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Mass Spectrometric Characterization of Synthetic Steroid Derivatives

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Introduction

The problem of “designer steroids” triggered an avalanche in sport as well as the scientific world in October 2003, when the doping control laboratory at UCLA identified a compound related to gestrinone[1]. Its hydrogenation at the ethinyl residue results in a steroid hormone termed *tetrahydrogestrinone* (THG), which is considered an analogue to the anabolic agent trenbolone, but physiological effects and side effects of THG were never clinically studied. Commonly employed strategies in doping control laboratories analyzing mainly approved or currently investigated remedies may need amelioration to cope with the willingness of some athletes to cheat in order to win by a short head against competitors. Unknown derivatives of remedies, such as THG, are invested it with invisibility for conventional procedures.

In the present study, commercially available steroid hormones of particular structures (e.g. trenbolone, gestrinone, etc.), synthesized analogues (e.g. THG, ethyltestosterone), known or putative metabolites as well as stably deuterated analogues were studied by means of electrospray ionization tandem mass spectrometry providing detailed structure information and possibilities to characterize unknown compounds related to steroid hormones.

Experimental

Steroids. 5α -Androst-1-en-17 β -ol-3-one, gestrinone and altrenogest were obtained from Thinker Chemical Co. Ltd. (Hangzhou, China). Trenbolone, norgestrel, ethisterone, norethisterone, testosterone, nortestosterone, metenolone and methyltestosterone were purchased from Sigma-Aldrich (Steinheim, Germany). 19-d_3 -Testosterone was a generous gift from Dr. R. Kazlauskas from the Australian Drug and Sport Testing Laboratory (ADSTL). 5α -Androst-1-en-3,17-dione was obtained from Steraloids (Newport, Rhode Island), and ethyltestosterone, d_4 -ethyltestosterone, norbolethone, dihydrogestrinone, tetrahydrogestrinone, d_4 -tetrahydrogestrinone, propyltrenbolone, and $1\alpha,2\alpha$ -dideutero-testosterone were prepared in our laboratory.

Mass spectrometry. Commercially obtained as well as synthesized steroids were dissolved in a mixture of 0.1% acetic acid and acetonitrile (1:1, v:v) at concentrations of 5 $\mu\text{g/mL}$ and analyzed by electrospray ionization (ESI) and collisionally activated dissociation (CAD) on an Applied Biosystems QTrap instrument (Darmstadt, Germany). Nitrogen was employed as curtain and collision gas ($5.33\text{e-}3$ Pa) delivered from a Whatman K75-72 nitrogen generator, and samples were introduced into the mass spectrometer by means of a syringe pump at a flow rate of 5 $\mu\text{L/min}$. Declustering potentials were optimized for respective protonated molecules, and collision energies (CE) were adjusted to enable efficient fragmentation of analytes retaining the precursor ion at a relative abundance of approximately 10% in product ion spectra. Unit resolution was employed for mass selection in Q1, and the linear ion trap was operated at a scan speed of 1000 u/s.

Aliquots of H/D-exchange experiments were dissolved only in acetonitrile and introduced into the mass spectrometer according to the conditions described above.

Results

ESI-product ion experiments with all analytes were performed at room temperature, and in Figure 1, the resulting MS/MS spectra of gestrinone, THG, norgestrel and ethyltestosterone are shown exemplarily.

