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Screening for the calstabin-ryanodine receptor complex stabilizers JTV-519 and S-107 in doping control analysis

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Recent studies outlined the influence of exercise on the stability of the skeletal muscle calstabin1-ryanodine receptor1-complex, which represents a major Ca^{2+} release channel. The progressive modification of the type-1 skeletal muscle ryanodine receptor (RyR1) combined with reduced levels of calstabin1 and phosphodiesterase PDE4D3 resulted in a Ca^{2+} leak that has been a suggested cause of muscle damage and impaired exercise capacity. The use of 1,4-benzothiazepine derivatives such as the drug candidates JTV-519 and S-107 enhanced rebinding of calstabin1 to RyR1 and resulted in significantly improved skeletal muscle function and exercise performance in rodents. Due to the fact that the mechanism of RyR1 remodelling under exercise conditions were proven to be similar in mice and humans, a comparable effect of JTV-519 and S-107 on trained athletes is expected, making the compounds relevant for doping controls. After synthesis of JTV-519, S-107, and a putative desmethylated metabolite of S-107, target compounds were characterized using nuclear magnetic resonance spectroscopy and electrospray ionization (ESI) – high-resolution/high-accuracy Orbitrap mass spectrometry. Collision-induced dissociation pathways were suggested based on the determination of elemental compositions of product ions and H/D-exchange experiments. The most diagnostic product ion of JTV-519 was found at m/z 188 (representing the 4-benzyl-1-methyl piperidine residue), and S-107 as well as its desmethylated analog yielded characteristic fragments at m/z 153 and 138 (accounting for 1-methoxy-4-methylsulfanyl-benzene and 4-methoxy-benzenethiol residues, respectively). The analytes were implemented in existing doping control screening procedures based on liquid chromatography, multiple reaction monitoring and simultaneous precursor ion scanning modes using a triple quadrupole mass spectrometer. Validation items such as specificity, recovery (68–92%), lower limit of detection (0.1–0.2 ng/mL), intraday (5.2–18.5%) and interday (8.7–18.8%) precision as well as ion suppression/enhancement effects were determined.

Keywords: doping; sport; mass spectrometry; fragmentation; orbitrap; labelling; deuterium

Introduction

The ryanodine receptor (RyR), a 565 kDa protein, plays an important role in contributing with four monomers to the formation of macromolecular complexes (2.2 mDa) that constitute scaffolds of intracellular Ca^{2+} release channels in skeletal and cardiac muscle.[1,2] These channels are essential for excitation-contraction coupling (ECC), which is initiated by membrane depolarization and the resulting activation of dihydropyridine receptors. Subsequently, the RyRs located at the sarcoplasmic reticulum are activated to release Ca^{2+} and allow muscle contraction.[3] An additional key factor responsible for stabilizing RyR-based Ca^{2+} channels is the FK506-binding protein (FKBP12), more recently referred to as calstabin1. Calstabin1-deficient mice as well as wild-type rodents that underwent pharmacological depletion of calstabin1 from type-1 skeletal muscle RyR (RyR1) showed an impaired function of Ca^{2+} channels, altered local subcellular Ca^{2+} release events ('leaking') and aberrant calcium sparks in myofibers, which were reportedly able to trigger fatal cardiac arrhythmias[4–8] and potentially responsible for a decreased exercise capacity.[9] The use of orally available 1,4-benzothiazepine drug candidates such as JTV-519 and S-107 (Fig. 1, **1** and **2**, respectively) were able to stabilize the calstabin-RyR complex by increasing the affinity of calstabin for RyR and, thus, promoting the closed state of the Ca^{2+} channel.

Intense exercise was proved to influence the RyR1 by progressive hyperphosphorylation, S-nitrosylation and depletion of calstabin1 as well as the phosphodiesterase PDE4D3, which also results in leaky calcium release channels and, consequently, in impaired exercise capacities. Recent investigations based on a three-week daily swimming protocol for mice, however, outlined the potential to counteract the exercise-induced reduction of endurance performance using the compounds JTV-519 and S-107 (Fig. 1). Wild-type animals were placed in a pool and induced to swim twice daily, and exercise capacities were assessed once per week by means of treadmill run to exhaustion. A significant increase of the endurance capacity was observed after treatment with S-107 (implanted osmotic pump, continuous delivery of 2.5 µg/h), which allowed the treated mice to run approximately 13 min

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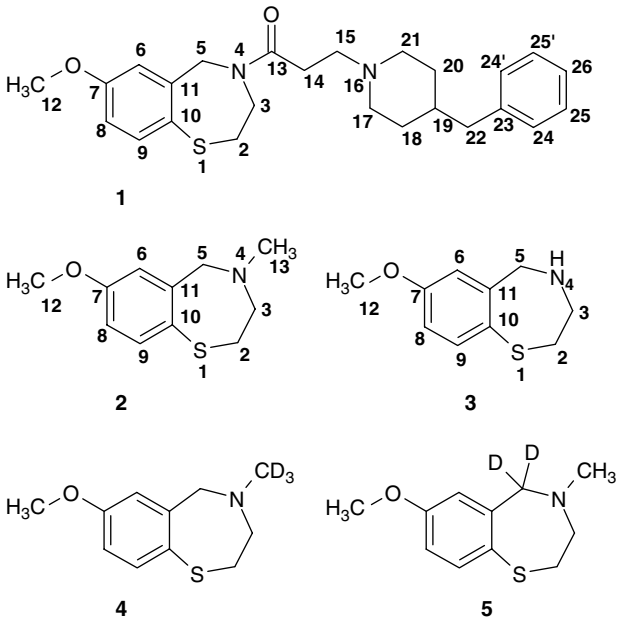


Figure 1. Chemical structures of JTV-519 (**1**, mol. wt = 424), S-107 (**2**, mol. wt = 209), the putative desmethylated metabolite of **2** referred to as **3** (mol. wt = 195), and triply (**4**) as well as doubly (**5**) deuterated analogs to **2**.

longer (77.7 ± 4.6 min *vs* 64.7 ± 1.4 min) than the control group, which received the vehicle only. The authors concluded that the reduction of exercise-induced Ca^{2+} leakage by drugs inhibiting the calstabin1 depletion from RyR1 prevents muscle damage, enhances muscle function and exercise capacity.[9] Consequently, sports drug testing authorities might have to consider these new, emerging therapeutics for future doping control purposes. Although not yet commercially available, preventive doping research approaches aim for an implementation of compounds into regular screening procedures as soon as these enter advanced clinical trials.[10,11] Due to the fact that various substances were misused in sports in the past without having (ever) received clinical approval,[12–17] a timely development of detection strategies for drug candidates with potential to artificially increase athletic performance is required. Hence, the reference substances JTV-519 and S-107 as well as a putatively desmethylated metabolite of S-107 (Fig. 1) were synthesized and characterized using nuclear magnetic resonance spectroscopy (NMR) and high-resolution/high-accuracy tandem mass spectrometry. Based on the obtained mass spectrometric data, the analytes were included into existing assays employing liquid chromatography-tandem mass spectrometry (LC/MS/MS), and items such as specificity, recovery, lower limit of detection, intraday and interday precision as well as ion suppression/enhancement effects were validated.

