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Review

Mass spectrometry of selective androgen receptor modulators

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Nonsteroidal selective androgen receptor modulators (SARMs) are an emerging class of drugs for treatment of various diseases including osteoporosis and muscle wasting as well as the correction of age-related functional decline such as muscle strength and power. Several SARMs, which have advanced to preclinical and clinical trials, are composed of diverse chemical structures including arylpropionamide-, bicyclic hydantoin-, quinoline-, and tetrahydroquinoline-derived nuclei. Since January 2008, SARMs have been categorized as anabolic agents and prohibited by the World Anti-Doping Agency (WADA). Suitable detection methods for these low-molecular weight drugs were based on mass spectrometric approaches, which necessitated the elucidation of dissociation pathways in order to characterize and identify the target analytes in doping control samples as well as potential metabolic products and synthetic analogs. Fragmentation patterns of representatives of each category of SARMs after electrospray ionization (ESI) and collision-induced dissociation (CID) as well as electron ionization (EI) are summarized. The complexity and structural heterogeneity of these drugs is a daunting challenge for detection methods. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: sport; doping; mass spectrometry; SARMs; orbitrap; anabolics

INTRODUCTION

The desire to reverse or slow age-related maladies in men has been stimulus for scientific research for more than 140 years. Reports from 1869, about suggestions to inject semen into the blood of elderly men to improve mental and physical powers, and subsequent experiments with saline extracts of dog testicles demonstrated the overwhelming desire to discover a chemical fountain of youth.[1] Although these attempts were not successful for a variety of endocrinological and chemical reasons, the hormone quest had started.[2] Following the discovery of the anabolic androgenic principal testosterone in 1935,[3,4] a major goal of early[5,6] and recent[7,8] research in steroid biochemistry was the separation of anabolic and androgenic effects, as well as probing for tissue selectivity of potential therapeutics that would enable the treatment or prevention of debilitating diseases, e.g. muscular dystrophy, benign prostate hyperplasia[9] or osteoporosis.[10–12] A scientific breakthrough in this regard was accomplished in 1998 with the determination of anabolic properties of nonsteroidal agents that were derived from androgen receptor (AR) antagonists such as bicalutamine (Fig. 1, **1**)[13] and flutamide (Fig. 1, **2**),[9] both of which include an arylpropionamide nucleus. The first selective androgen receptor modulators (SARMs) resulting from these studies were S-1 and S-4 (Fig. 1, **3** and **4**, respectively), which were later termed Andarine and Ostarine, respectively. In addition to these SARMs, numerous other chemical structures were found to possess SARM-like activities,[14] and currently at least four categories of nonsteroidal AR agonists have entered preclinical or clinical trials. These groups of compounds are classified, on the basis of their chemical core structures, into (1) arylpropionamides, (2) bicyclic hydantoins, (3) quinolines, and (4) tetrahydroquinolines; however, new substances with SARM properties are constantly reported, and there is great medicinal interest in this novel class of therapeutics.[12] Major advantages of SARMs over steroids in replacement therapies are the considerably reduced undesirable effects such as hepatic toxicity, decreased levels of HDL cholesterol, gynecomastia, and negative influences on prostate and cardiovascular systems.[12] Moreover, SARMs have demonstrated full anabolic activity in target tissues such as muscles and bones, as well as a considerable gain in lean body mass concomitant with a dose-dependent increase in functional performance.[15] On the basis of these facts, the World Anti-Doping Agency (WADA) added SARMs to the Prohibited List in January 2008.[16] Subsequently, doping control laboratories were urged to establish screening and confirmation procedures and/or implement the newly

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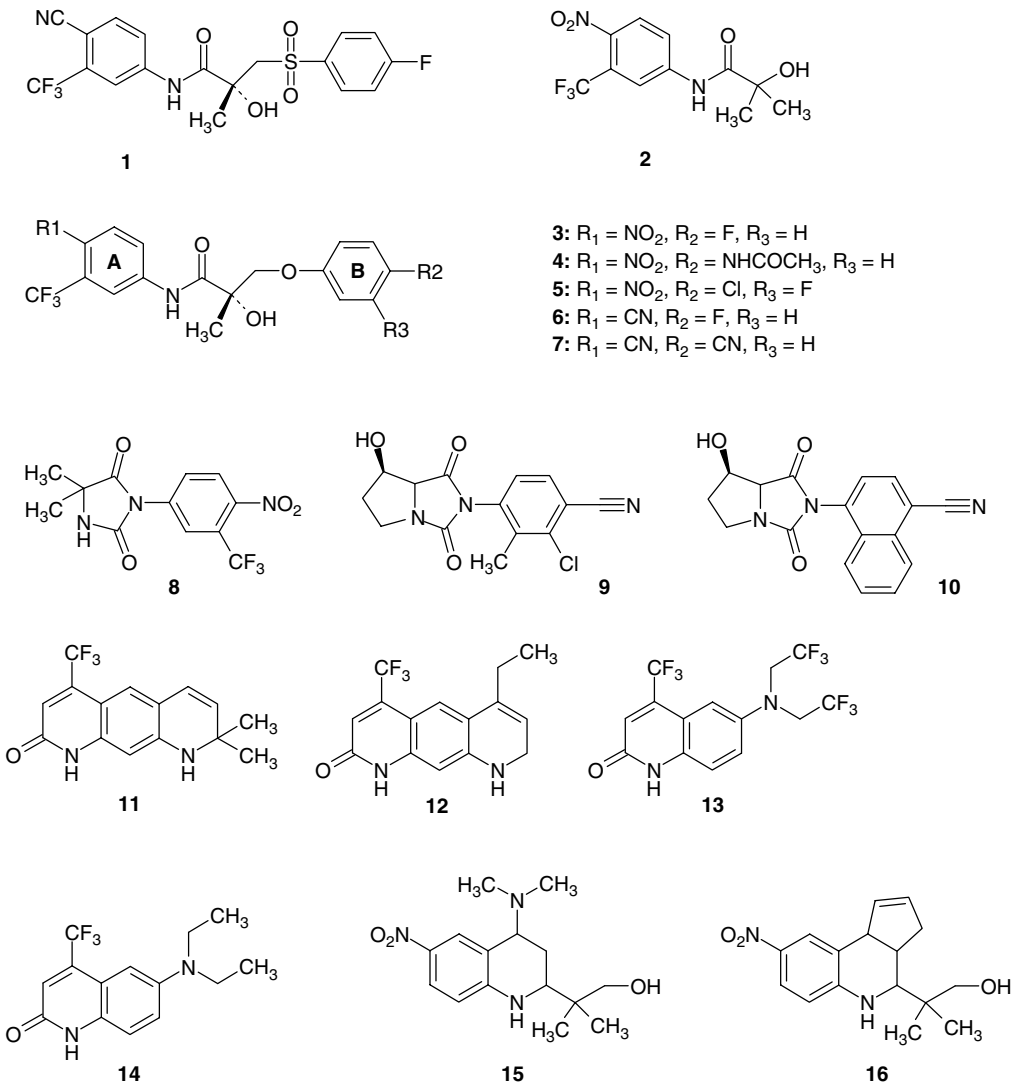

Figure 1. Chemical structures of arylpropionamide-derived selective androgen receptor modulators with antagonistic [**1** (Bicalutamide) and **2** (Flutamide)] and agonistic activity (**3**–**7**) – advanced representatives of the latter are Andarine (**3**) and Ostarine (**4**); hydantoin-derived androgen receptor antagonists [Nilutamide (**8**)] and agonists [BMS-564 929 (**9**) and **10**]; 2-quinolinone-derived SARMs with antagonistic (**11**) and agonistic [LG 121 071 (**12**), LGD 2226 (**13**), and **14**] activity; and tetrahydroquinoline-derived SARMs with bicyclic (S-40 503, **15**) and tricyclic (**16**) nuclei.

