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Characterisation of chemical and pharmacological properties of new steroids related to doping of athletes

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In the USA, a female cyclist, T. Thomas, was banned for the use of norbolethone, a steroid which has been studied by the pharmaceutical industry in the 60s but not commercialised[1]. Later in 2004, following the characterisation of THG[2] from a oily residue given to the American testing authorities, and in collaboration with the international athletic federation, several elite athletes' samples were tested and found to contain that "designer" steroid. The properties of tetrahydrogestrinone were subsequently studied; it appears that THG is a potent androgen and progestin[3]. Public comments made by individuals involved in the "Balco" scandal were adding to the information coming from other sources, mainly informants, to the effect that other "designer", potent and undetecTable steroids have been prepared and would be available to certain athletes. This has become a certainty in December 2003, following a seizure at the Canadian border of hGH, and two steroid-products one of which found to be THG. The identification of the second one, DMT (17α-methyl-5α-androst-2-en-17β-ol major isomer along with the 3-en isomer), is the subject of the first part of this work; picture of the bottle seized is shown at Figure 1.

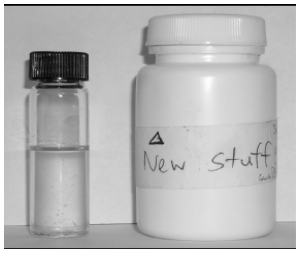

Figure 1: Plastic bottle seized at the Canadian border containing 3g per 100 mL of a steroid identified as 17α-methyl-5androst-2 (-3)-en-17-ol (DMT) in vegetable oil.

Experimental: Cryopreserved hepatocytes from a single donor or a pool of five were purchased from In vitro Technologies. In a typical experiment, the hepatocytes preserved at -150°C are thawed in a water bath at 37°C. The cells are suspended in In vitro GRO HT® Medium and centrifuged at 50 g for 5 min. The supernatant is discarded, the cells are suspended in 1 mL of KHB buffer. Cells are counted using Trypan Blue exclusion method and concentration is adjusted to 2 millions cells per mL with KHB buffer. Incubations are done for 5 hours; the cells are precipitated and the steroids are extracted after solid phase extraction on Sep Pak plus C_{18} cartridges, liquid-liquid extraction with diethyl ether and TMS-ether, TMS-enol formation.

Assessment of hormonal properties: All compounds were dissolved in 95% ethanol and the final concentration of the vehicle in the cell culture medium was 0.1%. For each assay, we included a standard curve with the following agonists: dihydrotestosterone (DHT) for the androgenic assay, progesterone for the progestagenic assay and estradiol for the estrogenic assay. A minimum of 5 concentrations were used to construct dose response curves for each substance. Measurements were made in triplicates and at least three independent experiments were conducted. The specificity of the response in each system was tested by using specific antagonists: hydroxyflutamide (anti-androgen), ICI 182 720 (anti-oestrogen) and mifepristone (antiprogestin).

Dose-response relations were fitted to a four parameter sigmoid curve using the Slide Write Plus for Windows software (version 6.00, Advanced Graphics Software Inc., Encinitas, CA). EC_{50} values (the concentration that yields a response equal to 50% of the maximum response induced by the agonist of reference) were intrapolated from these modeled curves. The efficacy of compounds in each assay was also calculated relative to that of the reference agonist.

Characterisation:

The chemical synthesis of DMT (3a and 3b) from epiandrosterone (1) is summarised in Figure 2, mass spectra of the TMS-derivatives of the two isomers present in the “designer” product are shown at Figure 3.

