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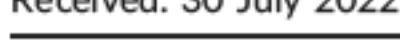

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SPECIAL ISSUE - SHORT COMMUNICATION

Detection of 18-methyl steroids: Case report on a forensic urine sample and corresponding dietary supplements

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Abstract

The detection of a putative 18-methyl-19-nortestosterone metabolite in a forensic bodybuilder's urine sample collected as part of a criminal proceeding has triggered a follow-up investigation. Four different dietary supplements in the possession of the suspect were examined with regard to possible precursor steroids. This led to the detection of the declared ingredient methoxydienone, which was confirmed by both, GC–MSMS and LC-HRMSMS. As neither 18-methyl-testosterone, nor 18-methyl-19-nortestosterone were detectable in the supplements, the possibility that the metabolite originates from methoxydienone was investigated. For this purpose, the metabolic fate of methoxydienone was studied in vitro using human HepG2 cells and in vivo by a single oral administration. While the 18-methyl-19-nortestosterone metabolite was not generated by HepG2 cells incubated with methoxydienone, it was observed in the urine samples collected at 2, 6, 10 and 24 h after methoxydienone administration. Moreover, the potential binding of methoxydienone as ligand to the human androgen receptor was modelled in silico in comparison with 18-methylnandrolone, for which androgen receptor activation had been shown in an in vitro approach before. In conclusion, we could ascribe the presence of the 18-methyl-19-nortestosterone metabolite in a forensic urine sample to originate from methoxydienone present in dietary supplements. Methoxydienone was observed to slowly degrade by demethylation of the methoxy substituent in liquid solutions. While no compound-specific intermediates were identified that allowed differentiation from other 18-methyl steroids, the 18-methyl-19-nortestosterone metabolite proved to be a suitable marker for reliable detection in doping analysis.

KEYWORDS
18-methyl steroids, doping analysis, methoxydienone, molecular modelling, nutritional supplements

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1 | INTRODUCTION

Designer anabolic steroids are available as labelled ingredient or nonlabelled impurities in over-the-counter dietary supplements. One group amongst compounds that are assigned as 'designer steroids' is characterized by an additional methyl group at position C18. First synthesis of 18-methyl steroids and description of their biological activities date back to the 1960s.[1 4] After that time, only one single study was examining the androgen receptor (AR) activating potency of 18-methylnandrolone in vitro.[5] Another 18-methyl steroid is methoxydienone, also referred to as methoxygonadiene, which is chemically characterized as 13β-ethyl-3-methoxygona-2,5(10)-dien-17-one. Regarding methoxydienone, there are no data available, on whether it is orally active, nor on its metabolism in humans. Interpretation of metabolic patterns in forensic samples is impeded by the fact that users are typically multidrug abusers and that respective doping agents traded on the black market are mostly cross-contaminated.[6] Beside different in vitro models used to study the receptor binding and activation of potential ligands, computer-based approaches have been developed to study interaction between different receptor ligand binding sites and compounds of interest. These allow for an estimation of the interaction by comparison with the binding data from endogenous ligands as well as other ligands known to activate receptor-mediated effects.

The present study started from the examination of a bodybuilder's urine in coincidence with the corresponding confiscated dietary supplements containing amongst others methoxydienone. As no 18-methyl-19-nortestosterone was detectable in any of the confiscated supplements, we wanted to investigate whether the 18-methyl-19-nortestosterone metabolite observed in the urine sample could originate from methoxydienone. For this purpose, we performed preparative HPLC fractionation of the dietary supplements and investigated its biotransformation in a cell-based model and by self-administration. Moreover, we studied the methoxydienone binding to the human AR in an in silico approach, in order to get data on the potential biological activity as an AR ligand.

