENT-M3a DENT-M3b	Not detected
 ***d*$_3$-DMT**	*d*$_3$-DMT-M1a *d*$_3$-DMT-M1b *d*$_3$-DMT-M1c	*d*$_3$-DMT-M2a *d*$_3$-DMT-M2b	*d*$_3$-DMT-M3a *d*$_3$-DMT-M3b	*d*$_3$-DMT-M4a *d*$_3$-DMT-M4b *d*$_3$-DMT-M5

* Confirmed with reference standards.

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Derivatisation for GC–MS analysis

TMS derivatives were prepared by adding either BSTFA (5% TMCS, 30 μl) or MSTFA/NH_4I/DTE (MND; 1000:2:4, *v/w/w*, 30 μl) to the dried residue. The mixture was incubated at 60 °C for 30 min or 80 °C for 30 min for respectively BSTFA or MND derivatisation. The resulting mixture was injected directly for GC–MS analysis.

Instrumentation

An Agilent 6890N Network GC system coupled to an Agilent 5973 Network Mass Selective Detector (Agilent Technologies, CA, USA) was used. Separation was performed on an HP-1MS (~30 m × 0.25 mm, 0.25 μm film thickness; Agilent Technologies, CA, USA) column with a constant helium flow of 1.2 ml/min. The oven temperature was set initially at 60 °C for 1.0 min, increased to 120 °C at 30 °C/min and then to 300 °C at 15 °C/min and finally held at 300 °C for 6 min. Samples (1 μl) were injected at 250 °C in the splitless mode. All GC–MS analyses were performed in the EI mode with full-scan acquisition.

Results and discussion

Synthesis of DMT and its five analogues

DMT and its five analogues were custom synthesised by the Hong Kong Polytechnic University. The synthetic route is shown in Fig. 1. Epiandrosterone was used as the starting material. The hydroxyl group at C3 was tosylated. Elimination of toluenesulfonic acid gave a carbon double bond in the A-ring. The mixture was then alkylated at C17 using the appropriate Grignard reagents to give the final products. In the case of **DT**, the 17β-hydroxy functional group

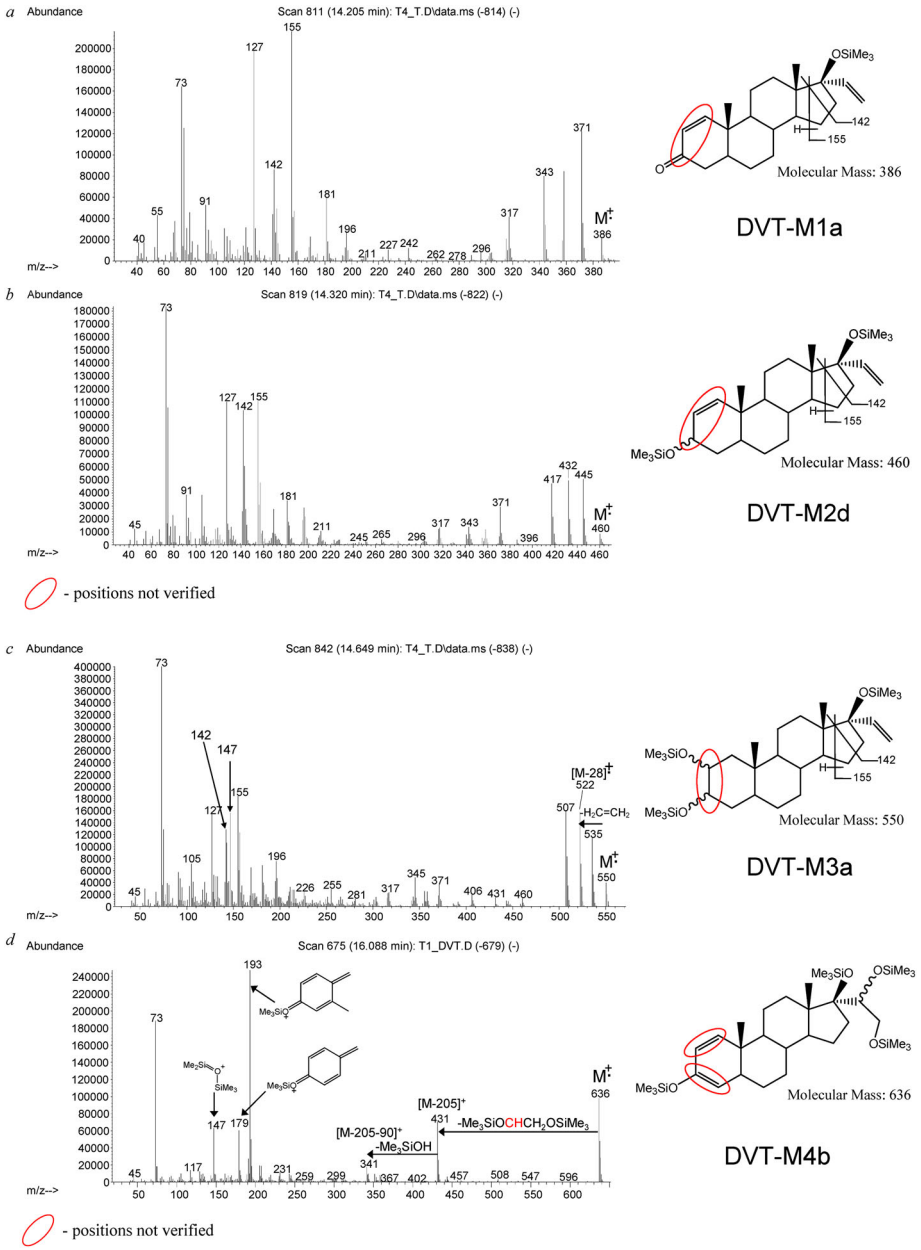

Figure 2. EI mass spectra of trimethylsilylated (a) **DVT-M1a**, (b) **DVT-M2d**, (c) **DVT-M3a**, (d) **DVT-M4b**, (e) **DVT-M5a**, (f) **DVT-M6**, (g) **DVT-M7a** and (h) **DVT-M8** obtained from the *in vitro* incubation mixture of **DVT** with horse liver microsomes. All TMS derivatives were prepared using MND except for **DVT-M1a** where BSTFA (5% TMCS) was used.

was formed by reduction of the 17-keto group with sodium borohydride. As the elimination step was not regiospecific, both the Δ^2 and Δ^3 isomers were formed in the final products (in a ratio of approximately 3:1). This was consistent with the sample of **DMT** seized by government officials at the US–Canadian border reportedly containing around 25% of the Δ^3 isomer.[3] The resulting **DMT** and its analogues were used for the subsequent *in vitro* and *in vivo* studies without further purification.