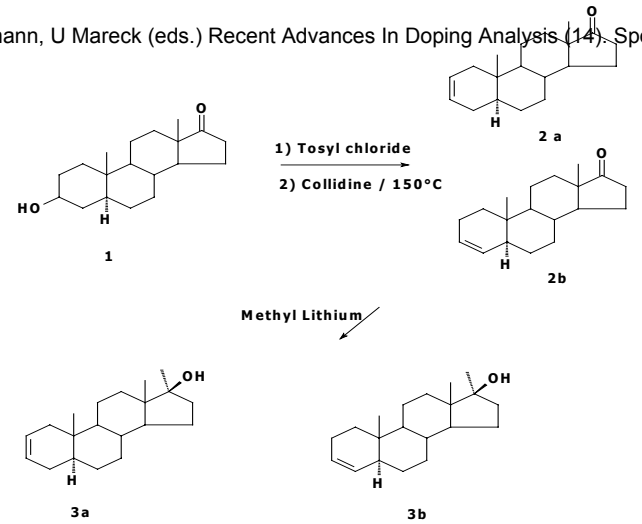

Figure 2: Scheme of chemical synthesis of 17α-methyl-5androst-2 -en-17-ol (DMT) (3a) from epiandrosterone (1) *via* 5α-androst-2-en-17-one (2a; major)

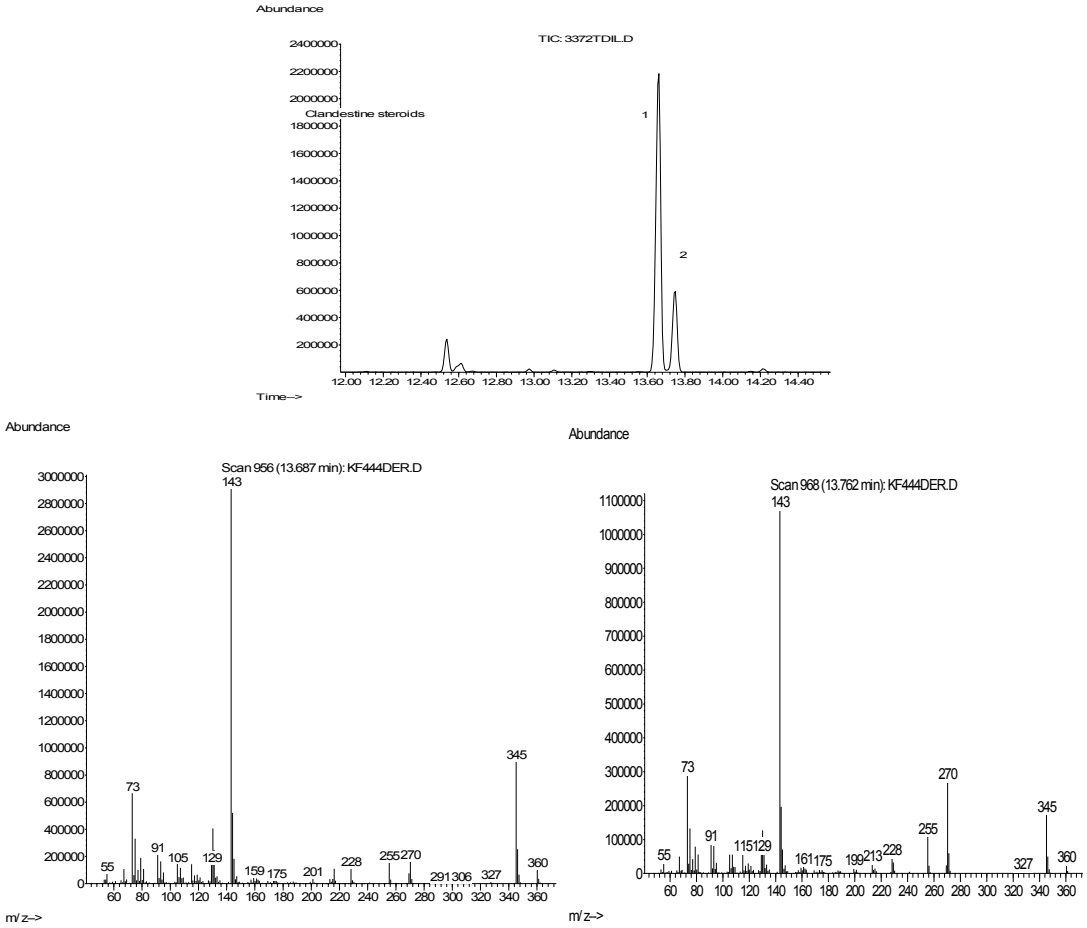

Figure 3: GC/MS analysis of the clandestine steroid product (upper panel) and mass spectra of the TMS-derivatives of the authentic synthesised standards of the two isomers (peaks 1 and 2) of DMT 3a (2-en major at left) and 3b.