2 | MATERIALS AND METHODS

2.1 | Materials

Acetonitrile and methanol (both liquid chromatography–mass spectrometry [LC–MS] grade) and ethanol (EtOH, ≥99.9%, analytical grade purity) were purchased from Th. Geyer (Berlin, Germany). Ammonium acetate and acetic acid (>98%), propane-2-thiol, and ammonium iodide (NH_4I) were purchased from Merck (Darmstadt, Germany). *t*-Butylmethylether (MBTE, 99.8%) was provided by Sigma-Aldrich (Taufkirchen, Germany). For solid-phase extraction (SPE), the Oasis HLB SPE cartridges (1 cm^3, 30 mg) were provided by Waters (Milford, USA). Ultrapure water (H_2O) was prepared using the Milli-Q® Integral System (Merck Millipore, Darmstadt, Germany). β-Glucuronidase (from *Escherichia coli* K 12, 80 units/mg protein) was purchased from Roche (Mannheim, Germany). *N*-Methyl-trimethylsilyltrifluoracetamide (MSTFA) was purchased from Macherey-Nagel (Düren, Germany). Sodium bicarbonate ($NaHCO_3$, reagent grade) and potassium dihydrogen phosphate (KH_2PO_4, reagent grade) were obtained by KMF (Lohmar, Germany) and potassium carbonate (K_2CO_3, reagent grade) by Carl Roth (Karlsruhe, Germany). Sodium hydrogen phosphate (Na_2HPO_4, reagent grade) was purchased from Serva (Heidelberg, Germany).

2.2 | Metabolism of methoxydienone

In vitro metabolism was investigated by incubation of HepG2 cells with methoxydienone. Cultivation and incubation were performed as described before.[7] In vivo biotransformation was investigated by self-administration of 0.2 mg methoxydienone by a healthy male volunteer (the project leader of this study). Due to the lack of reference material, it was attempted to estimate the total dosage of methoxydienone (i.e., parent plus desmethyl), by comparing the desmethyl moiety (i.e., cleavage of methoxy group) with structurally similar steroids. As similarity criterion, it was assumed that its ionization should be best comparable with other A-ring reduced 3-OH-steroids. In addition to a blank urine sample collected before administration, urine samples were collected 2, 6, 10 and 24 h post administration.

2.3 | GC–MS

The per-TMS derivatives of compounds of interest were prepared. Samples (forensic urine sample, urine samples from self-administration, HPLC fraction from dietary supplement putatively containing methoxydienone) were evaporated to dryness in a vacuum chamber (50°C, 45 min), then reconstituted in 40 μl of the mixture of MSTFA/NH4I/propane-2-thiol 1000:5:1 v/m/v and heated (55°C, 30 min). GC separation was achieved on a Phenomenex ZB-1ms column (10 m × 0.18 mm and 0.18 μm film thickness) applying temperature programming. The following conditions were applied: 130°C to 186°C at 56°C/min, then to 206°C at 2°C/min, then to 221°C at 5°C/min and then to 326°C at 35°C/min (held 1.5 min), the back flush of a ZB-1ms precolumn (1 m) 2.5 ml/min at 16 min, the He flow 1.0 ml/min. GC–MS Triple Quad 7890B/7010B system was used for analyses (Agilent, Santa Clara, CA, USA). Mass spectrometric data were obtained under electron ionization (EI) conditions. Two approaches were used: the targeted one in the MS/MS mode, MRMs of the expected phase I metabolites were monitored (1 μl injected, split 15:1) and the untargeted one in the Q1 scan mode 50–550 Da (1 μl injected, split 10:1 or splitless). The MassHunter software B.07.06 was used for data acquisition and QQQ B.08.00 for quantitative analysis (Agilent, Santa Clara, CA, USA).

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2.4 | LC–MS

Extraction of the urine and cell culture media samples with and without hydrolysis was carried out as described previously.[7,8] The LC-HRMS screening procedure of the confiscated dietary supplements comprises approximately 120 analytical targets categorized as anabolic, peptides (including growth hormone) and metabolic modulators, which are monitored and quantified as mandatory compounds according to German Anti-Doping Law. Briefly, the extracts of the dietary supplements were examined with a Q-TOF mass spectrometer (TripleTOF® 6600, AB Sciex, Darmstadt, Germany) by isolating relevant precursors in the mass range 100–800 Da, subsequent fragmentation at a collision energy of 35 (±10) eV and recording resulting product ion spectra in high resolution mode.

In addition to this survey analysis method, a sensitive LC-HR/MSMS method was established to detect methoxydienone (301 → 93, CE = 63 eV, high sensitivity mode) and the corresponding desmethyl artefact (287 → 211, CE = 25 eV) as targets of interest in urine samples.

