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Screening for quinolinone-derived selective androgen receptor modulators using GC-MS and LC-MS/MS

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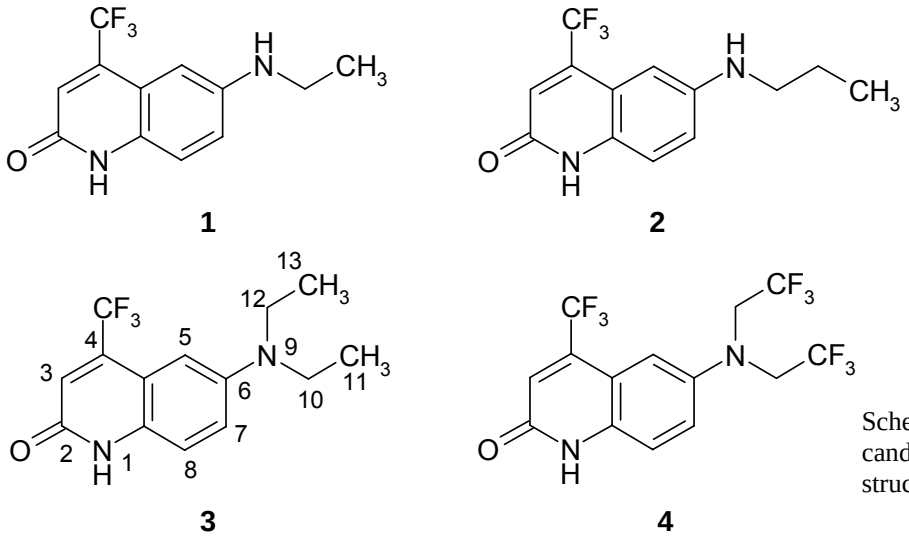
Introduction

The development of new compounds potentially complementing or replacing anabolic steroids is of great interest for pharmaceutical companies. Since 1998, non-steroidal anabolic agents termed selective androgen receptor modulators (SARMs) have been successfully introduced and investigated, and few have advanced to clinical trials[1]. One class of SARMs is based on a 2-quinolinone nucleus, and the lead drug candidate LGD-2226 has demonstrated great anabolic potential and tissue selectivity[2-4]. In order to control a possible misuse of these agents, reference material and model compounds (Scheme 1) were synthesized, characterized using mass spectrometric techniques, and validated detection methods were established using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS)[5].



Experimental

Reference compounds were synthesized as described elsewhere[2]. In brief, the common nucleus of all quinolinone-based SARMs was prepared by condensing aniline with ethyl-4,4,4-trifluoroacetoacetate followed by 6-nitrosylation of the resulting 4-trifluoromethylquinolin-2(*1H*)-one. The nitro residue was reduced to its corresponding amino function, which was subsequently alkylated to 6-*N*- monoethyl-, monopropyl-, bisethyl- and bistrifluoroethyl-amino-4-trifluoromethylquinolin-2(*1H*)-ones (Scheme 1, **1**, **2**, **3** and **4**, respectively). Final products were purified by flash chromatography on silica gel using *n*-hexane and ethyl acetate (4:1, *v:v*).

Synthesized compounds were characterized by nuclear magnetic resonance spectroscopy (NMR) with ^{1}H, H,H-COSY, H,C HMQC, and H,C HMBC experiments employing a Bruker II 600 MHz instrument (Bruker, Bremen, Germany) equipped with a 5mm TBI probehead (z-gradient coil). Approximately 10 mg of each compound was dissolved in deuterated dimethylsulfoxide (d_6-DMSO) or methanol, and spectra were recorded at room temperature. In addition, the elemental composition of each substance were determined using high resolution/high accuracy mass spectrometry utilizing an LTQ Orbitrap (Thermo, Bremen) at a resolving power of 30.000.

Electrospray ionization-tandem mass spectrometry.