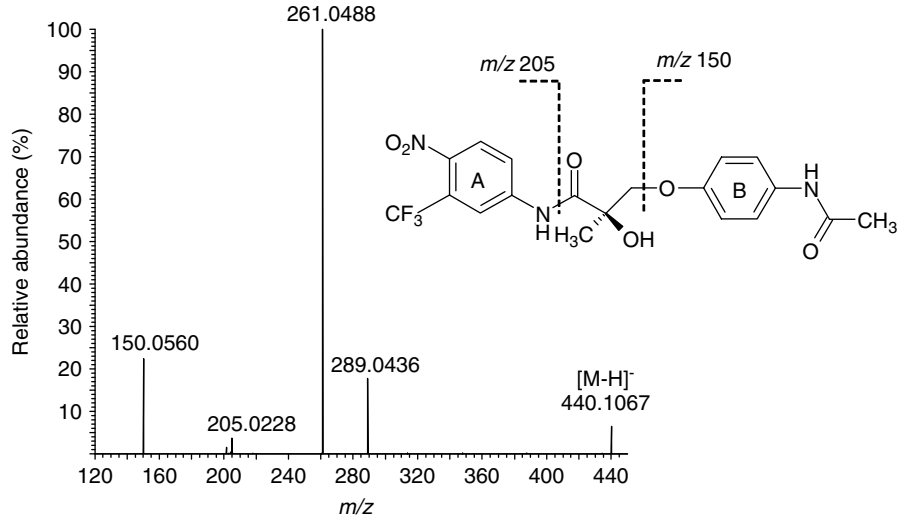

Figure 2. ESI product ion spectrum of $[M - H]^- = 440$ of Ostarine recorded on an LTQ Orbitrap.

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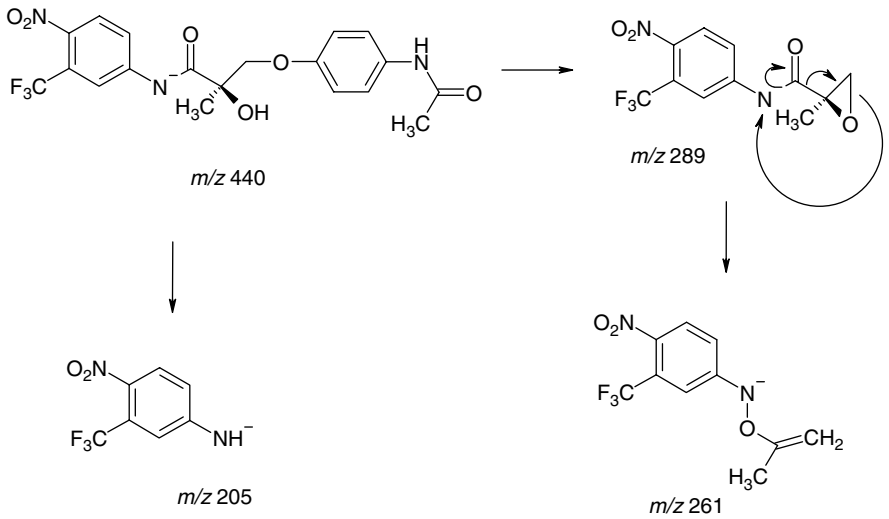

Scheme 1. Principal dissociation pathways of arylpropionamide-based SARMs demonstrated with Ostarine (**4**).

added target compounds into existing assays.[17] As most detection methods applied to doping control samples are based on mass spectrometric approaches,[18–20] information about the behavior of these new analytes under different ionization and dissociation conditions are of utmost importance. In this review, we present the mass spectra of selected SARMs with arylpropionamide-, bicyclic hydantoin-, quinoline-, and tetrahydroquinoline-based structures using electrospray ionization (ESI) with collision-induced dissociation (CID) or electron ionization (EI).

ELECTROSPRAY IONIZATION – MASS SPECTROMETRY

Using soft ionization techniques such as ESI, protonated or deprotonated molecules of the selected SARMs were generated. The CID of these precursor ions in MS/MS and MS^n experiments provided comprehensive structural information, which will facilitate the identification of such compounds, related substances and potential metabolic products in doping control samples.

Arylpropionamide-derived SARMs

Several arylpropionamide-derived SARMs, described as promising drug candidates,[21] have structural differences limited to the number and nature of ring substituents as outlined with selected examples in Fig. 1 (**3**–**7**). All of these substances are efficiently deprotonated, using ESI, yielding abundant $[M - H]^-$ ions.[22] Employing CID, diagnostic product ions were generated that allowed the characterization of both aromatic ring systems (A and B) as illustrated with the product ion mass spectrum of Ostarine (Fig. 2). Deprotonation of SARMs bearing propionanilide-derived nuclei is suggested to occur at the amide nitrogen due to the acidity of the respective hydrogen, which results from significant electron-withdrawing inductive effects (–I-effects) exerted by substituents such as the trifluoromethyl- and nitro-functions. The deprotonated molecule of **4** eliminates *N*-(4-hydroxyphenyl)-acetamide yielding the product ion at m/z 289,[23] which corresponds to m/z 269 in case of analytes with a cyano residue located at R_1, and the loss of

