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Short communication

Electrospray tandem mass spectrometric analysis of duboscic acid, exploring the structural features of a new class of triterpenoids, dubosane

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Dedicated to Prof. Dr. Muhammad Iqbal Choudhary H.I. S.I., T.I. (at the occasion of his 52nd birthday).

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ABSTRACT

Duboscic acid belongs to a new class of triterpenoids dubosane, isolated from *Duboscia macrocarpa*. Gas-phase fragmentation of duboscic acid was studied using positive ion electrospray ionization quadropole time-of-flight mass spectrometry (ESI-QqTOF-MS/MS) hybrid instrument. ESI-QqTOF-MS (positive ion mode) showed the presence of the protonated molecule $[M+H]^+$ which under low-energy collision-induced dissociation tandem mass spectrometric (CID-MS/MS) analysis showed the characteristic losses of methoxy, hydroxyl and carboxylic groups. Our results demonstrated the characteristic fragments of this new class of triterpenoids which are formed due to the cleavage of seven membered unsaturated ring C. The fragmentation pathways of characteristic fragments were proposed with the aid of HRMS and computational studies. The knowledge of the fragmentation pattern and key fragment ions of duboscic acid, i.e. gas phase fragmentation behavior of unique dubosane structure, will be useful for further exploration of the related species of the same genera for the characterization of novel members of this class of compounds.

1. Introduction

Triterpenoids are ubiquitous non-steroidal secondary metabolites that are found in terrestrial and marine flora and fauna, occurring in the free form as well as in the forms of ether, ester and glycoside [1]. Triterpenoids exhibit important biological properties such as anti-tumor, anti-viral, antibacterial, anti-inflammatory, immune-regulatory [2], anti-HIV protease [3], antiandrogenic [4], antioxidant [5], anticomplement [6], antimicrobial [7] and angiotensin converting enzyme-inhibitory activities [8]. We have recently isolated duboscic acid, a terpenoid with unique carbon backbone from *Duboscia macrocarpa* Bocq. (Tiliaceae). Different parts of this plant are traditionally used for the treatment of tuberculosis, cough, tooth and abdominal problems. It is also used as vermifuge for children [9]. It is the first member of a new class of triterpenoids, for which the name "dubosane" is proposed. Duboscic acid has a potent α-glucosidase inhibition, and its structure was unambiguously deduced by a single-crystal X-ray diffraction study [10].

To our knowledge, only one report on the phytochemical investigation of *Duboscia macrocarpa* has been published by utilizing classical phytochemical method [10]. This trivial phytochemical method consumes large amounts of plant extracts which obtained from bulk raw material (in tons). However, to preserve the endemic and non endemic plant species and their sustainability, the quantity of plant material has to be limited to the analytical level. Therefore, a sensitive analytical and high-throughput strategy like LC–MS/MS for the characterization of compounds in complex mixtures is needed to be developed. Electrospray ionization mass spectrometry (ESI-MS) with collision induced dissociation (CID) has been developed as a powerful technique for the identification and characterization of molecules in a mixture [11,12]. However, to structurally characterize a compound from its MS/MS data, a previous knowledge of the fragmentation pathways of homologous compounds exhibiting a conserved structural core is required [13], while ESI-MS/MS characterization of duboscic acid has not been studied yet. Therefore, to obtain sufficient information on the structure elucidation of this new class of compound, the detailed fragmentation patterns of duboscic acid was studied using ESI-quadropole time-of-flight mass spectrometry in positive ion mode. Characteristic fragments of duboscic acid will be helpful for the identification of its congeners in other plant species.

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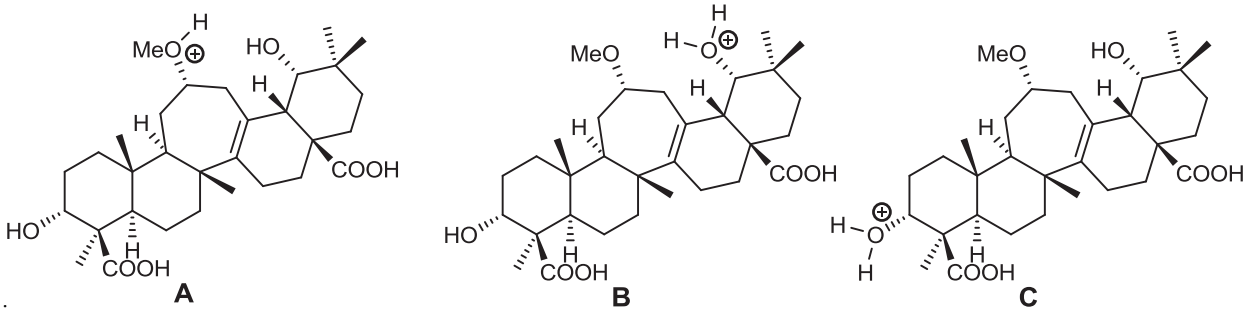
Table 1
Calculated energies for protonated duboscic acid at basis set 6-31G*:



Neutral *E* (Hartree)	Cation	*E* (Hartree)	Energy difference (Hartree) from (**A**)
−1736.79713	A	−1737.18048	0.00
	B	−1737.16750	0.01298
	C	−1737.15700	0.02348

2. Experimental

2.1. Chemicals and reagents

Chemicals and solvents were of analytical and HPLC grades, respectively and were purchased from Aldrich–Sigma (USA). Deionized water (Milli-Q) was used throughout the study. The isolation procedure and spectroscopic data of the duboscic acid has already been reported [10].

2.2. ESI-QqTOF-MS analysis:

The compound was dissolved in methanol (0.2 $\mu g\,\mu L^{-1}$) and working dilution was prepared in 95:5 acetonitrile–water containing 0.1% formic acid and analyzed by electrospray ionization (ESI) and collision-induced dissociation (CID), positive ion mode, on Qq-TOF-MS/MS instrument (QSTAR XL mass spectrometer Applied Biosystem/MDS Sciex, Darmstadt, Germany) at room temperature. High-purity nitrogen gas was used as the curtain gas and collision gas delivered from Peak Scientific nitrogen generator. The ESI interface conditions were as follows: ion spray capillary voltage of 5500 V, curtain gas flow rate 20 L min^{-1}, nebulizer gas flow rate 30 L min^{-1}, DP1 60 V, DP2 15 V, and focusing potential of 265 V. The collision energy was swept from 05 to 35 eV for MS/MS analysis. Calibration was performed using internal calibration process. Sample was introduced into the mass spectrometer using a Harvard syringe pump (Holliston, MA) at a flow rate of 5 $\mu L\,min^{-1}$. MS^2 experiment was conducted by selecting the product ion. Computational studies were performed using DFT at the B3LYP level with 6-31G* basis set in Spartan 08 v 1.2.0 (Wavefunction, CA, USA). Theoretical fragmentation of protonated duboscic acid was evaluated by using ACD/MS Fragmenter software (ACD Labs).

3. Results and discussion

ESI-QqTOF-MS analysis of duboscic acid showed $[M+H]^+$ at *m/z* 533.3489 corresponding to the protonated molecular formula $C_{31}H_{49}O_7$ (calcd 533.3472). MS/MS analysis of $[M+H]^+$ ion showing interesting fragmentation pattern and the product ion abundance were found to be significantly influenced by the variation of collision energy. Therefore, MS/MS spectra of duboscic acid were screened against laboratory collision energies ranging from 5 to 35 eV. It was observed that the fragment ions that are formed due to the substituent losses are best appeared at collision energy 20 eV (Fig. 1A) while the collision energy 35 eV is the optimum energy for the fragment ions formed due to the cleavage of seven membered ring C (Fig. 1B). The HR-ESI-MS data of characteristic fragments is summarized in Supplementary material Table 1.

Major characteristic peaks were observed by the sequential removals of substituent groups from the $[M+H]^+$. A characteristic removal of MeOH that is $[M+H-32]^+$ was observed as a base peak (*m/z* 501) at collision energy 15 eV. Other characteristic peaks were observed due to the further losses of multiple hydroxyl and carboxylic groups at *m/z* 483 $[M+H-MeOH-H_2O]^+$, 465 $[M+H-MeOH-2H_2O]^+$, 455 $[M+H-MeOH-HCOOH]^+$, 437 $[M+H-MeOH-H_2O-HCOOH]^+$, 419 $[M+H-MeOH-2H_2O-HCOOH]^+$, 391 $[M+H-MeOH-H_2O-2HCOOH]^+$ and 373 $[M+H-MeOH-2H_2O-2HCOOH]^+$ (Supplementary material Fig. 1A). The seven membered unsaturated ring C has a great influence on the fragmentation pattern of this new class of compound. The characteristic fragments at *m/z* 277, 263, 237 and 223 were observed due to the cleavage of this ring C. While the other fragments that appeared at *m/z* 259, 245, 219, 217, 213, 191, 177, 173, 159 and 199 were formed due to the