Experimental

Chemicals and reagents

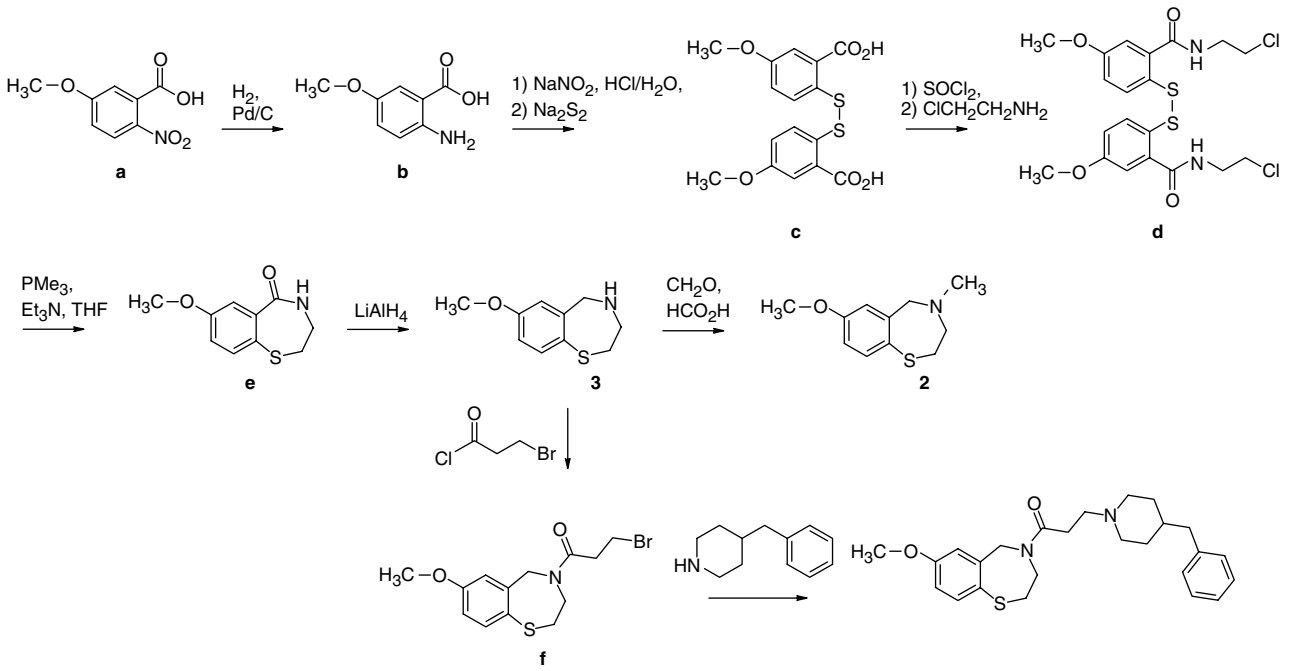
5-Methoxy-2-nitrobenzoic acid (97%), palladium (10% on charcoal), sodium nitrite (99%), sodium sulfide nonahydrate (98%), sulphur (99%), thionyl chloride (99%), 2-chloroethylamine (99%), triethylamine (99%), trimethylphosphine (1 M in THF), lithium aluminium hydride (95%), d_4-methanol (99%), d_2-formic acid (99%), lithium aluminium deuteride (95%), and deuterochloroform (99%) were purchased from Sigma (Deisendorf, Germany). Formaldehyde solution (37% in water, analytic grade), 4-benzyl piperidine (99%) and 3-bromopropionic chloride (95%) were from Acros (Nidderau, Germany) and sodium hydrogen chloride (99%) and sodium hydroxide (99%) from VWR (Darmstadt, Germany). Solvents (methanol, tetrahydrofurane, ethyl acetate, dimethylformamide, all analytical grade), hydrochloric acid (32% in water, analytical grade) and silica gel 60 (70-230 mesh) were obtained from Merck (Darmstadt, Germany), and deionized water used for sample preparation and buffer solutions was of MilliQ grade.

Synthesis and characterization of target compounds

The drug candidates JTV-519 (**1**), S-107 (**2**) as well as a putative metabolite (**3**) of S-107, were synthesized according to procedures described elsewhere.[9] In brief, 5-methoxy-2-nitrobenzoic acid (Scheme 1, **a**) was reduced to 2-amino-5-methoxybenzoic acid (Scheme 1, **b**) under a hydrogen atmosphere in the presence of a palladium catalyst (10% on active charcoal). Compound **b** was subsequently treated with sodium nitrite under acidic conditions and added to an aqueous solution of sodium disulphide to yield the disulphide dimer compound **c** (Scheme 1), which was converted into the amide (Scheme 1, compound **d**) by reaction with thionyl chloride and 2-chloroethylamine. After the cyclized amide **e** (Scheme 1) was generated from compound **d** by treatment with trimethylphosphine and triethylamine, 7-methoxy-2,3,4,5-tetrahydro-1,4- benzothiazepine (**3**), the supposed metabolite of S-107 (**2**), was liberated by reduction of **e** with lithium aluminium hydride. Methylation of **3** with aqueous formaldehyde and formic acid gave rise to 4-methyl-7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine (S-107, **2**). The drug candidate 4-[3-[1-(4-benzyl)piperidinyl]propionyl]-7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine (JTV-519, Fig. 1, **1**) was obtained by reaction of compound **3** with 3-bromopropionic chloride, followed by treatment with 4-benzyl piperidine (Scheme 1). Finally, the target compounds **1, 2**, and **3** were purified by column chromatography (ethyl acetate/triethylamine (1%) on silica gel). Accordingly, deuterated analogs were prepared by using deuterium-labelled formaldehyde and formic acid or lithium aluminium deuteride that allowed the introduction of a CD_3-residue or a deuterated methylene unit into S-107 (Fig. 1, compounds **4** and **5**). Structures were confirmed by nuclear magnetic resonance spectroscopy (NMR) with ^{1}H, H,H-COSY, H,C-HMQC, H,C-HMBC and ^{13}C-APT experiments employing a Bruker AV 600 MHz instrument (Bruker, Karlsruhe, Germany) equipped with a 5 mm inverse probe head (z-gradient coil). The spectra were recorded at room temperature from solutions at concentration levels of approximately 10 mg/mL. The elemental compositions of the synthesized substances were also determined, using high-resolution/high-accuracy mass spectrometry with a LTQ Orbitrap (Thermo, Bremen, Germany) at a resolving power of 30 000 (FWHM). A detailed description of further parameters is shown below.