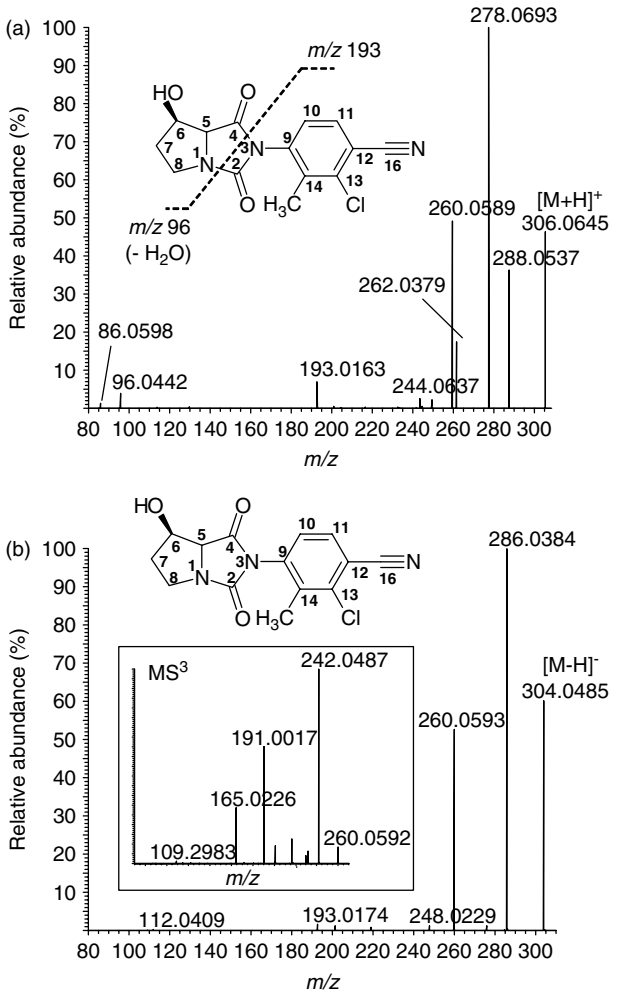

Figure 3. ESI product ion spectra of (a) $[M + H]^+ = 306$ and (b) $[M - H]^- = 304$ of BMS-564 929 recorded on an LTQ Orbitrap. The inset of (b) contains the MS^3 spectrum generated from *m/z* 260.

the B-ring is followed by the release of carbon monoxide giving rise to the most abundant product ion at m/z 261 (corresponding to m/z 241 with R_1 = CN).[24] The A-ring

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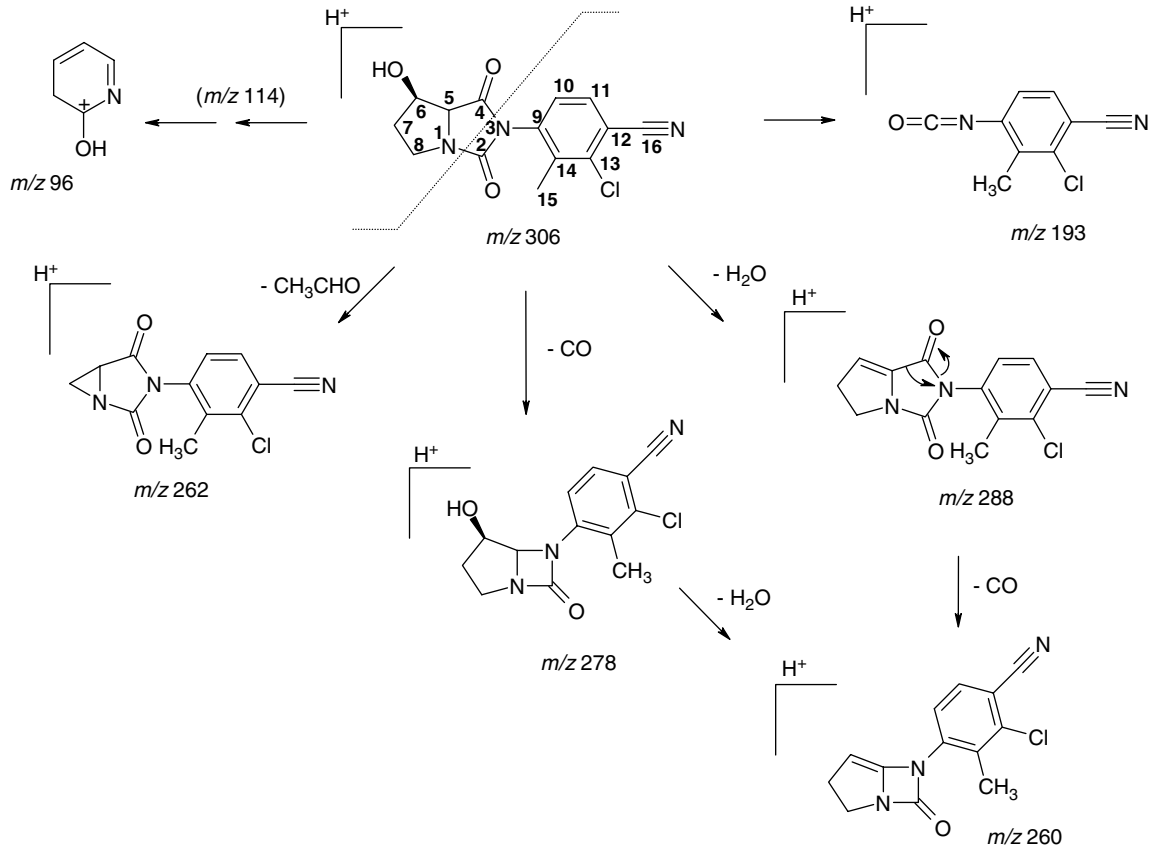
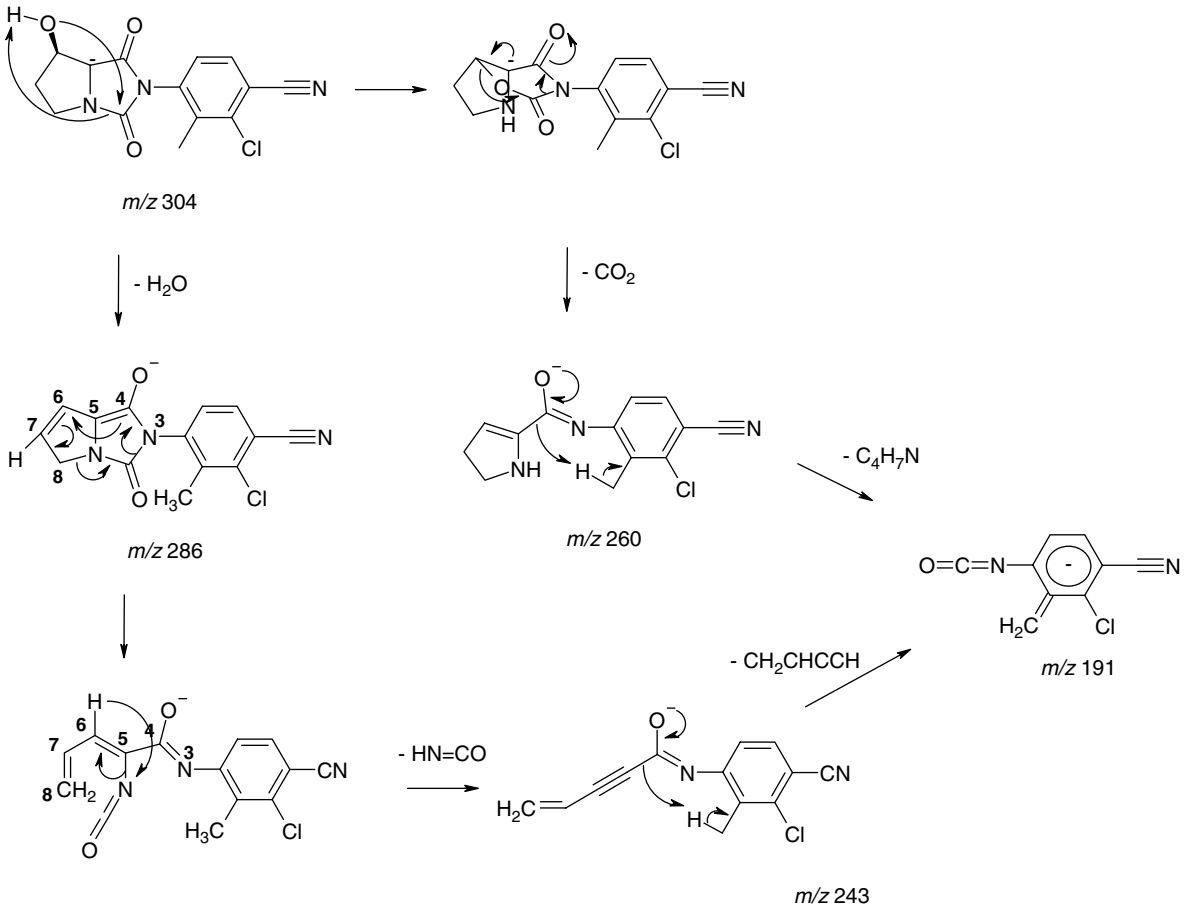


Scheme 2. Fragmentation pattern of the protonated hydroxybicyclic hydantoin-derived SARM BMS-564 929 (**9**).