TABLE 1 Comparison of ingredients declared and verified by LC-HRMS in the four confiscated dietary supplements

Declared ingredients	Verified (✓) or not detectable (Ø)
2,17α-Dimethyl-5α-androsta-1-en-17β-ol-3-one (methylstenbolone)	✓ (three supplements)
4-Chloro-17a-methyl-androst-1,4-diene-3b,17b-diol (halodrol)	✓ (five isomers) (one supplement)
13-Ethyl-3-methoxygona-2,5(10)-diene-17-one (MAX LMG)	✓ (one supplement)
6-Bromo-androstane-3-17-dione (6-BROMO)	Ø
2A,3A-Epithio-17A-methyl-5A-androstan-17B-ol (epistane)	Ø
1,4,6-Androstatriene-dione (ADT)	Ø

2.5 | Molecular modelling

The 3D structure of human AR with cocrystallized endogenous ligand dihydrotestosterone (DHT) with the highest resolution

FIGURE 1 GC-EI-MS spectra after trimethyliodosilane derivatization of the LC fraction showing (a) methoxydienone (RT = 6.637 min) and (b) the hydrolysis product 13-ethyl-gona-4-ene-3,17-dione (RT = 6.994 min). Spectra are in accordance with that described by Abbate et al.[16]

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available at Protein Data Bank (PDB)[9] of 1.55 Å (PDB entry: 3L3X (10)) was chosen for performing docking of methoxydienone and 18-methylnandrolone. DHT exhibits similar geometrical and chemical attributes compared with both ligands of interest; therefore, the protein structure with cocrystallized DHT is a viable model for docking methoxydienone and 18-methylnandrolone. Protein preparation was conducted in Molecular Operating Environment software v. 2020.0901.[10] The structure was protonated at pH 7 using Protonate3D[11] and 300 K, and the cocrystallized fragment of protein coactivator SRC3 and water molecules were removed except for water molecule HOH919 situated between Gln711, Arg752, Met745 and the 3-keto group of the cocrystallized ligand DHT. This water molecule is suspected to play a role in anchoring the ligands in the orthosteric pocket[12] as it participates in hydrogen bonding to the cocrystallized ligand DHT in the crystal structure (PDB entry: 3L3X (10)) and should therefore be considered when performing docking experiments.

Both methoxydienone and 18-methylnandrolone were docked into AR using GOLD v. 5.8.1.[13] The coordinates of C6 of DHT were used as centre of a docking sphere of 10 Å as this atom represents the geometric centre of the AR binding pocket for DHT. Search efficiency was set to 100% and the genetic algorithm (GA) was run 20 times. Early termination was disabled; otherwise, default settings were kept. The obtained conformations were evaluated using GoldScoreP450 scoring function. Energy minimization took place in the MMFF94 force field[14] in the LigandScout v. 4.4.3[15] framework within the AR. The poses were visually inspected and evaluated based on their interaction pattern and shape similarity to the cocrystallized ligand in LigandScout.

3 | RESULTS AND DISCUSSION

3.1 | GC–MS

Recently, the analysis of a bodybuilder's urine (testosterone/epitestosterone ratio of 46, which suggests an abuse of synthetic endogenous anabolic androgenic steroids) showed an interesting metabolite pattern, comprising steroid metabolites with an 18-methyl (13β-ethyl) structure. Those are targets in our screening procedures since 2003, based on a control urine (QA2003_3A) provided by the Australian Sports Drug Testing Laboratory Sydney and distributed by the World Association of Anti-Doping Scientists (WAADS). Besides, dehydrochloromethyltestosterone (DHCMT) metabolites and methylstenbolone and its respective metabolites were detected in the urine sample.

In order to clarify the origin of the urinary metabolites, we analysed the corresponding confiscated dietary supplements. The four preparations in question differed in their intended purpose and putative ingredients. However, three preparations were rather similar containing amongst others the declared methylstenbolone as major ingredient, out of which solely one contained the 18-methyl steroid methoxydienone. A fourth dietary supplement contained DHCMT. Conversely, neither 18-methyl-testosterone, nor 18-methyl-19-nortestosterone or any of the other indicated designer steroids could be detected in the supplements (Table 1). The urinary findings of DHCMT metabolites and methylstenbolone metabolites are in good accordance with the confiscated preparations.