ESI-MS(/MS) was performed on a Thermo LTQ Orbitrap mass spectrometer. The instrument was operated in positive ionization mode and calibrated using the manufacturer's calibration mixture (consisting of caffeine, MRFA and ultramark). Mass accuracies < 2 ppm (calculated from 30 averaged spectra) were accomplished for the period of analysis. Analytes were dissolved in acetonitrile/water (1:1, v:v) containing 0.1% formic acid at concentrations of 2 µg/mL and introduced into the mass spectrometer using a syringe pump at a flow rate of 5 µL/min. The ionization voltage was 3500 V, the capillary temperature was set to 290 °C, and protonated precursor ions were isolated using a width of 2 Da. The protonated species were dissociated at normalized collision energies between 25 and 35. Damping gas in the linear ion trap was helium (purity grade 5.0), and gas supplied to the curved linear ion trap (CLT) was nitrogen obtained from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany).

Liquid Chromatography-Tandem Mass Spectrometry.

All analyses were performed using an Agilent 1100 Series liquid chromatograph (Waldbronn, Germany) coupled to an Applied Biosystems API 4000 Qtrap mass spectrometer (Darmstadt, Germany) with electrospray ionization. The LC was equipped with a Macherey-Nagel Sphinx column (4.0 x 70 mm, 5 µm particle size), and the eluents used were 5 mM ammonium acetate containing 0.1% acetic acid (mobile phase A) and acetonitrile (mobile phase B). A gradient was employed starting at 30% B increasing to 100% B within 4.5 min followed by re-equilibration at 30% B for 1.5 min. The flow rate was set to 800 µL/min. The ion source was operated in the positive mode at 550°C using a spray voltage of 5500 V. All four analytes (Scheme 1) as well as the internal standard (ISTD, methyltestosterone) were detected by means of characteristic product ions formed from protonated molecules by collision-induced dissociation (CID) utilizing the multiple reaction monitoring mode (MRM). Nitrogen was employed as curtain and collision gas (5 x 10^{-3} Pa) delivered from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany), and collision offset voltages were optimized for each product ion.

GC/MS analysis

GC/MS measurements of all samples were conducted on an Agilent 6890 gas chromatograph interfaced to a 5973 mass selective detector. The GC was equipped with a HP-5MS column (inner diameter 0.2 mm, film thickness 0.2 µm, length 17 m), and a temperature program starting at 100°C increasing by 30°C/min to 320°C was employed. Helium was used as carrier gas (0.8 mL min^{-1}, constant pressure), and the injector temperature was set to 300°C, the interface temperature to 320°C and the ion source temperature to 230°C. Two µL of the derivatized sample were injected in split mode (1:10), and compounds were detected after electron ionization (EI) at 70 eV using selected ion monitoring (SIM) at dwell times of 10 ms each. Three diagnostic ions per analyte were considered for qualitative analyses according to current WADA guidelines (*m/z* 464, 449, and 297).

GC/MS/MS analysis

For the elucidation of dissociation pathways with MS/MS experiments, trimethylsilylated analytes were measured on a GCQ GC-ion trap mass spectrometer (Thermo, Dreieich, Germany). The GC temperature program was identical to the one described above, and

fragmentation studies were conducted with selected precursor ions. The isolation width was 1.0 Da, and the collision offset voltage energy was set to 1.0-1.3 V.

Sample Preparation

The sample preparation was performed according to a method described for the detection of selected anabolic androgenic steroids earlier[6]. Briefly, a volume of 2 mL of urine was buffered to pH 7.0 (750 µL of a sodium phosphate buffer (0.2 M, Na_2HPO_4: NaH_2PO_4, 1:2, *w:w*)) and 200 ng of methyltestosterone (20 µL of a 10 ng/µL solution) and 20 µL of β-glucuronidase were added. After incubation at 50°C for 1 h, liquid-liquid extraction (LLE) was performed at pH 9.6 (established by the addition of 200 mg of a mixture of K_2CO_3 and $NaHCO_3$ (2:1, *w:w*)) with 4 mL of *t*-butyl methyl ether by shaking the sample for 15 min and centrifuging at 620 x *g* for 5 min. The organic layer was transferred into a fresh test tube, evaporated to dryness employing a rotary evaporator at reduced pressure, and the dry residue was reconstituted in either 100 µL of water/acetonitrile (4:1, *v:v*) or MSTFA for LC-MS/MS or GC-MS measurements.

