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SPECIFIC DETECTION OF FOOD SUPPLEMENT STEROIDS

ABSTRACT

Since the nineties steroid containing food supplement products have become widely available. After the introduction of these products, a frequently occurring abuse has been suspected in the sports community. Steroids present in food supplements are 19C-steroids as dehydroepiandrosterone (DHEA), androst-4-ene-3,17-dione (Δ4-AEDIONE) and 19-norsteroids as 19-norandrost-4-ene-3,17-dione. In this chapter an overview is given on the metabolism of food supplement steroids with DHEA and Δ4-AEDIONE in particular.

The detection of DHEA or Δ4-AEDIONE abuse in doping analysis is usually based on easily available metabolic pathways to *e.g.* androst-5-ene-3β,17β-diol, testosterone, epitestosterone, androsterone, etiocholanolone and androstanediols. However, also oxygenation reactions form relevant routes of biotransformation. DHEA is mostly converted by respectively 7α-, 7β- and 16α-hydroxylase activity. Also 7-keto-metabolites can be detected. Oxygenating enzymatic activities related to Δ4-AEDIONE are 6β-, 7α-, 16α- and 16β-hydroxylase.

When Δ4-AEDIONE is converted to estrone by aromatase, small quantities of 19-hydroxy-androst-4-ene-3,17-dione and 19-nor-androst-4-ene-3,17-dione could possibly be produced as aromatase intermediate products or byproducts. Detection of these steroids could provide relevant information about the exogenous or endogenous origin of 19-norsteroids.

Scientific data does not describe occurrence of 4-hydroxy-androst-4-ene-3,17-dione (4-OH-Δ4-AEDIONE) as endogenous steroid. However, it cannot be excluded that 4-OH-Δ4-AEDIONE is produced as metabolite after Δ4-AEDIONE administration.

INTRODUCTION

Since the abuse of testosterone (T) was first suspected during the Olympic Games of Moscow in 1980, many other endogenous steroids have been introduced as ergogenic aid in sports. During the following decade, the abused steroids were mostly limited to T and 5α-dihydrotestosterone derivatives. During the nineties, steroid containing food supplement products became widely available. The first supplement steroids introduced were dehydroepiandrosterone (DHEA) and androst-4-ene-3,17-dione (Δ4-AEDIONE). One or two years later these steroids were followed by androst-5-ene-3β,17β-diol (Δ5-AEDIOL), androst-4-ene-3β,17β-diol (Δ4-AEDIOL) and androst-5-ene-3,17-dione (Δ5-AEDIONE). The common feature of these steroids is that they are biosynthetic precursors of T and they are claimed to metabolize to T after oral administration, without pertaining anabolic effects themselves.

Simultaneous to the developments of the mentioned 19C-steroids, 19-norsteroids became popular as food supplement steroids. Based on metabolic conversion to 19-nortestosterone (nandrolone), products were developed that contained steroids as 19-nor-androst-4-ene-3,17-dione, 19-nor-androst-4-ene-3β,17β-diol and 19-nor-androst-5-ene-3β,17β-diol. Nowadays, the concept of the endogenous origin of nortestosterone's main metabolite norandrosterone has gained interest and scientific acceptance [1,2]. This illustrates that besides the mentioned 19C-steroids, also the 19-norsteroids are metabolically related to the endocrine system.

Food supplement steroids are mostly sold in capsules or other formulations requiring oral administration. As no chemical modification has been performed, as for example has been the case for 17α-methyl-substituted anabolic steroids, extensive first-pass metabolism occurs after oral administration. Besides a low absorption, this leads to a high metabolic and renal clearance and a low half-life time.

In this short overview, a summary will be given of the metabolic pathways of DHEA and Δ4-AEDIONE. These will serve as model compounds for other supplement steroids mentioned above, as limited knowledge is available on the oxygenation of 19-nor steroids.

"NON-SPECIFIC" METABOLISM OF FOOD SUPPLEMENT STEROIDS

In doping analysis a limited number of analytical tools are available to tackle the detection of endogenous compounds or corresponding biosynthetic precursors. One of these is isotope ratio mass spectrometry that discriminates between endogenous

and exogenous origin of steroids [3]. This technique was initially developed for analytical confirmation of T administration [4] and is still under development as an efficient screening technique for T [5] and as a confirmation technique for other endogenous steroids [6,7].

