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HPLC-ESI-MS/MS characterisation of metabolites produced by *Pseudovibrio* sp. W64, a marine sponge-derived bacterium isolated from Irish waters

Short Title: ESI-MS/MS of marine bacteria *Pseudovibrio* sp. W64 metabolites

Alka Choudhary[1], Lynn M. Naughton[2], Alan D.W. Dobson[2,3] and Dilip K. Rai [1,*]

[1] Department of Food Biosciences, Teagasc Food Research Centre Ashtown, Dublin D15 KN3K, Ireland

[2] School of Microbiology, University College Cork, Western Road, T12 YN60, Cork City,

[3] Environmental Research Institute, University College Cork, Lee Road, Cork, T23 XE10, Ireland

***** Correspondence: dilip.rai@teagasc.ie; Tel.: +353-(0)-1805-9569; Fax: +353-(0)-1805-9550

Abstract

RATIONALE: In recent years**,** metabolites produced by *Pseudovibrio* species have gained scientific attention due to their potent antimicrobial activity. Recently we also have assessed antibacterial activities of *Pseudovibrio* sp. W64 isolates against *Staphylococcus aureus,* where only the dominant tropodithietic acid (TDA) was identified. However characterisation of other metabolites is necessary as these metabolites may also serve as potent antimicrobial agents.

METHODS: LC-MS/MS, aided by accurate mass measurements, was employed to screen and characterise a range of metabolites produced by *Pseudovibrio* sp. W64 via assessment of ethyl acetate fractions generated from bacterial cultures.

RESULTS: Thirteen metabolites unique to the bacterial culture were detected and their chemical structures were assigned by tandem mass spectrometry and accurate mass measurement. Among the thirteen metabolites, a methyl ester of TDA, a number of cholic acid derivatives, and amino diols and triols were characterised.

CONCLUSIONS: *Pseudovibrio* sp. W64 produces methylated TDA in addition to TDA, and metabolises lipids and amino acids in the cell-culture medium. To the best of our knowledge, this is the first report of methylated TDA, cholic acid and its various analogs, and sphinganine being detected in this *Pseudovibrio* strain. The data generated may help to better understand the biochemical processes and metabolism of bacterial strains towards discovery of antimicrobial agents from marine sources.

Keywords: *Pseudovibrio* sp.; Tropodithietic acid; cholic acid; sphinganine; HPLC-MS/MS

1. Introduction

Marine microorganisms possess such an inherent potential to produce chemically and stereo-chemically unique natural products with varying bioactivities that have fetched attention from researchers to uncover novel natural products [1]. Over the past few years, marine-derived *Pseudovibrio* species have been recognised due to the dominance of the genus amongst host-associated marine microbial communities, and more importantly due to the broad-spectrum antimicrobial capabilities of the strains [2-6]. Members of the *Pseudovibrio* genus belong to the Rhodobacteracea family, Rhodobacterales order and alphaproteobacteria class. These organisms are widely distributed among numerous marine sources such as the surfaces of tunicates [2], sea squirts [7], coral [8], seawater [9], algae [9] and are often found in close association with sponges, such as *Axinella corrugata*, *Mycale laxissima* [10]; *Erylus discophorus* [11]; *Irciniidae* sponges [12]; *Didiscus oxeata* [13]; *Paraleucilla magna*, *Clathrina aurea*, *Mycale microsigmato* [14]; *Axinella dissimilis*, *Polymastia boletiformis* and *Haliclona simulans* [15], *Suberites carnosus*, *Leucosolenia* sp and *Amphilectus fucorum* [16]. *Pseudovibrio denitrificans* (*P. denitrificans*) type strain DN34 was previously reported to produce the red pigment heptylprodigiosin possessing antimicrobial activity against *Staphylococcus aureus* (*S. aureus*) [2]. Vizcaino *et al*. reported the production of the polypeptide pseudovibrocin, which was isolated from three unique coral-derived bacteria [8]. Biosynthesis of tropodithietic acid (TDA), a tropolone antibiotic, has been observed in a number of strains of this genus [4]. TDA has been shown to be active against a broad spectrum of human and fish pathogens, including *Morganella morganii* and *Pandoraeae sputonum*, both multidrug resistant opportunistic pathogens commonly associated with nosocomial infections [17-19]. TDA-producing *Pseudovibrio* isolates also displayed antimicrobial activity against *Salmonella enterica* sp. arizonae and *S. aureus*, one of the top three pathogens of blood stream infections [20-22]. Wang *et al.* have isolated and characterised a number of diindol-3-ylmethanes with antifouling activities from the crude extract of *P. denitrificans* UST4-50 using NMR spectroscopy and mass spectrometry [23]. Recently, Nicacio *et al.* have detected several bromotyrosine-derived alkaloids in the cultures of *P. denitrificans* Ab134. Earlier, marine sponges were known to biosynthesise these types of secondary metabolites [24].

In previous work by Harrington *et al*. *Pseudovibrio* sp.W64 isolates had been reported to possess antibacterial activities against *S. aureus* and TDA was found to be the major compound responsible for the activity [25]. Production of TDA and expression of the *tda*A

and *tda*B genes involved in the production of TDA occurred following 24 h growth of strain W64 [25]. Following on from that, here we present the profile of metabolites of *Pseudovibrio* sp. W64 using high performance liquid chromatography quadrupole time of flight mass spectrometry (HPLC-Q-Tof-MS). Thirteen metabolites that are unique to the bacterial culture were characterised through accurate mass measurements and tandem mass spectrometry (MS/MS) analysis.

2. Experimental

2.1 Bacterial strains and culture conditions

Pseudovibrio sp. W64 was isolated from the marine sponge *Axinella dissimilis* and subjected to genetic, physiological and antimicrobial assessment as previously described by O' Halloran *et al*. [26]. *Pseudovibrio* sp. W64 was streaked from stock on marine agar (Difco) and incubated at 28°C for 48 h. An individual colony of the strain of interest was used to inoculate 50 mL of marine broth (Difco) (in duplicate) and growth was allowed to proceed for 24 h at 28°C with agitation (180 r.p.m). Cultures were centrifuged at 7,000 g for 10 minutes at 4°C using an Avanti J-E centrifuge and rotor JA-10. Following centrifugation, the resulting supernatant was subjected to filtration through 0.45 µm filters (Sarstedt). To ensure no cells were present in the filtrate, an aliquot (100 µL) of the supernatant was spread on marine agar plates, which were assessed following 48 h incubation at 28°C.