Another 17α-alkylated steroid, 2α,17α-dimethyl-5α-androstan-3-one-17-ol, 17-methyldrostanolone (5), was commercialised under the trade names of *Superdrol, Methasterone*. The GC/MS analysis of the content of one capsule of *Superdrol* seized at the

Canadian customs is shown at Figure 4 along with the mass spectrum of the TMS-ether,TMS-enol (d_9-TMS in parenthesis) derivative. The spectrum is characterised by the presence of ions at m/z 462 (molecular ion), 419 (-43, C1-C2-CH_3; A-ring), 157 and 141 (A-ring), 143 and 130 (D-ring).

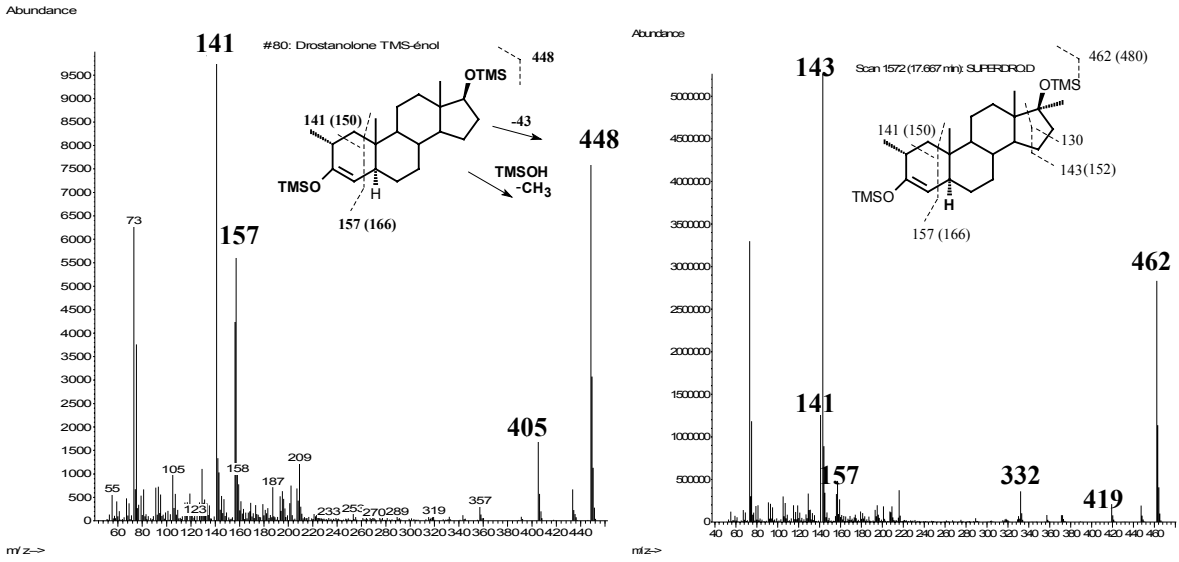

Figure 4: GC/MS analysis of the content of a capsule of commercial product, *Superdrol*; mass spectrum of the TMS-derivative of main peak (lower right) compared to that of drostanolone (4) (lower left).

A summary of the steps involved in the synthesis of 17-methyldrostanolone (5) from drostanolone (4) is shown at Figure 5 while Figure 6 presents the mass spectra of underivatised and TMS-ether derivative of the synthesised steroid (5). The chromatographic properties, NMR and mass spectrum were found to be identical to that of the steroid isolated from the commercial product.

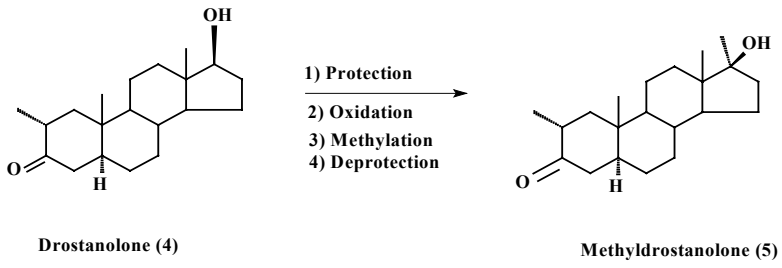

Figure 5: Synthesis of 17-methyldrostanolone (5) from drostanolone (4).