Other analytical tools are based on easily available metabolic pathways of the food supplement steroids. This mostly implies the metabolism as summarized in Figure 1. The presented model is limited to 19C-steroids, but the enzymatic activities can be extrapolated to 19-norsteroids. Metabolites that are mostly studied in relation to administration of 19C-steroids are T, E, androsterone (AO), etiocholanolone (EO), 5α- and 5β-isomers of dihydrotestosterone (5α-DHT and 5β-DHT) and different stereoisomers of androstanediol (5α,3α-ADIOL; 5α,3β-ADIOL; 5β,3α-ADIOL and 5β,3β-ADIOL).

Based on the presented metabolism, steroid ratios have been introduced for the detection of T [8,9] and 5α-DHT [10,11]. Several studies have focussed on the effect of administration of food supplement 19C-steroids on the ratio of testosterone/epitestosterone (T/E ratio) [12-16]. Although the T/E ratio mostly increases, the response is limited. Also the AO/EO ratio or the corresponding 5α/5β-ratio for androstanediols has been studied [17]. Saturation of 5α-reductase by androst-4-ene steroids could lead to an increased activity of 5β-reductase. Administration of high dose levels can therefore be expected to decrease the AO/EO (5α/5β) ratio. So far, the AO/EO ratio has mostly been studied in a case study design [15-17] and requires additional investigation.

The main virtue of the described analytical tools is the general detection of unknown 19C-steroids or 19-norsteroids, by applying a rather arbitrary and qualitative pattern recognition methodology [15,16]. It is difficult to obtain sufficient specific information from a steroid profile based on the steroids shown in Figure 1, to identify the steroid that has been administered. The large number of steroids that are available today as supplement products challenges for more specific analytical methodologies. Additionally, an increasing number of claims are made of unintentional steroid administration, due to contamination of permitted (non-steroid) food supplement products. In general, these claims are supported by some scientific observations [18,19]. This illustrates a need for more specificity in doping analysis.