Assays for LC-MS/MS and GC-MS were validated in terms of specificity, lower limit of detection (LLOD), recovery, intraday- and interday-precision applying ICH guidelines.

Results and discussion

Electrospray ionization-tandem mass spectrometry.

The CID behavior of model target analytes **1-4** was studied employing high resolution/high accuracy (tandem) mass spectrometry using a hybrid linear ion trap-orbitrap instrument. Product ion mass spectra are depicted in Figure 1 (a-d), and major fragmentation pathways are presented using compound **3**. The protonated molecule of **3** $(M+H)^+$ at *m/z* 285 dissociates efficiently under CID conditions as shown in Figure 1c. Protonation is suggested to occur at the bis-alkylated amino function initiating the homolytic or heterolytic cleavage of a C-N bond yielding the product ions $[M+H]^+$ – 29 Da and – 28 Da at *m/z* 256 and 257, respectively (Scheme 2). The formation of odd-electron product ions from an even-electron precursor ion is usually not favored under ESI/CID conditions but explained by an extensive conjugated π-electron system that comprises the entire quinolinone structure of the analytes. The resulting radical cation at *m/z* 256 subsequently eliminated a methyl radical (-15 Da) yielding *m/z* 241 as demonstrated in MS^3 experiments, which subsequently released a methylimine radical (- 28 Da). A principally identical behaviour was observed also with the bis-trifluoroethylated analogue (compound **4**) that underwent consecutive losses of trifluoroethyl (-83 Da, yielding

m/z 310) and trifluoromethyl (-69 Da) radicals giving rise to the common product ion at *m/z* 241 (Figure 1d). However, the precursor ion at *m/z* 393 demonstrated a considerably higher stability than its non-halogenated counterpart **3** due to the exchange of mobile hydrogen by fluorine atoms, and higher collision energies were required to generate the spectrum illustrated in Figure 1d.