2.2 Chemicals

Marine agar and marine broth were purchased from Difco and prepared according to the manufacturer's instructions. Commercial standards of cholic acid, glycocholic acid and sphinganine were procured from Sigma Aldrich, Wicklow, Ireland. HPLC-grade acetonitrile, ethyl acetate and formic acid were purchased from Sigma Aldrich, Wicklow, Ireland. Ultra-pure water (18.2 MΩ) was generated in the laboratory by using a Milli-Q Plus water purification system.

2.3 Extraction of metabolites

Metabolites from the cell free supernatants were extracted twice with equal volumes of ethyl acetate. The upper organic layers were separated and dried under a gentle flow of nitrogen at room temperature. The dried ethyl acetate fractions were further reconstituted in acetonitrile

filtered through 0.22 µm polytetrafluoroethylene (PTFE) filters before injecting into the HPLC-MS system.

2.4 HPLC-MS and HPLC-MS/MS analysis

HPLC-MS and MS/MS analyses were performed on a Q-Tof Premier mass spectrometer (Waters Corporation, Milford, MA, USA) coupled to an Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA). The Q-Tof Premier is equipped with a lockspray source where an internal reference compound (Leucine-Enkephalin) was introduced simultaneously with the analytes for accurate mass measurements. Separation of compounds was achieved on an Atlantis T3 C18 column (Waters Corporation, Milford, USA, 100 mm x 2.1 mm; 3 µm particle size) using 0.1 % aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Column temperature was maintained at 40°C. A stepwise gradient from 10% to 90% solvent B was applied at a flow rate of 0.3 mL/min for 60 min. Electrospray mass spectra data were recorded in positive and negative ionisation mode for a mass range *m/z* 100 to *m/z* 1000. Capillary voltage and cone voltage were set at 3 kV and 30 V, respectively. Collision induced dissociation of the analytes was achieved using 12 eV to 20 eV energy with argon as the collision gas. HPLC-MS chromatograms of growth medium and bacterial culture were compared in order to detect unique *m/z* signals in bacteria culture.

3. Results and Discussion

Since marine broth contains large amounts of salt and other minerals, ethyl acetate was chosen to extract the metabolites due to its moderate polarity and low boiling point. These ethyl acetate extracts were then evaluated by HPLC-MS/MS for metabolite profiling.. The HPLC-ESI-MS chromatograms (+ and – ion modes) of sterile marine broth and W64 strain cultures were compared in an effort to identify chromatographic peaks that are unique to the bacterial culture (Figure 1). In the full scan mass spectra, the protonated molecules $[M+H]^+$ and alkali metal adducts $[M+Na]^+$ and $[M+K]^+$ were observed in positive ionisation mode, while the deprotonated molecules $[M-H]^-$ were detected in negative ionisation mode. Tentative identifications of the compounds **1-13** (Figure 2) unique to the bacterial culture were reached through accurate mass measurements of precursor ions and their MS/MS fragment ions (Table 1 and Supplementary Information). The identities of metabolites, glycocholic acid (**4**), cholic acid (**8**) and sphinganine (**10**) were confirmed using the commercially available standards.

As previously mentioned *Pseudovibrio* species produce TDA (**1**) as one of their major metabolites. This compound was indeed detected as a major metabolite at t_R 12.3 min in the W64 cultures when screened for metabolites using the photodiode array (PDA) detector (200-800 nm). The characteristic UV spectra of the HPLC-PDA peak displaying absorption maxima at 242, 303, 356 and 450 nm (Figure 3) was in corroboration with that of pure TDA [27]. The identity of the compound was further confirmed based on its accurate mass determination and collision induced dissociation (CID) pattern (Figure 4). The ions at *m/z* 212.9684 corresponded to the protonated molecular $[M+H]^+$ species of TDA and also observed by Bruhn *et al.* [28].. The product ion scan of the precursor ions at *m/z* 212.97 generated *m/z* 194.96, 166.96, 150.97 ions, which were assigned respectively to have the following chemical compositions: $[C_8H_3O_2S_2]^+$, $[C_7H_3OS_2]^+$ and $[C_7H_3S_2]^+$. The proposed low-energy CID-MS/MS fragmentation pattern suggests that the protonated molecule undergoes dehydration $[M+H-H_2O]^+$ to form a stable carbonium product ion at *m/z* 194.96. Subsequent loss of 28 Da from the carbonium ion was due to neutral loss of a carbon monoxide molecule yielding a tropodithietane cation $[M+H-H_2O-CO]^+$ at *m/z* 166.96. Elimination of carbon monoxide (28 Da) from the seven member tropone cation resulted in the formation of a six membered benzo-dithietane cation at *m/z* 138.97. The product ion at *m/z* 150.97 was proposed to be formed from tropodithietane cation through the loss of a hydroxyl group as suggested by its predicted molecular formula. The product ions at *m/z* 167 and 151 have been previously described in the literature as CID fragment ions of TDA, however, the mechanism of fragmentation has not been proposed [29]. Characterisation of compound **1** has also been previously reported by NMR spectroscopy establishing its accurate structure and position of functional groups [30].

Compound (**2**) at t_R 11.0 min showed *m/z* 226.9841 $[M+H]^+$ fitting to molecular formula $[C_9H_7O_3S_2]^+$, along with the sodium adduct at *m/z* 248.97. Low energy CID-MS/MS analysis of the precursor protonated molecules $[M+H]^+$, shown in Figure 5, produced a series of major product ions with a mass difference of 59 Da at *m/z* 167.97 attributed to loss of methyl-carbonyl $[M+H-COOCH_3]^+$ moiety assigned as $[C_7H_4OS_2]^+$. This cation further lost a molecule of carbon monoxide, i.e. $[M+H-COOCH_3-CO]^+$, to produce the product ions at *m/z* 139.97 $[C_6H_4S_2]^+$ and its subsequent dehydration yielded *m/z* 150.21. Minor product ions including *m/z* 109.01 and 122.02 fitting to molecular formula $[C_6H_5S]^+$ and $[C_7H_6S]^+$ were also observed in the CID-MS/MS spectrum. The CID-MS/MS pattern (Figure 5) of compound **2** was found to be similar to the known molecule TDA (**1**). Hence, based on the CID-MS/MS fragmentation pattern and accurate mass determination, the molecule was

tentatively characterised as a methyl ester of TDA. Methanol was not used throughout the sample preparation step nor as a HPLC solvent to ensure that the ions *m/z* 227.98 are not due to an artefact [31] formed by methylation of TDA during the extraction protocol.