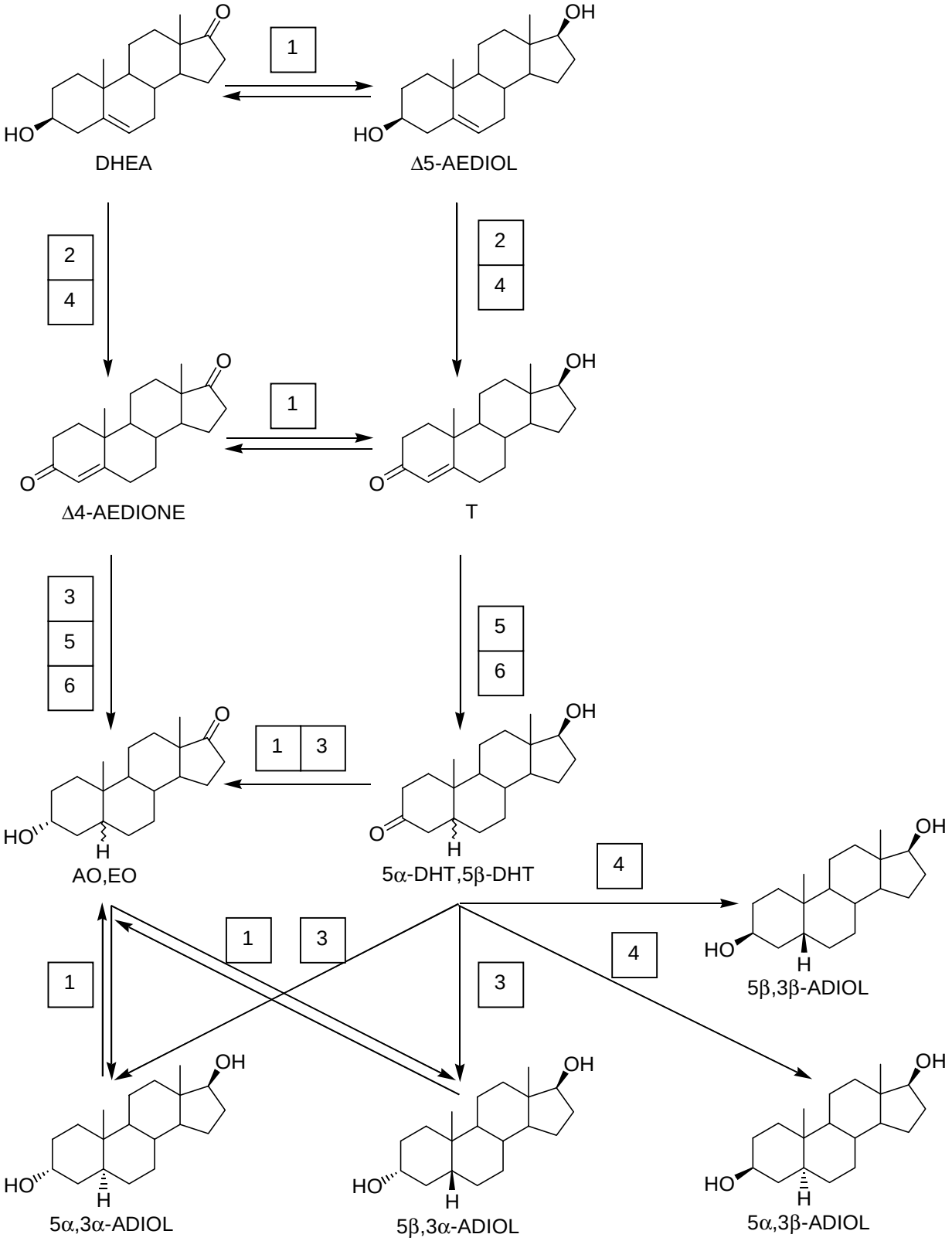

Figure 1: "Non-specific" metabolism of DHEA and Δ4-AEDIONE. Enzymatic activities involved are: (1) 17β-dehydrogenase; (2) 3,4-isomerase; (3) 3α-dehydrogenase; (4) 3β-dehydrogenase; (5) 5α-reductase; (6) 5β-reductase.