Stock and working solutions

All solutions of target analytes were prepared in acetonitrile and stored at 2–8 °C. The concentrations of stock and working solutions were 1 mg/mL and 1 µg/mL, respectively. Over a period of four weeks no degradation was observed in either of the solutions.



Scheme 1. Route of synthesis of target analytes.

H/D-exchange experiments

In order to provide more detailed mass spectrometric information for product ion characterization and suggestion of dissociation pathways, hydrogen/deuterium-exchange was performed by dissolving 1 mg of compound **2** in 1 mL of deuterated methanol (MeOD) and deuterium oxide (D_2O) (1 : 1, v : v). After incubation at room temperature for 10 min, the solution was diluted 1 : 50 in MeOD/D_2O (1 : 1, v : v) and introduced into the LTQ Orbitrap mass spectrometer via the syringe pump. Analyses were carried out under identical conditions as applied for compound **2** without H/D-exchange.

Electrospray ionization-tandem mass spectrometry

ESI-MS(/MS) was performed on a 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 <5 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 1 µg/mL and introduced into the mass spectrometer using a syringe pump at a flow rate of 5 µL/ min. The ionization voltage was 4200 V, the capillary temperature was set to 290 °C, and for MS^n experiments the protonated precursor ions were isolated using a width of 1.5 Da. The protonated species were dissociated at normalized collision energies between 17 and 25 (arbitrary units, Xcalibur software version 2.0, Thermo, Bremen, Germany). 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 × 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 15% B increasing to 100% B within 8 min, maintained at 100% B for 0.5 min and followed by re-equilibration at 15% 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 three analytes (Fig. 1, **1**–**3**) 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) as listed in Table 1. Nitrogen was employed as curtain and collision gas (5×10^{-3} Pa) delivered from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany), and collision offset voltages were optimized for each product ion (Table 1).

Sample preparation

The sample preparation was performed according to a method described for the detection of selected anabolic androgenic steroids earlier.[17] Briefly, an aliquot of 2 mL of urine was adjusted to pH 7.0 using a 0.2 M sodium phosphate buffer (Na_2HPO_4: NaH_2PO_4, 1 : 2, w : w), and 200 ng of methyltestosterone (ISTD, 20 µL of a 10 ng/µL solution) and 20 µL of β-glucuronidase were added. After one hour of incubation at 50 °C, liquid-liquid extraction (LLE) was performed at pH 9.6 (buffered 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. The organic layer was transferred into a fresh test tube, evaporated to dryness, and the dry residue

Table 1. MS parameters for multiple reaction monitoring of three 1,4-benzothiazepine derivatives

Compound	Declustering potential (V)	Ion transition (m/z)	Collision offset voltage (V)	Dwell time (ms)
1	91	425–188	41	40
	91	425–115	127	40
	91	425–96	65	40
2	71	210–153	25	40
	71	210–138	35	40
	71	210–109	37	40
3	61	196–153	21	40
	61	196–138	31	40
	61	196–109	35	40
methyltestosterone	70	303–109	40	40

was reconstituted in 100 µL of water/acetonitrile (4 : 1, *v* : *v*). The solution was transferred to HPLC vials, and 10 µL were injected into the LC-MS/MS system.

Assay validation

The qualitative determination of two calstabin1-RyR1 complex stabilizers and one putative metabolite in human urine was validated regarding specificity, recovery, lower limit of detection (LLOD), intraday and interday precision according to ICH guidelines.[18] Respective items were defined and tested as follows.

Specificity and lower limit of detection (LLOD)

Ten different blank urine specimens (5 male and 5 female urine samples) were prepared as described in order to probe for interfering peaks, i.e. to demonstrate sufficient specificity, and to calculate average noise levels in the selected ion chromatograms at the expected retention times for all target analytes. As the LLOD was defined as the 'lowest content that can be measured with reasonable statistical certainty'[19] at a signal-to-noise ratio $\geq$3, the same ten blank urine samples were fortified with 1 ng/mL of the compounds **1–3**, prepared and analysed according to the established protocol providing the data necessary to estimate the LLOD.

Recovery

The recovery of all analytes was determined at 50 ng/mL considering the above-mentioned animal studies where approximately 3 mg/kg/day were administered.[9] Six blank urine specimens were fortified with the target analytes before sample preparation, and another six blank samples were extracted according to the described protocol followed by addition of the analytes into the final sample extract. To both sets of samples, 200 ng of methyltestosterone (ISTD) were spiked into the ether layer before evaporation. Recoveries were calculated by comparison of mean peak area ratios of analytes and ISTD of samples fortified prior to and after LLE.

Intraday and interday precision

Within one day as well as on three consecutive days, ten urine samples of low (1 ng/mL), medium (10 ng/mL), and high (100 ng/mL) concentrations of all three analytes were prepared and analysed (n = 30 + 30 + 30) and the intraday and interday precision was calculated for each concentration level.

Test for ion suppression/enhancement effects

In order to estimate ion suppression effects possibly caused by matrix interference, four different blank urine samples and solvent only were analysed with continuous co-infusion of the target analytes (solution concentration 0.5 pg/µL, flow rate 5 µL/ min) via T-connector according to literature recommendations.[20,21]

Administration study urine samples

Assay validations commonly include analyses of administration study urine specimens. The presented compounds, however, have not completed clinical trials or entered the pharmaceutical market yet and thus no authentic urine samples but only spiked specimens were available and used to establish a screening tool for a new set of compounds potentially challenging the doping control system. *In vitro* metabolism studies will provide deeper insights into potentially additional target compounds in the near future to complement the group of analytes used to detect the misuse of compounds such as JTV-519, S-107 and related derivatives.

Results and Discussion

The timely development of doping control screening methods necessitates adequately characterized reference compounds, which is particularly arduous if the substances are not clinically approved and/or commercially available. In order to reduce or even eliminate any competitive edge that cheating athletes might be given by misusing new, emerging drugs, preventive doping research and early implementation of potentially performance-enhancing substances into doping controls is required. The validation of new or expanded procedures and proof-of-concept studies of the method capabilities are prerequisites in sports drug testing as well as numerous other fields of analytical chemistry.