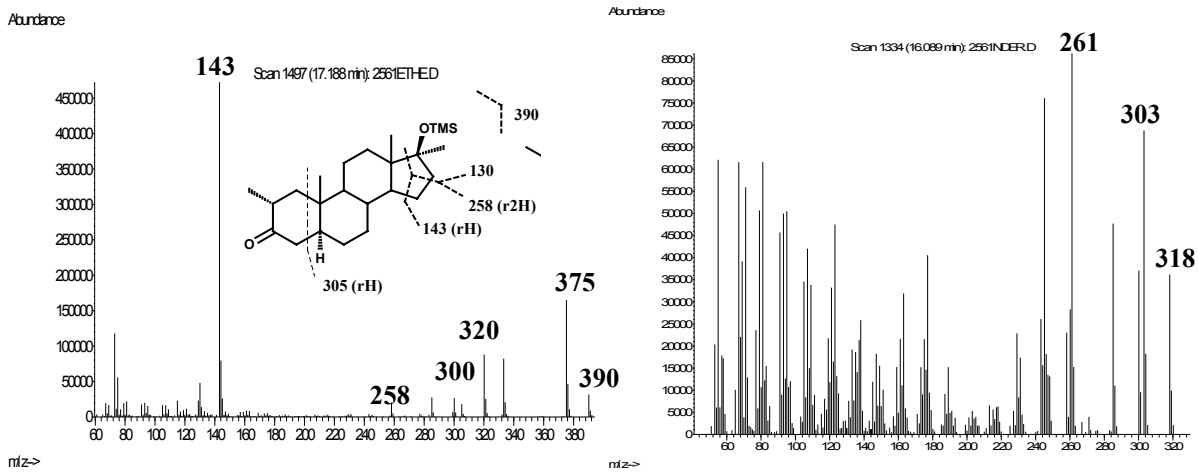

Figure 6: Mass spectrum of synthesised 2α,17α-dimethyl-5α-androstan-3-one-17-ol, TMS-ether (left) and underivatised (right) and proposed fragmentation pathways.

Identification of urinary metabolites: For this purpose we have used cryopreserved human hepatocytes which were reported to be a suiTable model and to produce results comparable to *in vivo* experimentation. More specifically, human hepatocytes have been shown to produce phase I and phase II metabolites similar, qualitatively to those produced *in vivo* for androstenedione, norandrostenedione[4] and THG[5]. Recently, freshly isolated human hepatocytes were successfully cryopreserved, thus maintaining their viability and showing comparable drug-metabolizing enzyme activities to freshly isolated ones[6].

We were not able to get phase II metabolites in significant amounts under those conditions. Using the molecule of 17-methyldrostanolone as a model compound, we have nonetheless isolated reduced and hydroxylated metabolites in the supernatant. The in vitro experiments lead to clean extracts which were easier than urine to analyse; the reconstructed total ion chromatogram of a representative result is shown at Figure 7. The mass spectrum of the 3α-(major) reduced metabolite is shown as well along with the spectra of 16-hydroxylated metabolites of parent compound and reduced in C-3.