In vitro biotransformation studies

Results of the *in vitro* studies of **DMT** and its five analogues are summarised in Table 1. All proposed metabolites are 5α-steroids unless they have a carbon double bond at the C5 position. The major biotransformations of **DMT** and its five analogues can be categorised into a combination of enone formation, hydroxylation and reduction in the A-ring. In the case of **DVT**, **DMT**, **DET** and ***d*$_3$-DMT**, additional metabolites derived from other biotransformation pathways have been identified. For simplicity, results and mass spectral interpretations for *in vitro* metabolites of the other five analogues will be discussed using **DVT** as a model compound and divided into four groups as follows.

(i) Enone metabolites

In order to facilitate the interpretation of the TMS derivatives of the M1-enone metabolites, a mild TMS derivatising reagent (BSTFA with 5% TMCS) was used to suppress the formation of enol-TMS derivatives in the A-ring. The TMS derivatives of **DVT-M1** (four isomers) had molecular ions at *m/z* 386, which was consistent with the addition of a keto function to **DVT** (Fig. 2(a)). The characteristic pair of D-ring fragments at *m/z* 155 and *m/z* 142 confirmed that the D-ring structure of **DVT** remained intact. The mechanism for the formation of such D-ring fragments was first investigated by Middleditch *et al.*[16] The *in vitro* studies of the other five analogues **DMT**, **DT**, **DET**, **DENT** and ***d*$_3$-DMT** gave similar enone metabolites, **DMT-M1a** and **DMT-M1b**, **DT-M1a** and **DT-M1b**, **DET-M1a** and **DET-M1b**, **DENT-M1a** and **DENT-M1b**, and ***d*$_3$-DMT-M1a** to ***d*$_3$-DMT-M1c**, respectively. The mass spectra of the TMS derivatives

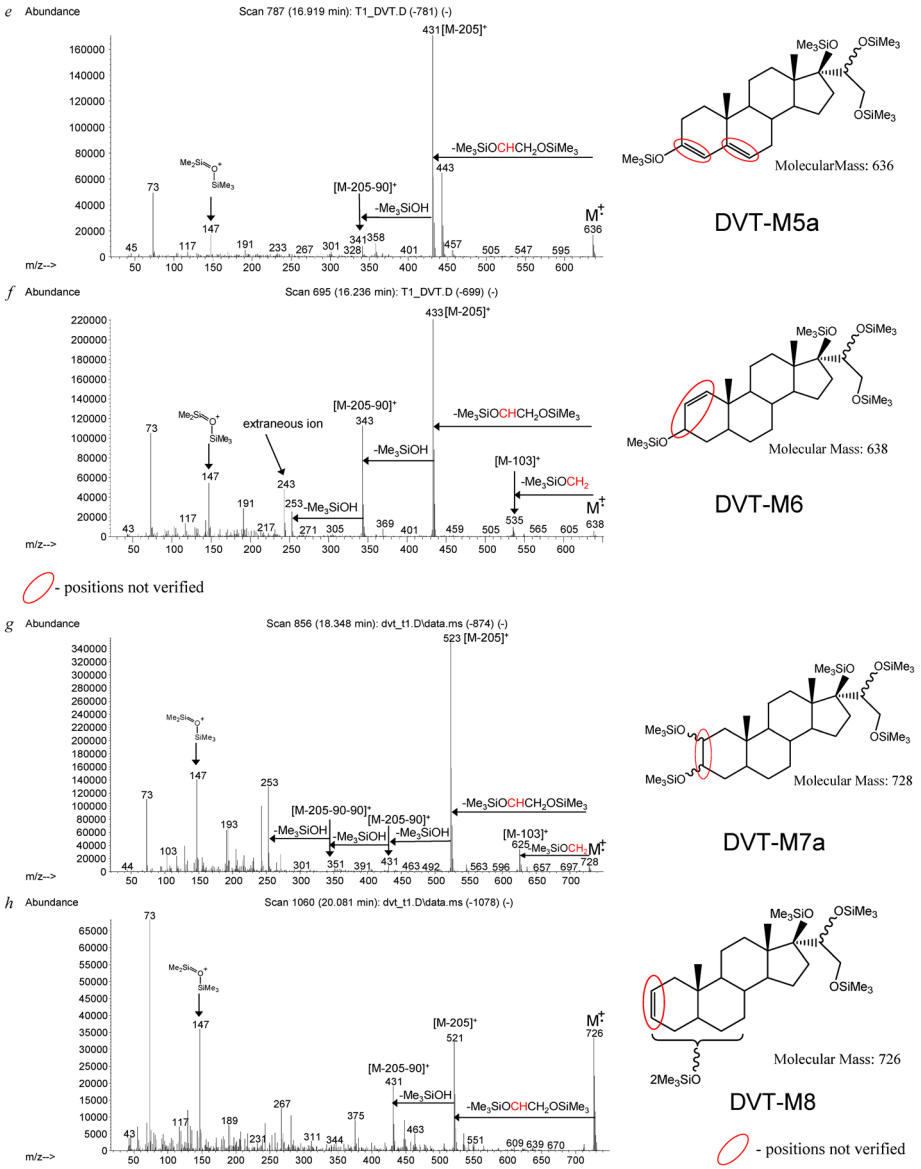

Figure 2. (Continued).