Compound (**3**) at t_R 14.3 min showed protonated $[M+H]^+$ ions at *m/z* 464.3033 along with a sodium ion adduct at *m/z* 486.28 $[M+Na]^+$. The presence of this compound was also observed in negative ionisation mode at *m/z* 462.28 $[M-H]^-$. The low energy CID-MS/MS analysis of the precursor protonated molecules $[M+H]^+$, produced a series of major product ions at *m/z* 446.29 ($C_{26}H_{40}O_5N^+$) for $[M+H-H_2O]^+$, 428.28 ($C_{26}H_{38}O_4N^+$) for $[M+H-2H_2O]^+$, 410.27 ($C_{26}H_{36}O_3N^+$) for $[M+H-3H_2O]^+$ and 335.24 ($C_{24}H_{31}O^+$) for $[M+H-3H_2O\text{-glycine}]^+$ (Table 1). Product ions at *m/z* 76.0397 ($C_2H_6NO_2^+$) corresponding to protonated glycine suggested the glycine conjugation of the molecule. The loss of the side-chain containing pentanoic acid linked to glycine, i.e. ($-CH(CH_3)CH_2CH_2CONHCH_2COOH$), resulted the steroidal core ions at *m/z* 253. Fragment ions corresponding to sequential loss of alkyl units (loss of 14 Da) indicated fragmentation from four rings of the steroidal core were present in the MS/MS spectrum. Fission of ring D produced the product ions *m/z* 227 and 209. Base peak with ions *m/z* 209 formed upon cleavage of ring D is characteristic of sterol triene compounds and hence suggests the presence of three double bonds in the sterol ring system [32]. Subsequent fragmentation of ring C resulted in ions at *m/z* 171, 143 and 105; ring B at *m/z* 157, 131, 119 and 95 and ions at *m/z* 183 and 199 were produced due to cleavage of ring A. This type of charge driven fragmentation is reported for steroidal compounds including cholesterol and bile acids [33]. The MS/MS fragment ions and accurate mass determination led to tentative characterisation of the molecule as 3,7,12-trihydroxychol-5-enoylglycine [34]. This compound is structurally analogous to cholic acid, which along with several other derivatives is widely distributed among eukaryotes, however, few reports detail its biosynthesis in prokaryotes. *Streptomyces faceum* AN21 has previously been reported to produce methylcholate which exhibits antibacterial activity against Gram-positive and negative bacteria [35]. Other cholanic acid derivatives including; cholic acid, deoxycholic acid, glycocholic acid, deoxyglycocholic acid have been detected in *Myroides* sp. Marine bacteria are believed to produce cholanic acid metabolites by conversion of cholesterol from the culture media to cholic acid [36]. Kim *et al.* have also reported biosynthesis of cholanic acid derivatives in the marine bacterium *Donghaeana dokdonensis* and indicated that these metabolites are produced by a variety of bacteria in sea waters around the world [37].

Compound (**4**) at t_R 15.4 min displayed ions *m/z* 466.3184 $[M+H]^+$ and 488 $[M+Na]^+$ in positive ionisation mode, and 464.30 $[M-H]^-$ in negative mode. The low energy CID-MS/MS

of $[M+H]^+$ ions corresponding to loss of one, two and three molecules of water were also present in the MS/MS spectrum at *m/z* 448.30 ($C_{26}H_{42}O_5N^+$) for $[M+H-H_2O]^+$, *m/z* 430.29 ($C_{26}H_{40}O_4N^+$) for $[M+H-2H_2O]^+$, *m/z* 412.28 ($C_{26}H_{38}O_3N^+$) for $[M+H-3H_2O]^+$, respectively. The CID-MS/MS gave product ions at *m/z* 337.25 ($C_{24}H_{33}O^+$) for $[M+H-3H_2O\text{-glycine}]^+$ and *m/z* 76.0397 ($C_2H_6NO_2^+$) for protonated glycine. The proposed MS/MS fragmentation of the molecule showing fragmentation of steroidal rings A, B, C and D is presented in Figure 6. The molecule was tentatively identified as glycocholic acid (GCA) based on the above information and accurate mass determination [37, 38]. For further confirmation of the identified compound, a commercial standard of glycocholic acid was analysed under same conditions, which eluted at same retention time and produced identical MS/MS fragmentation pattern as that of compound **4**. GCA has been previously reported to be produced by *Pseudovibrio* sp.[39].

The compound (**5**) appearing at t_R 16.2 min in the HPLC-MS chromatogram showed ions for $[M+H]^+$ at *m/z* 407.2781 while its sodium adduct ions at *m/z* 429.26 were observed in higher abundance. ESI negative mode mass spectra presented ions *m/z* 405.26 corresponding to $[M-H]^-$ of the parent molecule. Similar to compound (**4**), the low-energy CID-MS/MS of the protonated precursor ions at *m/z* 407.28 created the product ions *m/z* 389.27, 371.28, and 353.25 due to loss of one, two and three water molecules, and resembled the fragmentation pattern of the cholic acid nucleus as discussed above.. Based on the MS/MS results and accurate mass measurement data, the tentative identification of compound (**5**) was reported as 3,7,12-trihydroxy-5-cholenoic acid [40].