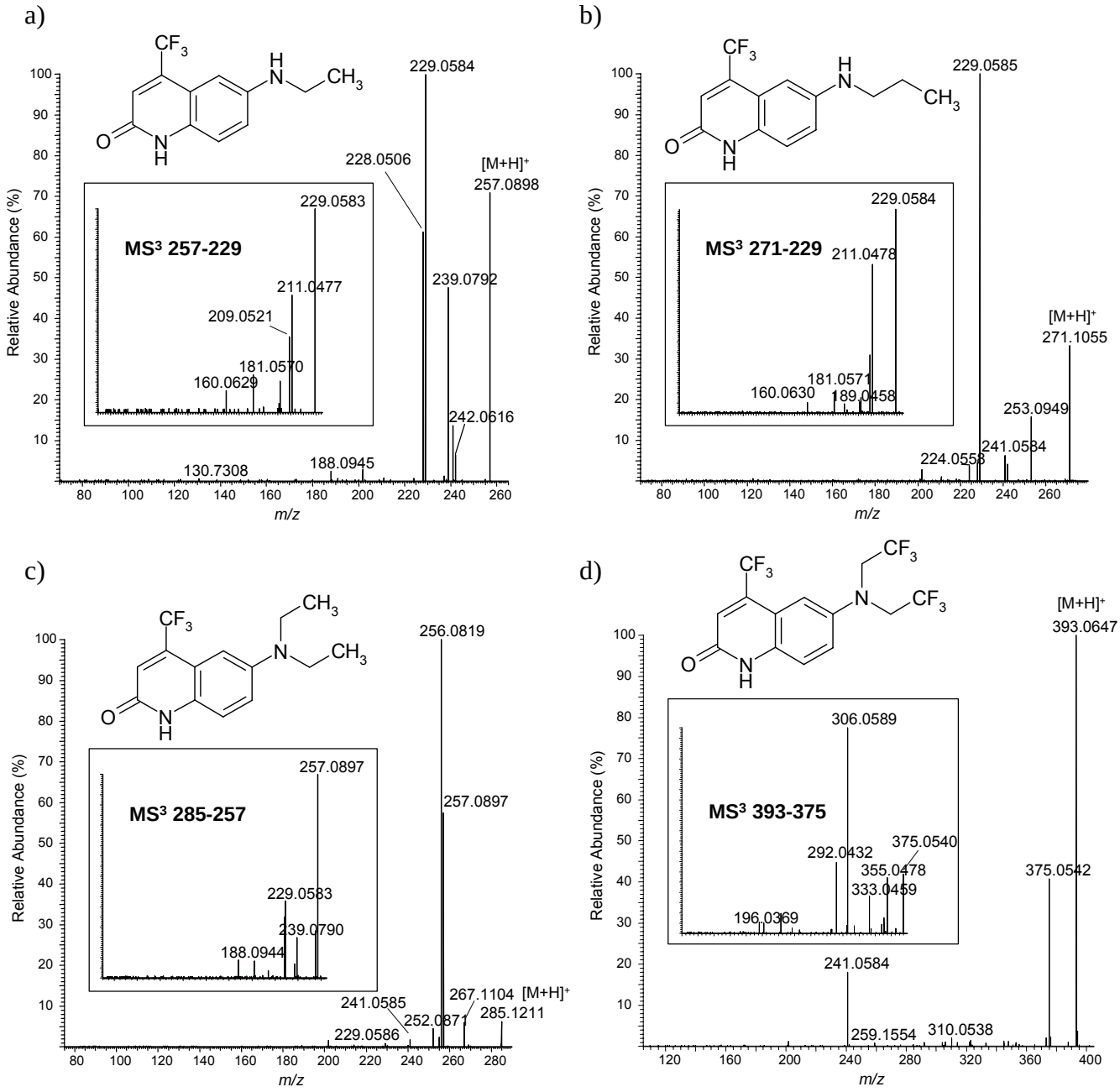


Figure 1: ESI-MS/MS spectra of 2-quinolinone-based SARMs using an LTQ-Orbitrap mass spectrometer.

In contrast to these common features of **3** and **4**, the elimination of ethylene (-28 Da) from **3** yielding the even-electron product ion at *m/z* 257 was not observed with substance **4**

accordingly. However, the product ion at *m/z* 257 resembles the protonated precursor ion of compound **1**. The neutral loss of C_2H_4 did not trigger the loss of a methyl radical from the remaining alkyl side chain in MS^3 experiments but another neutral loss of ethylene producing the protonated 6-amino-quinolin-2-one at *m/z* 229 (Scheme 2). This behavior was also observed in MS/MS measurements of **1** presumably comprising the same composition and structure as the product ion at *m/z* 257 derived from **3**. This product ion gave rise to further dissociation products originating from the elimination of water (-18 Da, *m/z* 239) as well as the release of a trifluoromethyl radical (-69 Da, *m/z* 188) as illustrated in the inset of Figure 1a. A summary of the proposed dissociation pathway is presented in Scheme 2, and the major outcome of the mass spectrometric study of dissociation pathways of these model SARMs is the generation of common product ions at *m/z* 241 and *m/z* 229 from bis-alkylated and mono-alkylated 6-amino-2-quinolinone derived selective androgen receptor modulators, respectively.