The unknown metabolic fate as well as the chemical diversity and, thus, virtually unlimited variety of related drugs, complicate a targeted screening. Generalized and comprehensive methods as for instance enabled by precursor or neutral loss scan experiments using product ions characteristic for common 1,4-benzothiazepine nuclei might become necessary – an approach that has been successfully demonstrated for other classes of compounds such as synthetic anabolic steroids,[17,22] corticosteroids,[23] or selective androgen receptor modulators.[24–26]

Synthesis and characterization of 1,4-benzothiazepine derivatives

The syntheses yielded the intended structures with overall yields ranging from 15–20%, which provided sufficient amounts of pure analytes necessary for method development and structure characterization. All analytes were characterized by NMR analyses, and only compound **1**, which comprises a partial double-bond character at the amide bond, gave rise to two isomer forms at room temperature. Consequently, this substance yielded doubled signals in the ^{1}H- and ^{13}C-NMR spectra, while all other compounds were characterized with one particular structure:

Compound **1** (doubled signals observed at room temperature, as indicated by "/"), ^{1}H-NMR, 600 MHz, d_1-chloroform, δ: 7.48/7.41 (*d*, J = 8.6 Hz, 1H, H-9), 7.29–7.24 (*m*, 2H, H-25), 7.21–7.15 (*m*, 1H, H-26), 7.15–7.12 (*m*, 2H, H-24), 7.13/6.89 (*d*, J = 3.2 Hz, 1H, H-6), 6.72/6.68 (*dd*, J = 8.6/3.2 Hz, 1H, H-8), 4.65 (*s*, 2H, H-5), 4.05/3.95 (*s*, broad, 2H, H-3), 3.78 (*s*, 3H, H-12), 2.90–2.82 (*m*, 2H, H-17/H-21), 2.75 (*d*, J = 4.2 Hz, 2H, H-2), 2.68–2.61 (*m*, 2H, H-15), 2.54–2.49 (*m*, 2H, H-22), 2.46 (*t*, J = 7.5 Hz, 2H, H-14), 1.98–1.89 (*m*, 2H, H-17/H-21), 1.65–1.57 (*m*, 2H, H-18/H-20), 1.56–1.46 (*m*, 1H, H-19), 1.35–1.20 (*m*, 2H, H-18/H-20); ^{13}C-NMR, 150 MHz, d_1-chloroform, δ: 171.52/170.42 (C-13), 159.37/159.22 (C-7), 143.83/143.08 (C-11), 140.54/140.51 (C-23), 134.68/133.81 (C-9), 129.08 (C-24), 128.15 (C-25); 126.89/126.09 (C-10), 125.80/125/78 (C-26), 116.87/116.42 (C-6), 113.27/112.27 (C-8), 55.39–55.36 (C-12), 54.20/52.30 (C-5), 54.02 (C-15), 53.83/53.92 (C-17/C-21), 52.83/50.38 (C-3), 43.08 (C-22), 37.68 (C-19), 33.75/35.12 (C-2), 31.91 (C-18/C-20), 31.39/31.24 (C-14).

Compound **2**, ^{1}H-NMR, 600 MHz, d_4-methanol, δ: 7.43 (*d*, J = 8.3 Hz, 1H, H-9), 6.93 (*d*, J = 2.8 Hz, 1H, H-6), 6.78 (*dd*, 8.3 Hz/2.8 Hz, 1H, H-8), 4.05 (*s*, broad, 2H, H-5), 3.81 (*s*, 3H, H-12), 3.23–3.18 (*m*, 2H, H-3), 2.80–2.74 (*m*, 2H, H-2), 2.31 (*s*, 3H, H-13); ^{13}C-NMR, 150 MHz, d_4-methanol, δ: 161.18 (C-7), 145.01 (C-11), 134.93 (C-9), 129.56 (C-10), 118.51 (C-6), 114.05 (C-8), 62.65 (C-5), 61.64 (C-3), 56.11 (C-12), 42.29 (C-13), 31.56 (C-2).

Compound **3**, 600 MHz, d_1-chloroform, δ: 7.48 (*d*, J = 8.4 Hz, 1H, H-9), 6.80 (*d*, J = 2.7 Hz, 1H, H-6), 6.68 (*dd*, 8.4 Hz/2.7 Hz, 1H, H-8), 4.10 (*s*, broad, 2H, H-5), 3.79 (*s*, 3H, H-12), 3.38 (*t*, J = 4.7 Hz, 2H, H-3), 2.70 (*t*, J = 4.7 Hz, 2H, H-2); ^{13}C-NMR, 150 MHz, d_1-chloroform, δ: 159.21 (C-7), 148.21 (C-11), 134.06 (C-9), 127.74 (C-10), 115.33 (C-6), 112.01 (C-8), 55.34 (C-12), 55.31 (C-5), 53.23 (C-3), 36.55 (C-2).

Electrospray ionization-tandem mass spectrometry

The CID behaviour of compounds **1**–**3** 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 Fig. 2(a-c). Calculated elemental compositions of product ions as obtained from MS^n measurements are listed in Table 2, and major fragmentation pathways are suggested for **1** and **2** as substantiated by deuterium labelling.

Compound 1 (JTV-519)

The protonated molecule of **1** $(M + H)^+$ at *m/z* 425 dissociates under CID conditions primarily to one abundant product ion at *m/z* 188 as shown in Fig. 2a. It is suggested that protonation occurs at the piperidine nitrogen, which exhibits a higher proton affinity (E_{pa}) than the amide function or the sulphur atom included in the thiobenzene structure. According to literature data, the E_{pa} of piperidine (954.0 kJ/mol) is considerably higher than those of, for example, methylthiobenzene (E_{pa} = 872.6 kJ/mol) and dimethylpropenamide (E_{pa} = 904.3 kJ/mol),[27] which represent specific parts of the benzothiazepine nucleus of **1** that are also amendable for protonation. Nevertheless, the mobility of a proton introduced by ESI, in particular in the course of collision-induced dissociation (CID), was described in numerous cases[28] and would allow locating the positive charge of the precursor ion also at other sites of the molecule prior to dissociation. The elimination of 4-acetyl-7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine (−237 Da) from compound **1** yields a product ion with the elemental composition of $C_{13}H_{18}N$, presumably composed by the 4-benzyl-1-methylene piperidine residue (Scheme 2a). Subsequently, neutral losses of toluene (−92 Da) or ethyl-methylamine (−59) from *m/z* 188 are observed that generate product ions at *m/z* 96 and 129, respectively (Table 2), which are suggested to comprise 1-methylene-tetrahydropyridine and buta-1,3-dienyl benzene structures (Scheme 2a). From the elimination of 4-benzylpiperidine (−175 Da) from the protonated molecule of compound **1**, a structure-characteristic ion is found at *m/z* 250 (Table 2, Scheme 2b); however, its abundance remained low at any chosen collision offset voltage.

Compound 2 (S-107)

In contrast to compound **1** (JTV-519), S-107 (compound **2**) does not contain an amide function in the 1,4-benzothiazepine nucleus but a tertiary amine, the proton affinity of which was estimated from hexahydroazepine (E_{pa} = 956.7 kJ/mol)[27] and, thus, higher than the one attributed to the sulphur atom (see above). Following protonation via ESI, CID of **2** yielded abundant and characteristic product ions, the proposed generation of which is depicted in Scheme 3. The loss of methylamine (−31 Da) from the precursor ion at *m/z* 210 generates a product ion at *m/z* 179 that is suggested to comprise a charged 1-methoxy-4-vinylsulfanyl-cycloheptane structure, which subsequently eliminates a methyl radical under MS^3 conditions (Scheme 3a). Supporting information for the suggested dissociation route was obtained from analyses of **4** and **5** (Table 2), which proved the initial loss of methylamine and the retention of two deuterium atoms located at C-5.