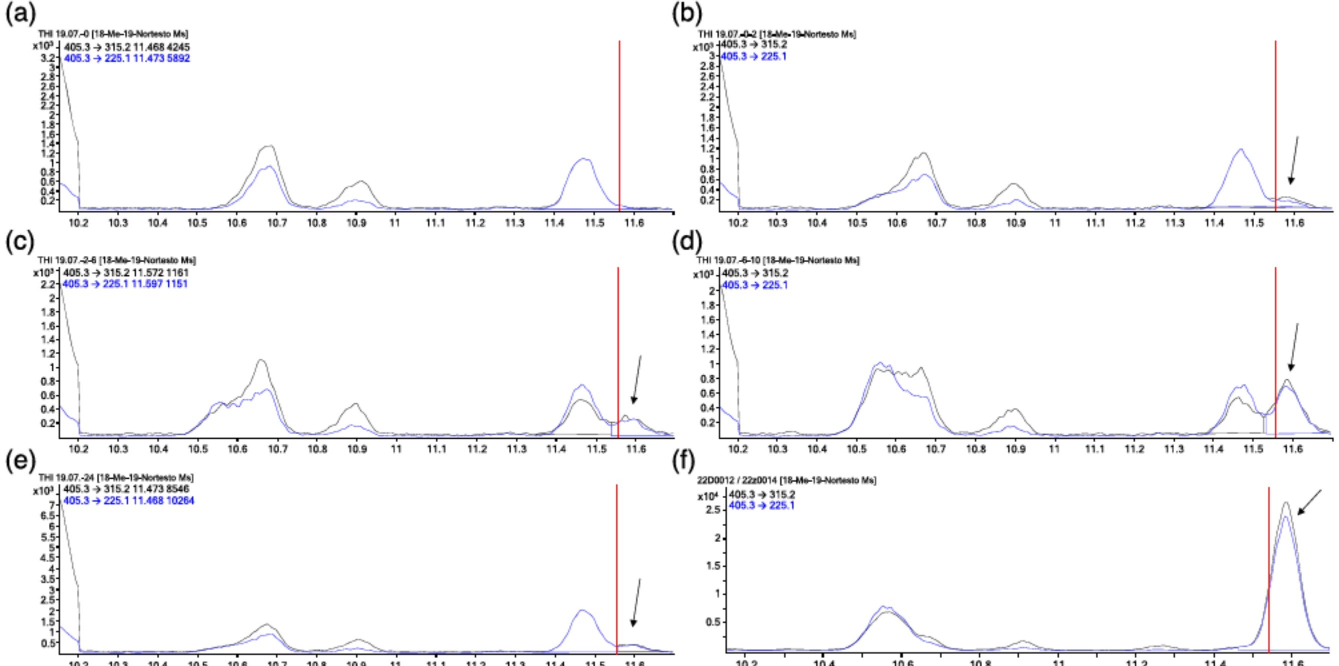

FIGURE 2 Time-dependent excretion of the 18-methyl-19-nortestosterone metabolite after oral methoxydienone self-administration (a–e) in comparison with its detection in the bodybuilder sample (f). GC-EI-MS chromatograms are shown for urine samples collected (a) at 0 h before administration, (b) 2, (c) 6, (d) 10, and (e) 24 h after administration.