Electron ionization-mass spectrometry

The trimethylsilylated 2-quinolinone-derived SARM LGD-2226 (compound **3**), which is obtained by enolization of the 2-keto function, yielded the EI mass spectrum presented in Figure 2. The molecular ion is found at *m/z* 464, and characteristic losses of 69 ($\cdot CF_3$), 84 ($F_3C\text{-}CF_3$), and 152 ($F_3C\text{-}CH_2\text{-}CF_3$) with or without the elimination of a methyl radical were observed. Consequently, informative fragment ions were generated at *m/z* 449 (M^+-15), 395 (M^+-69), 365 (M^+-15 - 84), 311 (M^+-69 - 84), 297 (M^+-15 - 152), and 269 (M^+-15 -152 - 28), which are proposed to originate from dissociation processes as depicted in Scheme 3. The initial loss of a methyl radical from the TMS residue triggered a dissociation pathway that first yielded the fragment ion at *m/z* 449. Subsequently, 1,1,1,3,3,3-hexafluoropropane (- 152 u, Scheme 2, route b) or 1,1,1-trifluoroethane (- 84 u, Scheme 3, route d), were eliminated giving rise to fragment ions at *m/z* 297 and 365, respectively. Consecutively, both product ions formed the ion at *m/z* 269 by losses of a methyleneamine radical (-28 u, Scheme 3, route c) and 2,2,2-trifluoroethylideneamine radical (-96 u, Scheme 3, route e), respectively. All fragmentation cascades were proven by MS/MS experiments.

In addition to the dissociation route initiated by the loss of a methyl radical, the release of a trifluoromethyl radical from the molecular ion generated the fragment ion at *m/z* 395 (Scheme 3, route f). Here, the formation of a cross-conjugated π-electron system necessitating the opening of the ring structure is suggested as the trifluoromethyl group located at C-4 was

identified as the leaving group. The assumption was substantiated by the subsequent elimination of 1,1,1-trifluoroethane (- 84 u, Scheme 3, route g) yielding the ion at *m/z* 311.

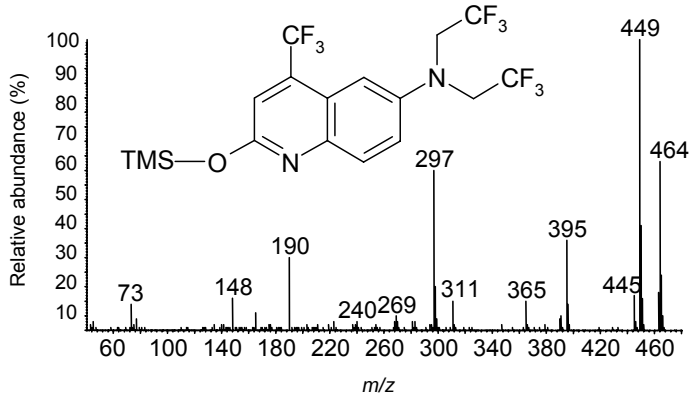


Figure 2: EI-MS spectrum of trimethylsilylated LGD-2226

Assay validation

Two detection approaches based on LC-MS/MS and GC-MS were validated for LGD-2226 as summarized in Table 1. LC-MS/MS appeared to be superior to GC-MS in terms of LLOD, which was estimated at 0.05 ng/mL (compared to 0.2 ng/mL), but the overall results demonstrated that both assays enable a sensitive and robust detection of the lead drug candidate LGD-2226 in human urine.

Table 2: Summary of assay validation results

Method	LLOD (ng mL^{-1})	Recovery (%) at 100 ng mL^{-1}	Intraday precision (n=24)		Interday precision (n=72)	
			Concentration (ng mL^{-1})	CV (%)	Concentration (ng mL^{-1})	CV (%)
GC-MS	0.2	85	2	9.0	2	14.6
			10	8.2	10	12.7
			50	11.6	50	14.4
LC-MS/MS	0.05	81	1	8.0	1	16.6
			10	8.5	10	15.1
			100	5.8	100	11.4

Conclusion

Quinolinone-derived SARMs such as LGD-2226 are efficiently detected using GC-MS as well as LC-MS/MS approaches. Due to the inclusion of SARMs in the prohibited list as issued by WADA in 2008, such compounds (even though not yet commercially available) need consideration in screening procedures and can be implemented into existing assays based on either gas- or liquid-chromatographic methods. Detection limits of conventional instruments are sufficient to uncover the misuse of LGD-2226, although studies on the metabolic fate will be required to demonstrate whether the active drug or its metabolite(s) will be the best targets for doping control purposes.