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of representative **M1** metabolites, **DMT-M1b**, **DT-M1b**, **DET-M1a**, **DENT-M1a** and ***d*$_3$-DMT-M1c**, are shown in Figs 3(a)–7(a), respectively. All these spectra showed peaks at their corresponding molecular ions and characteristic pairs of D-ring fragments. Of these metabolites, the identities of **DMT-M1b** and **DT-M1b** were confirmed with authentic reference standards of 17α-methyl-1-testosterone and testosterone, respectively, suggesting that enone formation was in the A-ring bearing either the 1-ene-3-one or 4-ene-3-one structure. As the retention time of trimethylsilylated ***d*$_3$-DMT-M1c** matched well with that of trimethylsilylated 17α-methyl-1-testosterone, and its mass spectrum (Fig. 7(a)) also closely resembled that of **DMT-M1b** (Fig. 3(a)) with a shift of 3 *m/z* units at their molecular ions and some fragment ions,

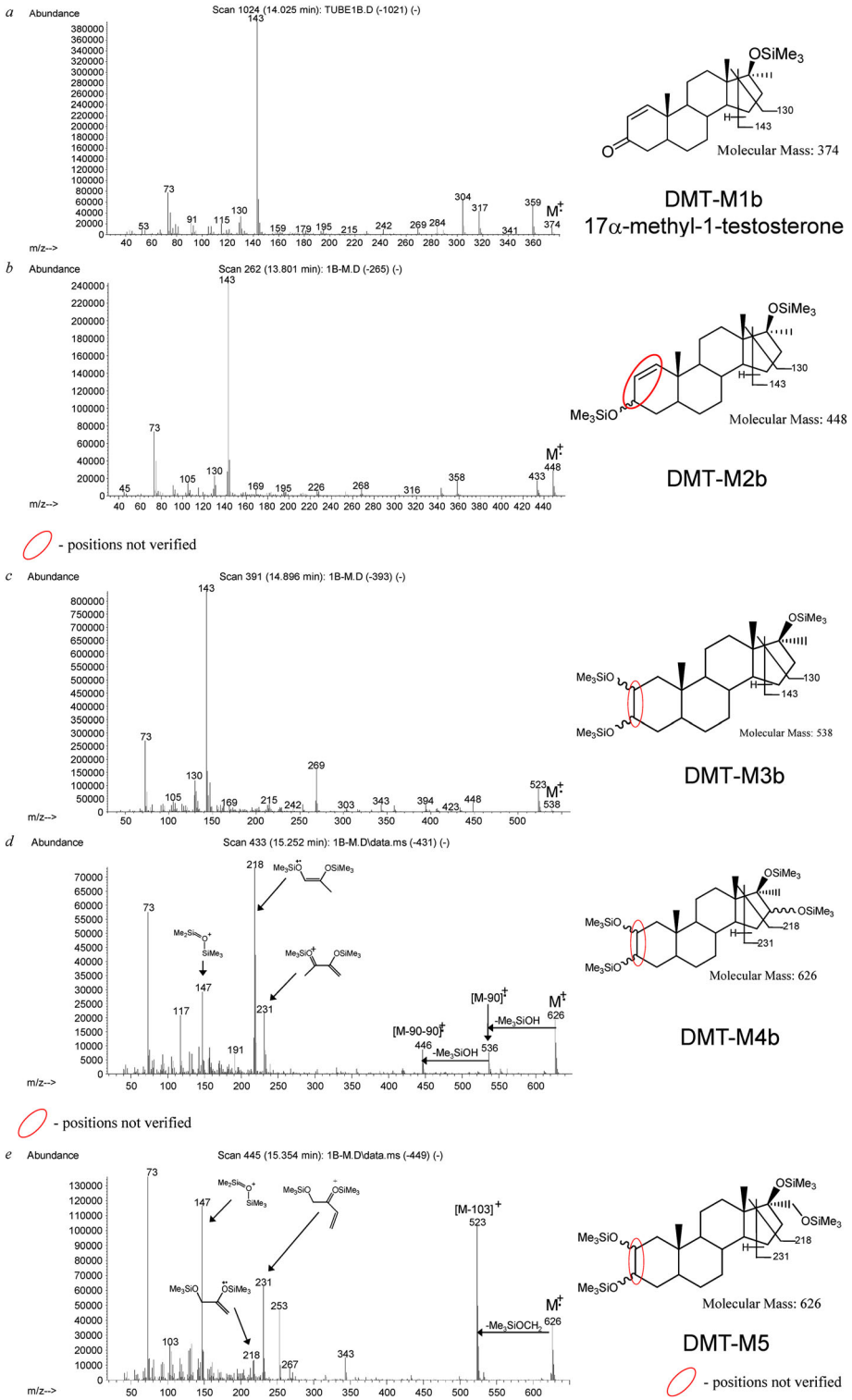

Figure 3. EI mass spectra of trimethylsilylated (a) **DMT-M1b** (**17α-methyl-1-testosterone**), (b) **DMT-M2b**, (c) **DMT-M3b**, (d) **DMT-M4b** and (e) **DMT-M5** obtained from the *in vitro* incubation mixture of **DMT** with horse liver microsomes. All TMS derivatives were prepared using MND except for **DMT-M1b** where BSTFA (5% TMCS) was used.

