


Detection of designer steroids

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Illicit use of performance-enhancing steroids has proliferated among a wide range of professional and amateur athletes. This problem has attracted broad public attention and has led the United States Congress to draft legislation that proposes frequent testing of athletes. However, current testing protocols are inadequate as athletes can evade detection by using novel steroids that are unknown to authorities. We have developed a strategy that overcomes this limitation by virtue of its ability to detect “designer steroids” without prior knowledge of their existence.

Received August 19th, 2005; Accepted October 14th, 2005; Published October 21st, 2005 | **Abbreviations:** AR: androgen receptor; DHT: dihydrotestosterone; GC/MS: gas chromatography/mass spectrometry; HR: House of Representatives; LC/MS: liquid chromatography/mass spectrometry; NCAA: National Collegiate Athletic Association; T: testosterone; THG: tetrahydrogestrinone; WADA: World Anti-Doping Agency | Copyright © 2005, Yuan and Forman. This is an open-access article distributed under the terms of the Creative Commons Non-Commercial Attribution License, which permits unrestricted non-commercial use distribution and reproduction in any medium, provided the original work is properly cited.

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Introduction

The use of performance-enhancing drugs has proliferated among elite professional athletes, Olympians and even high-school amateurs. Use of these agents are of particular concern as they are administered in the absence of the type of efficacy and safety testing that would be required for FDA approval. Thus, the ongoing abuse of these potentially toxic agents has aroused widespread public attention. On October 22, 2004, President George W. Bush signed the Anabolic Steroids Control Act into federal law. This act extended the definition of anabolic steroids to include “any drug or hormonal substance, chemically and pharmacologically related to testosterone”. As a result, compounds that meet this definition are now considered a controlled substance under federal law. Continued abuse by professional athletes and others has prompted two separate Congressional committees to draft additional legislation that is further aimed at curtailing this problem. If passed into law, the Clean Sports Act (HR 2565) and the Drug-Free Sports Act (HR 1862) would mandate recurrent testing of professional athletes and would impose extended suspensions or lifelong bans for those who test positive [USHR, 2005].

The success of this legislation and/or other interventions assumes the availability of robust screens that can detect the presence of all performance-enhancing drugs. In the case of anabolic steroids, current approaches utilize sensitive assays such as GC/MS or ELISA to detect the presence of known steroids in biological samples. These assays suffer from a severe limitation: they require pre-existing knowledge of the precise compounds that are being abused. An athlete can evade detection by using an anabolic steroid that is not known to authorities [Handelsman, 2004]. Indeed, an emerging clandestine industry has allowed athletes to evade detection via the use of novel “designer steroids”. Several test-evading designer steroids have been identified in the past 3 years including two existing compounds that were never marketed [Sekera et al., 2005] and a novel chemical entity

that was specifically synthesized to evade detection (tetrahydrogestrinone, THG) [Catlin et al., 2004]. The latter drug has been implicated in the BALCO investigation.

Anabolic steroids function by binding to the nuclear androgen receptor (AR) [Handelsman, 2004]. A bewildering array of diverse synthetic ligands have been identified for AR and other nuclear receptors [Buijsman et al., 2005] and hundreds-to-thousands of never marketed AR ligands have been described in the patent and scientific literature. Moreover, existing high-throughput screening strategies could allow rogue scientists to produce additional designer steroids. It is possible that test-evading steroids are currently in use as these compounds cannot be identified until a “whistle-blower” steps forward and provides suspect samples to the authorities. It is evident that novel strategies must be developed that can detect illicit steroids without prior knowledge of their chemical structure.

Reagents and Instruments

Gas chromatography/mass-spectrometry (GC/MS)

A ThermoFinnigan Trace DSQ GC/MS system was used for these studies. Gas chromatographic separation was performed with a Phenomenex ZB-5 (5% phenyl-95% dimethyl-polysiloxane) column (15 meters, 0.25 mm ID, film thickness 0.50 μ m). Following data acquisition, total ion spectra found in the samples were compared to known spectra contained in the NIST 98 Library using the Finnigan Xcalibur software.