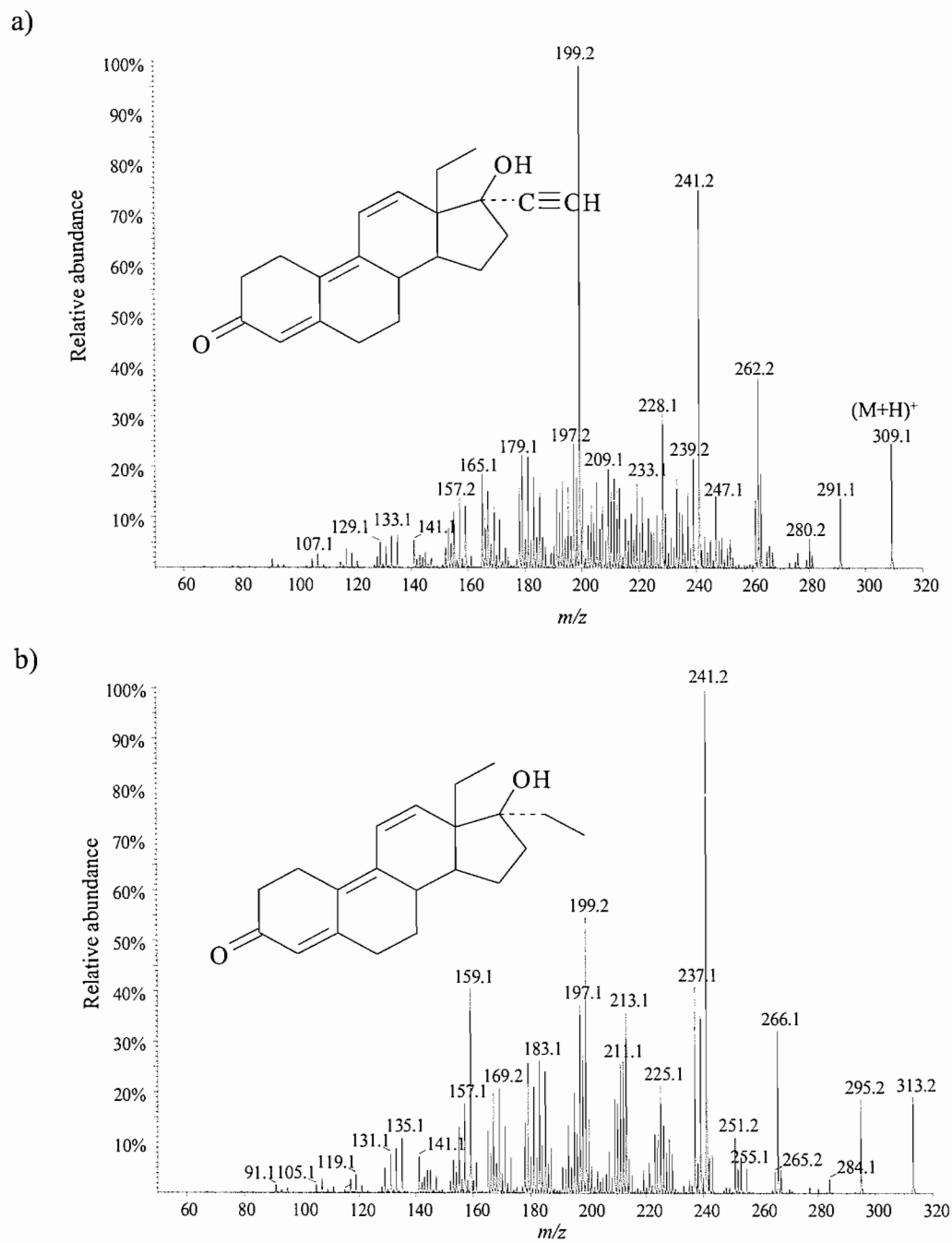


Figure 1: ESI-product ion spectra of a) gestrinone (mol wt = 308, CE = 30), and b) tetrahydrogestrinone (mol wt = 312, CE = 30).