The same dissociation behaviour is observed in case of compound **3**, which initially loses ammonia (−17 Da) instead of methylamine and subsequently CH_3• (−15 Da) to produce the ions at *m/z* 179 and 164, respectively (Table 2). The protonated molecules of **2** and **3** further eliminate a species with the composition C_3H_7N (−57 Da), which presumably constitutes 1-methyl-aziridine (Scheme 3b) because the leaving group retains the proton that was introduced during the ionization process as demonstrated with H/D exchange experiments (data not shown). Moreover, the analyses of the doubly and triply deuterated S-107 (compounds **4** and **5**, respectively, Fig. 2d-e) provided information supporting the proposed rearrangements. In case of compound **4**, an abundant fragment at *m/z* 154 is detected that retains one of the deuterium atoms formerly located at the *N*-linked methyl function, and compound **5** gives rise to a product ion at *m/z* 155 that evidently contains both deuterium atoms introduced at C-5. As such, the release of 1-methyl-aziridine from the precursor ion of compound **2** is suggested to include the migration of a hydrogen atom from the *N*-linked methyl residue to C-5, followed by the cleavage of the S-1–C-2 linkage (Scheme 3b) to yield the ion at *m/z* 153. Subsequently, a methyl radical (−15 Da), potentially originating from the methoxy group or C-5, is lost, which generates the product ion at *m/z* 138. The fact that several options are feasible is supported by MS^3 experiments of the

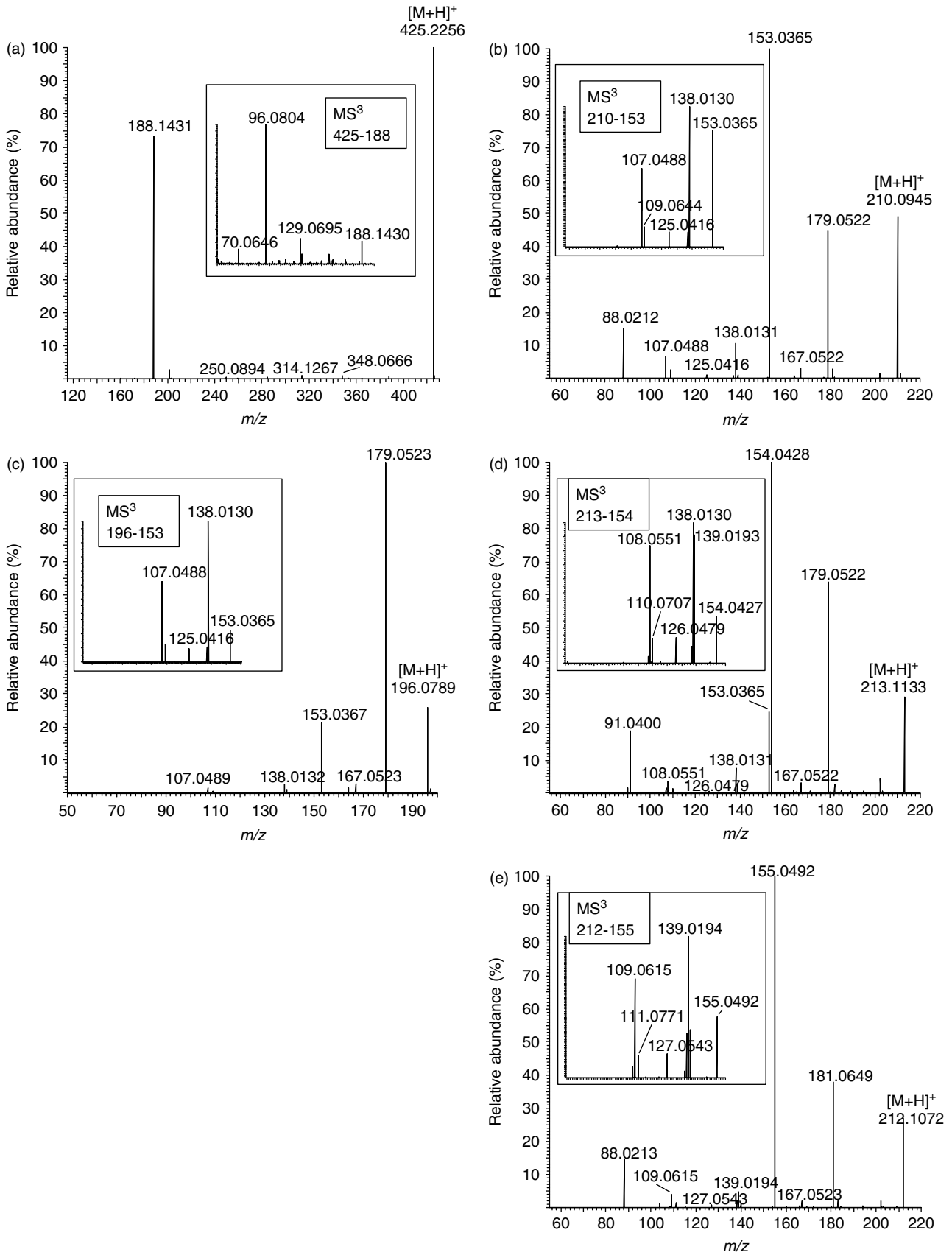
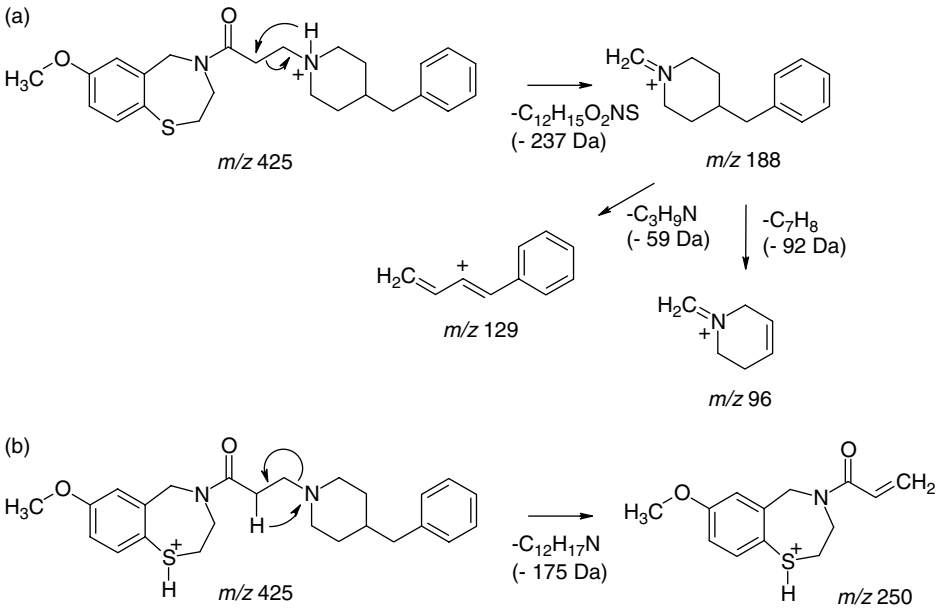