Compound (**6**) at t_R 17.1 min showed ions with *m/z* 274.2738 $[M+H]^+$. The low energy CID-MS/MS fragmentation generated product ions at *m/z* 102.09, 88.07 and 70.06 corresponding to the molecular formulae $C_5H_{12}NO^+$, $C_4H_{10}NO^+$ and $C_4H_8N^+$, respectively. Accurate mass determination for this compound and its typical MS/MS fragment ions suggested to be hexadecasphinganine. The similar MS/MS fragmentation pattern of this compound has also been reported by Gong *et al.* in the rice metabolome [41]. Sphinganines are the dihydro derivative of the long chain amino alcohol sphingosine. Sphingosine is the core moiety of complex lipids called sphingolipids. Sphingolipids are ubiquitous elements in eukaryotic membranes and are also found in some bacterial, fungal and viral species [42]. Bacteria that are known to produce sphingolipids include *Sphingomonas* sp. and *Myxobacterium sp*., *Bacteroides fragilis, Porphyromonas gingivalis, Burkholderia pseudomallei* and *Bacillus cereus strain S2* [43, 44]. Sphingoid bases have been reported to possess an array of

biological activities including antiseptic, anti-inflammatory [45] and antimicrobial activity against Gram-positive and negative bacteria [46].

Another compound (**7**) eluting at t_R 17.2 min showed precursor ions at *m/z* 318.3005 $[M+H]^+$ proposing the elemental composition $C_{18}H_{40}NO_3$. The low energy CID-MS/MS fragmentation of the compound was similar to hexadecasphinganine with product ions at *m/z* 102.09, 88.07, 70.07 and 57.11 indicating it to be related to previously identified aminodiol compound. The additional oxygen atom in the proposed molecular formula, as opposed to sphinganine molecules, could be due to the presence of a hydroxyl group on the fatty acid chain or due to *N*-alkyl hydroxyl as in *N*-ethanolamines, since both of them correspond to the same molecular formula. The possibility of *N*-ethanolamine was ruled out due to the absence of ions at *m/z* 62 (the diagnostic ion formed due to cleavage of an ethanolamine head group). The fragmentation pattern and molecular formula were typical of the molecule 4-hydroxysphinganine [47].

Compound (**8**) at t_R 18.1 min showed abundant ions corresponding of sodium adducts at *m/z* 431.27 $[M+Na]^+$ and less abundant $[M+H]^+$ ions at *m/z* 409.29. The negative mode mass spectrum of the molecule showed ions at *m/z* 407.28 $[M-H]^-$. The low energy CID-MS/MS of the $[M+H]^+$ yielded major product ions at *m/z* 355.26 corresponding to loss of three molecules of water, along with minor product ions *m/z* 391.28, 373.27 depicting the loss of one and two water molecules, respectively. Similar to compounds **3-5** discussed earlier, the CID-MS/MS fragmentation of this compound also produced an MS/MS spectrum typical for a sterol nucleus. These observations along with the molecular formula deduced following accurate mass determination led to the assignment of this molecule as cholic acid [40]. The confirmation of identity of compound **8** was established with the HPLC-ESI-MS/MS characteristics of a commercially available cholic acid.

The compound (**9**) at t_R 19.3 min showed protonated ions $[M+H]^+$ at *m/z* 450.32 along with sodium adduct ions at *m/z* 472.30, while in negative mode corresponding deprotonated ions were observed at *m/z* 448.31. The low energy CID-MS/MS of the $[M+H]^+$ ions generated predominant product ions at *m/z* 414.30 attributed to loss of two water molecules, i.e. $[M+H-2H_2O]^+$. The MS/MS fragment pattern was identical to fragmentation of the steroidal ring described earlier in compounds **3** and **4**. These observations together with the accurate mass formula led to the prediction of the molecule as glycodeoxycholic acid, which has been reported to be produced by marine bacterium [36, 40].

At t_R 22.4 min, *m/z* 459.2727 ($C_{25}H_{40}O_6Na^+$) in positive mode and *m/z* 435.2750 ($C_{25}H_{39}O_6^-$) in negative mode were observed. These ions suggested the presence of a compound with the

molecular formula $C_{25}H_{40}O_6$. The compound has a molecular weight 28 Da higher than cholic acid and differs by CO atoms in terms of their molecular formula as predicted by accurate mass measurements. Moreover no ions corresponding to *m/z* 76 or 126 were observed in the spectrum indicating an absence of glycine or taurine conjugation in the CID-MS/MS spectrum. The MS/MS of $[M+H]^+$ showed product ions corresponding to loss of two water molecules at *m/z* 401.2697 along with predominant product ions *m/z* 355.2624 corresponding to molecular formula $C_{24}H_{35}O_2^+$. Other product ions corresponding to fission of sterol rings at *m/z* 55/199, 67/185, 81/171, 95/159, 105/145, and 119/131 were also observed in the MS/MS spectrum. MS/MS of *m/z* 435.27 $[M-H]^-$ yielded product ions *m/z* 407.28 $[M-H-CO]^-$ and 389.27 $[M-H-CO-H_2O]^-$ as major fragment ions. Based on the MS and MS/MS data, the compound was proposed to be a cholic acid derivative; however the exact structure of the compound (**11**) could not be determined.

Protonated ions t_R 21.3, 27.6 and 28.1 min displayed a similar fragmentation pattern to aminodiols as discussed above and was tentatively identified as sphinganine (**10**) [48], C_{20} sphinganine (**12**) [49] and hydroxy C_{22} sphinganine (**13**) [50] as presented in figure 2.

4. Conclusions

Pseudovibrio sp. W64, previously isolated from a marine sponge in Irish waters was found to produce a variety of metabolites and novel compounds. Based on high-resolution mass spectrometry analysis, thirteen metabolites were detected from the ethyl acetate fraction from the bacterial culture. The identified metabolites included a new secondary metabolite, i.e. a methyl ester of TDA, several cholanic acid derivatives, amino diols and triols, which have not been previously reported from this microorganism. The mechanism of fragmentation of these compounds has been proposed, which assisted in their structural elucidation. HPLC-MS/MS proved to be an efficient tool for rapid screening and characterisation of the molecule without actual purification. However further scale-up studies to isolate pure compounds and their absolute characterisation using NMR spectroscopy holds an encouraging scope to discover novel molecules from this bacterium.

Supplementary Materials: MS/MS spectra and accurate mass measurements of the MS/MS fragment ions of compounds **1-13** are available.