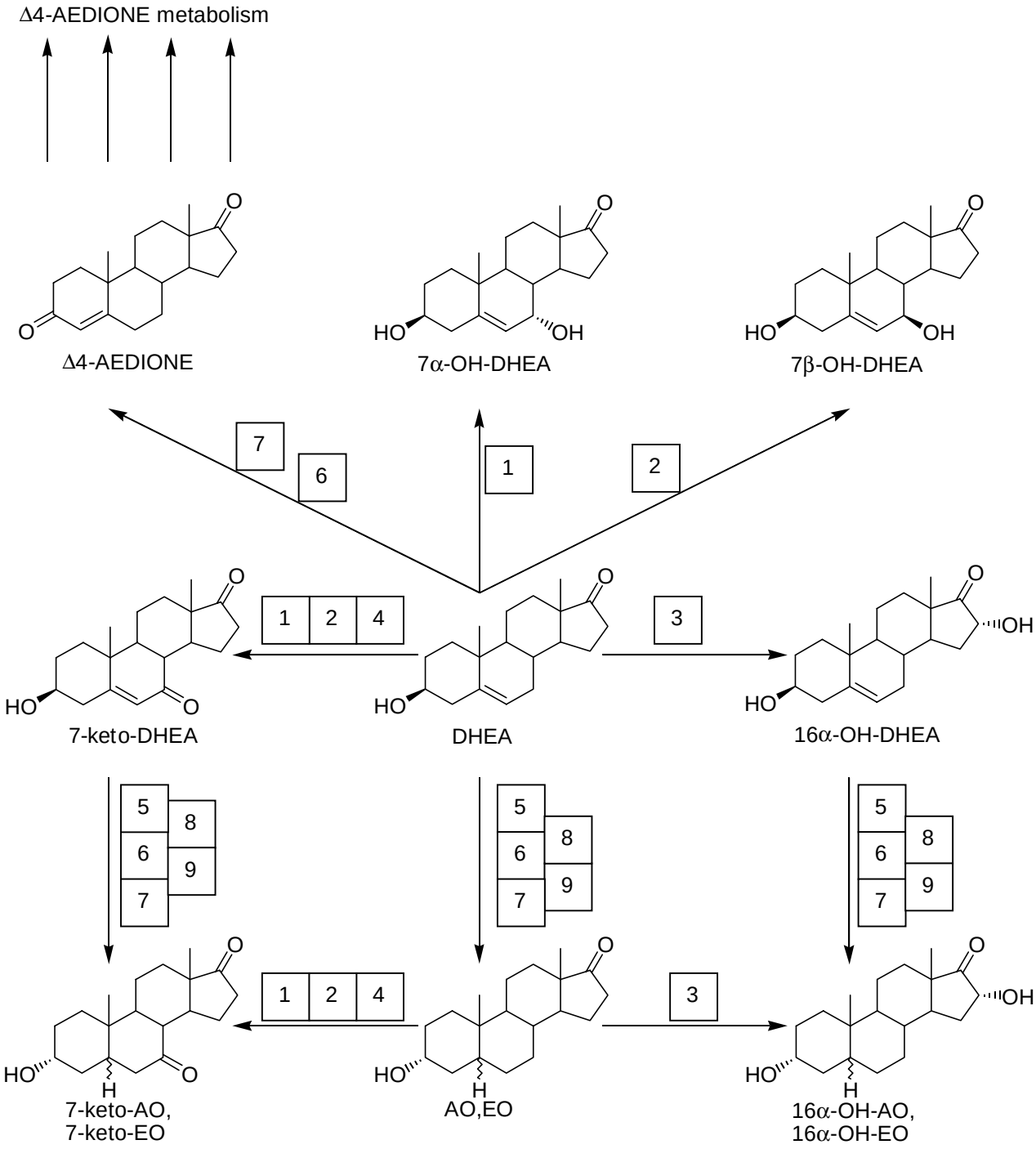

Figure 2: Suggested model for "specific" metabolism of DHEA. Involved enzymatic activities are: (1) 7α-hydroxylase; (2) 7β-hydroxylase; (3) 16α-hydroxylase; (4) 7-hydroxy dehydrogenase; (5) 3α-dehydrogenase; (6) 3β-dehydrogenase; (7) 4-5 isomerase; (8) 5α-reductase; (9) 5β-reductase

"SPECIFIC" METABOLISM OF DHEA AND Δ4-AEDIONE

Endoplasmic reticulum-bound cytochrome P-450 plays a central role in the oxidative metabolism of lipophilic compounds as steroids [20]. In mammals, the microsomal cytochromes are predominantly present in hepatic tissues, where they catalyze NADPH-dependent monooxygenation, *e.g.* hydroxylation. There are multiple pathways for this kind of steroid biotransformation, with a high degree of specificity, depending on the chemical properties of the steroid. Usually, not much knowledge is present about the possible endocrine function of such metabolites.

DHEA is readily converted to several oxygenated metabolites, as summarized in Figure 2. The main hydroxylation pathways are 7α-, 7β- and 16α-hydroxylation, resulting in 7α-hydroxy-DHEA (7α-OH-DHEA), 7β-hydroxy-DHEA (7β-OH-DHEA) and 16α-hydroxy-DHEA (16α-OH-DHEA) [21-25]. 7α-Hydroxylation is the first and rate-limiting step in the metabolic pathway of steroids, leading to bile acids [26]. Only suggestive biological relevance has been described for 7α- or 7β-hydroxylation of DHEA, as immunomodulatory action of 7α- and 7β-OH-DHEA in semen [27] and increased production of 7α-OH-DHEA in Alzheimer's disease [28].

Also 7-keto-DHEA has been detected in human urine samples [29]. It can be expected that 7-keto-DHEA is produced through 7α-, or 7β-hydroxy dehydrogenation of the mentioned 7α- or 7β-OH-metabolites of DHEA. Although no biological effect is known, this steroid is sold nowadays as food supplement steroid, either as 7-keto-DHEA or 3-acetyl-7-keto-DHEA (see Appendix). The commercially based claim for the biological effects are the same as for DHEA, but excluding androgenic side effects. No literature is available about the metabolism to 7-keto-androsterone (7-keto-AO) and 7-keto-etiocholanolone (7-keto-EO). Based on the described metabolic steps, these steroids can be expected as main metabolites of 7-keto-DHEA. However, the presence of these metabolites in human plasma or urine could also originate from direct 7-oxygenation of AO and EO.

16α-Hydroxylation of DHEA to 16α-OH-DHEA is one of the most described hydroxylation reactions of DHEA, as this metabolite is present in relatively high concentrations in urine [23,30,31]. It is a known intermediate product of estriol (1,3,5(10)-estratriene-3,16α,17β-triol) during pregnancy. Based on the presented metabolic steps, it is suggested that 16α-hydroxy-androsterone and -etiocholanolone (16α-OH-AO and 16α-OH-EO) could be detected as main metabolites of 16α-OH-DHEA. However, as mentioned before, these metabolites could also be produced by direct 16α-hydroxylase conversion of AO and EO [32].

Another reported minor oxygenation pathway is 18-hydroxylation, as determined in *in vitro* incubation experiments with human liver microsomes [33]. No further *in vivo* data are available.