Acknowledgments

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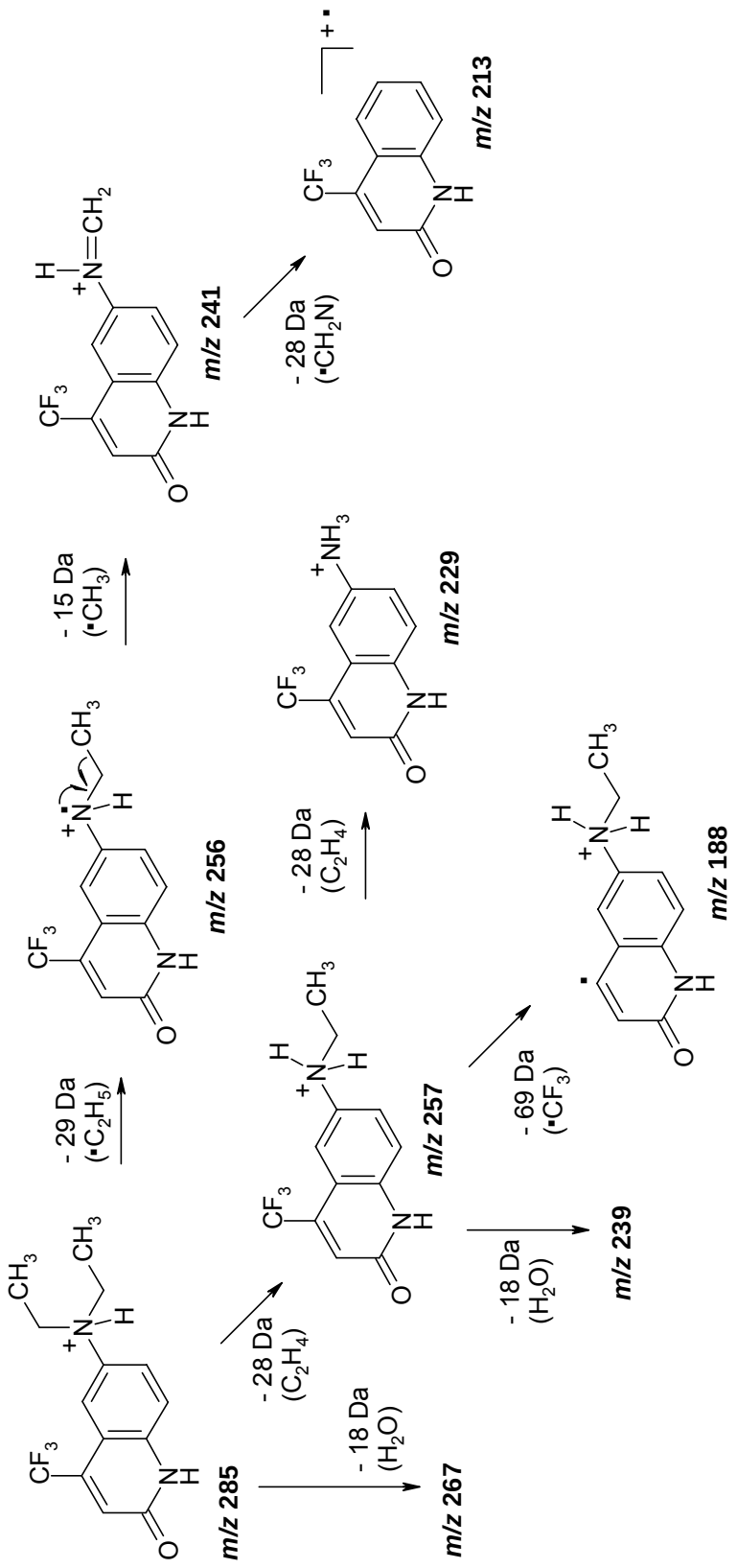
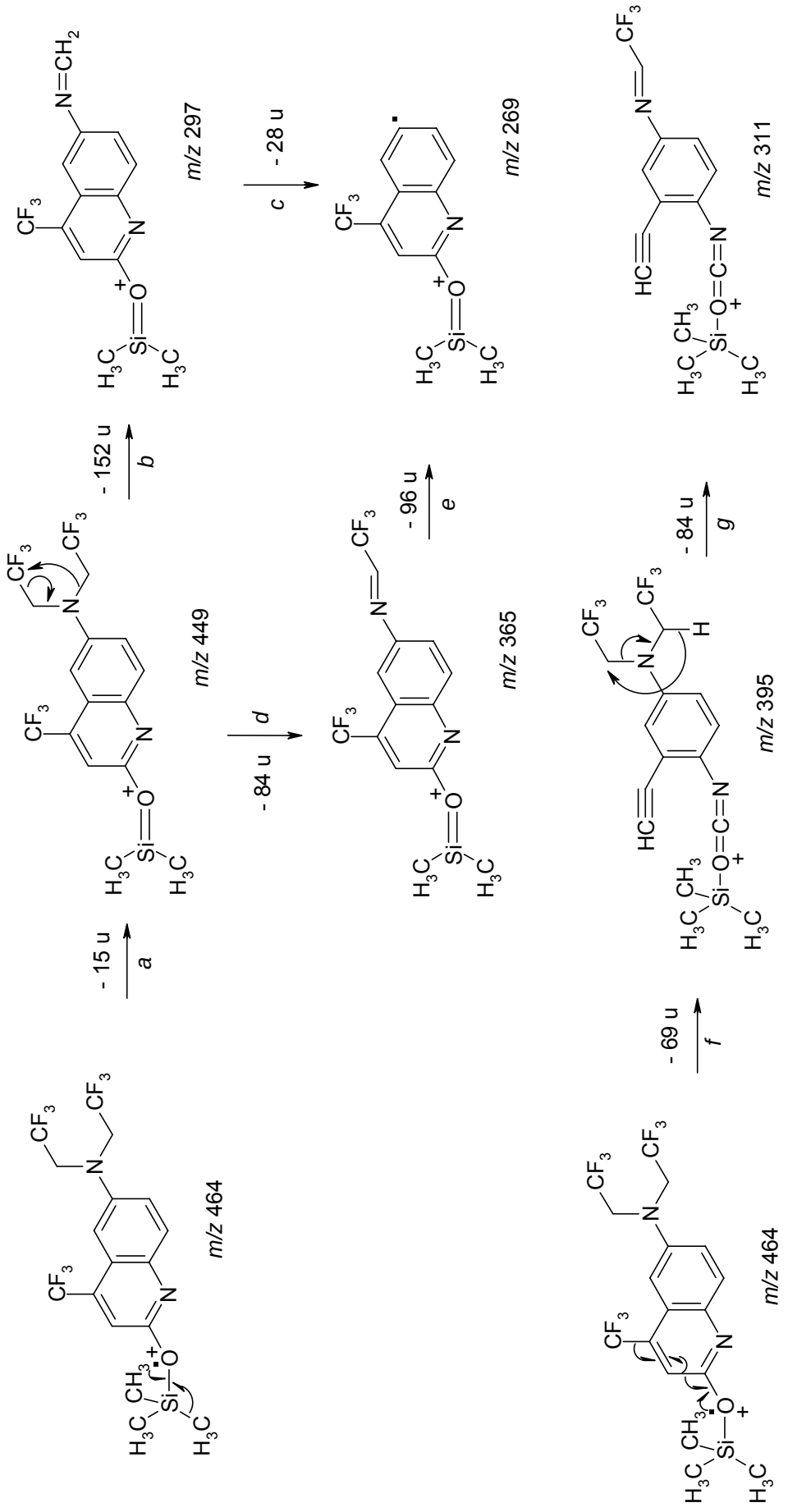


Scheme 2: Proposed dissociation route of a 2-quinolinone-derived SARM after ESI and CID



Scheme 3: Proposed dissociation pathway of LGD-2226 after trimethylsilylation and EI.