Methods

Isolation and identification of anabolic steroids

A single lot of normal human male serum (Gemini cat#100-512, Lot# H01904Y) and a single lot of normal human female serum (Gemini cat#100-110F, Lot# H00903Y) were used throughout these experiments in order to exclude interindividual differences as a source

of variation. Where indicated, serum was spiked with 0.1-10 nM nandrolone (Steraloids cat# E4050-000), 0.1-10 nM methandienone (Steraloids cat# A0130-020) and in some cases female serum was adjusted to contain concentrations of testosterone (T, 15 nM) and dihydrotestosterone (DHT, 3 nM) that are within the range normally present in healthy adult men [Griffen and Wilson, 1998]. For analysis, serum (2.5 ml) was extracted two-times with 2 volumes of ethyl acetate. The two ethyl acetate fractions were pooled, washed twice with water and then dried under nitrogen.

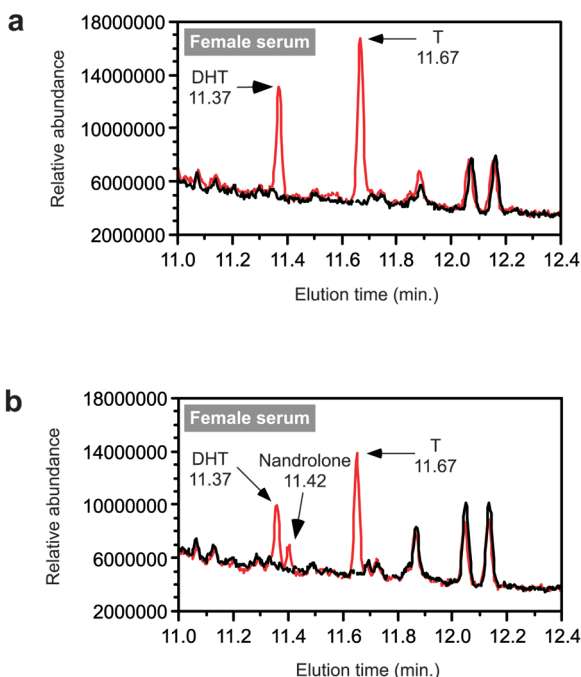


Figure 1. Detection of anabolic steroids in female serum. (a) Normal human female serum spiked with T (15 nM) and DHT (3 nM) was extracted and incubated with control (black line) or AR-containing beads (red line). Eluates were analyzed by GC/MS and the chromatograms were overlaid. The peaks corresponding to DHT (11.37 min) and T (11.67 min) were confirmed by comparing elution times and mass spectra with known standards. (b) Female serum containing 15 nM T, 3 nM DHT and 1 nM (0.27 ng/ml) nandrolone was analyzed as in (a). The elution time and mass spectrum of the 11.42 min. peak is indistinguishable from nandrolone.

The steroid-screening assay was performed by incubating the ethyl acetate fraction with agarose-bound androgen receptor ligand binding domain (AR). To do so, thioredoxin-linked AR (~30 μ g total protein; specific activity 2.2 pmol bound ligand/ μ g, Invitrogen) was incubated with 40 μ l Ni-NTA agarose (50% slurry) in a total volume of 300 μ l of buffer A (20mM Tris, pH 8, 500mM NaCl, 5mM imidazole) at 4 $^{\circ}$ C for 1 hr. Ni-NTA beads were pelleted and washed once with 1 ml of ligand binding buffer (30 mM Tris pH 8.0, 150 mM KCl) then resuspended in 300 μ l of ligand binding buffer containing 0.4 mg/ml ovalbumin. Immediately prior to assay, the dried ethyl acetate serum fraction was dissolved in 2 ml of ligand binding buffer by incubating for 30 min at room temperature and then incubated with the immobilized AR beads for 1 hr at room temperature followed by 1 hr at 4 $^{\circ}$ C. Immobilized AR was then pelleted and washed once

with 2 ml ligand binding buffer to remove any unbound ligand. To dissociate the bound ligands from receptor, immobilized AR was incubated for 30 min. at 65 $^{\circ}$ C in 400 μ l PBS and the released ligands were collected in the supernatant. This elution was repeated and the two PBS- fractions were pooled, filtered through a 0.22 μ m PTFE filter, and extracted two-times with 3-volumes of ethyl acetate. The organic phases were pooled and dried under nitrogen.