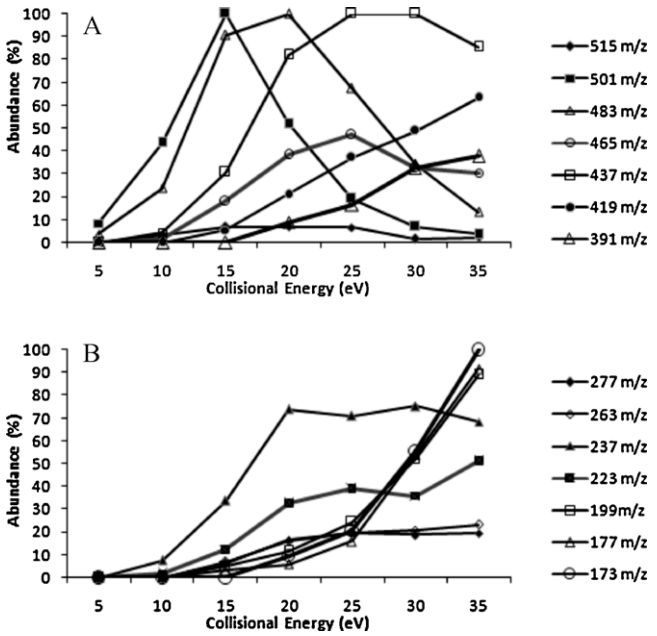
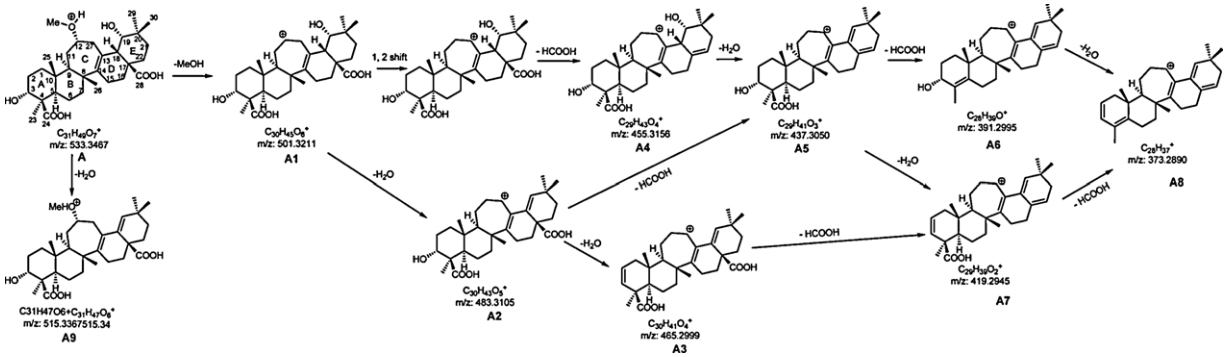


Fig. 1. (A) Relative abundances of fragment ions which are formed due to the substituent losses vs. collisional energies of duboscic acid, (B) relative abundances of fragment ions formed due to the cleavage of seven membered ring C vs. collisional energies of duboscic acid.



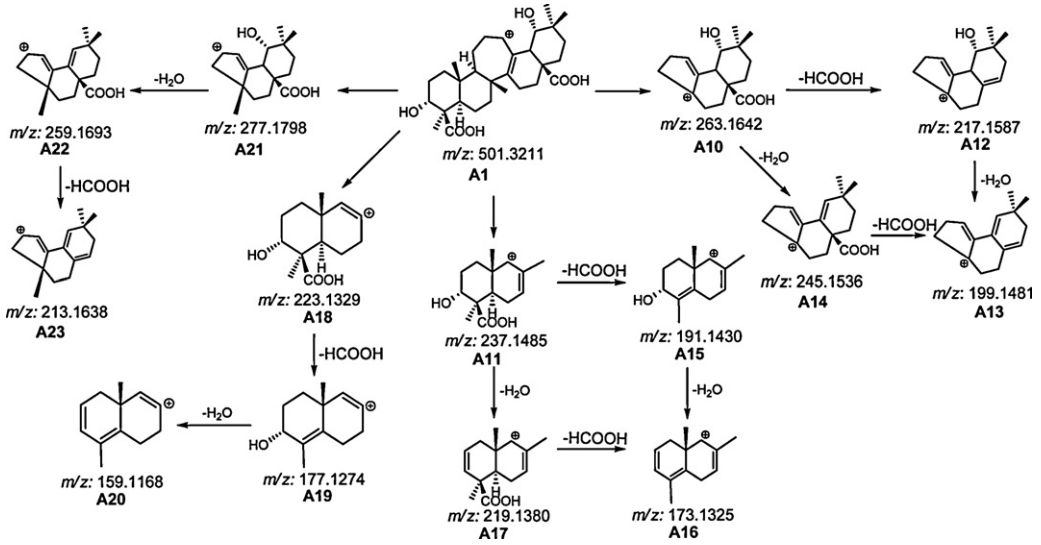
Scheme 1. Proposed fragmentation pathway for the fragments formed due the loss of substituents.

further losses of hydroxyl and carboxylic groups from the above fragments (Supplementary material Fig. 1B).