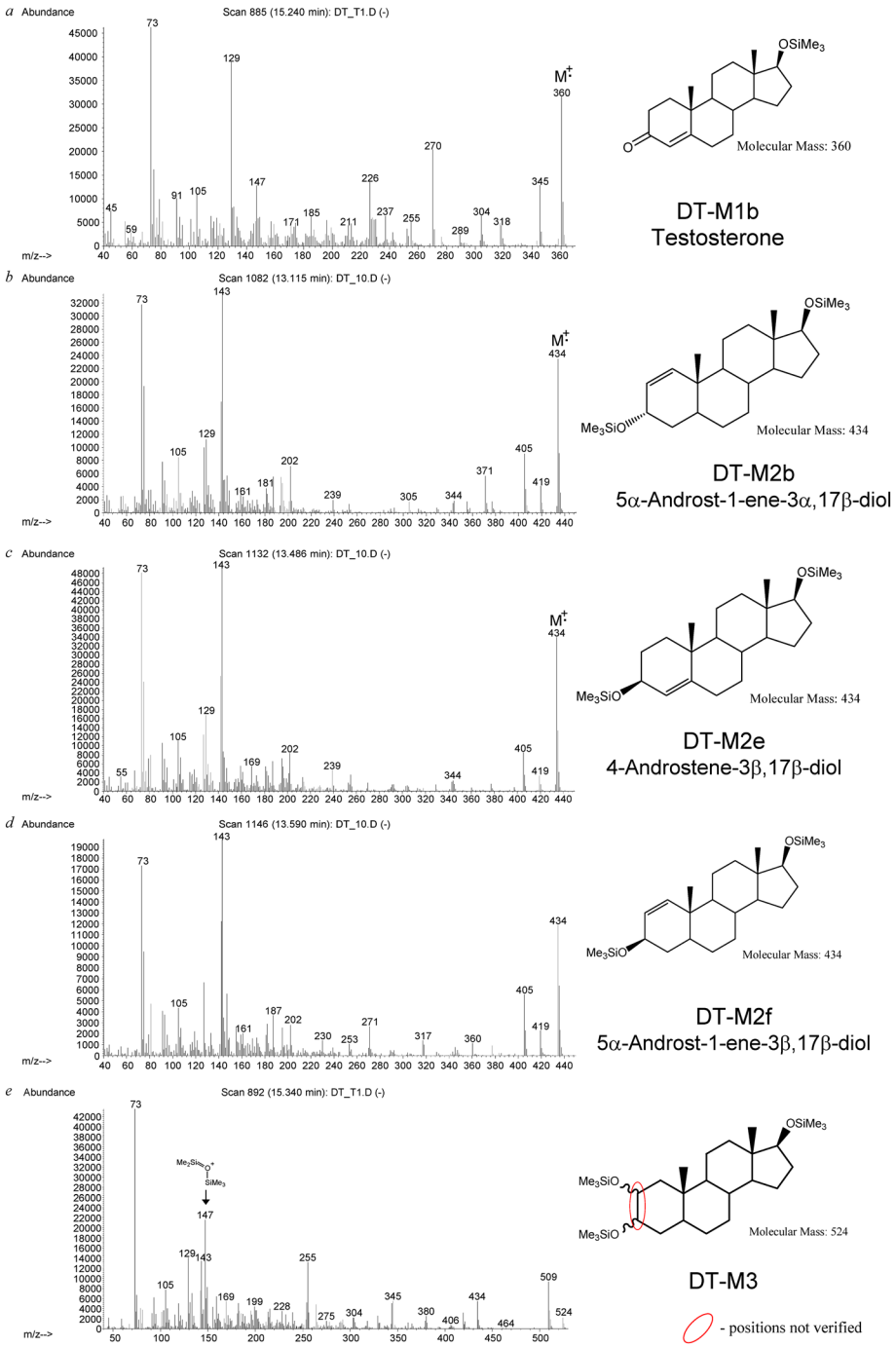

Figure 4. EI mass spectra of trimethylsilylated (a) **DT-M1b** (**testosterone**), (b) **DT-M2b** (**5*α*-androst-1-ene-3*α*,17*β*-diol**), (c) **DT-M2e** (**4-androstene-3*β*,17*β*-diol**), (d) **DT-M2f** (**5*α*-androst-1-ene-3*β*,17*β*-diol**) and (e) **DT-M3** obtained from the *in vitro* incubation mixture of **DT** with horse liver microsomes. All TMS derivatives were prepared using MND except for **DT-M1b** where BSTFA (5% TMCS) was used.

***d*$_3$-DMT-M1c** could be assigned as 17α-$^2H_3$-methyl-1-testosterone. However, the mass spectra did not allow categorical assignment of the enone position in the A-ring of other **M1** metabolites. The existence of approximately 25% of the Δ^3-isomer in each parent substance (Fig. 1) further complicated the structural interpretation of the *in vitro* metabolites. A similar metabolic pathway has been reported for ethylestrenol in horses,[17] where the corresponding enone (norethandrolone) was the major urinary metabolite. In addition, the formation of the enone metabolite from **DMT** has also been reported in human urine.[18]

(ii) Hydroxylated metabolites

The TMS derivatives of **DVT-M2** (four isomers) had molecular ions at *m/z* 460, which was consistent with the addition of a hydroxyl group to **DVT** (Fig. 2(b)). Diagnostic ions at *m/z* 155 and *m/z* 142 confirmed that the D-ring was intact. The lack of a prominent *m/z* 147 (Me_3SiO^+=$SiMe_2$) indicated that the two trimethylsiloxyl groups were not in close proximity. The *in vitro* studies of the other analogues except for **DET** gave similar mono-hydroxylated **M2** metabolites (**DMT-M2a** and **DMT-M2b**, **DT-M2a** to **DT-M2f**,

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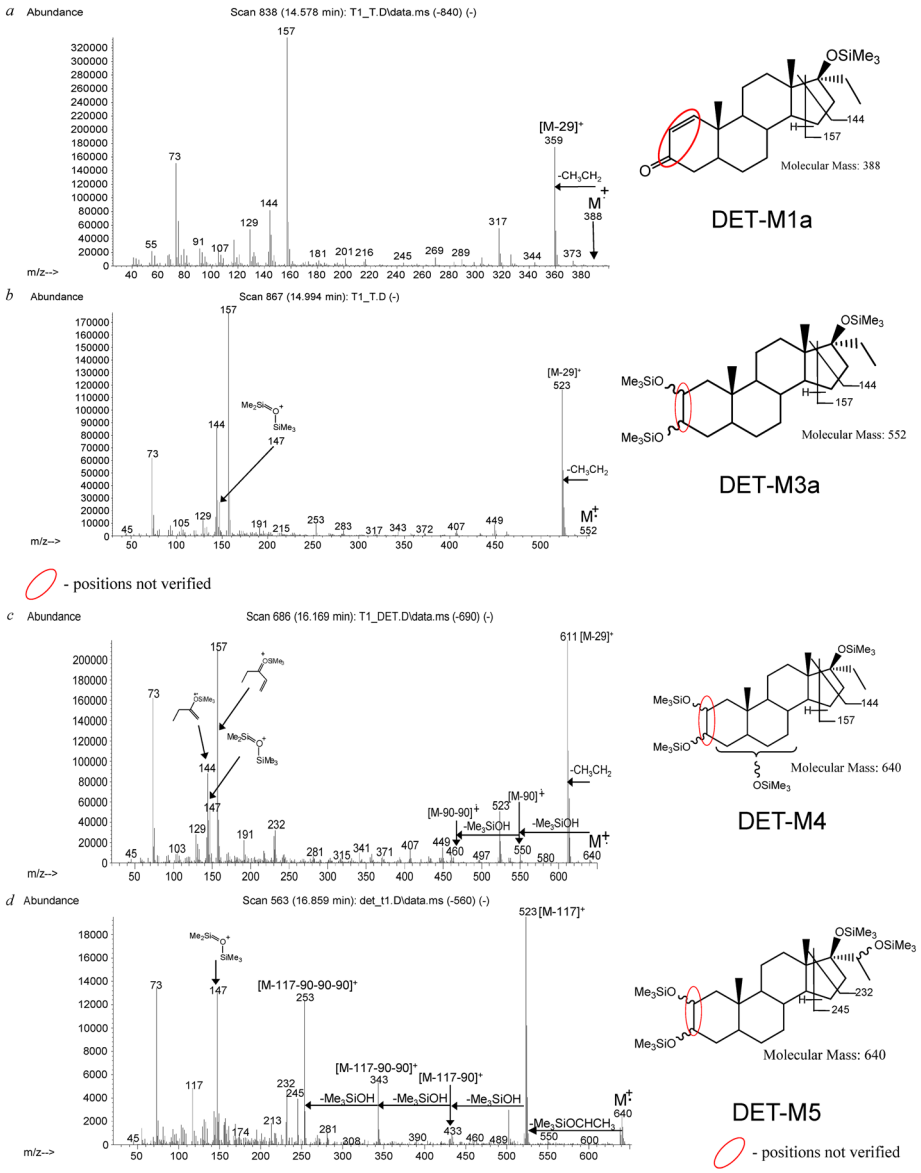