Scheme 3. Dissociation pathways of deprotonated hydroxybicyclic hydantoin-derived SARMs BMS-564 929 (**9**).

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is further characterized by the formation of product ions corresponding to bisubstituted and deprotonated anilines, e.g. 4-nitro-3-trifluoromethyl-aniline at m/z 205 in case of **4** (Fig. 2). A corresponding product ion at m/z 185 was found in CID-spectra of **6**. The elucidation of the B-ring structures resulted from the generation of product ions representing the deprotonated and substituted hydroxyphenyl residues, which yielded a product ion at m/z 150 in case of **4** (Fig. 2, R_2 = $NHCOCH_3$, R_3 = H).[23,24] The principal dissociation route of arylpropionamide-derived SARMs is summarized in Scheme 1. Using a few but diagnostic product ions, modifications of either ring system can be detected, allowing the identification of metabolic products[23,25,26] as well as modified designer analogs.

Bicyclic hydantoin-derived SARMs

Hydroxybicyclohydantoin-derived SARMs are structurally related to hydantoin-based AR antagonists such as Nilutamide (Fig. 1, **8**), but the presence of a hydroxylated five-membered ring, e.g. in BMS-564 929 (Fig. 1, **9**), enables excellent AR binding affinities with activating properties and high muscle-tissue selectivity.[27–29] Drug candidates with bicyclic hydantoin core (Fig. 1, **9** and **10**) are readily protonated as well as deprotonated using ESI, and product ion mass spectra are diagnostic (Fig. 3).

Hydantoins are likely protonated at either of the nitrogens or the carbonyl oxygens[30] with a thermodynamically favored initial *O*-protonation.[31] The complex structure of respective SARMs, however, modifies the proton affinity of N-3 resulting in a preferred protonation at N-1 and carbonyl residues as substantiated by density functional theory (DFT) calculations.[32] Still, the mobile nature of protons (mobile proton model[33,34]), particularly after the excitation of ionized molecules under CID conditions, allows dissociation pathways starting from both options. BMS-564 929 (**9**) yields a protonated molecule at m/z 306, which eliminated water (18 u) and carbon monoxide (−28 u) in either sequence to generate product ions at m/z 288, 278 and 260 (Fig. 3(a)). Moreover, the loss of acetaldehyde is characteristic of a 6-hydroxylated bicyclic hydantoin such as **9**, while other analogs with 7-hydroxylation, for instance, are lacking this particular fragment in CID spectra.[32] Additional product ions found at m/z 193 and 96 are complementary fragments originating from a cleavage of the hydantoin core following the fission of the linkages between N-1 and C-2 as well as N-3 and C-4.[30] Evidence for this dissociation route was obtained by the analysis of stable isotope-labeled analogs to **9** (Scheme 2).[32]

Using negative ionization, deprotonation of **9** is suggested to result from the abstraction of the protons located either at C-5 or the hydroxyl function at C-6. Under CID conditions, the MS^2 product ion spectrum contains only few but informative ions, specifically characterizing the structure of BMS-564 929 (Fig. 3(b)), which was substantiated by MS^3 experiments. The loss of carbon dioxide (−44 u) from $[M-H]^-$ (m/z 304) yielding the product ion at m/z 260 requires a complex rearrangement involving an intermediate six-membered ring structure. The hydroxyl function located at C-6 plays a key role as analogs lacking this residue do not show the loss of CO_2. Subsequently, m/z 260 releases the newly formed 2,3-dihydro-1H-pyrrole (−69 u) residue

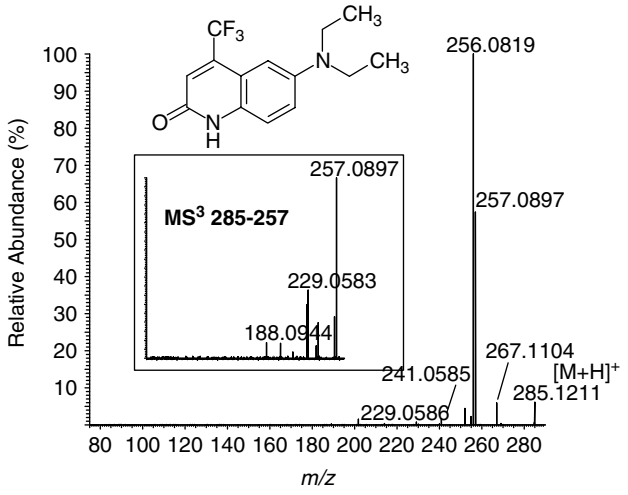
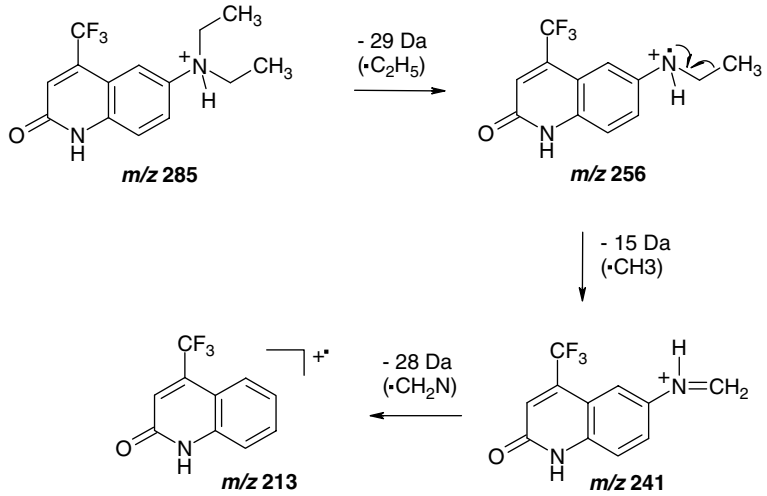


Figure 4. ESI product ion spectrum of $[M+H]^+ = 285$ of a bisalkylated 2-quinolinone-derived SARM recorded on an LTQ Orbitrap.



Scheme 4. Principal dissociation routes of protonated 2-quinolinone-derived SARMs demonstrated with compound **14**.