In silico studies were performed to investigate the most probable protonation site in duboscic acid. Minimum energy conformation of the neutral molecule was first optimized and every possible protonation site was then individually analyzed. There are three possible protonation sites in duboscic acid, the methoxy oxygen at C-12 (A), the hydroxyl oxygen at C19 (B) and the hydroxyl oxygen at C3 (C). The energy optimization was done by DFT at basis set 6-31G* level. It was found that the protonation at methoxy oxygen (A) showed minimum energy and therefore it was the most favourable site for protonation among the three possible sites, while the hydroxyl oxygen at C19 (B) was the second most favourable site of protonation. Form A showed energy of −1737.18048 Hartree while form B showed energy of −1737.16750 Hartree. Both forms have an energy difference of approximately 0.01298 Hartree (8.14 kcal mol^{-1}) (Table 1). Therefore, the major fragmentation pathway of duboscic acid was most likely to be initiated from A.

As depicted by energy calculations the most feasible site for protonation is at methoxy oxygen in ring C therefore loss of methanol was observed from $[M+H]^+$ to form fragment A1 at *m/z* 501 followed by the loss of water molecule either from C-3 or C-19 to give the product ion at *m/z* 483 (fragment A2) but the loss from C19 seems to be more feasible because it forms conjugate system. This fragment at *m/z* 483 could also be simultaneously formed by another pathway in which protonation occurs at hydroxyl at C-19. The protonated form A could also leads to the formation of fragment A9 by losing water molecule at *m/z* 515. The ion abundance of fragment A9 is low while the fragment A1 appeared as a base peak at collision energy 15 eV. Fragment A7 at *m/z* 419 could be formed by both pathways. In one of which fragment A7 is formed by the sequential loss of water molecule fragment from A2 giving product ion at *m/z* 465 (fragment A3) followed by the loss of formic acid. While in other possible pathway there is a loss of formic acid to gives the fragment A4 at *m/z* 455 from fragment A1 followed by the removals of two water molecules to gives the fragment A7. After the formation of product ion at *m/z* 419 further loss of formic acid gives fragment A8 at *m/z* 373. Fragment A8 could also be formed by the water removal from A6 at *m/z* 391 which formed from fragment A5 by the removal of formic acid (Scheme 1). The ion abundances of fragments A6 and A8 are low at 20 eV and these fragments are more prominent on increasing collision energy.

Fragments which are formed due to the cleavage of seven membered ring C are more prominent on increasing collision energy. Only the fragment A11 at *m/z* 237 also appeared on low collision energy. MS/MS analysis of fragment A1 at *m/z* 501 showed the cleavage of ring C which leads to the formation of fragment A10 (*m/z* 263), A11 (*m/z* 237), A18 (*m/z* 223) and A21 (*m/z* 277). Fragments A10 and A11 showed further losses of formic acid and water molecules to afforded fragments A12 (*m/z* 217), A13 (*m/z* 199), A14 (*m/z* 245) and A15 (*m/z* 191), A16 (*m/z* 173), A17 (*m/z* 219), respectively. In a similar manner fragments A18 and A21 showed further losses of formic acid and water molecules to afforded fragments



Scheme 2. Proposed fragmentation pathway for the fragments formed by the cleavage of ring C.

A19 (*m/z* 177), A20 (*m/z* 159) and A22 (*m/z* 259), A23 (*m/z* 213), respectively (Scheme 2).

4. Conclusion

In conclusion, fragmentation pattern of new class of compound, duboscic acid has been studied using ESI-QqTOF-MS/MS. It has been observed that many characteristic neutral losses and formation of key fragment ions can provide important structural information of the basic skeleton having seven membered ring and its substituents. The knowledge of fragmentation pattern of this new class will be helpful for the rapid identification and characterization of dubosane type triterpenoids through liquid chromatography coupled with mass spectrometry in complex mixtures such as plant extracts or herbal formulations by utilizing their analytical amount.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2011.11.007.

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