Figure 5. EI mass spectra of trimethylsilylated (a) **DET-M1a**, (b) **DET-M3a**, (c) **DET-M4** and (d) **DET-M5** obtained from the *in vitro* incubation mixture of **DET** with horse liver microsomes. All TMS derivatives were prepared using MND except for **DET-M1a** where BSTFA (5% TMCS) was used.

DENT-M2a and **DENT-M2b**, ***d*$_3$-DMT-M2a** and ***d*$_3$-DMT-M2b**). The mass spectra of TMS derivatives of representative **M2** metabolites, **DMT-M2b** (Fig. 3(b)), **DT-M2b** (Fig. 4(b)), **DT-M2e** (Fig. 4(c)), **DT-M2f** (Fig. 4(d)), **DENT-M2a** (Fig. 6(b)) and ***d*$_3$-DMT-M2b** (Fig. 7(b)), are shown in the corresponding figures. All these spectra show peaks at their corresponding molecular ions, $[M\text{-}90]^{+\bullet}$ ions (neutral loss of $HOSiMe_3$ from the molecular ions), and characteristic pairs of D-ring fragments. Of these metabolites, **DT-M2b**, **DT-M2e** and **DT-M2f** were confirmed with authentic reference standards to be 5α-androst-1-ene-3α,17β-diol, 4-androstene-3β,17β-diol and 5α-androst-1-ene-3β,17β-diol, respectively. Based on these findings, one of the hydroxylation sites of **M2** metabolites should be at the C3 position in the A-ring. In the case of **DMT-M2b**, the mass spectrum of its TMS derivative resembled that reported by Gauthier *et al.*[18] and Rodchenkov *et al.*,[19] although the hydroxylation site of this human metabolite proposed by Gauthier *et al.* was at C4. It was noteworthy that both the mass spectrum and retention time of trimethylsilylated ***d*$_3$-DMT-M2b** (Fig. 7(b)) matched well with those of trimethylsilylated **DMT-M2b** (Fig. 3(b)) with shifts of 3 *m/z* units at their molecular ions and some fragment ions. Therefore, the sites of hydroxylation in ***d*$_3$-DMT-M2b** and **DMT-M2b** should be the same.

(iii) Reduced and hydroxylated metabolites

The TMS derivatives of **DVT-M3** (four isomers) had molecular ions at *m/z* 550, which was consistent with the addition of two hydroxyl groups together with reduction of the carbon double bond in the A-ring (Fig. 2(c)). Again, diagnostic ions at *m/z* 155 and *m/z* 142 confirmed that the D-ring was intact. The presence of a prominent *m/z* 147 indicated that the two trimethylsiloxyl groups were in close proximity, possibly formed by reduction and di-hydroxylation of the carbon double bond in the A-ring. The *in vitro* studies of the other five analogues all gave similar **M3** metabolites (**DMT-M3a** and **DMT-M3b**, **DT-M3**, **DET-M3a** to **DET-M3c**, **DENT-M3a** and **DENT-M3b**, ***d*$_3$-DMT-M3a** and ***d*$_3$-DMT-M3b**). The mass spectra of TMS derivatives of representative **M3** metabolites, **DMT-M3b** (Fig. 3(c)), **DT-M3** (Fig. 4(e)), **DET-M3a** (Fig. 5(b)), **DENT-M3a**

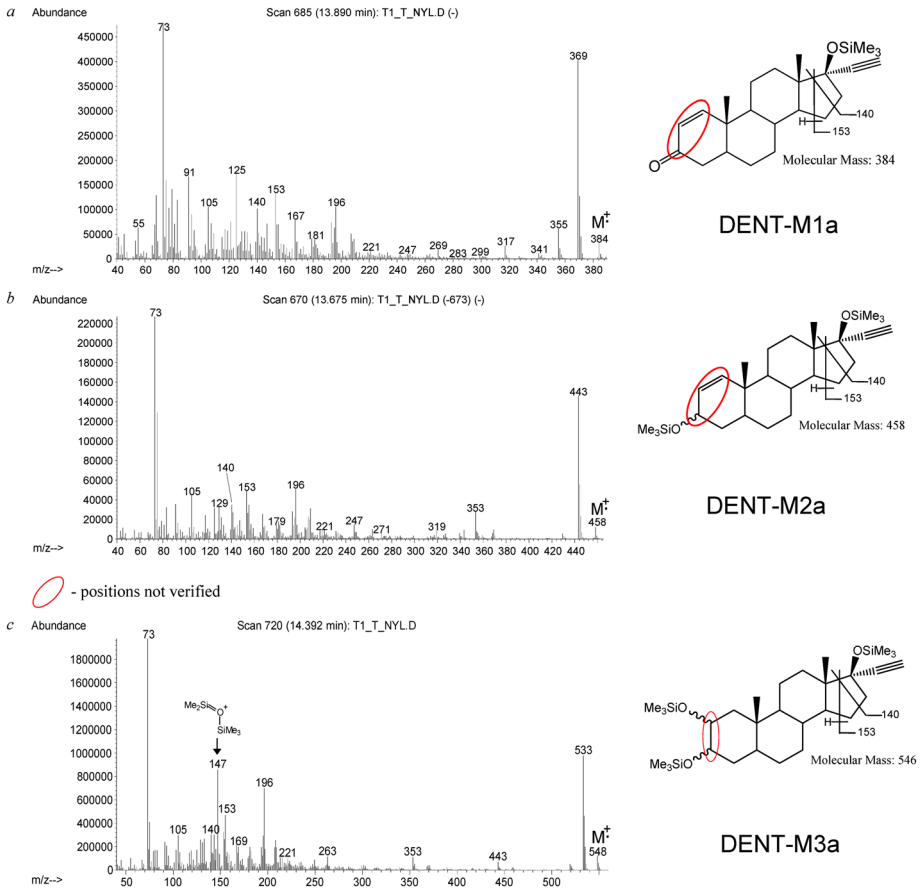

Figure 6. EI mass spectra of trimethylsilylated (a) **DENT-M1a**, (b) **DENT-M2a** and (c) **DENT-M3a** obtained from the *in vitro* incubation mixture of **DENT** with horse liver microsomes. All TMS derivatives were prepared using MND except for **DENT-M1a** where BSTFA (5% TMCS) was used.