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yielding the product ion at m/z 191 (Fig. 3(b), inset). Complementarily, the deprotonated molecule of **9** eliminates water (−18 u), and consecutive losses of imino-methanone (−43 u) and but-1-en-3-yne (−52 u) also give rise to m/z 191, as summarized in Scheme 3.

Quinolinone-derived SARMs

Several bi- and tricyclic quinoline derivatives have agonistic SARM-like activity,[35–40] two of which (LG 121 071 and LGD 2226) are depicted in Fig. 1 (**12** and **13**, respectively). In analogy to AR antagonists such as LG 120 907 (Fig. 1, **11**), these include a 4-trifluoromethyl-2-quinolinone nucleus, but different C-ring substituents enable the activation of AR. The keto-function of the A-ring and the ethyl residue at the C-ring presumably mimic the 3-keto- and 17-OH-functions of testosterone.[21] Instead of a C-ring, LGD 2226 (Scheme 6, **13**) includes a 6-located bis(trifluoroethyl)amine residue at the 4-trifluoromethyl-2-quinolinone nucleus and demonstrated considerable tissue selectivity and AR binding affinities.[41,42] A comprehensive series with alternative alkylations such as bisethylation of the amino function (Fig. 1, **14**) has been tested.[40]

Quinolinone-derived SARMs are efficiently ionized using positive ESI, and common as well as unique dissociation pathways were observed for these compounds (e.g. substance **14**, Fig. 1).[43,44] The protonated molecule (m/z 285), preferably eliminated ethylene (−28 u) and an ethyl radical (−29 u), yielding the product ions at m/z 256 and 257 (Fig. 4). While the first mentioned pathway follows the commonly accepted even-electron rule,[43,45] the loss of the ethyl radical is attributed to the conjugated π-electron

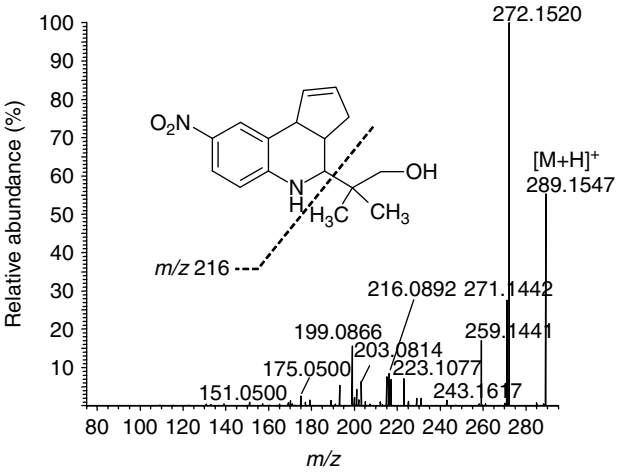
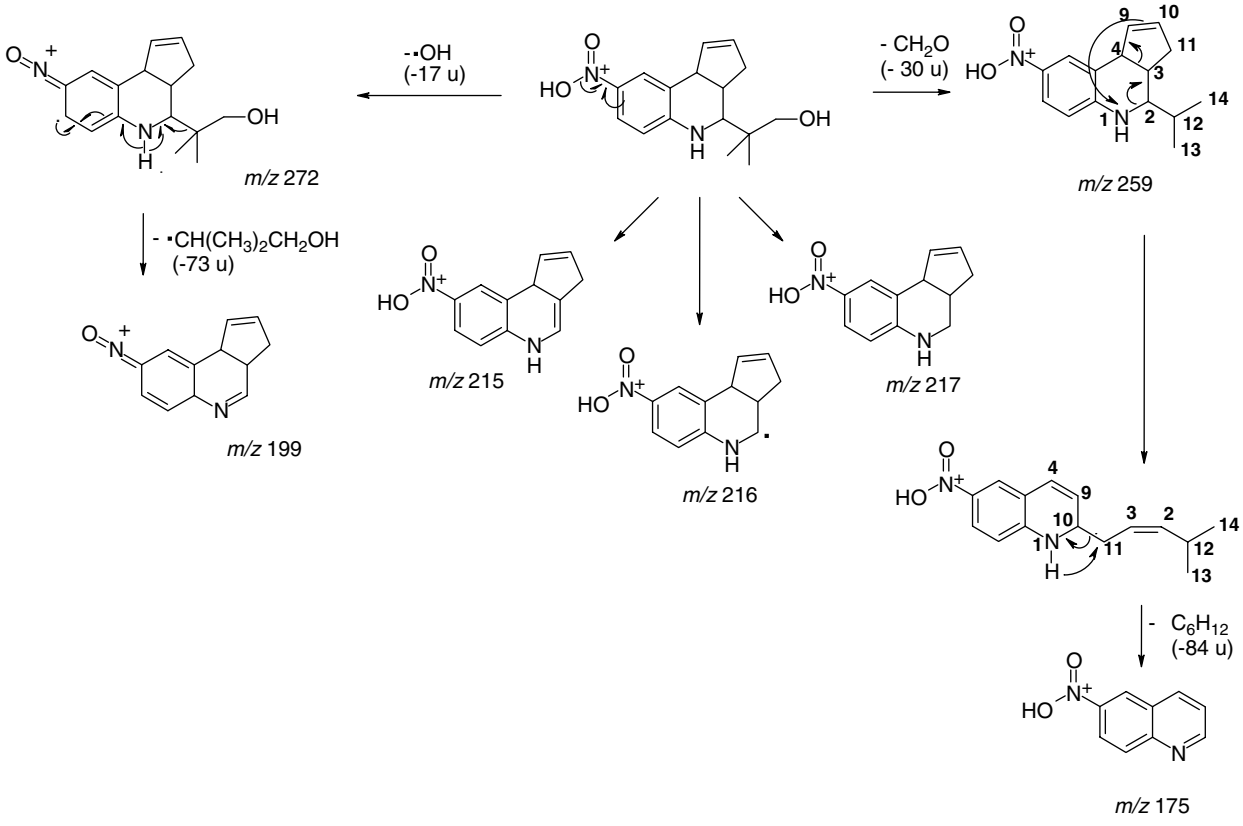


Figure 5. ESI product ion spectrum of $[M + H]^+ = 289$ of a tricyclic tetrahydroquinolinone-derived SARM recorded on an LTQ Orbitrap.

system of 2-quinolinones that promotes the generation of radical cations under CID conditions. The resulting odd-electron ion at m/z 256 further dissociates by eliminating a methyl radical (−15 u) to yield a core product ion at m/z 241 that represents the common nucleus of bisalkylated 4-trifluoro-2-quinolinones.[46] In subsequent MS^3 experiments, the even-electron product ion at m/z 241 released a methyleneamine radical (−28 u) giving rise to the product ion at m/z 213 (Scheme 4). The alternation between even- and odd-electron ions may be due to the unusual properties of 2-quinolinones to form stable radical cations. In contrast



Scheme 5. Principal dissociation pathways of protonated tricyclic tetrahydroquinoline-based SARMs as shown for compound **16**.