Several oxygenation routes are described for Δ4-AEDIONE. A summary is given in Figure 3. Based on *in vitro* experiments with liver microsomes the best-described reactions are 6β-, 7α-, 16α- and 16β-hydroxylation [20,34]. The fact that the same pathways are found for the hydroxylation of T [34,35] suggests that these reactions are specific for androst-4-ene-3-one steroids. For both steroids, the major part is accounted by 6β-hydroxylation (≥ 70% for Δ4-AEDIONE [20]). This is also the case for some synthetic derivatives of T [36]. No data is available about 6α-hydroxylation and metabolic conversion to 6-keto-Δ4-AEDIONE. However, analogous to DHEA metabolism, the presence of 6-keto-metabolites could occur after dehydrogenation of 6β-OH-Δ4-AEDIONE.

In order of decreasing conversion rate, hydroxylation at C^{16} of Δ4-AEDIONE is the second route [20,34,35]. In contrast to DHEA metabolism, 16α- as well as 16β-hydroxylation takes place at Δ4-AEDIONE. Also, multiple hydroxylation to 6β,16α-diol and 6β,16β-diol metabolites has been reported for rats [37]. As is also suggested above for the metabolism of DHEA, likely products to be expected are the 16α- and 16β-hydroxy metabolites of AO and EO.

Described by Ryan *et al.* [38], local production of estrogens occurs from androgens, catalyzed by an aromatase complex in human placenta tissue. However, this reaction has also been established *in vitro* in microsomes obtained from other tissues [38]. The conversion of Δ4-AEDIONE to estrone (3-hydroxy-1,3,5(10)-oestratriene-17-one) is expected to occur with 19-hydroxy-Δ4-AEDIONE (19-OH-Δ4-AEDIONE) as one of the intermediate products or byproducts [39,40]. Kelly *et al.* [41] showed that 19-OH-Δ4-AEDIONE is readily excreted in urine as glucuronide and sulfate conjugate. The conversion of Δ4-AEDIONE to 19-OH-Δ4-AEDIONE is, however, relatively small and it is expected that little 19-OH-Δ4-AEDIONE leaves the site of aromatization. It cannot be ruled out that conjugation occurs at C^{19} [40], and it is unknown whether these conjugates can be deconjugated by the applied enzymatic β-glucuronidase and solvolysis methods of hydrolysis [40,41].