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Table 1: LC-MS/MS profiling of metabolites found in ethyl acetate extract of *Pseudovibrio* sp. W64

Compound no.	Retention time (min)	ESI (+) MS Observed *m/z*	Molecular Formula	Δppm	ESI (+) MS/MS *m/z*	Tentative Identity	Ref.
2	11.0	226.9841	$C_9H_7O_3S_2$	1.8	168, 150, 140, 121, 109, 95	Tropodithietic acid (TDA) - methyl ester	-
1	12.3	212.9684	$C_8H_5O_3S_2$	1.9	195, 167, 151, 139, 111	TDA	[26,27]
3	14.3	464.3033	$C_{26}H_{42}NO_6$	4.5	353, 335, 317, 253, 249, 235, 227, 209, 199, 183, 171, 157, 143, 131, 119, 105, 95, 85, 76	3,7,12-trihydroxychol-5-enoylglycine.	[33]
4	15.4	466.3184	$C_{26}H_{44}NO_6$	3.2	412, 337, 319, 255, 227, 209, 199, 185, 171, 159, 145, 133, 119, 107, 95, 81, 76, 67, 55	Glycocholic acid	[36,37]
5	16.2	407.2781	$C_{24}H_{39}O_5$	-3.9	353, 335, 317, 235, 227, 211, 209, 199, 183, 171, 157, 145, 133, 119, 105, 95, 85, 81	3,7,12-trihydroxy-5-cholenoic acid	[39]
6	17.1	274.2738	$C_{16}H_{36}NO_2$	-2.9	102, 88, 70, 57	Hexadecasphinganine	[40]
7	17.2	318.3005	$C_{18}H_{40}NO_3$	-0.9	256, 102, 88, 70, 57	4-hydroxysphinganine	[46]
8	18.1	409.2947	$C_{24}H_{41}O_5$	-1.7	373, 355, 337, 319, 273, 254, 227, 213, 119, 185, 171, 159, 145, 133, 119, 107, 95, 81, 67, 55	Cholic acid	[39]
9	19.3	450.3206	$C_{26}H_{44}NO_5$	-2.9	414, 339, 321,239, 229, 215, 211, 201, 175, 161, 158, 149, 135, 119, 107, 95, 81, 76, 67	Glycodeoxycholic acid	[36,40]
10	21.3	302.3055	$C_{18}H_{40}NO_2$	-1.3	106, 102, 88, 70, 57	Sphinganine	[47]
11	22.4	459.2727	$C_{25}H_{40}O_6Na$	-1.1	355, 239, 227, 213, 199, 185, 171, 159, 145, 131, 119, 105, 95, 81, 67, 55	Cholic acid derivative	-

12	27.6	330.3372	$C_{20}H_{44}NO_2$	-3.0	106, 102, 88, 70, 57	C_{20} sphinganine	[48]
13	28.1	374.3620	$C_{22}H_{48}NO_3$	-3.7	312, 102, 88, 70, 57	4-hydroxy C_{22}sphinganine	[49]

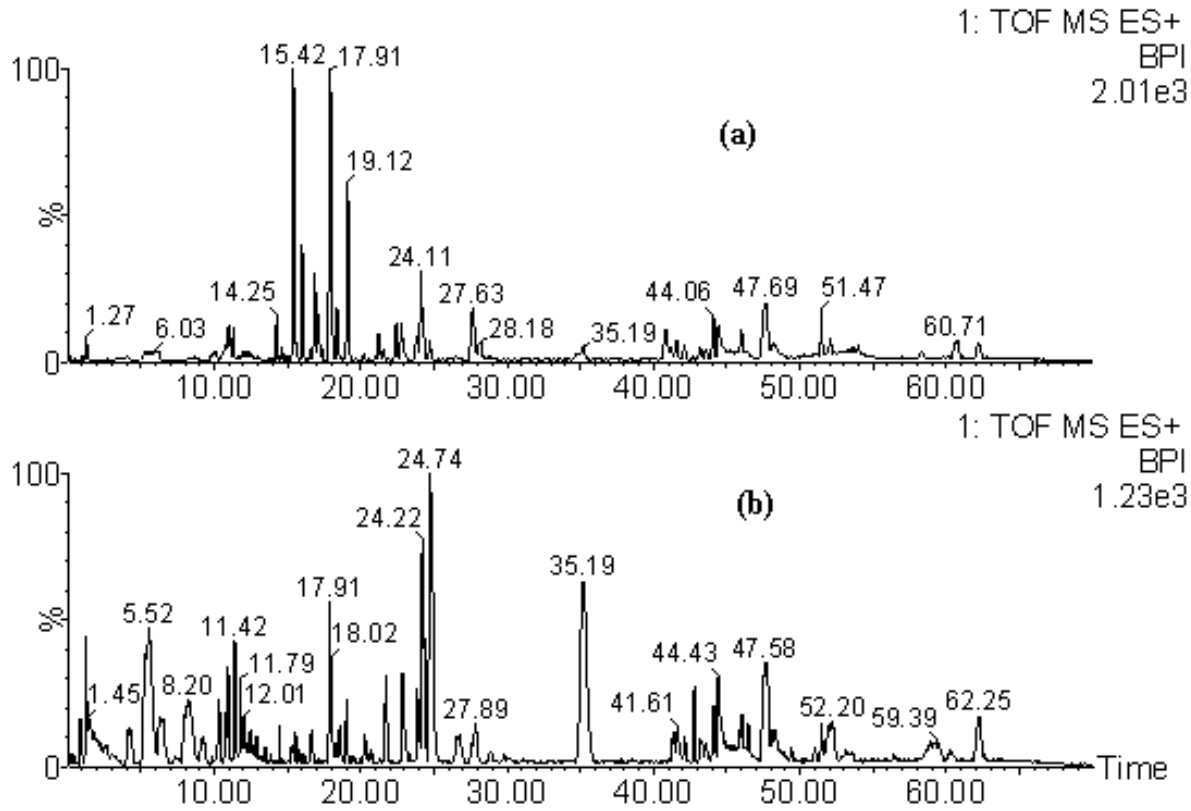

Figure 1: LC-MS chromatogram of ethyl acetate fractions of (a) culture supernatant from W64 strain grown in marine broth (b) Marine broth only