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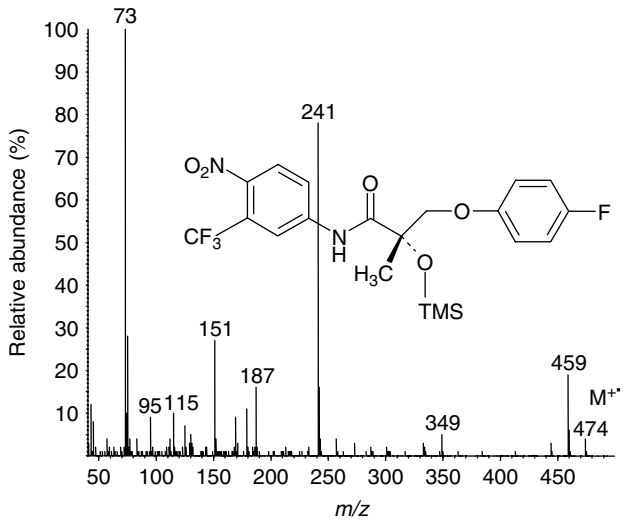


Figure 6. EI mass spectrum of the TMS-derivative of Andarine recorded on an Agilent 6890/5973 GC–MSD.

to bisalkylated 2-quinolinones, monoalkylated analogs were reported to yield a common product ion at m/z 228 (instead of m/z 241), which represents the 6-amino-4-trifluoromethyl-1H-quinolin-2-one core.[46] Precursor ion scanning utilizing diagnostic product ions such as m/z 241 and 228 should enable the detection of these known compounds as well as unknown, structurally related substances and metabolites.

Tricyclic tetrahydroquinoline-derived SARMs

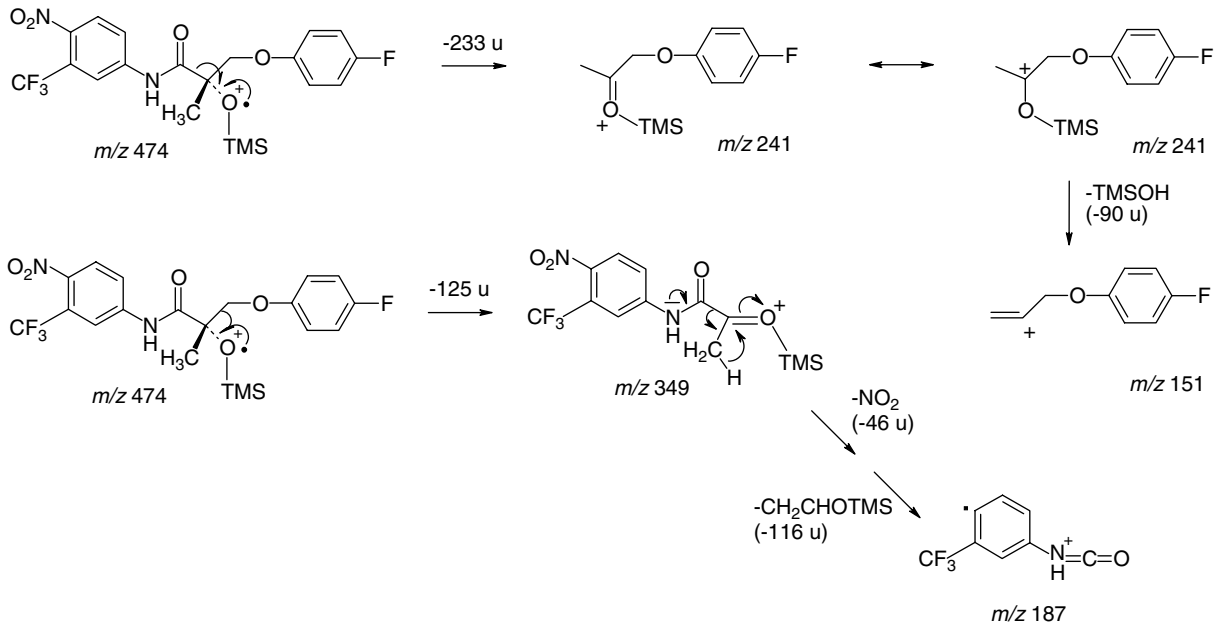
In addition to quinoline-based SARMs, tetrahydroquinoline-derived drug candidates were reported to possess tissue-selective AR agonist activity.[47,48] Two representatives are depicted in Fig. 1 (compounds **15** and **16**), and mass spectral data are available for the tricyclic derivative **16** (Fig. 5).[49] The protonated molecule at m/z 289 dissociates under CID conditions by the loss of a hydroxyl radical (−17 u) originating from the nitro function.[44] The site of initial protonation is not clear as the proton affinity of 1,2,3,4-tetrahydroquinoline of 225 kcal/mol[50] is higher than the corresponding affinity of nitrobenzene (164 kcal/mol),[51] but the –I-effect caused by the NO_2 residue is not accounted for. Moreover, based on the mobile proton model,[33] CID can induce proton migration and trigger dissociation processes at various sites of the molecule. Hence, the loss of the OH-radical might result from immediate protonation at the nitro function or after proton transfer. In addition to the loss of •OH, losses of water (−18 u), formaldehyde (−30 u), and the two-linked side chain with homolytic or heterolytic cleavages are observed yielding the product ions at m/z 271, 259 and 217, 216 and 215, respectively (Fig. 4). The elimination of the hydroxyl radical (m/z 272) is followed by the loss of a 2-methyl propanol radical (−73 u) yielding m/z 199, and the release of formaldehyde from the precursor ion resulting in the fragment at m/z 259 is followed by the loss of 4-methylpent-2-ene (−84 u) yielding 6-nitroquinoline (m/z 175), which necessitates an intramolecular rearrangement (Scheme 5). Dissociation pathways were supported by high-resolution/high-accuracy mass spectrometry and analysis of chemically synthesized standards.

ELECTRON IONIZATION – MASS SPECTROMETRY

GC–MS still plays an important role in sports drug testing. Most of the commonly employed doping control analytical methods using GC–MS require conversion of target analytes to trimethylsilylated (TMS) derivatives.[18,20,52–55] Elucidation of EI mass spectra of target analytes after trimethylsilylation is of great interest since the existing GC–MS-based procedures can be adapted for the analysis of many SARMs.

Arylpropionamide-derived SARMs

Andarine and Ostarine (Fig. 1, **3** and **4**), which represent advanced arylpropionamide-derived SARMs, have a



Scheme 6. Principal fragmentation routes of the TMS-derivative of compound **3** after EI.

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free hydroxyl function that is readily derivatized using trimethylsilylating agents. Consequently, the molecular weight of the analytes is incremented by 72 u, and dissociation pathways are considerably influenced by the presence of TMS residues (e.g. Andarine, Fig. 6). The molecular ion observed at m/z 474 eliminated a methyl radical (−15 u), yielding the ion at m/z 459, but cleavages of C–C bonds comprising the central chiral carbon atom predominated. The loss of a 1-fluoro-4-methoxy-benzene radical yielded the fragment ion at m/z 349, while the release of a *N*-(4-nitro-3-trifluoromethyl-phenyl)formamide radical gave rise to m/z 241.[49] Subsequent dissociations of m/z 349 or 241 yielded the ions at m/z 187 or 151 by losses of NO_2 (−46 u) and $CH_2CHOTMS$ (−116 u) or TMSOH (−90 u), respectively, as demonstrated in MS^3 experiments (Scheme 6).