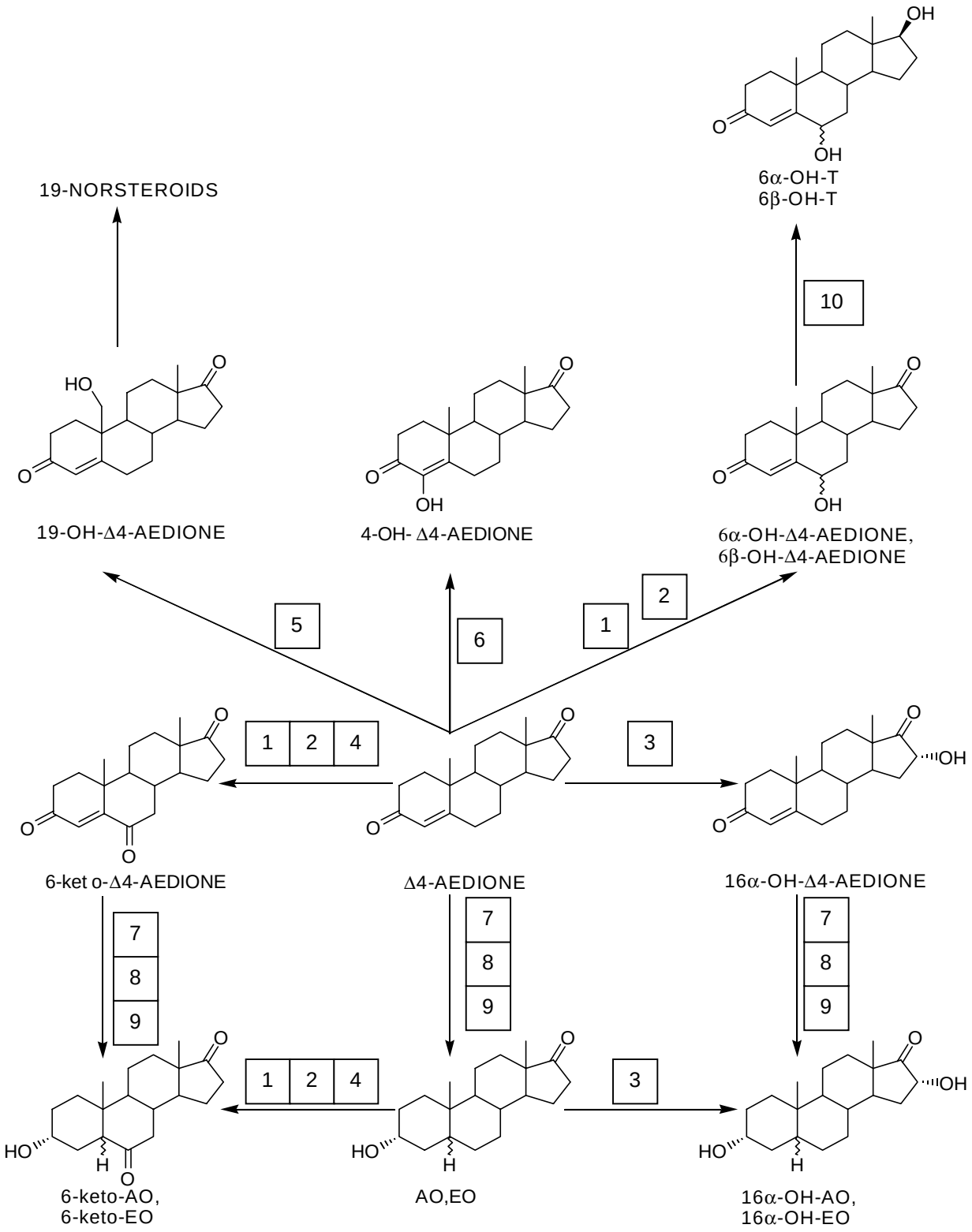

Figure 3: Suggested model of "specific" metabolism of Δ4-AEDIONE. Supposedly involved enzymatic activities are: (1) 6α-hydroxylase; (2) 6β-hydroxylase; (3) 16α-hydroxylase; (4) 6-hydroxy dehydrogenase. (5) 19-hydroxylase; (6) 4-hydroxylase; (7) 3α-dehydrogenase; (8) 5α-reductase; (9) 5β-reductase; (10) 17β-dehydrogenase

During aromatization also small quantities of 19-norsteroids (19-nortestosterone and 19-nor-Δ4-AEDIONE) are produced. This has been shown by *in vitro* tests on aromatase rich tissues as the ovarian follicle [42] and by the placenta [43]. Dehennin *et al.* [1] posed the production of small quantities of 19-nor-steroids by less aromatase rich tissues as adipose tissue, skin, testis, adrenal, liver and muscle. Based on this theory, production of 19-norsteroids could be accompanied by production of similar quantities of 19-OH-Δ4-AEDIONE and 19-OH-T. Detection of these steroids could perhaps aid the analytical discrimination between urine samples taken after 19-norsteroid administration and samples containing only 19-norsteroids of endogenous origin. For this purpose, quantification of 19-hydroxy-steroids down to low ng/ml levels is necessary.

Metabolism of estrogens by hydroxylation at C^4 is an important metabolic step, leading to the production of catecholestrogens [44,45]. Hydroxylation at the Δ4-double bond in Δ4-AEDIONE, leading to 4-hydroxy-Δ4-AEDIONE (4-OH-Δ4-AEDIONE) has never been reported. During development studies on this steroid as aromatase inhibitor for treatment of breast cancer, no 4-OH-Δ4-AEDIONE was detected as endogenous substance [46]. A limit of quantification of 0.3 ng/ml was reported by Dowsett *et al.* for the applied radioimmunoassay of non-conjugated 4-OH-Δ4-AEDIONE [47]. This was done by an assay for Δ4-AEDIONE that showed 25% cross-reactivity for 4-OH-Δ4-AEDIONE. This suggests that 4-hydroxylation either does not occur, or in very small amounts, or that conjugation prevents radioimmunoassay detection.

Poon *et al.* [48] showed that orally administered 4-OH-Δ4-AEDIONE shows extensive first and second phase metabolism. This suggests that if production of 4-OH-Δ4-AEDIONE would occur after Δ4-AEDIONE administration, this would probably lead to efficient excretion as conjugates. As conjugation occurs at C^4, it is not clear whether enzymatic deconjugation results in a high recovery. Concluding, insufficient results are available to exclude the endogenous origin of 4-OH-Δ4-AEDIONE and the conversion to 4-OH-Δ4-AEDIONE after oral administration of Δ4-AEDIONE.

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