The dried samples were derivatized with 20 μ l of N,O-bis (trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (Pierce) at 55 $^{\circ}$ C for 6 hr, then 4 μ l were analyzed by GC/MS run in scanning mode over a M/Z range of 50-700. At the start of each run, the column temperature was held at 50 $^{\circ}$ C for 1 minute, increased using a gradient of 25 $^{\circ}$ C /min up to 300 $^{\circ}$ C, and held at 300 $^{\circ}$ C for an additional 8 minutes.

Results

We sought to develop a novel strategy to detect illicit steroids without prior knowledge of their chemical structure. To be useful in an anti-doping setting, these assays must also distinguish illicit androgens from their naturally occurring counterparts: testosterone (T) and dihydrotestosterone (DHT). To address this need, we sought to establish a sensitive assay that identifies anabolic steroids by virtue of their ability to bind to their pharmacologic target, AR. We then analyze the bound ligands by GC/MS which distinguishes the illicit drug from endogenous androgens. This strategy is distinct from existing strategies as detection is based on AR-binding activity, as opposed to chemical identity.

Our proposed strategy cannot be tested with actual samples due to legal restrictions on banned substances and the obvious difficulties in obtaining specimens from athletes who are actively in violation of competitive guidelines. Instead, we performed a proof-of-principle experiment using nandrolone (4-estren-17 β -ol-3-one) [Holterhus et al., 2002], a commercially available anabolic steroid that is prohibited by the World Anti-Doping Agency (WADA), the National Collegiate Athletic Association (NCAA) and by Anabolic Steroids Control Act of 2004. Human female serum was adjusted to contain concentrations of testosterone (T, 15 nM) and dihydrotestosterone (DHT, 3 nM) that are within the range normally present in healthy adult men [Griffen and Wilson, 1998]. This test serum was then incubated with agarose-bound AR or control beads. The beads were collected, washed and AR-bound ligands were eluted and analyzed by GC/MS. As expected, peaks corresponding to DHT (11.37 min) and T (11.67 min) were detected when using AR, but not with control beads (Figure 1a) or beads linked to estrogen receptor α (data not shown). It is important to note that no other endogenous steroids (e.g. glucocorticoids, estrogens, progestins, mineralocorticoids, etc.) were detected with AR despite the presence of these steroids in normal serum. Furthermore, AR failed to detect the presence of 17 β -estradiol even when excess quantities of this steroid (10 nM) were intentionally spiked into the serum (data

not shown). Taken together, these findings demonstrate that the proposed strategy selectively identifies natural androgens from the diverse background of endogenous compounds that are present in normal human serum.