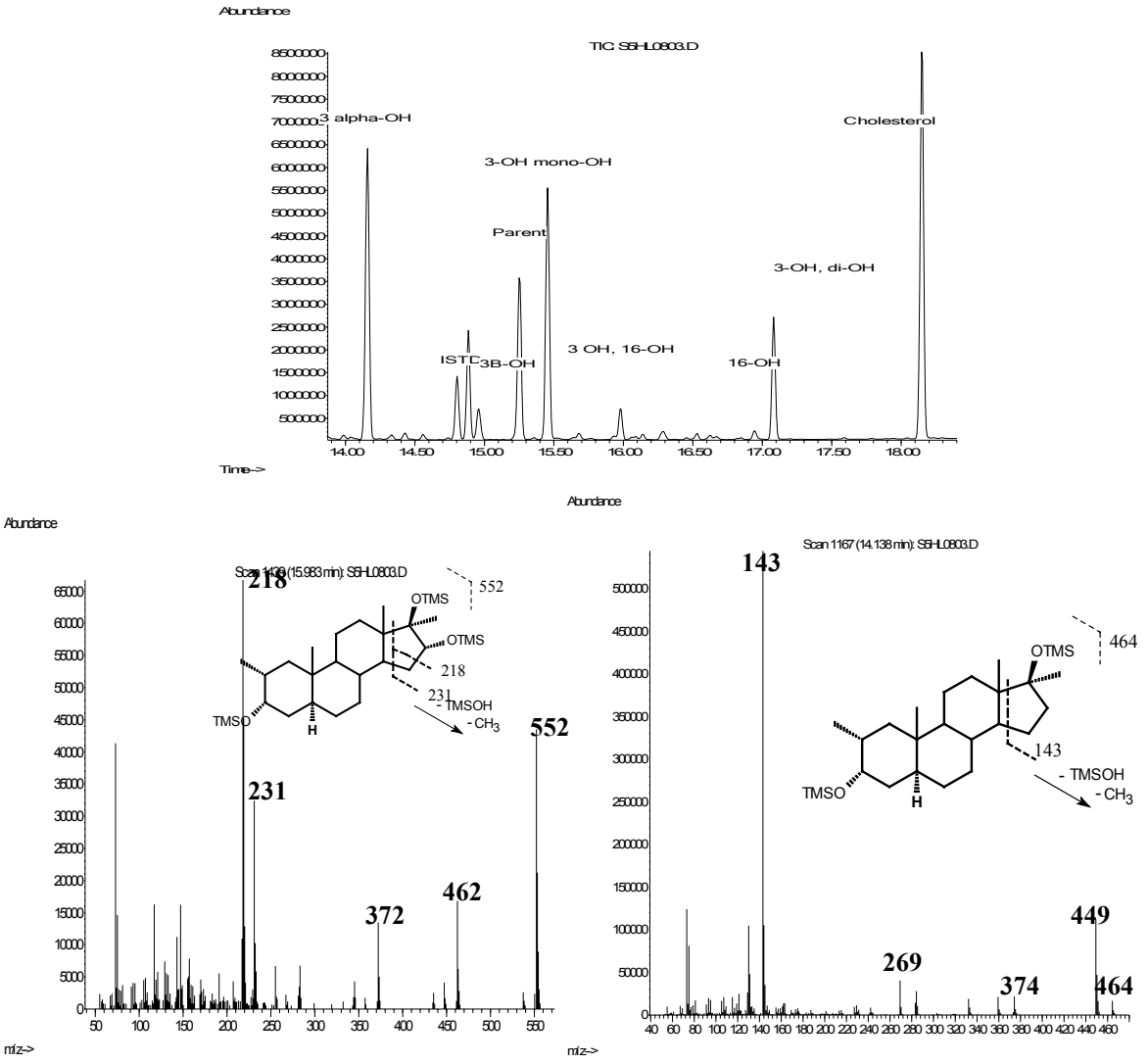

Figure 7: GC/MS Analysis of supernatant fraction of hepatocyte with 17-methyldrostanolone (5) (upper): mass spectra of 3α-reduced and 16-OH hydroxylated metabolites (lower).

Hormonal properties: Some of the steroids offered to athletes *via* this underground market have been synthesised and studied by researchers of pharmaceutical companies in the early 60s. Their androgenic/anabolic properties have been estimated using techniques available at the time. DMT (17α-methyl-5α-androst-2-en-17β-ol) for example, was reported in two studies to possess 2 to 5 times the anabolic activity of methyltestosterone and half of its androgenicity[7]. Employing different bioassays, another group reported for the same steroid, 12 times the anabolic activity and less than twice the androgenic activity of methyltestosterone[8]. Recently, the hormonal properties of THG have been assayed *in vitro* using yeast-based bioassays[3] and novel microarray approaches[9]. Using mammalian-cell based reporter gene bioassays, we investigated the hormonal properties of tetrahydrogestrinone and other designer steroids that are suspected of being used to enhance

performance and included steroids of known activity for comparison: desoxymethyltestosterone (DMT), ethylestrenol, mestanolone, methyldrostanolone, methyltestosterone, metribolone, and mibolerone.