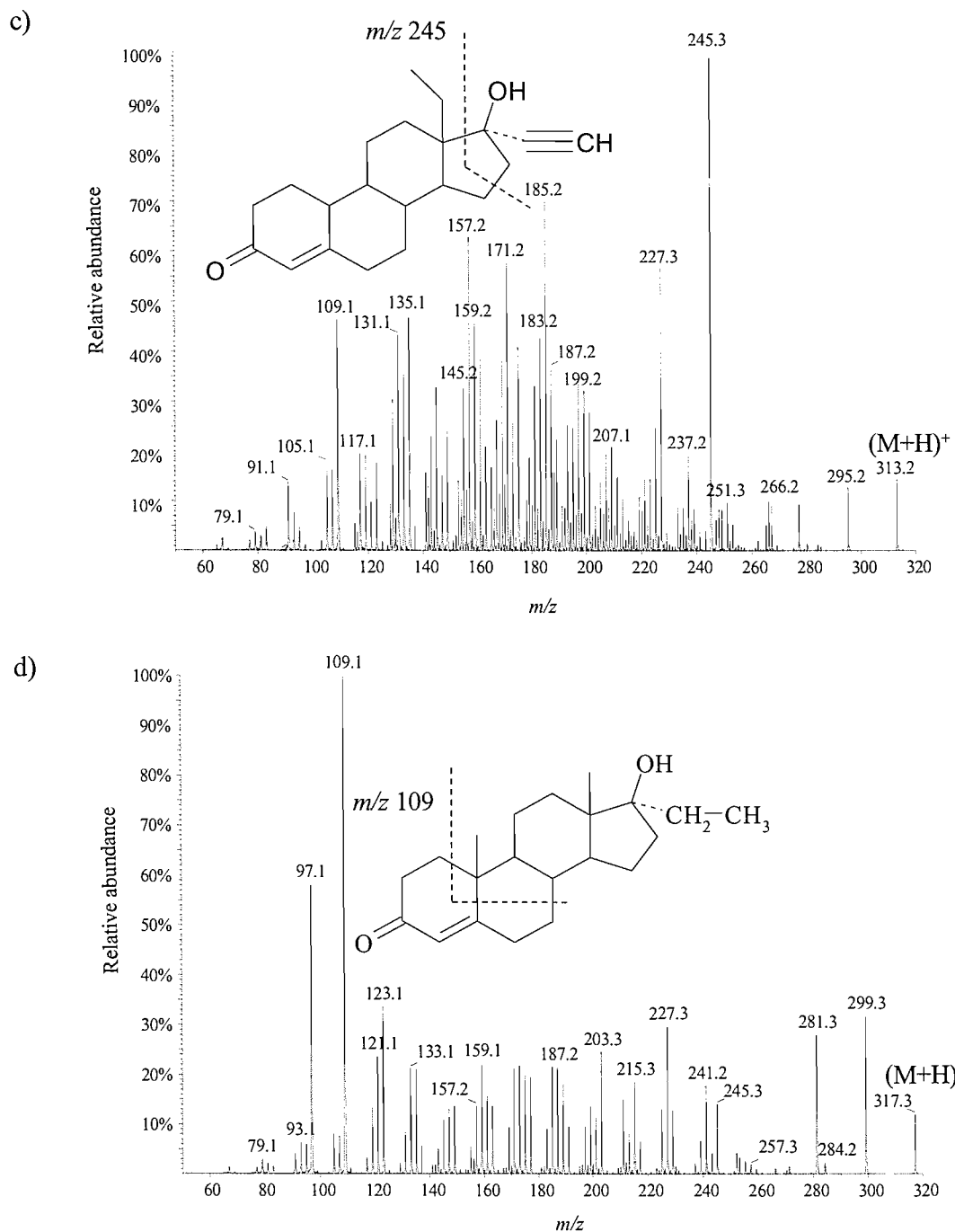
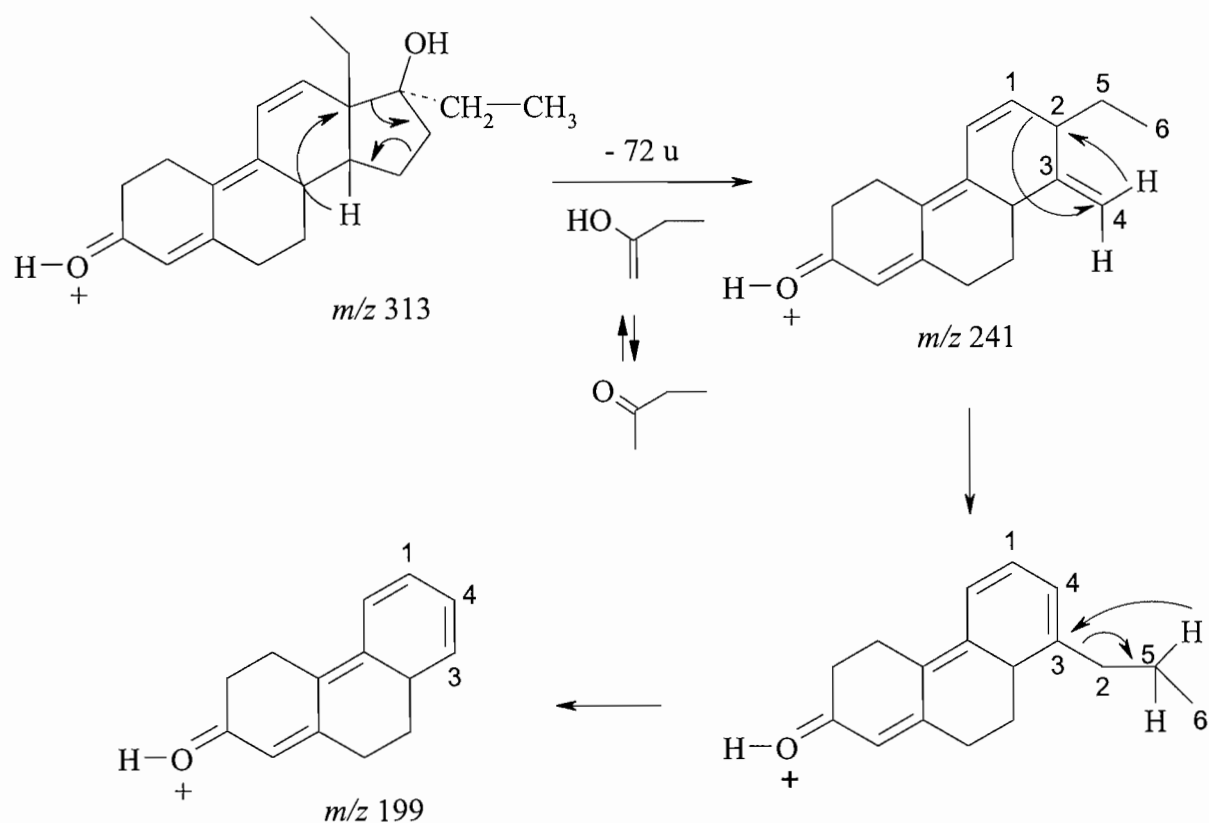