(Fig. 6(c)) and ***d*$_3$-DMT-M3b** (Fig. 7(c)), are shown in the corresponding figures. All these spectra showed peaks at their corresponding molecular ions, *m/z* 147 (Me_3SiO^+=$SiMe_2$), and characteristic pairs of D-ring fragment ions. It was reported that **DMT-M3** was one of the major urinary metabolites of **DMT** in human, and the mass spectrum of trimethylsilylated **DMT-M3b** (Fig. 3(c)) matches well with those reported.[18–20] Moreover, ***d*$_3$-DMT-M3b** should have the same configuration as **DMT-M3b** because their retention times and mass spectra matched well with each other except for a shift of 3 *m/z* units at their molecular ions and some fragment ions.

(iv) Additional metabolites

Eight additional *in vitro* metabolites (**DVT-M4** to **DVT-M8**) were detected for **DVT** (Table 1). These metabolites were believed to arise from the reduction and di-hydroxylation of the 17α-vinyl group in addition to the three metabolic pathways described earlier. This metabolic pathway was unique to **DVT** and was not observed in the other five analogues studied. The mass spectra of TMS derivatives of representative metabolites **DVT-M4** to **DVT-M8** (Fig. 2(d) to (h)) all contained a prominent characteristic $[M\text{-}205]^+$ ion, representing the loss of a $Me_3SiOCHCH_2OSiMe_3$ radical from the corresponding molecular ion. A similar loss of the trimethylsilylated 17α-hydroxyalkyl moiety had been observed for the metabolites of norethandrolone,[17] 17α-methyltestosterone[21] and turinabol.[22] Based on their trimethylsilylated mass spectra, **DVT-M4b** (Fig. 2(d); two isomers) and **DVT-M5a** (Fig. 2(e); two isomers) were assigned to be the enone metabolites of **DVT** (**DVT-M1**) with additional modification of the 17α-vinyl group to a 17α-(1,2-dihydroxyethyl) group. Similarly, **DVT-M6** (Fig. 2(f)) and **DVT-M7a** (Fig. 2(g); two isomers) were assigned to be the 17α-(1,2-dihydroxyethyl) analogues of **DVT-M2** and **DVT-M3**, respectively. The trimethylsilylated **DVT-M8** had a molecular ion at *m/z* 726 (Fig. 2(h)). The prominent ion of $[M\text{-}205]^+$ indicated that this metabolite also contained a 17α-(1,2-dihydroxyethyl) group. Based on these data, **DVT-M8** was assigned to be a dihydroxylated **DVT** with a 17α-(1,2-dihydroxyethyl) group. However, the exact locations of the two hydroxyl groups could not be determined. In summary, the proposed structures of the 20 **DVT** *in vitro* metabolites are shown in Fig. 8.

In the case of **DMT**, three more minor *in vitro* metabolites **DMT-M4a**, **DMT-M4b** (Fig. 3(d)) and **DMT-M5** (Fig. 3(e)) were observed. **DMT-M4a** and **DTM-M4b** were two stereoisomers having similar mass spectra of their TMS derivatives. The TMS derivatives of **DMT-M4** and **DMT-M5** had molecular ions at *m/z* 626, which was consistent with the addition of three hydroxyl groups together with reduction of the double bond in the A-ring (Fig. 3(d) and (e)). The diagnostic D-ring fragment ions *m/z* 231 and *m/z* 218 indicated that one hydroxylation occurred in the D-ring, and the characteristic ion *m/z* 147 indicated that at least two trimethylsiloxyl groups were in close proximity with each other. Therefore, **DMT-M4a** and **DMT-M4b** were possibly formed from **DMT-M3** with additional hydroxylation at C16 in the D-ring. The structures of **DMT-M4** were confirmed by matching their mass spectra with those reported by Rodchenkov *et al.*[19] as one of the urinary metabolites in human.[18,20] A prominent characteristic ion $[M\text{-}103]^+$, representing the loss of a Me_3SiOCH_2 radical from the molecular ion (*m/z* 626),