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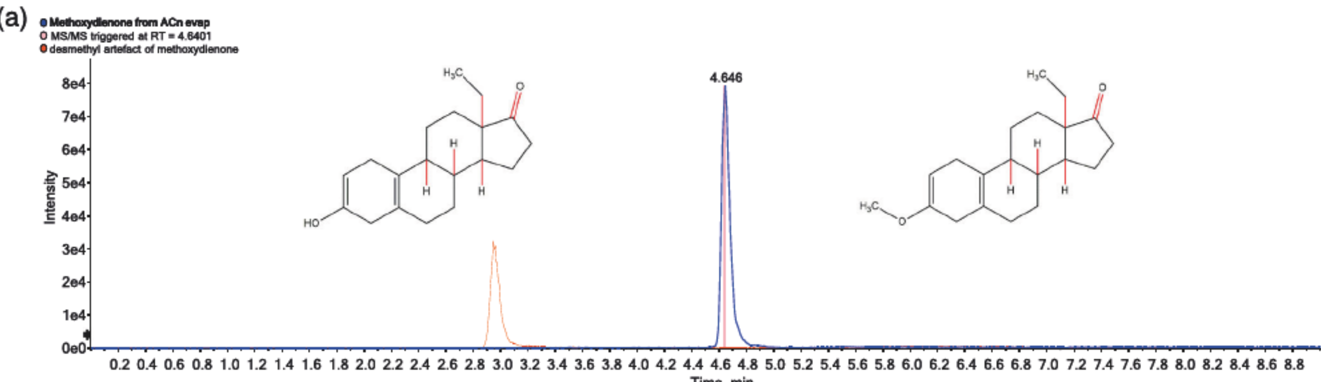
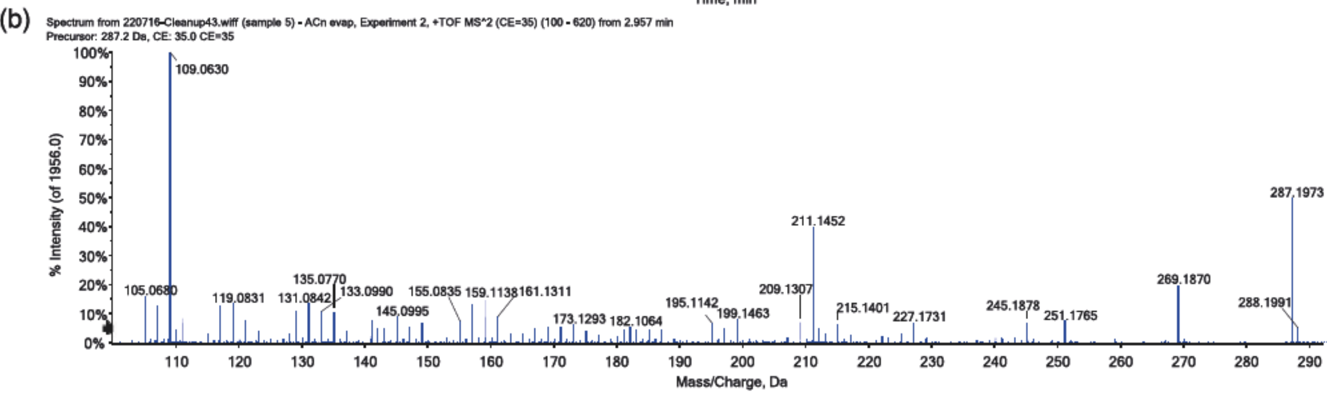
In contrast, the absence of 18-methyl-testosterone and 18-methyl-19-nortestosterone in the supplements rose the question of the origin of the 18-methyl steroid metabolite detected in the urine sample. In order to elucidate whether methoxydienone is the possible source of the respective metabolite, we attempted to purify methoxydienone from the dietary supplements by preparative HPLC fractionation. For fragmentation information, the fraction was analysed by GC–MS analysis showing one mono- and one di-hydroxylated structure. The molecular ion of the mono-TMS derivatives was identified as methoxydienone (RT = 6.637 min, *m/z* 372, Figure 1a) and the molecular ion of the bis-TMS derivative (RT = 6.944 min, *m/z* 430, Figure 1b) putatively results from the hydrolysis of methoxydienone yielding the 3-hydroxy structure 13-ethyl-gona-4-ene-3,17-dione. Our observations are in accordance with those described before by Abbate et al. for methoxydienone isolated from a dietary supplement.[16]

While the putative methoxydienone metabolite was not generated by HepG2 cell incubation (data not shown), we detected the metabolite between 2 and 24 h after voluntary administration peaking in the urine sample collected at the period 6–10 h post administration (Figure 2). Hence, the 18-methyl steroid metabolite observed in the forensic urine sample can be very likely ascribed to methoxydienone, even though no intermediates were found.

3.2 | LC–MS

A component, whose mass spectrum matched consistently the declared methoxydienone was found in one of the supplements examined (Figure 3a), in addition to the identification of its main ingredient methylstenbolone (Table 1).