Figure 1 (continued): ESI-product ion spectra of c) norgestrel (mol wt = 312, CE = 30), and d) ethyltestosterone (mol wt = 316, CE = 30).

Steroids with 4,9,11-triene nucleus. This class of steroids includes compounds such as gestrinone, THG, trenbolone, altrenogest and propyltrenbolone, and the principal fragmentation behavior of representatives will be discussed by means of the product ion spectrum of THG (Figure 1, b). The protonated molecule $(M+H)^+$ at m/z 313 gives rise to a

variety of abundant fragment ions upon CAD. The neutral loss of water (-18 u), presumably originating from C-17, generates the product ion at m/z 295. Here, no hydrogen from the ethyl residue linked to C-17 is involved in the elimination process as the deuterated analogue (d_4 -THG, Figure 2a) also releases 18 u proving an origin of the hydrogen necessary for the neutral loss of water from a position remote of the ethyl group at C-17. The fragment at m/z 295 (Figure 1, a) subsequently releases an ethyl radical generating the ion at m/z 266. All gestrinone analogues expel 29 u from the fragment ion obtained from respective protonated molecules after elimination of water. Hence, the primary origin of the ethyl radical is proposed to be C-13 that is located in allylic position to a large conjugated 8- π -electron system explaining the stable character of the generated radicalic cation. THG and its deuterated analogue comprise additional ethyl side chains at C-17 amenable for elimination, and the product ion spectrum of d_4 -THG (Figure 2a) demonstrates that m/z 266 of THG is obtained by release of an ethyl residue originating either from C-13 or C-17 as product ions are found at m/z 270 and 266 upon CAD of d_4 -THG accounting for the loss of C_2H_5 and C_2D_4H , respectively.