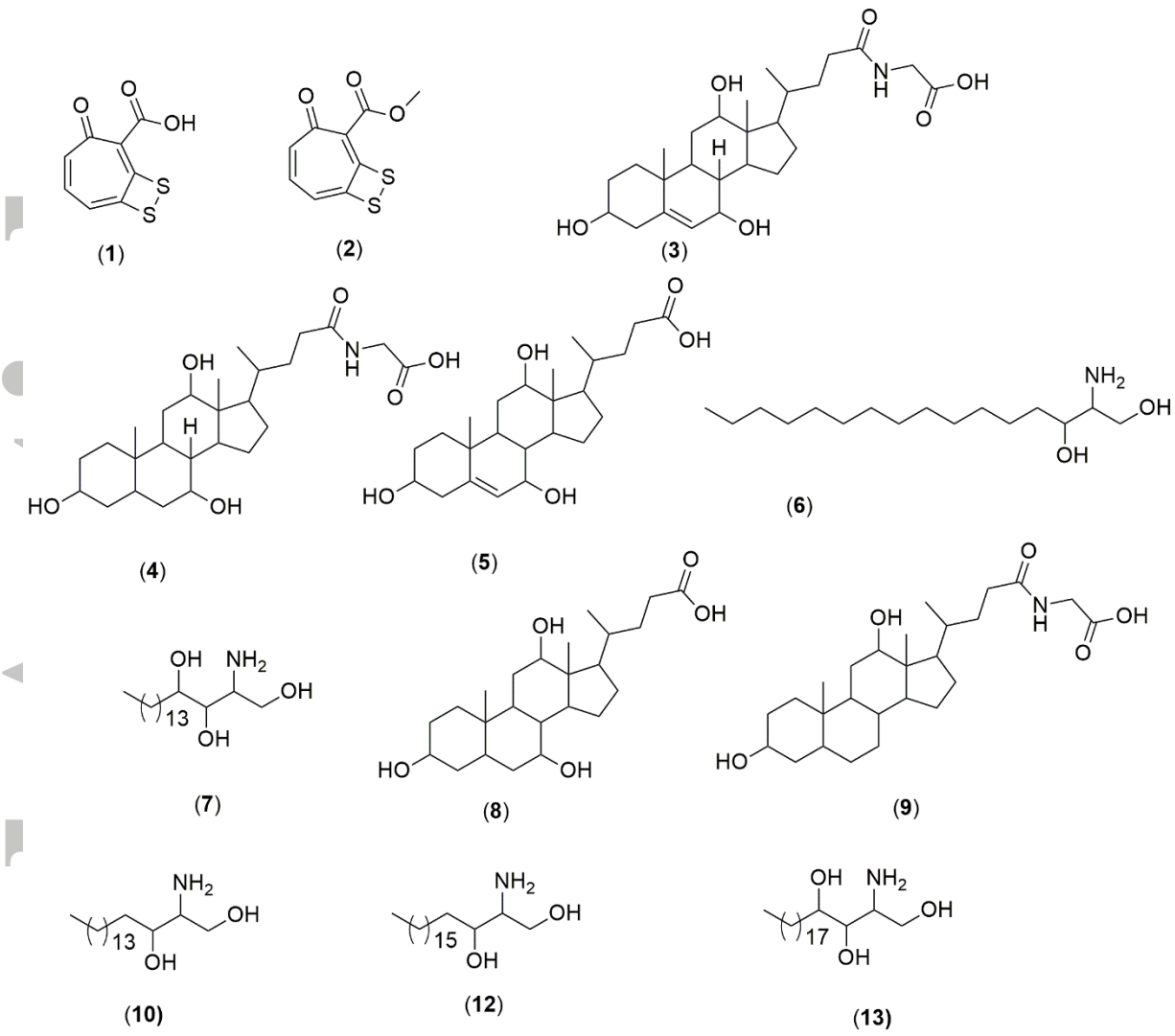

Figure 2: Proposed chemical structures of metabolites detected in W64 strain

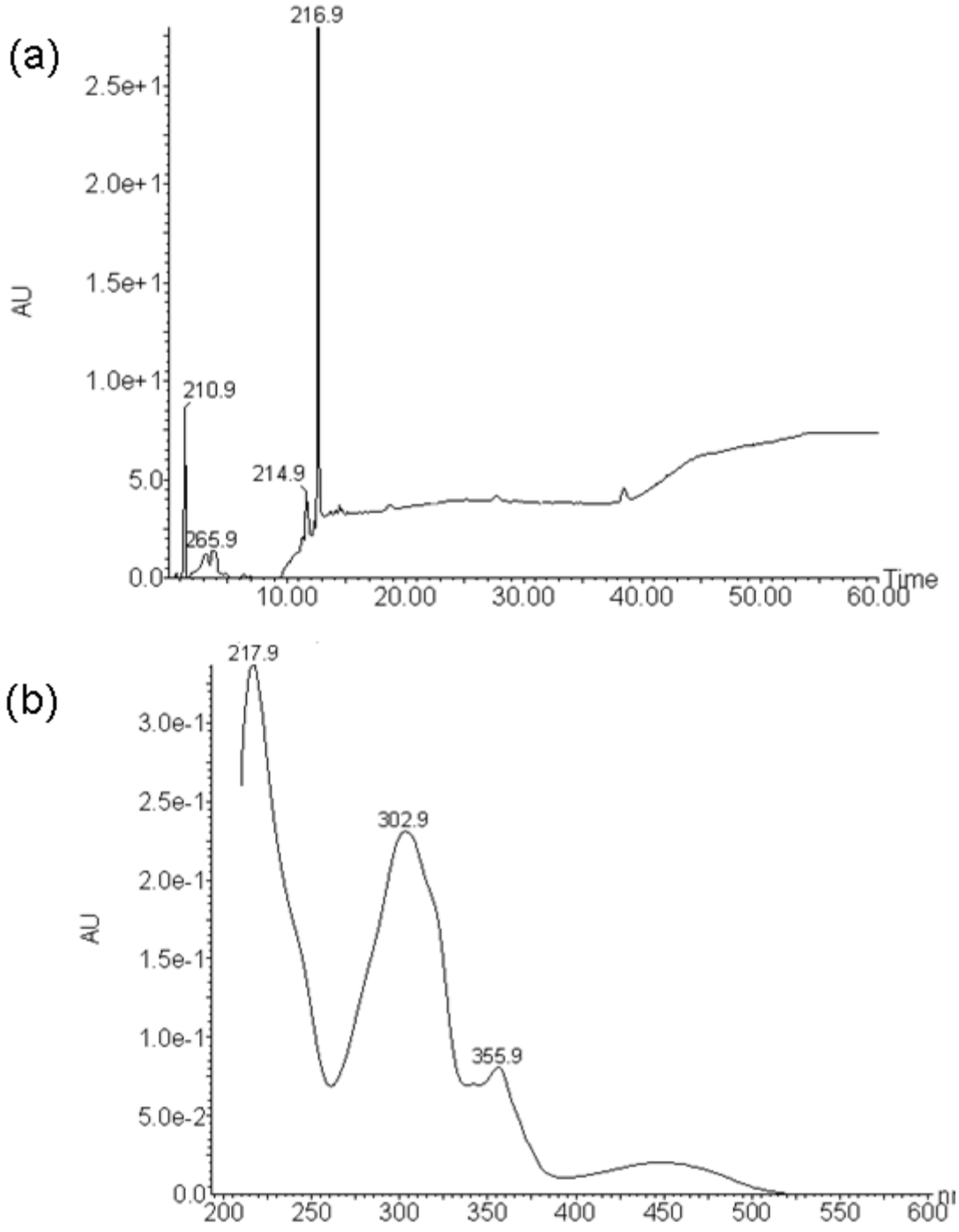

Figure 3: (a) HPLC-PDA chromatogram of ethyl acetate