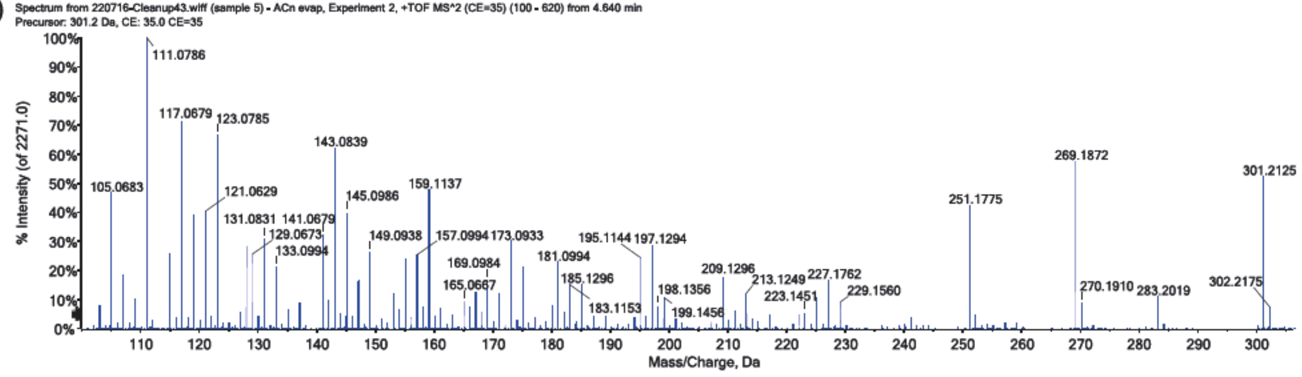


FIGURE 3 (a) LC-HRMS examination of the relevant dietary supplement. (b) Product spectrum of $m/z = 287.2$ at RT = 2.957 min. (c) Product spectrum of $m/z = 301.2$ at RT = 4.640 min

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The subsequent examination of the corresponding isolated LC fraction allowed an unambiguous identification of the active ingredient comparing EI-MS spectra with literature data (Figure 1).[16] Whereas the ingredient was found to be stable in the solid state, it was found to be slowly degraded by demethylation of the methoxy substituent in liquid solutions (e.g., mobile phase) with an apparent half-life of several days (Figure 3b). Moreover, it was rapidly decomposed when evaporated to dryness, which could be either due to heat and/or gradual increase of acid concentrations during evaporation.

3.3 | Molecular modelling

We reassessed the assumptions on the biological activity of methoxydienone and 18-methylnandrolone applying an in silico approach. In order to obtain a plausible binding hypothesis of methoxydienone and 18-methylnandrolone to AR, the final conformation was picked based on the similarity of both interaction pattern within the binding pocket and shape similarity to cocrystallized and endogenous ligand DHT. DHT, methoxydienone and 18-methylnandrolone occupy the orthosteric binding pocket. Particularly, the docking pose of 18-methylnandrolone and the conformation of cocrystallized DHT display high alignment. This observation can be traced back to the molecules' similar binding hydrogen bond interaction patterns. The binding mode of DHT in its binding pocket (Figure 4a) as obtained from the crystal structure (PDB code: 3L3X (10)) shows that the 3-keto group of DHT acts as a hydrogen bond acceptor to HOH919 and Gln711 epsilon nitrogen. This water molecule has been obtained from the AR crystal structure[17] and is suspected to be involved in the formation of a hydrogen bond network facilitating the binding of several AR ligands.[12] Additional hydrogen bonds are established from DHT to Thr877 gamma-hydroxyl group and Asn705 delta oxygen with the ligand's 17-hydroxyl group as hydrogen bond donor. The binding hypothesis for 18-methylnandrolone (Figure 4c) shows the molecule forming the same hydrogen bonds with HOH919 and Gln711 epsilon nitrogen via its 3-keto group, as well as with Thr877 hydroxyl group and Asn705 delta oxygen via the 17-hydroxyl group. Comparable interaction patterns correspond to a high scaffold congruence and are in good alignment with activity data obtained for 18-methylnandrolone at human AR in previous research.[5] The binding hypothesis for methoxydienone (Figure 4b) deviates from the highly aligned poses of DHT and 18-methylnandrolone. This can be attributed to the missing hydrogen bond donor moiety at Position 17 and the replacement of the 3-keto group in DHT and 18-methylnandrolone by the 3-methoxy group. In the methoxydienone docking pose, the hydrogen bonds to HOH919 and Thr877 are present, as well, with the ligand's 3-methoxy group and 17-keto group acting as hydrogen bond acceptors, respectively. The methoxy oxygen forms a hydrogen bond to the terminal NH nitrogen of Arg752 instead of the epsilon nitrogen Gln711. This indicates that methoxydienone might protrude deeper into the binding pocket. The hydrogen bond to Asn705 present in DHT and 18-methylnandrolone is missing, as methoxydienone features no hydrogen bond donor moiety at position 17 to fulfil this interaction. Thus, it is plausible for methoxydienone to assume a binding position shifted towards Arg752. Due to their similar binding modes, the conformations of DHT, methoxydienone, and 18-methylnandrolone are seamed by the same lipophilic residues contributing to the molecules' binding. Hydrophobic residues forming