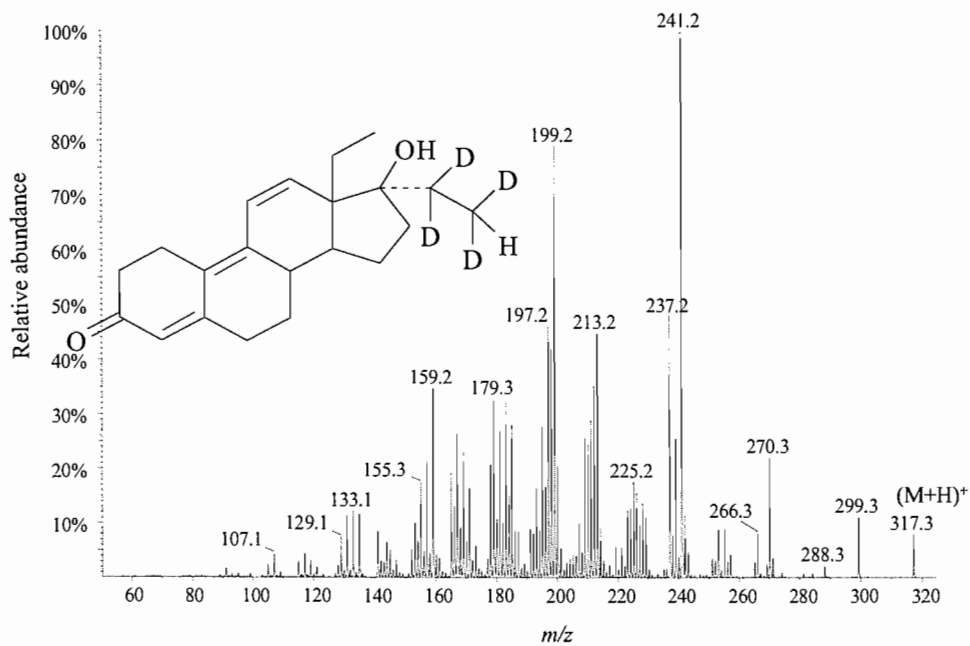
In contrast to the fragments generated by the losses of water and ethyl residues, ions at m/z 241 and 199 are present in all gestrinone analogues requiring a structure independent from the steroidal D-ring. Hence, a neutral loss of C-16 and C-17 including their substituents is postulated giving rise to m/z 241 as demonstrated in Scheme 1. The ion at m/z 199 results from a neutral loss of 42 u from m/z 241, also proven by MS^3 experiments. Here, an elimination of propene is suggested including the carbons C-13, C-18 and C-19, which requires a rearrangement of the C-ring. This fragment at m/z 199 in particular is observed in all product ion spectra of steroids with a 4,9,11-triene nucleus investigated in this study, whereas compounds bearing a methyl residue at C-13 instead of an ethyl group give rise to a fragment at m/z 227 instead of m/z 241 as for instance in case of propyltrenbolone (Figure 2b). These phenomena are in accordance to the fragmentation pathway proposed for the collisionally activated dissociation of gestrinone analogues, where m/z 241 includes the C-13-linked alkyl residue, and m/z 199 is generated by removal of this particular part of the molecule.