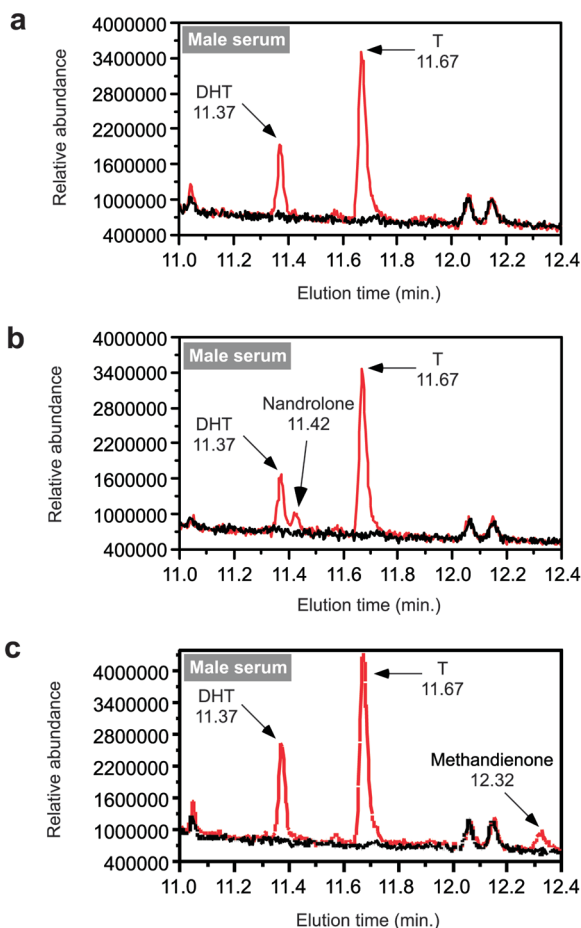


Figure 2. Detection of anabolic steroids in male serum. (a) Normal human male serum was extracted and incubated with control (black line) or AR-containing beads (red line). Eluates were analyzed by GC/MS and the chromatograms were overlaid. The peaks corresponding to endogenous DHT (11.37 min) and T (11.67 min) were confirmed by comparing elution times and mass spectra with known standards. (b) Male serum containing 1 nM (0.27 ng/ml) nandrolone was analyzed as in (a). The elution time and mass spectrum of the 11.42 min. peak is indistinguishable from nandrolone. (c) Male serum containing 1 nM (0.30 ng/ml) methandienone was analyzed as in (a). The elution time and mass spectrum of the 12.32 min. peak is indistinguishable from methandienone.

A separate experiment was performed to determine if this approach could be used to identify banned steroids. Female serum with T & DHT was spiked with 1 nM (0.27 ng/ml) nandrolone to mimic a “tainted” serum sample. As described below, 1 nM represents a very low concentration of drug – i.e., a concentration that is below the detection limits established for doping-controls labs. This low concentration was intentionally selected in order to examine the sensitivity of the assay. When this “tainted” sample was tested, an additional AR-specific peak (Figure 1b, 11.42 min) was identified that does not correspond to endogenous androgens (Figure 1a). Since this material binds AR but does not correspond to the endogenous androgens, one can conclude an illicit steroid is present in this sample.

In order to further validate the assay, we performed similar experiments using normal male serum. As expected, AR specifically captured endogenous T and DHT from normal male serum (Figure 2a). Since the serum used in this experiment was not modified in any way, this analysis further demonstrates that our assay is very robust, i.e. it can easily identify the physiological levels of endogenous androgens that are normally present in male serum.

To confirm that illicit steroids can also be identified in male serum, we asked whether we could identify a foreign peak in male serum that had been tainted by addition of 1 nM nandrolone. As was the case for female serum, when this “tainted” sample was tested, an additional AR-specific peak (Figure 2b, 11.42 min) was identified that is distinct from the endogenous androgens seen in normal serum (Figure 2a). The presence of a foreign peak that specifically bound to AR leads to the conclusion that an illicit anabolic steroid is present.

To further extend the usefulness of this approach, we asked whether our assay could detect other anabolic steroids. Methandienone (metandienone, dianabol, 17 β -hydroxy-17 α -methylandrosta-1,4-dien-3-one) provides an example of another commercially available steroid that is banned by WADA and the NCAA and controlled by the Anabolic Steroids Control Act of 2004. Again, very low concentrations of the drug (1 nM, 0.30 ng/ml) were intentionally used in order to challenge the sensitivity limits of the assay. As seen in Figure 2c, a foreign peak (12.32 min) is also detected in normal male serum spiked with 1 nM methandienone. Thus, our assay can detect low levels of several anabolic steroids that are banned by major athletic oversight agencies.

The ability to easily detect endogenous androgens in normal male serum (Figure 2a-c) indicates that our assay has sufficient sensitivity to detect biologically relevant concentrations of androgens. As a further test of our assay, we wished to determine whether our assay detects pharmacologically relevant concentrations of illicit steroids. WADA has established minimum required performance limits for doping-control laboratories [World Anti-Doping Agency, 2004]. For most anabolic steroids this detection limit is set at 10 ng/ml (~33 nM) though a lower detection limit of 2 ng/ml (6.6 nM) has been established for methandienone and several other steroids. The data in Figures 1-2 demonstrate that we can detect nandrolone and methandienone at concentrations of 1 nM which is 6-30 fold lower than the minimum limits prescribed by WADA. To further quantify the sensitivity of our assay, we spiked normal female serum with 0.1, 1 and 10 nM concentrations of nandrolone and methandienone and examined our ability to detect the captured steroid. Figure 3 represents a plot of the peak area versus the concentration of illicit steroid. Although we were unable to detect 0.1 nM (0.030 ng/ml) methandienone, we could detect peaks for all other concentrations including 0.1 nM (0.027 ng/ml) nandrolone. The 0.1 nM nandrolone peak was at the very lower limit of detection suggesting that a more practical detection limit for nandrolone is somewhere between 0.1 and 1 nM

(0.027 – 0.27 ng/ml). Thus, in the case of nandrolone the sensitivity of our assay is over 33-fold greater than WADA requirements and at least 6-fold greater for methandienone.