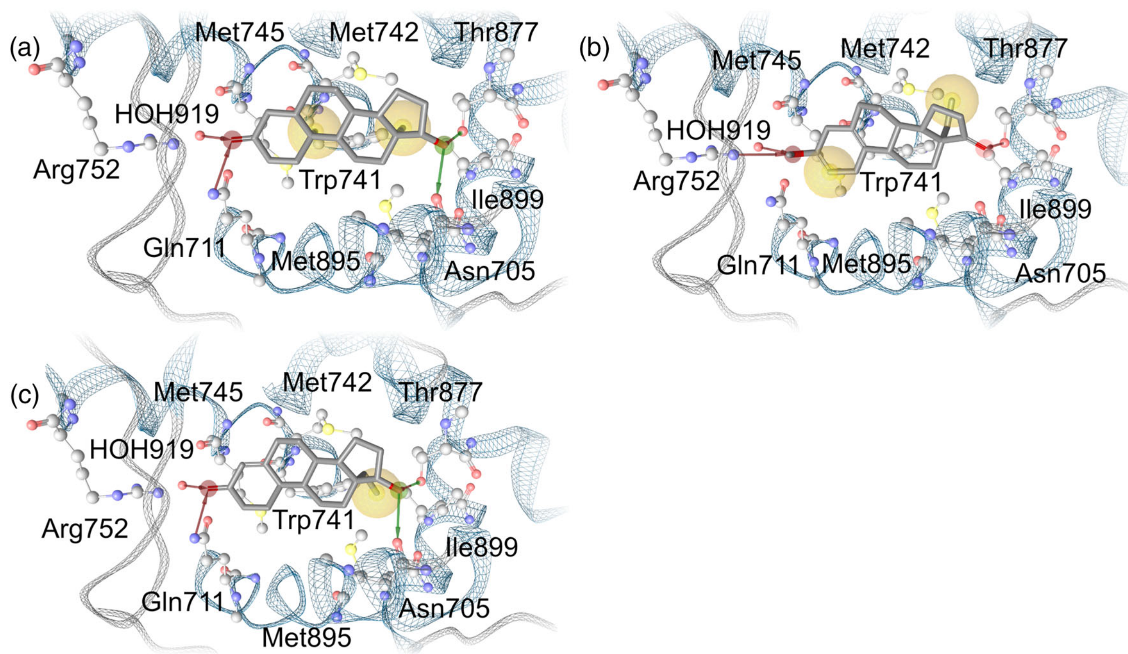

FIGURE 4 Comparison of the binding modes of (a) DHT (PDB code 3L3X [2]), (b) methoxydienone, and (c) 18-methylnandrolone in AR. Hydrogen bonds donors are depicted as green arrows, hydrogen bonds acceptors as red arrows and yellow spheres indicate lipophilic interactions.

lipophilic contacts with the steroids include Met742 for all three, as well as Met745, Trp741, Met895 and Ile899 for DHT; Met745 for methoxydienone; and Trp741, Met895 and Ile899 for 18-methylnandrolone.

4 | CONCLUSION

The relevance of atypical (so-called designer) steroids in the underground market is hard to evaluate. While the class of prohormones has almost disappeared, there is still a significant number of other steroids such methyl (nor)-steroids available. After decades of negative screenings, we have confirmed the excretion of the 18-methyl-19-nortestosterone metabolite resulting from methoxydienone for the first time. In addition to methyl steroids, questionable anabolic agents, such as 1-DHEA, 4-DHEA or estra-4,9-diene-3,17-dione, were recently identified in seized preparations. Hence, it seems to be worthwhile to maintain or extend respective screening procedures to cover not only methyl steroids substances.

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