Bicyclic hydantoin-derived SARMs

Trimethylsilylation of the hydroxybicyclohydantoin-derived SARM BMS-564 929 (Fig. 1, **9**) yields a molecule with a monoisotopic mass of 377 u, which decomposes using EI to product ions at m/z 362, 349, 321, and 129 (Fig. 7). The first two ions are attributed to the losses of a methyl radical (−15 u) and a molecule of carbon monoxide (−28 u), respectively. The latter was reported in earlier studies on the fragmentation of hydantoins,[56] but the elimination of 56 u yielding the ion at m/z 321 is distinctive for bicyclic hydantoins. This may result from the loss of propenal that necessitates the migration of the TMS residue from the hydroxyl function to the C-3-linked oxygen (Scheme 7). The most abundant fragment at m/z 129 is proposed to consist of the carbons C-6–C-8 including the O-TMS residue. This ion derived from the hydroxylated and condensed five-member ring structure was also described for various steroids bearing a 17-hydroxylated and TMS D-ring[18,57–59] and is characteristic of EI mass spectra derived from 6-hydroxylated bicyclic hydantoins.

Quinolinone-derived SARMs

The dissociation route of TMS-derivatives of 2-quinolinone-based SARMs after EI is primarily characterized by the loss

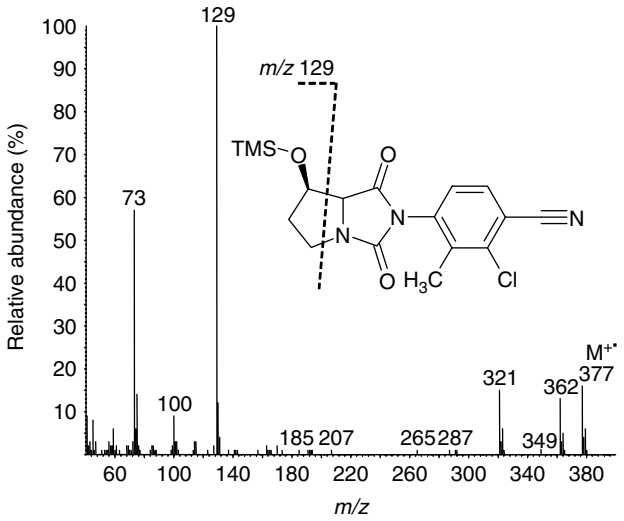
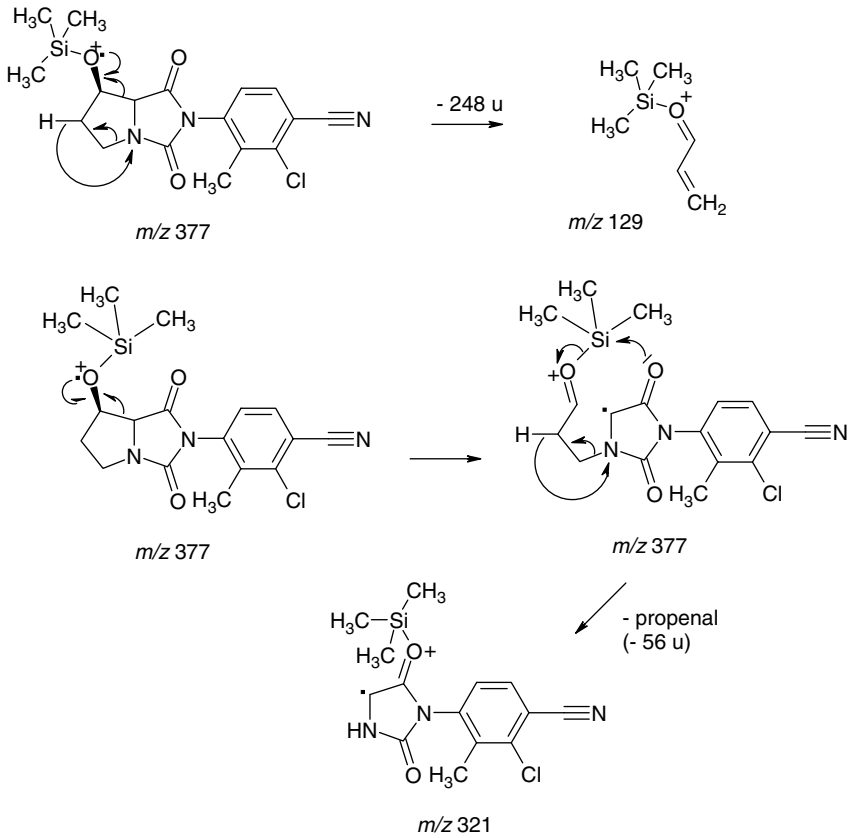


Figure 7. EI mass spectrum of the TMS-derivative of BMS-564 929 recorded on an Agilent 6890/5973 GC–MSD.



Scheme 7. Principal fragmentation routes of the TMS-derivative of compound **9** after EI.

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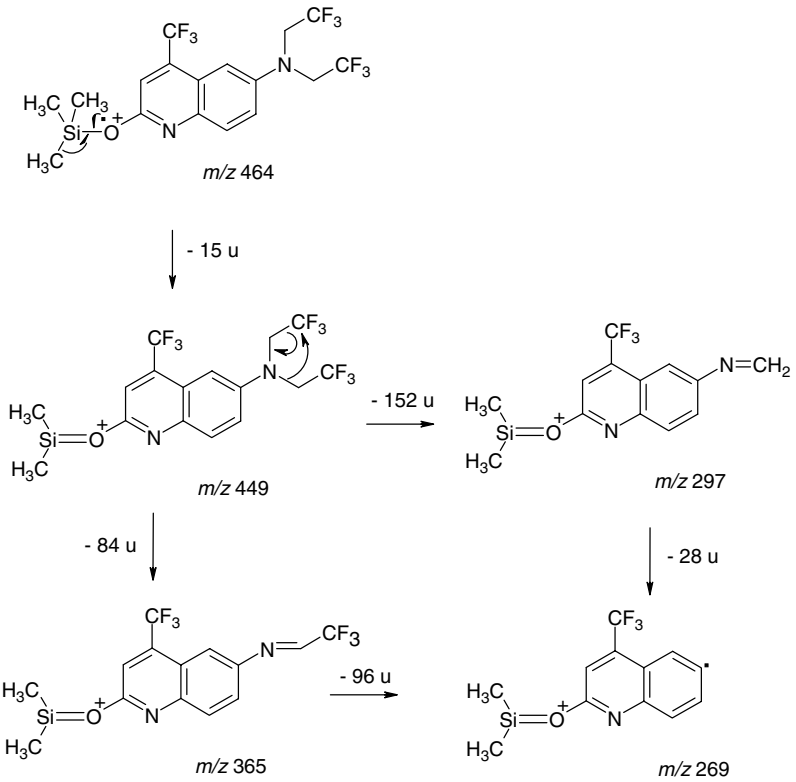

Scheme 8. Principal fragmentation routes of the TMS-derivative of compound **13** after EI.