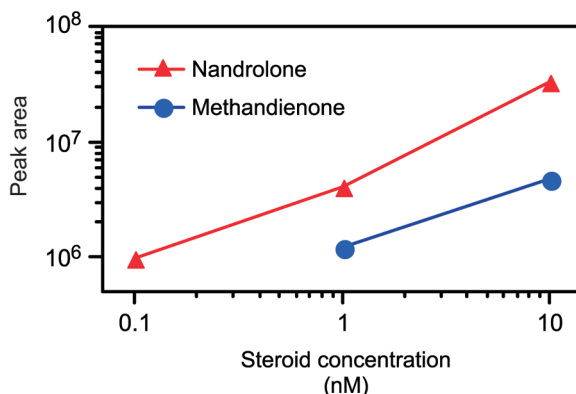


Figure 3. Sensitivity of anabolic steroid detection. Normal female serum was spiked with 0.1-10 nM (0.027 - 2.74 ng/ml) nandrolone or with 0.1-10 nM (0.030-3.00 ng/ml) methandienone and analyzed as in Figure 1a. The peaks areas corresponding to nandrolone (11.42 min) and methandienone (12.32 min) are plotted vs. steroid concentration.

A recent survey has suggested that athletes may resort to polypharmacy when abusing steroids [Perry et al., 2005]. This prompted to us ask whether our assay could simultaneously detect two banned substances in a single sample. In principle, detection of multiple substances should be possible provided that the amount of AR in the assay is sufficiently high relative to the total androgen pool in the tainted sample. The final concentration of active AR in our assay is 29 nM (see Methods). This concentration is higher than the low nanomolar concentrations of steroids that would typically be present in our assay conditions. To examine this situation experimentally, we spiked normal female serum (Figure 4a) and normal male serum (Figure 4b) with 1 nM of both nandrolone and methandienone and asked whether two foreign peaks could be identified in an AR-specific manner. In both cases, the assay was able to detect the presence of foreign peaks that do not correspond to the endogenous androgens. These data further demonstrate the utility of our assay in detecting small amounts of illicit steroids from both male and female serum.

Many anabolic steroids like nandrolone and methandienone possess high affinity (low nanomolar) for the androgen receptor [Holterhus et al., 2002]. As with other steroid-related drugs, higher affinity AR ligands would generally be preferred pharmaceutical agents as they are less likely to bind to and modulate other related nuclear receptors. Nonetheless, it remains possible that lower affinity designer steroids could potentially be introduced to athletes. As a final test of our assay we asked whether a lower affinity compound could be detected. Previous studies have shown that 5 α -androstan-3 α ,17 β -diol can activate AR though high concentrations (1 μ M) are required for maximal activation [Forman et al., 1998]. As shown in Figure 5, our assay could successfully detect the presence of 1 μ M 5 α -androstan-3 α ,17 β -diol in male serum. Thus, even

lower affinity AR ligands can be detected by this methodology.