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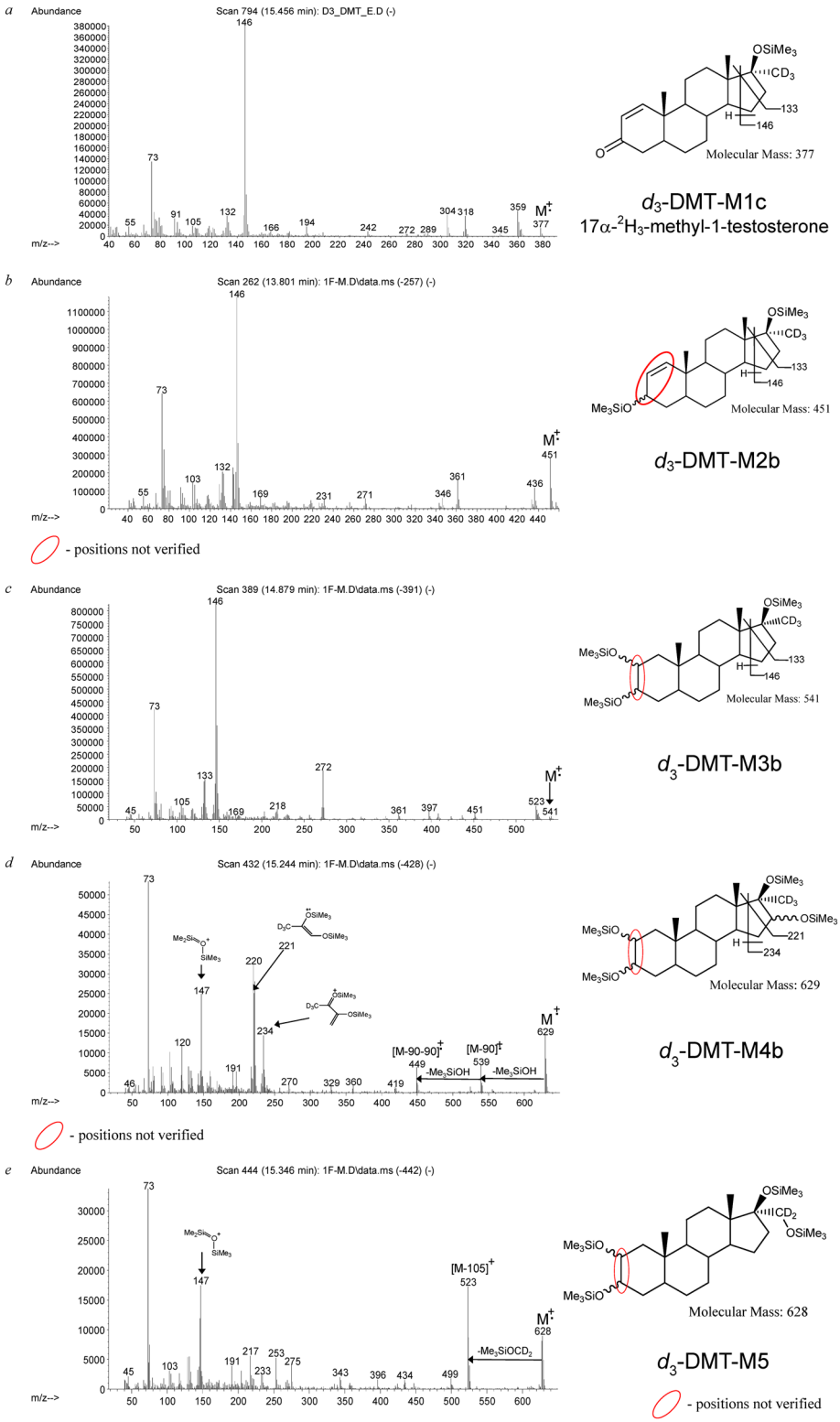

Figure 7. EI mass spectra of trimethylsilylated (a) ***d*$_3$-DMT-M1c** (**17α-^{2}H$_3$-methyl-1-testosterone**), (b) ***d*$_3$-DMT-M2b**, (c) ***d*$_3$-DMT-M3b**, (d) ***d*$_3$-DMT-M4b** and (e) ***d*$_3$-DMT-M5** obtained from the *in vitro* incubation mixture of ***d*$_3$-DMT** with horse liver microsomes. All TMS derivatives were prepared using MND except for ***d*$_3$-DMT-M1c** where BSTFA (5% TMCS) was used.

was observed in the mass spectrum of trimethylsilylated **DMT-M5** (Fig. 3(e)), indicating that the hydroxylation occurred at the 17α-methyl group, similar to the additional metabolites of **DVT** (**DVT-M4** to **DVT-M8**). To our knowledge, this novel 17-hydroxymethyl metabolite **DMT-M5** has not been reported previously.

In the case of ***d*$_3$-DMT**, three similar minor metabolites ***d*$_3$-DMT-M4a**, ***d*$_3$-DMT-M4b** and ***d*$_3$-DMT-M5** were observed. The presence of the diagnostic ions *m/z* 147 ($Me_3SiO^+{=}SiMe_2$), *m/z* 234 and *m/z* 221 (characteristic D-ring fragments) in the mass spectrum of trimethylsilylated ***d*$_3$-DMT-M4b** (Fig. 7(d)) further supported its proposed structure.

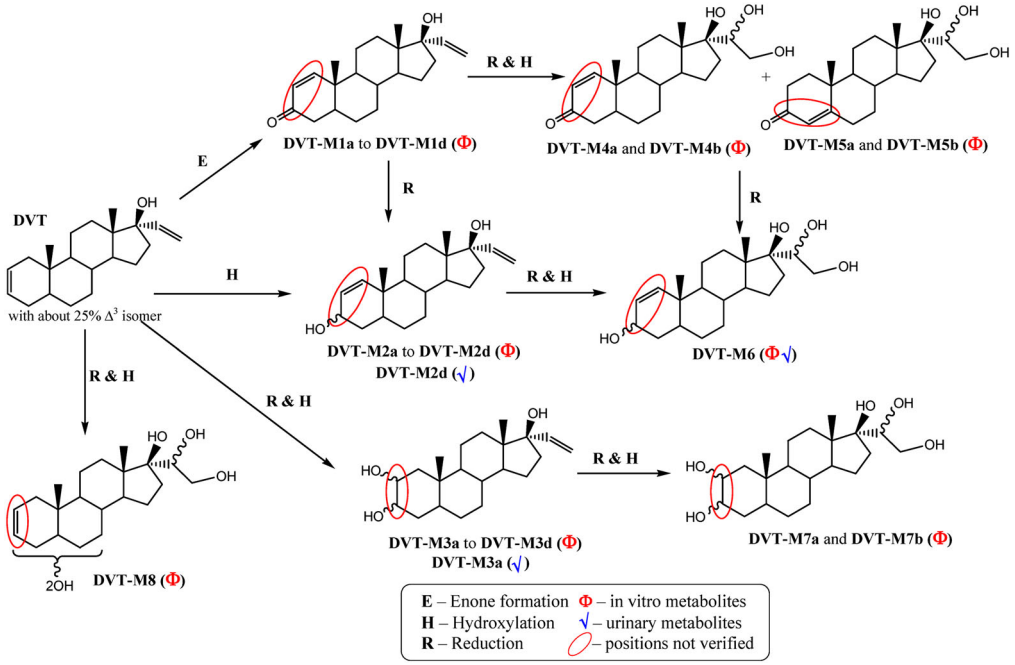

Figure 8. A proposed scheme for the phase I metabolism of **DVT** in horse.

The mass spectrum of tetrakis-trimethylsilylated ***d*$_3$-DMT-M4a** was highly similar to that of ***d*$_3$-DMT-M4b** (Fig. 7(d)). In addition, the retention times of trimethylsilylated ***d*$_3$-DMT-M4a**, ***d*$_3$-DMT-M4b** and ***d*$_3$-DMT-M5** matched well with those of trimethylsilylated **DMT-M4a**, **DMT-M4b** and **DMT-M5**, respectively; and their mass spectra (Fig. 7(d) and (e)) closely resembled those of trimethylsilylated **DMT-M4b** (Fig. 3(d)) and **DMT-M5** (Fig. 3(e)), respectively, with the corresponding deuterium mass shift at their molecular ions and some fragment ions. Therefore, the additional hydroxylation in ***d*$_3$-DMT** should also occur at the C16 and 17α-methyl positions in the D-ring.