Figure 2. Electrospray ionization product ion spectra of protonated molecules of three 1,4-benzothiazepine derivatives and one deuterium-labeled analog measured on an LTQ-Orbitrap: a) compound **1**, collision energy = 17 arbitrary units; b) compound **2**, collision energy = 25 arbitrary units; c) compound **3**, collision energy = 25 arbitrary units; d) compound **4**, collision energy = 23 arbitrary units; and e) compound **5**, collision energy = 23 arbitrary units. MS^3 experiments yielded spectra shown as insets in respective MS/MS product ion spectra.

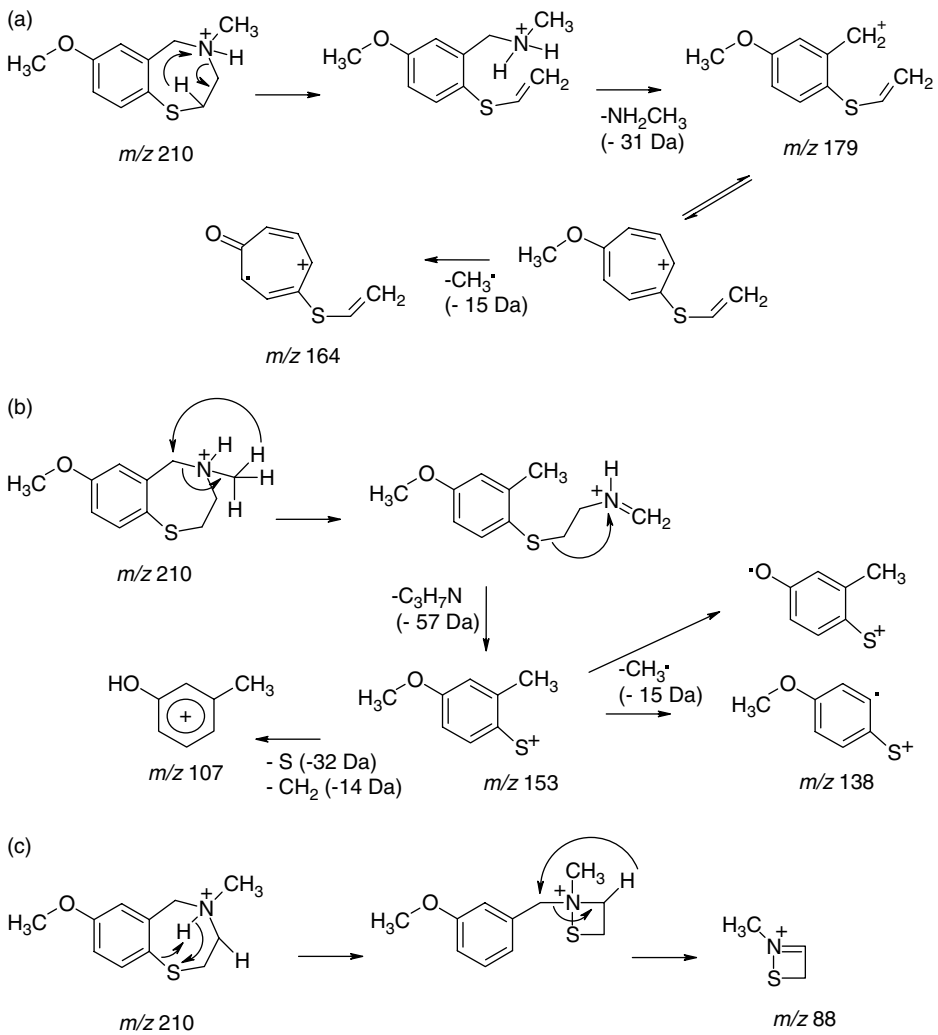
Table 2. Elemental compositions of protonated molecules of **1** – **3** and resulting product ions (>5% of relative abundance) using high resolution/high accuracy MS^n experiments