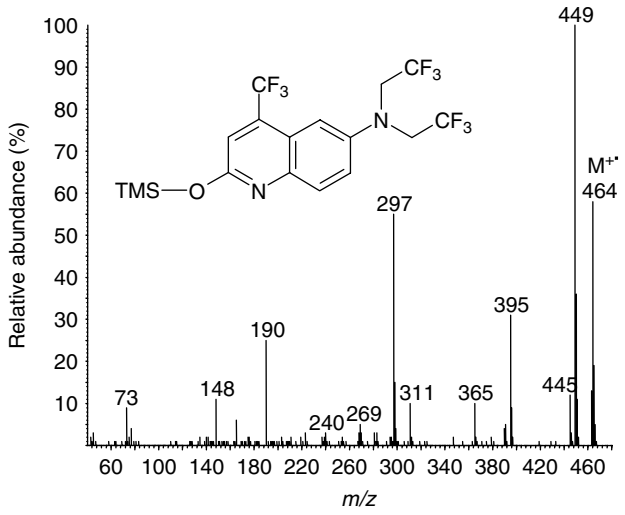

Figure 8. EI mass spectrum of the TMS-derivative of LGD-2226 recorded on an Agilent 6890/5973 GC–MSD.

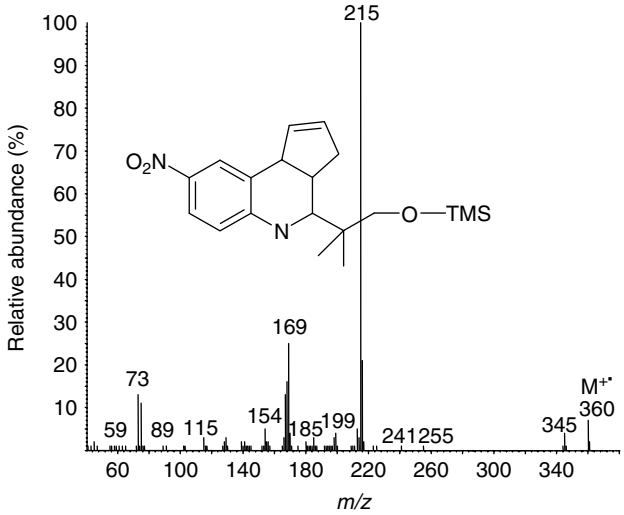

Figure 9. EI mass spectrum of the TMS-derivative of compound **16** recorded on an Agilent 6890/5973 GC–MSD.

of a methyl radical (−15 u) and subsequent fragmentation of the N-linked alkyl side chains, e.g. ions are observed for compound **13** (Fig. 1) at *m/z* 365, 297 and 269 (Fig. 8).[60] The release of a methyl group from the TMS functionality is followed by the eliminations of 1,1,1,3,3,3-hexafluoropropane (−152 u) or trifluoroethane (−84 u) yielding *m/z* 297 and 365, respectively. Both fragment ions were shown to produce *m/z* 269 by the losses of methyleneamine- or 2,2,2-trifluoroethylideneamine-radicals, respectively (Scheme 8). In addition to these dissociation routes, the loss of a trifluoromethyl radical (−69 u) was observed leading to the fragment ion at *m/z* 395.

Tricyclic tetrahydroquinoline-derived SARMs

The EI mass spectra derived from tricyclic tetrahydroquinoline-based SARMs such as compound **16** (Fig. 9) produced product ions due to the tricyclic nucleus after elimination of the side chain. Such ions are found at *m/z* 215 and 169, which are proposed to consist of the tetrahydrocyclopenta-quinoline core with and without nitro function, respectively. The molecular ion (Fig. 9, *m/z* 360) was commonly observed as well as an M-15 ion, which was found to be independent from trimethylsilylation.[49]

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Table 1. Summary of typical product ions derived from representatives of four classes of SARMs using ESI–MS/MS or EI–MS

			ESI				EI (TMS-derivative)				
Compound	Class	Mol wt (monoisotopic)	Precursor ion (m/z)	Major product ions (m/z)	Analyzer/ Dissociation	Molecular ion (m/z)	Major fragment ions (m/z)		Analyzer	References
3	Arylpropionamide	402	401 $[M-H]^-$	289 261	205 Ion trap/CID	474	459	241 151	Quadrupole	24,49
4	Arylpropionamide	441	440 $[M-H]^-$	289 261	205 Ion trap/CID	585	570	480 222	Quadrupole	24,49
9	Bicyclic hydantoin	305	306 $[M+H]^+$	288 278	260 Ion trap/CID	377	362	321 129	Quadrupole	32,49
		305	304 $[M-H]^-$	286 260	248 Ion trap/CID					32,49
13	2-quinolinone	392	393 $[M+H]^+$	375 310	241 Ion trap/CID	464	449	395 297	Quadrupole	46,60
16	Tricyclic tetrahydroquinoline	288	289 $[M+H]^+$	272 271	259 Ion trap/CID	360	345	215 169	Quadrupole	49

ANALYTICAL CHALLENGE

The enormous structural heterogeneity of SARMs, the limited knowledge of their metabolism and the fact that drugs, which have been misused in sports are not necessarily pharmaceutically and/or clinically approved,[17,61–64] presents a challenge for doping control authorities. Comprehensive screening for representatives of each category of emerging SARMs requires concerted analytical activities including LC–MS/MS and GC–MS and, thus, detailed information on the mass spectrometric behavior under a variety of ionization and dissociation conditions summarized in Table 1.

Arylpropionamide-based SARMs (e.g. compounds **3** and **4**) were selectively and sensitively analyzed using LC–MS/MS-based assays[24] with limits of detection (LODs) below 1 ng/ml of urine. Their poor gas-chromatographic properties with or without derivatization did not allow for adequate LODs using GC–MS procedures. In contrast, the rather low-ionization efficiency of bicyclic hydantoins such as compound **9** using positive or negative ESI necessitated the use of adduct ion formation to screen for this drug candidate and related compounds at LODs of at least 20 ng/ml.[65] Here, GC–MS yielded better results improving the detection limit to 10 ng/ml.[49] 2-Quinolinone- and tricyclic tetrahydroquinolinone-based SARMs were efficiently analyzed with established GC–MS[60] and LC–MS/MS[46] methods. GC–MS-based procedures were slightly better due to the considerable volatility of 2-quinolinone-derived SARMs, particularly of compound **13**, due to the presence of nine fluorine atoms. LODs below 1 ng/ml in spiked urine specimens were obtained by both analytical approaches.

CONCLUSION

Mass spectrometry is an indispensable tool for sports drug testing. Details on the dissociation behavior of new, emerging drugs under various ionization and fragmentation conditions is needed to comprehensively screen for these compounds in doping control samples. Rapid implementation of new analytes into detection assays will minimize their use by amateur and professional athletes. Structural characteristics of classes of compounds that enable the identification of conserved features or molecular nuclei, providing a mass spectrometric 'signature' of substances and their metabolic products in complex biological matrices, will allow their sensitive and specific detection.

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