fraction of W64 strain. (b) UV spectrum of the peak appearing at t_R= 12.3 min

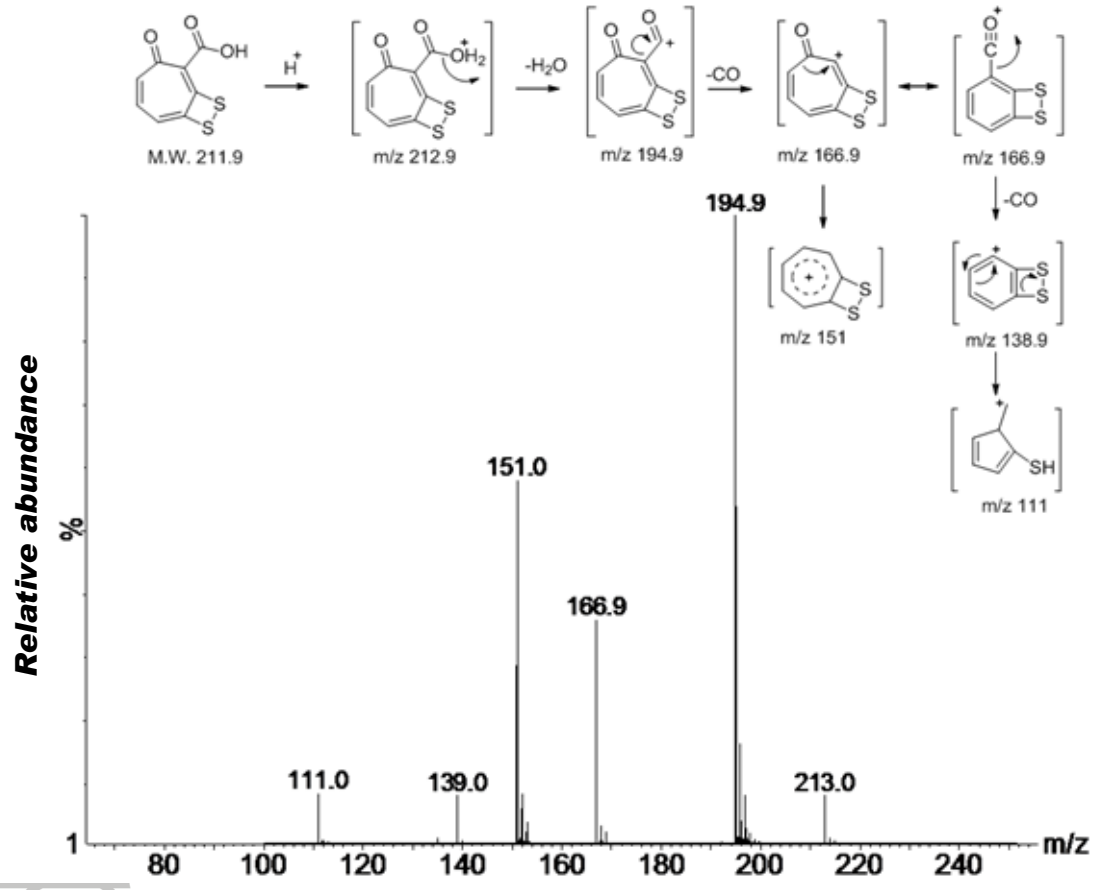

Figure 4: Proposed ESI-MS/MS fragmentation pattern of Tropodithietic acid (TDA) (**1**)