Compound	Precursor ion (m/z) MS^2	MS^3	Elemental comp. (exp.)	Error (ppm)	Collision energy (arb. units)	Product ion (m/z)	Elemental comp. (exp.)	Error (ppm)	Cleaved species
1	425.2252		$C_{25}H_{33}O_2N_2S$	−1.2	17	250.0894[a]	$C_{13}H_{16}O_2NS$	−1.1	$C_{12}H_{17}N$
						188.1431	$C_{13}H_{18}N$	−1.5	$C_{12}H_{15}O_2NS$
		188.1430	$C_{13}H_{18}N$	−1.8	25	129.0695	$C_{10}H_9$	−2.9	C_3H_9N
						96.0804	$C_6H_{10}N$	−3.8	C_7H_8
2	210.0947		$C_{11}H_{16}ONS$	−0.3	25	179.0521	$C_{10}H_{11}OS$	−2.2	CH_3NH_2
						153.0365	C_8H_9OS	−2.1	C_3H_7N
						138.0131	C_7H_6OS	−2.2	$C_4H_{10}N$
						107.0488	C_7H_7O	−2.8	C_4H_9NS
						88.0212	C_3H_6NS	−4.1	$C_8H_{10}O$
		179.0521	$C_{10}H_{11}OS$	−2.2	25	164.0286	C_9H_8OS	−2.5	CH_3•
		153.0365	C_8H_9OS	−2.1	20	138.0130	C_7H_6OS	−2.6	CH_3•
						125.0416	C_7H_9S	−2.8	CO
						109.0644	C_7H_9O	−3.2	CS
						107.0488	C_7H_7O	−3.1	CH_2S
3	196.0789		$C_{10}H_{14}ONS$	−0.7	25	179.0523	$C_{10}H_{11}OS$	−1.1	NH_3
						153.0367	C_8H_9OS	−1.2	CH_3NCH_2
						138.0132	C_7H_6OS	−1.7	C_3H_8N
		179.0522	$C_{10}H_{11}OS$	−1.5	25	164.0287	C_9H_8OS	−1.8	CH_3•
		153.0365	C_8H_9OS	−2.1	20	138.0130	C_7H_6OS	−2.6	CH_3•
						125.0416	C_7H_9S	−2.8	CO
						109.0645	C_7H_9O	−2.3	CS
						107.0488	C_7H_7O	−3.1	CH_2S
4	213.1133		$C_{11}H_{13}{}^2H_3ONS$	−1.1	23	179.0522	$C_{10}H_{11}OS$	−1.5	$C^2H_3NH_2$
						154.0428	$C_8H_8{}^2HOS$	−2.1	$C_3H_5{}^2H_2N$
						153.0366	C_8H_9OS	−1.7	$C_3H_4{}^2H_3N$
						138.0131	C_7H_6OS	−2.2	$C_4H_7{}^2H_3N$
						108.0551	$C_7H_6{}^2HO$	−2.6	$C_4H_7{}^2H_2NS$
						91.0400	$C_3H_3{}^2H_3NS$	−3.6	$C_8H_{10}O$
		179.0521	$C_{10}H_{11}OS$	−2.2	25	164.0286	C_9H_8OS	−2.5	C^2H_3•
		154.0427	$C_8H_8{}^2HOS$	−2.7	20	139.0193	$C_7H_5{}^2HOS$	−2.8	CH_3•
						138.0130	C_7H_6OS	−2.6	$CH_2{}^2H$•
						126.0479	$C_7H_8{}^2HS$	−2.8	CO
						110.0707	$C_7H_8{}^2HO$	−3.0	CS
						108.0551	$C_7H_6{}^2HO$	−2.9	CH_2S
		153.0365	C_8H_9OS	−2.1	20	138.0130	C_7H_6OS	−2.6	CH_3•
						125.0416	C_7H_9S	−2.8	CO
						109.0645	C_7H_9O	−2.3	CS
						107.0488	C_7H_7O	−3.1	CH_2S
5	212.1072		$C_{11}H_{14}{}^2H_2ONS$	−0.4	23	181.0649	$C_{10}H_{11}OS$	−0.8	CH_3NH_2
						155.0492	$C_8H_7{}^2H_2OS$	−1.5	C_3H_7N
						139.0194	$C_7H_5{}^2HOS$	−1.9	$C_4H_9{}^2HN$
						138.0132	C_7H_6OS	−1.3	$C_4H_8{}^2H_2N$
						109.0615	$C_7H_5{}^2H_2O$	−2.1	C_4H_9NS
						88.0213	C_3H_6NS	−3.3	$C_8H_8{}^2H_2O$
		181.0649	$C_{10}H_{11}OS$	−0.8	20	166.0414	$C_9H_6{}^2H_2OS$	−1.3	CH_3•
		155.0492	$C_8H_7{}^2H_2OS$	−1.5	20	140.0257	$C_7H_4{}^2H_2OS$	−1.6	CH_3•
						139.0194	$C_7H_5{}^2HOS$	−1.9	$CH_2{}^2H$•
						138.0132	C_7H_6OS	−1.3	CH^2H_2•
						127.0543	$C_7H_7{}^2H_2S$	−1.8	CO
						111.0771	$C_7H_7{}^2H_2O$	−2.1	CS
						109.0615	$C_7H_5{}^2H_2O$	−2.0	CH_2S

[a] Abundance < 5%



Scheme 2. Proposed dissociation pathway of JTV-519 (**1**).



Scheme 3. Proposed dissociation pathway of S-107 (**2**).

Table 3. Summary of assay validation results

Compound	LLOD (ng/mL)	Recovery (%) at 100 ng/mL	Intraday precision (n = 30)		Interday precision (n = 30 + 30 + 30)	
			Concentration (ng/mL)	CV (%)	Concentration (ng/mL)	CV (%)
1	0.1	92	1	10.1	1	10.0
			10	7.4	10	8.7
			100	9.1	100	9.4
2	0.1	74	1	18.5	1	17.8
			10	16.1	10	15.8
			100	5.2	100	9.6
3	0.2	68	1	18.3	1	18.8
			10	9.2	10	13.1
			100	9.7	100	8.8