Scheme 1: Proposed generation of the fragment ions at m/z 241 and 199 of tetrahydrogestrinone

Steroids with 3-keto-4-ene nucleus. Testosterone and 19-nortestosterone analogues comprise 3-keto-4-ene nuclei and demonstrate a distinct difference in dissociation behavior compared to the class of 4,9,11-triene steroids. Product ion spectra of compounds related to testosterone contain abundant fragments at m/z 97 and 109 as found in the spectrum of ethyltestosterone (Figure 2d) for instance, the proposed generation of which was described for testosterone by Williams *et al.* in 1999[2]. To a smaller extent, also fragment ions generated by D-ring dissociation are found in respective product ion spectra, comparable to dissociation routes observed with steroids composed by a 4,9,11-triene nucleus. A characteristic A- and B-ring dissociation of 19-nortestosterone analogues is observed with the fragment at m/z 109. This ion corresponds to m/z 123 of testosterone analogues generated from A- and B-ring cleavages and is detected in all spectra of the investigated compounds related to 19-nortestosterone regardless of C-13 or C-17 substitution.

a)



b)

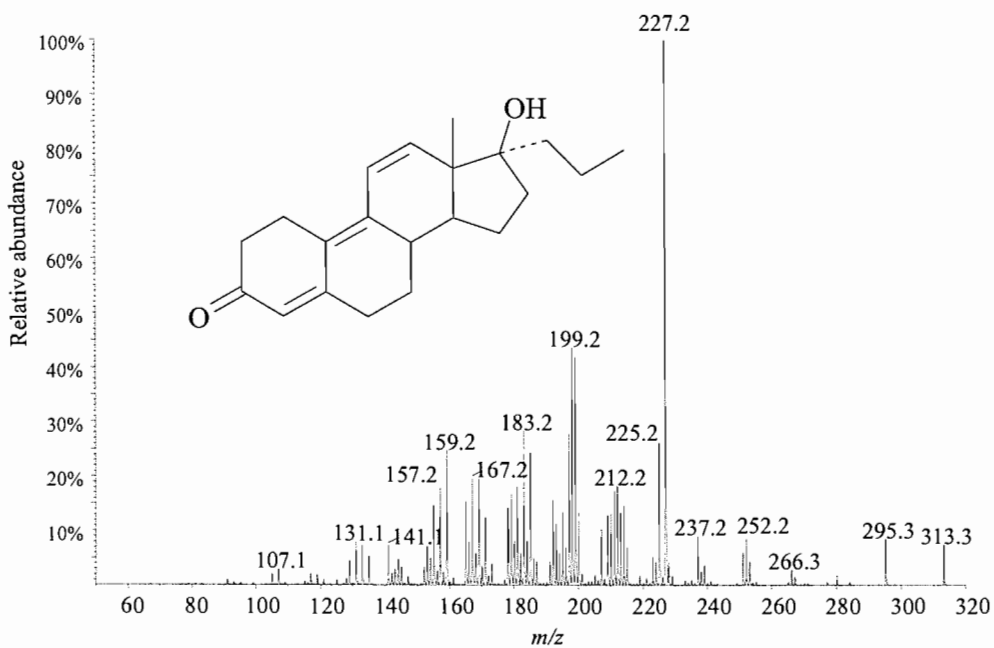


Figure 2: ESI-product ion spectra of a) d₄-THG (mol wt = 316, CE = 30), and b) propyltrenbolone (mol wt = 312, CE = 30).

Conclusion

ESI-CAD-MS/MS spectra of steroids with a 4,9,11-triene or a 3-keto-4-ene nucleus provide distinct information about the principle structure of the analyte. The detection of abundant product ions at m/z 241 and 199 or m/z 227 and 199 indicate the presence of a 4,9,11-triene steroid with a C-13-linked ethyl or methyl group, respectively. In addition, the neutral loss of 68 u from the protonated molecule is generally observed in case of a D-ring bearing a 17-ethynyl and 17-hydroxyl function. In contrast, in product ion spectra of testosterone analogues comprising a 3-keto-4-ene structure fragments generated by A- and B-ring cleavages are predominant, in particular the ions at m/z 97 and 109. With removal of the angular 19-methyl group giving rise to molecules related to 19-nortestosterone, product ion spectra are obtained that are not prevailed by A- and B-ring fragment ions but contain an ion at m/z 109 (corresponding to m/z 123 of testosterone analogues) as well as fragments generated by commonly observed D-ring fragmentation releasing C-16 and C-17 including their substituents.

Acknowledgments

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