Androgenic activity: The androgenic activity of the designer steroids was determined using CAMA-1 breast cancer cells transiently transfected with a reporter plasmid, which comprises the androgen-responsive mouse mammary tumor virus promoter in front of the luciferase gene (MMTV-luc). CAMA-1 cells express a functional androgen receptor.

The EC_{50} and efficacy values are listed in Table 1. Mibolerone, metribolone, mestanolone, tetrahydrogestrinone and methyldrostanolone were potent full agonists in this assay. Methyltestosterone and desoxymethyltestosterone were somewhat less potent androgens. Ethylestrenol was 400-fold less potent than mibolerone and only showed a 50% efficacy. Progesterone did not display any androgenic activity. We tested the potential of hydroflutamide (1 μM), a potent antiandrogen, to block the androgenic response induced by steroids in this assay. We used concentrations of steroids that yielded near maximal response. In those conditions, hydroxyflutamide reduced the response of all steroids by more than 85%, indicating that our assay was specific for androgens (data not shown).

Table 1. EC_{50} and efficacy values of designer steroids in CAMA-1 cells transiently transfected with the MMTV-luc plasmid.

Compound	**EC_{50} (nM)**	**Efficacy (%)**
Dihydrotestosterone	0,30 ± 0,03	100
Mibolerone	0,10 ± 0,02	90 ± 6
Metribolone	0,26 ± 0,09	82 ± 19
Mestanolone	0,40 ± 0,14	84 ± 15
Tetrahydrogestrinone	0,54 ± 0,10	103 ± 10
Methyldrostanolone (5)	0,79 ± 0,17	88 ± 10
Methyltestosterone	1,67 ± 0,40	67 ± 16
DMT (1)	2,50 ± 0,87	92 ± 3
Ethylestrenol	40,8 ± 6,8	52 ± 7
Progesterone	>1000	3 ± 1

Estrogenic activity: The estrogenic activity of the compounds was assessed using MCF-7 breast cancer cells transiently transfected with a reporter vector that contains two copies of the

vitellogenin consensus estrogen response elements fused to a 105-bp thymidine kinase promoter and the luciferase gene [(ERE)2-vit-TK-luc]. MCF-7 cells express a functional estrogen receptor (ER-α). In both androgenic and estrogenic cell transfection assays, a second plasmid containing another reporter gene (Renilla) was co-transfected to control for the efficacy of the transfection.

As shown in Table 2, listing the EC_{50} and efficacy values, all steroids exhibited estrogenic activity, albeit at concentrations greater than 10 nM. The most potent compounds were tetrahydrogestrinone and mibolerone, followed by metribolone, desoxymethyltestosterone and mestanolone. EC_{50} values for ethylestrenol, methyltestosterone, and methyldrostanolone were greater than 250 nM, hence some 30000-fold less potent than estradiol. We tested the potential of ICI 182 720 (1 μM), a selective estrogen receptor blocker, to antagonise the estrogenic response induced by steroids in this assay. We used concentrations of steroids that yielded near maximal response. ICI 182 720 reduced the response of all steroids by more than 95%, indicating that our assay was specific for estrogens (data not shown).

Table 2. EC_{50} and efficacy values of designer steroids in MCF-7 cells transiently transfected with the (ERE)2-vit-TK-luc plasmid.

Compound	**EC50 (nM)**	**Efficacy (%)**
Estradiol	0,008 ± 0,002	100
Tetrahydrogestrinone	36,1 ± 5,5	104 ± 3
Mibolerone	38,7 ± 8,5	71 ± 6
Metribolone	94,0 ± 9,3	105 ± 13
DMT (1)	96,2 ± 13,6	103 ± 12
Mestanolone	118,3 ± 13,3	98 ± 6
Ethylestrenol	257,7 ± 34,1	84 ± 10
Methyltestosterone	304,0 ± 99,2	43 ± 2
Methyldrostanolone (5)	383,9 ± 55,2	64 ± 19

Progestagenic activity: **T**he progestagenic activity of the steroids was evaluated by measuring the activity of alkaline phosphatase (AP) in T47D breast cancer cells, a breast

cancer cell line that express a functional progesterone receptor. T47D cells express AP when exposed to a progestagen[10].