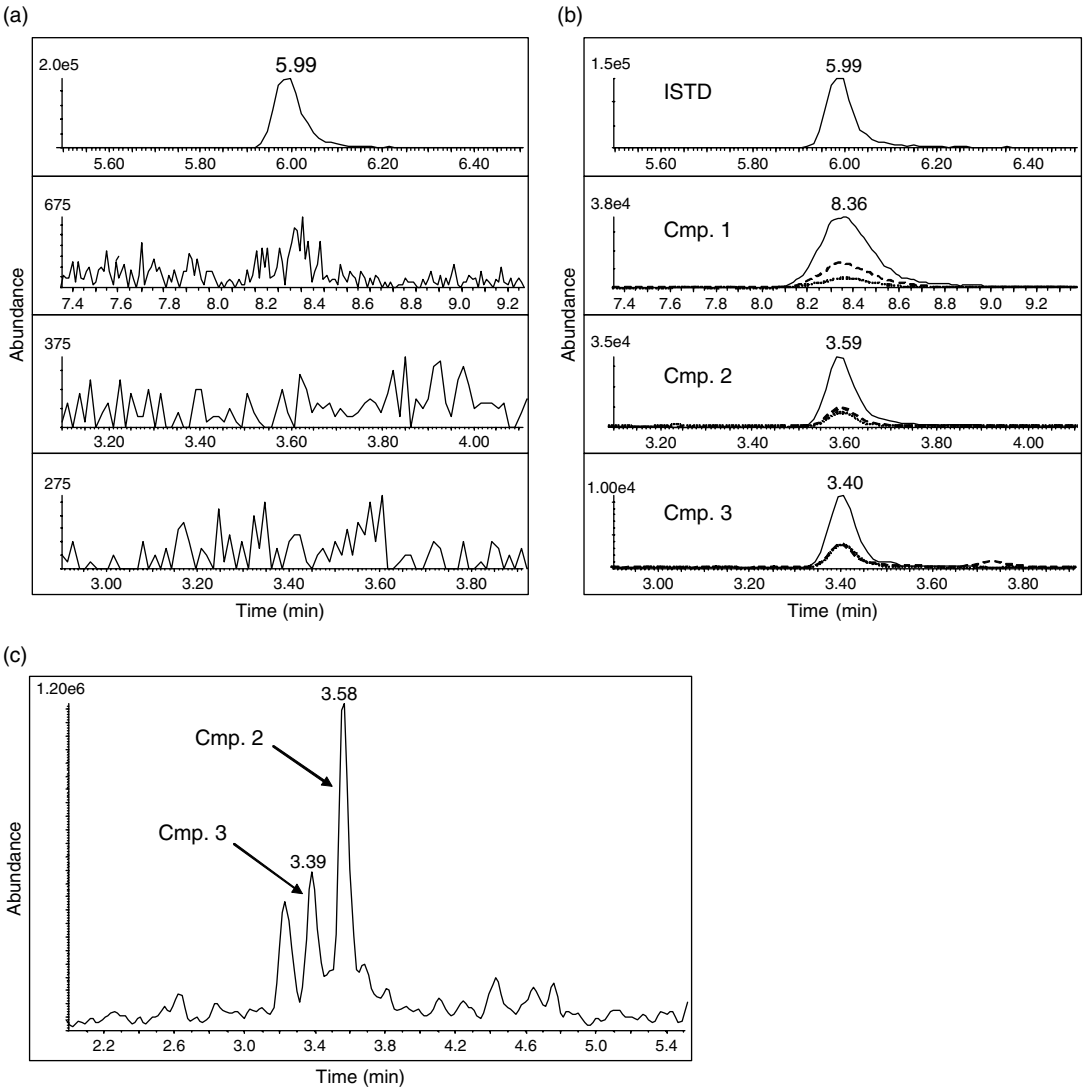


Figure 3. Extracted ion chromatograms of a) blank urine containing the internal standard (methyltestosterone) only, and b) urine specimen spiked with 1 ng/mL of each target compound measured on an Agilent 1100 HPLC (Waldbronn, Germany) interfaced to an Applied Biosystems API 4000 QTrap (Darmstadt, Germany). All analytes were determined using three diagnostic ion transitions (see Table 1) employing multiple reaction monitoring. Precursor ion scan experiments were conducted on *m/z* 153 and required approx. 10 ng/mL of analyte in order to generate abundant signals as illustrated in c).

labelled analogs **4** and **5** (Table 2) that showed corresponding product ions with and without the retention of deuterium atoms. The ion at *m/z* 107 is also obtained from *m/z* 153, presumably by the loss of carbene (−14 Da) and sulphur (−32 Da). Based on the information obtained from accurate mass measurements (Table 2), which prove the nominal loss of CH_2S, the deuterium labelling experiments with compounds **4** and **5** (Fig. 2d and e), which demonstrate a shift of *m/z* 107 to 108 and 109, respectively, and the fact that the elimination of carbene was observed in earlier studies,[29] a structure of 3-methyl phenol is proposed.

It is suggested that the product ion at *m/z* 109 (Fig. 2b) originates from the protonated molecule as well as from *m/z* 153, being composed of protonated methoxybenzene (C_7H_9O), as proved by accurate mass measurements (Table 2).

The product ion mass spectra derived from compounds **2** and **3** yielded highly comparable dissociation patterns except for one product ion at *m/z* 88 that was generated by compound **2** only (Figs 2b and 2c). Its composition was determined as C_3H_6NS (Table 2), and H/D exchange experiments proved the absence of the introduced proton in the product ion, which is suggested to bear a 2-methyl-4H[1,2]-thiazete structure (Scheme 3c). Further evidence for the intact methyl function was obtained by the analysis of the triply deuterated analog of S-107 (Fig. 1, compound **4**), which yielded a corresponding ion at *m/z* 91 (Fig. 2d).

Assay validation

Based on the mass spectrometric data, an assay for the qualitative determination of the selected 1,4-benzothiazepines was established and validated for doping control purposes. The validation results are summarized in Table 3. In order to include this new class of target compounds into existing sports drug testing procedures, the sample preparation strategy was adapted from established screening and confirmation methods applied for designer anabolic steroids.[17] The target analytes were analysed by LC-MS/MS employing an Agilent 1100 Series liquid chromatograph coupled to an Applied Biosystems API 4000 QTrap mass spectrometer in multiple reaction monitoring mode (MRM), and typical extracted ion chromatograms obtained from a blank urine specimen and a sample enriched with 1 ng/mL of each compound are illustrated in Fig. 3. Moreover, a precursor ion scan experiment was implemented using the core ion of 1,4-benzothiazepines at *m/z* 153, which allows a broader screening for structurally related or modified drugs and respective metabolic degradation products. Using spiked urine samples containing 10 ng/mL of compounds **2** and **3**, abundant signals are obtained that indicate the presence of substances related to 1,4-benzothiazepines (Fig. 3c) and might also support the identification of metabolic products of compound **1**. This strategy has been used frequently in metabolite identification studies as well as doping control procedures.[30–34]

Specificity and lower limit of detection

No interfering signals at expected retention times were observed for any of the target analytes (Fig. 3a). The compounds **1,2** and **3** were identified at estimated LLODs of 0.1–0.2 ng/mL of urine (Table 3).

Recovery

Recoveries of all tested compounds ranged from 68% to 92% as outlined in detail in Table 3.

Intraday precision and interday precision

The intraday and interday precision were determined at three concentrations of all target analytes and ranged from 10.0 to 18.8%, 7.4 to 16.1% and 5.2 to 9.7% for low (1 ng/mL), medium (10 ng/mL), and high (100 ng/mL) concentrations, respectively (Table 3).

Test for ion suppression/enhancement effects

Four different blank urine samples (two male, two female donors) were prepared for analysis and measured as described. Via T-connector all four analytes were continuously infused and ion suppression/enhancement effects were less than 10% at retention times of the target compounds.

Administration study urine samples/metabolite identification

Administration study urine samples are common and important items of method validations in order to demonstrate the applicability of new procedures to authentic specimens. In this particular case, such samples are not available as drugs are still undergoing clinical trials and not yet approved to enter the market. However, *N*-dealkylation is a major degradation pathway as observed with numerous drugs bearing mono- or bis-alkylated amino functions.[35–39] Hence, a putative desmethylated metabolite of S-107 was synthesized and the screening procedure, based on MRM and precursor ion scan experiments, established to enable the detection of known target analytes using specific and diagnostic product ions as well as the determination of unknown related drugs and/or metabolites due to a characteristic product ion. However, future *in vitro* and *in vivo* studies will be required to complement the list of target compounds and to evaluate the metabolism of these drugs in humans.

Conclusions

The issue of doping and manipulation has always been an unpopular companion of sports. Constantly increasing knowledge of biochemical, physiological and medicinal options for influencing pathways of energy expenditure and, thus, of possibilities for increasing athletic performance artificially has necessitated new strategies in the fight against doping. One approach consists of preventive doping research, which aims to incorporate new, emerging drugs into sports drug-testing procedures as soon as the potential for misuse of novel therapeutics is evident and clinical trials have advanced to phase II or later. The major problem is the availability of reference material, which is required to establish reliable and sensitive detection assays. Hence, drug candidates and metabolites need to be synthesized using either chemical and/or enzymatic methodologies. Studies on mass spectrometric behaviours are conducted and existing screening protocols are expanded to include new substances or their degradation products. In the present investigation, new compounds presumably increasing athletic performance by correcting malfunctions of calcium-channels in muscle tissue after intense exercise were prepared, characterized and implemented in existing analytical assays for human sports drug testing. In combination with results of future *in vitro*/*in vivo* studies, the data obtained will facilitate the identification of known analytes in urine samples as well as the characterization of unknown metabolites by means of diagnostic dissociation patterns.

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