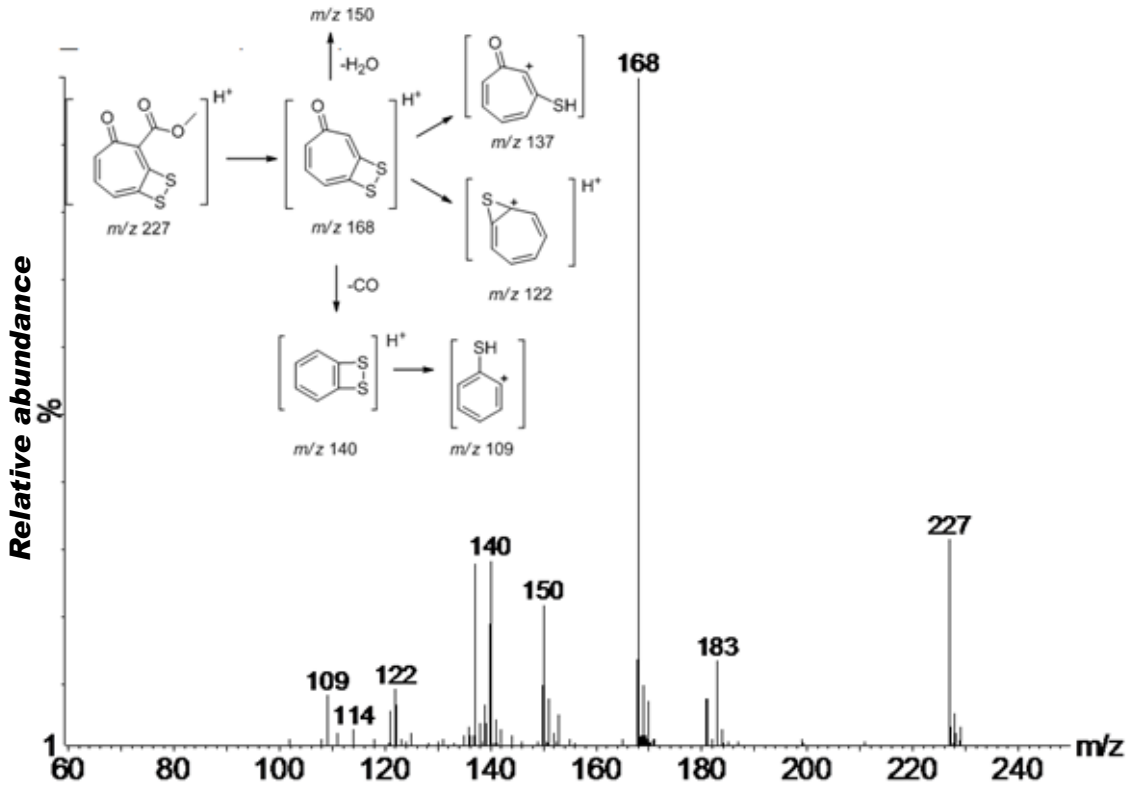

Figure 5: Proposed ESI-MS/MS fragmentation pattern of methyl ester of TDA (**2**)

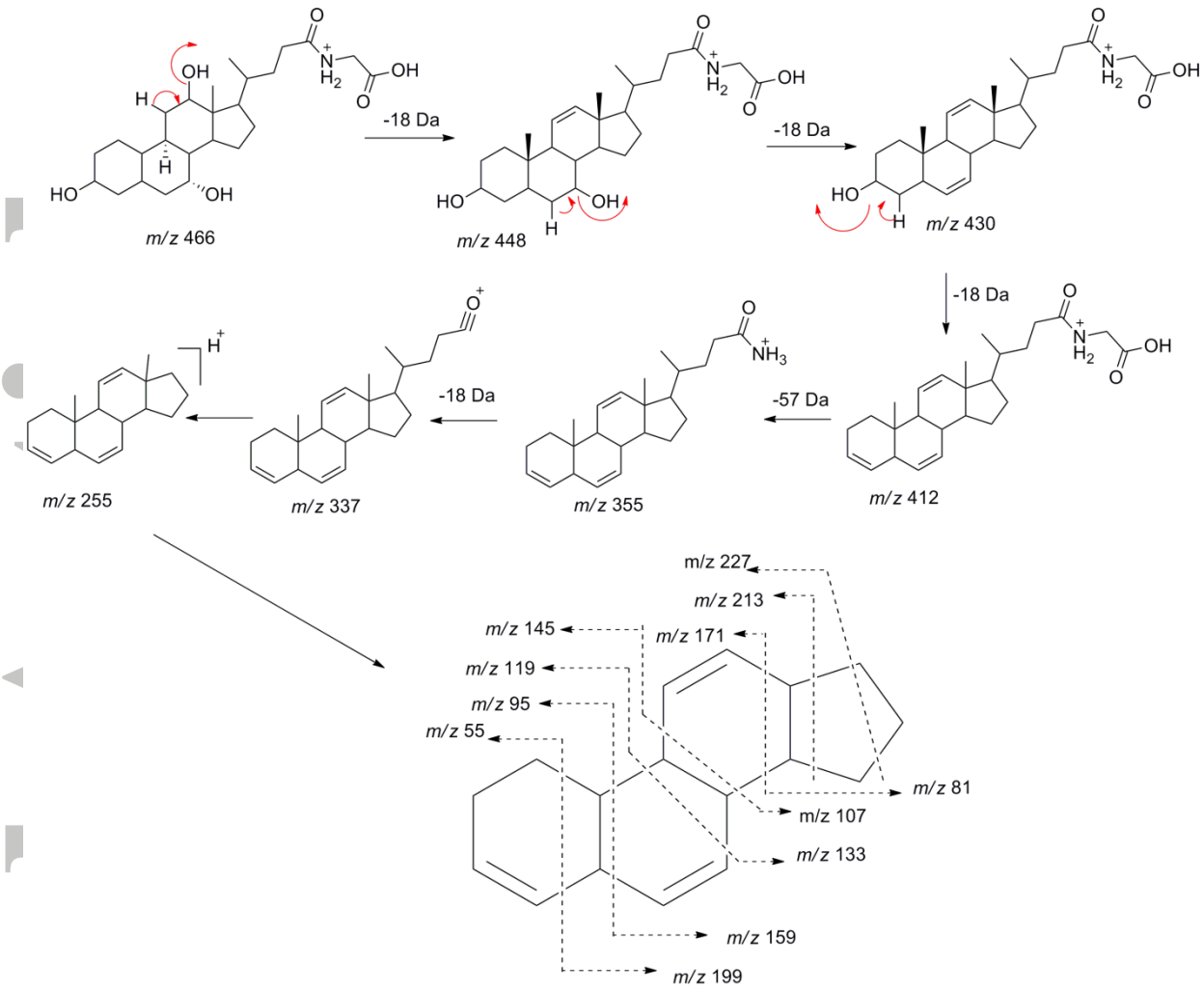

Figure 6: Proposed ESI-MS/MS fragmentation pattern of Glycocholic acid (**4**)