Metribolone, mibolerone and tetrahydrogestrinone were potent progestins in this assay, with EC_{50} values two-fold lower or more compared to that of progesterone (see Table 3). However, these compounds were only partial agonists (efficacy values of 57%, 68% and 28%, respectively). Ethylestrenol was 6.8-fold less potent than progesterone but had a similar efficacy. Mestanolone, methyltestosterone, and desoxymethyltestosterone were also full agonists in this assay but were an order of magnitude less potent than ethylestrenol. We tested the potential of mifepristone (1 μM), a selective progesterone receptor blocker, to antagonise the progestagenic response induced by steroids in this assay. We used concentrations of steroids that yielded near maximal response. Mifepristone reduced the response of all steroids by more than 85%, indicating that our assay was specific for progestins (data not shown).

Table 3. EC_{50} and efficacy values of designer steroids for the induction of alkaline phosphatase expression in T47D cells

Compound	**EC50 (nM)**		**Efficacy (%)**	
Progesterone	0,40	± 0,09	100	
Metribolone	0,13	± 0,03	57	± 10
Mibolerone	0,13	± 0,01	68	± 4
Tetrahydrogestrinone	0,19	± 0,03	28	± 5
Ethylestrenol	2,73	± 0,33	112	± 15
Mestanolone	28,5	± 11,4	99	± 20
Methyltestosterone	31,5	± 8,1	104	± 16
DMT (1)	50,4	± 6,6	104	± 7
Methyldrostanolone (5)	457,4	± 42,0	54	± 5

Acknowledgments

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Reference

[1] Catlin et al., *Detection of norbolethone, an anabolic steroid never marketed, in athlete's urine.* Rapid Communication Mass Spectrom., (2002) 16, 1273-5
[2] Catlin et al., *Tetrahydrogestrinone : discovery, synthesis, and detection in urine,* Rapid Commun. Mass Spectrom., 18, 1245 (2004)
[3] Death AK, McGrath KC, Kazlauskas R, Handelsman DJ. *Tetrahydrogestrinone is a potent androgen and progestin*. Rapid Communications, J Clin Endocrinol Metab. 2004;89:2498-500
[4] Lévesque J.-F. et al., *Evaluation of human hepatocyte incubation as a new tool for metabolism study of androstenedione and norandrostenedione in a doping control perspective,* J. Chromatogr. B., 780, 145 (2002)
[5] Levesque JF, Templeton E, Trimble L, Berthelette C, Chauret N., *Discovery, biosynthesis, and structure elucidation of metabolites of a doping agent and a direct analogue, tetrahydrogestrinone and gestrinone, using human hepatocytes*. Anal Chem. 2005 15;77(10):3164-72.
[6] Li. A. P. et al., *Present status of the application of cryopreserved hepatocytes in the evaluation of xenobiotics: consensus of an international expert panel*, Chem.-Biol. Interactions, 121:1, 117 (1999)
[7] Bowers A. et al., *Steroids. CCV. Ring A modified hormone analogs. Part I. Some ring A olefins,* J. Med. Chem., 6, 156 (1962).
[8] Counsell R. E. et al., *Anabolic agents. 19-nor- and 19-substituted 5□-androst-2-ene derivatives*, J. Med. Chem., 9, 685 (1966)
[9] Labrie F. et al., Tetrahydrogestrinone (THG) induces a genomic signature typical of a potent anabolic steroid, J. Endocrinol., 184, 427–433 (2005)
[10] Dong Y, Roberge JY, Wang Z, Wang X, Tamasi J, Dell V, Golla R, Corte JR, Liu Y, Fang T, Anthony MN, Schnur DM, Agler ML, Dickson JK Jr, Lawrence RM, Prack MM, Seethala R, Feyen JH. *Characterization of a new class of selective nonsteroidal progesterone receptor agonists*. Steroids. 2004;69:201-17