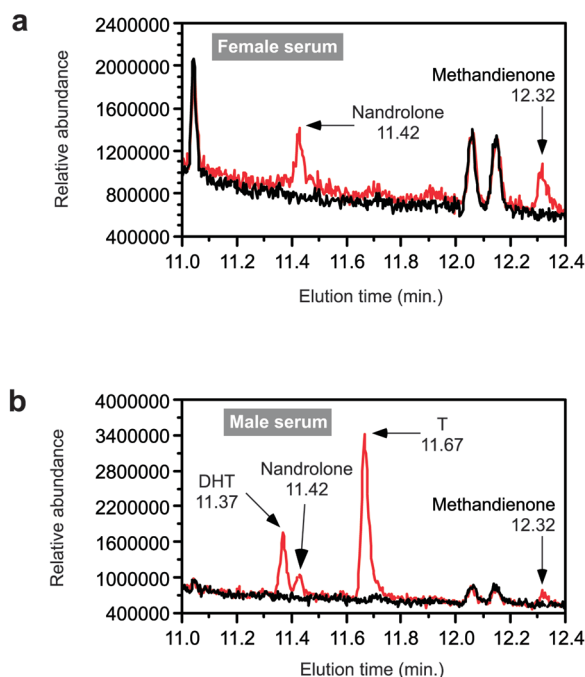


Figure 4. Simultaneous detection of multiple anabolic steroids. (a) Normal female serum was simultaneously spiked with 1 nM nandrolone (0.27 ng/ml) and 1 nM methandienone (0.30 ng/ml) and analyzed as in Figure 1a. (b) Normal male serum spiked as in (a). The peaks corresponding to nandrolone (11.42 min) and methandienone (12.32 min) are noted. Control beads: black line; AR-containing beads: red line.

Discussion

The above findings demonstrate the unique utility of our strategy relative to current steroid testing strategies. Existing approaches screen serum samples for known chemical substances. As a result, athletes can effectively evade the current testing regimens by utilizing novel compounds that are not yet known to the authorities. The demand for such material has spawned a rogue pharmaceutical industry whose sole purpose is to supply athletes with anabolic steroids that go under the radar of existing detection strategies.

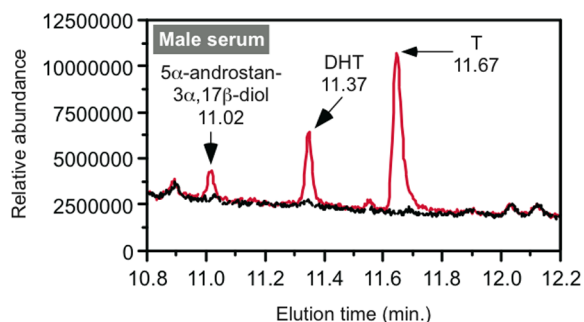


Figure 5. Detection of low affinity anabolic steroids. Normal male serum was spiked with 1 μ M 5 α -androstan-3 α ,17 β -diol and analyzed as in Figure 1-2. Peaks corresponding to DHT (11.37 min) and 5 α -androstan-3 α ,17 β -diol (11.02 min) are noted. Control beads: black line; AR-containing beads: red line.

This underground industry has been remarkably successful. The availability of high-throughput AR-ligand screens and a vast scientific and patent literature on androgenic steroids, raises legitimate concern that other novel compounds could currently be in use, while others may be developed in the future. Indeed, the full-extent of the current problem is not fully known because the only designer compounds that can be identified are those where there was a “whistle-blower” who alerted authorities. Even in cases where such individuals exist and are willing to step forward, chemical characterization of the new steroid can only be accomplished if these individuals have access to fairly large quantities of relatively pure material. It is clear that a new strategy is required that can efficiently detect the levels of novel anabolic steroids that would be present in the biological fluids of abusing athletes. Furthermore, for such a strategy to be effective it must be able to distinguish synthetic and natural steroids and it must also detect the synthetic steroids without prior knowledge of their chemical structure.

The technique we present addresses these requirements. Rather than searching for the chemical signatures of known anabolic steroids, our approach identifies the illicit drug by virtue of its ability to bind AR. We could successfully detect pharmacologically relevant concentrations of nandrolone and methandienone in a background of normal human serum; detection limits were well within the 2-10 ng/ml range specified by WADA. For other compounds, the precise sensitivity of our approach will vary depending on the compound, the extraction scheme and/or the separation technique utilized (e.g. LC/MS vs. GC/MS). Nonetheless, our approach is likely to be of general utility as these same analytical techniques, and their corresponding limitations, are routinely applied toward the identification of known steroids in sample specimens. Thus, our strategy is likely to have an appropriate range of sensitivity for many compounds, but with the added value of detecting unknown steroids.

It should be noted that our assay is only designed to detect AR-binding compounds: it is not designed to detect inactive pro-drugs. This is unlikely to be a practical limitation as biological activity requires that sufficient quantities of the pro-drug be converted into the biologically active AR-binding compound. Thus, while our assay would miss the pro-drug, the data provided suggest that it would detect the active metabolite.

Our designer steroid assay deserves further consideration and/or field-testing as it has implications for emerging anti-doping legislation and for international athletic testing guidelines. This assay may also have utility for testing dietary supplements that may potentially contain designer steroids as undeclared ingredients [Maughan, 2005; Parr et al., 2004].

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