In contrast, similar 16-hydroxy metabolites were not observed in the *in vitro* study of **DET**, probably owing to steric hindrance from the 17α-ethyl group. Instead, **DET-M4** was detected, with a comparable abundance with those of **DET-M1** and **DET-M3**. Based on the mass spectrum of trimethylsilylated **DET-M4** (Fig. 5(c)), with a prominent $[M-29]^+$ ion representing the loss of ethyl radical from the molecular ion (*m/z* 640), and the characteristic D-ring fragments *m/z* 157 and *m/z* 144 showing the intact D-ring, the structure of **DET-M4** was proposed to be tri-hydroxy **DET**, possibly formed from **DET-M3** with additional hydroxylation at a site other than the D-ring. In addition, a minor metabolite **DET-M5** was observed, with hydroxylation possibly occurring at the α-carbon of the 17α-ethyl group, similar to the metabolic pathway of **DMT** to **DMT-M5**. The mass spectrum of trimethylsilylated **DET-M5** (Fig. 5(d)) showed a prominent characteristic ion $[M-117]^+$ representing the loss of a $Me_3SiOCHCH_3$ radical from the molecular ion *m/z* 640, fragment ions *m/z* 433, *m/z* 343 and *m/z* 253 (sequential losses of $HOSiMe_3$ from $[M-117]^+$), a pair of diagnostic D-ring fragments *m/z* 245 and *m/z* 232, and a prominent *m/z* 147 ($Me_3SiO^+{=}SiMe_2$), which further supported its proposed structure.

Oral administration study with DVT: phase I metabolism

Urine samples from oral administration of **DVT** were collected up to 8 days post-administration. The parent drug was not detected. Three metabolites (**DVT-M2d**, **DVT-M3a** and **DVT-M6**), derived respectively from (1) mono-hydroxylation in the A-ring, (2) di-hydroxylation with double-bond reduction in the A-ring and (3) mono-hydroxylation in the A-ring with di-hydroxylation of the reduced 17α-vinyl group, were observed in post-administration urine samples. All three *in vivo* metabolites were confirmed to be the same as those identified in the *in vitro* study. The other 17 *in vitro* metabolites identified could not be detected from the post-administration urine samples using the GC–MS methods in this study. This was possibly due to their low *in vivo* concentrations, especially after extensive distribution and hepatic as well as non-hepatic metabolism, and interferences from the horse urine matrix.

A phase I metabolic pathway for **DVT** in horses is proposed in Fig. 8. The major phase I metabolic processes involved a combination of enone formation, hydroxylation and reduction in the A-ring to give **DVT-M1**, **DVT-M2** and **DVT-M3**, respectively. Upon further di-hydroxylation of the reduced 17α-vinyl group, **DVT-M4** and **DVT-M5**, **DVT-M6** and **DVT-M7** could be formed from respectively **DVT-M1**, **DVT-M2** and **DVT-M3**. In addition, **DVT-M8** could be formed by di-hydroxylation of the reduced 17α-vinyl group together with di-hydroxylation elsewhere in the molecule.

Oral administration study with DVT: phase II metabolism

To study phase II metabolism, unconjugated, glucuronide-conjugated, sulfate-conjugated and sulfate glucuronide-conjugated metabolites were fractionated and deconjugated. Enzyme hydrolysis and solvolysis were used to deconjugate the urinary metabolites because **DVT** was found to be unstable under methanolysis condition.[23] Metabolites **DVT-M2d** and **DVT-M3a** were found exclusively in the glucuronide fraction, indicating that they were excreted

Table 2. Detection periods for the urinary metabolites of **DVT** by GC/MS

Metabolites of DVT	Horse A (h)	Horse B (h)
DVT-M2d	4–22	4–22
DVT-M3a	4–46	4–46
DVT-M6	4–70	4–46

mainly as glucuronide conjugates. Metabolite **DVT-M6** was excreted predominantly in the glucuronide fraction and to a smaller extent in the sulfate and/or sulfate-glucuronide fraction.

Target analytes for detecting DVT administration

Table 2 gives the detection times for the *urinary* metabolites of **DVT** by GC/MS. Both **DVT-M3a** and **DVT-M6** could be the target analytes to detect **DVT** administration as they could be detected consistently in urine from both horses for at least 46 h after administration. In view of the relatively short detection times for these metabolites, other alternatives, such as hair analysis and the use of steroidomics approach to establish a statistical model of biomarkers profile, are being explored in order to detect the abuse of DVT and its analogues more effectively in performance horses.

Conclusion

In vitro biotransformation studies of **DMT** and its analogues (**DVT**, **DT**, **DET**, **DENT** and d_3**-DMT**) using horse liver microsomes showed that the biotransformations occurred predominantly in the A-ring by way of a combination of enone formation, hydroxylation and reduction. In the case of **DVT** alone, additional biotransformations via reduction and di-hydroxylation of the 17α-vinyl group were observed; in the case of **DMT**, d_3**-DMT** and **DET**, additional biotransformations via hydroxylation of the 17α-alkyl group were observed; and in the case of **DMT** and d_3**-DMT**, additional biotransformations were observed via hydroxylation at C16. For the oral administration of **DVT** to horses, no parent drug was detected. Three urinary metabolites, namely **DVT-M2d**, **DVT-M3a** and **DVT-M6**, were observed in post-administration urine, mainly in the glucuronide fraction. The metabolic pathway for **DVT** was postulated. Metabolites **DVT-M3a** and **DVT-M6** are the analytes of choice for detecting **DVT** administration, as they gave the longest detection time in urine (up to a maximum of 70 h post-administration observed). The three urinary metabolites of **DVT** could all be produced from *in vitro* microsomal incubation. Although *in vivo* metabolism studies of **DMT** and its four analogues (**DT**, **DET**, **DENT** and d_3**-DMT**) in horses have not been carried out, inclusion of the mass spectra of their *in vitro* metabolites in a GC/MS library could permit to detect the use of these designer steroids in racehorses through library search. Furthermore, an isolate of *in vitro* metabolites from a microsomal incubation can also be used as an acceptable reference material.[24]

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