Hyphenated LC-ICP-MS/ESI-MS Identification of Halogenated Metabolites in South African Marine Ascidian Extracts

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ABSTRACT
Extracts of 13 species of marine ascidian collected in Algoa Bay were analyzed by LC-ICP-MS/ESI-MS. This technique allows parallel analysis of the molecular species and the presence of certain elements. The LC-ICP-MS/ESI-MS technique was used to target iodinated metabolites in this study. Three ascidian species afforded the known 3,5-diiodotyrosine (12), which was confirmed by the isolation of this metabolite from Aplidium monile. MS also suggested the presence of the known 3,5-dibromo-4-methoxypyphenethylamine (10) and the new 3-bromo-5-iodo-4-methoxypyphenethylamine (11) in the A. monile extract. The presence of the known 3,5-dibromotetramethyltyrosine (21) and the new 3-iodotetramethyltyrosine (23) in extracts of an unidentified Didemnum species was similarly proposed from MS evidence. This is the first report of the occurrence of iodinated metabolites in South African marine invertebrates.

KEYWORDS
Marine, ascidian, iodinated tyramine, iodinated tyrosine, LC-ICP-MS/ESI-MS.

1. Introduction
The sub-tidal rocky reefs along the western periphery of Algoa Bay, South Africa, provide a unique benthic habitat for a plethora of sessile filter feeders including diverse populations of endemic ascidians (also known as tunicates or sea squirts). The marine ascidians of Algoa Bay have recently been shown to be a source of halogenated secondary metabolites including the rubrolides (1–4) from Synoicum globosum7 and 3,6-dibromoindole (5), 6-bromo-3-chloroindole (6) and 6-bromo-2-oxindole (7) from Distaplia skoogi2 (Fig. 1).

As part of an ongoing search for new halogenated metabolites from the diverse ascidian populations of Algoa Bay, South Africa, we have expanded our search to target naturally occurring iodinated metabolites. Iodide and iodate ion concentrations in the ocean (c. 60 ppb) significantly exceed those in fresh water (c. 0.03–6 ppb).6,7 Of the 182 known iodinated secondary metabolites reported in a recent review of naturally occurring organo-iodine compounds, more than 80 % are marine.6 Given that over 5000 halogenated metabolites have been isolated from natural sources, organoiodines can be considered to be rare in nature.3 The first naturally occurring organoiodine to be identified, ‘jodgorgosaure’ (3,5-diiodotyrosine, 8), was originally isolated in 1896 from the marine octocoral (sea fan), Gorgonia carolini.8 3,5-Diiodotyrosine and its mono-iodo analogue, 3-iodotyrosine (9), have also been reported from other eukaryotic marine phyla, e.g. kelp (order Laminariales).4 Kelp species can accumulate iodide in concentrations 300 000 times higher than the iodide concentration in the surrounding seawater,5 and as much as 10 % of the iodine sequestered by kelp is incorporated into the organoiodines,8 and 9.5 A possible endocrine hormone role for 8 and 9 has been postulated in which these two compounds may mediate cell to cell communication in algae and control developmental processes in other eukaryote species.3,7

Rapid identification of iodinated metabolites, and their brominated congeners, in Algoa Bay ascidian extracts was facilitated by access to the hyphenated LC-ICP-MS/ESI-MS facilities at the Marine Biodiscovery Centre at the University of Aberdeen. The University of Aberdeen hyphenated LC-ICP-MS/ESI-MS facility has successfully been used to, inter alia, explore the distribution of naturally occurring organoarosenic compounds,9–11 metal chelated ascidian metabolites,12–16 and recently identifying new secondary metabolites containing heteroatoms, e.g. iodinated metabolites in marine extracts.17 Hyphenated LC-ICP-MS/ESI-MS, in which a high performance liquid inductively coupled plasma (ICP) and high resolution electrospray ionization (ESI) mass spectrometers arranged in parallel (Fig. 2), provides an opportunity to simultaneously acquire elemental and molecular information for the individual peaks separated by HPLC.

Received 26 September 2017, revised 14 August 2018, accepted 19 August 2018.

DOI: https://doi.org/10.17159/0379-4350/2018/v71a14

ISSN 0379-4350 Online / ©2018 South African Chemical Institute / http://saci.co.za/journal
2. Materials and Methods

2.1. General

LC-ICP-MS/ESI-MS analyses were carried out using an Agilent 1100 series HPLC with ESI-MS performed on a Thermo Orbitrap mass spectrometer whilst ICP-MS was performed on an Agilent 8800 triple quad ICP-MS, with micro-flow PFA nebulizer and Pt cones, and 7% O₂ reaction gas. Chromatography for LC-ICP-MS/ESI-MS analyses were carried out by injecting 100 µL of sample onto an analytical C18 Waters SunFire column with a solvent flow rate of 1 mL min⁻¹ and gradient profile of 100% H₂O to 100% MeOH over 20 min. HPLC solvents which were used for chromatography were made up with 0.1% formic acid (FA). A passive splitter was used to direct approximately 80–85% of the eluent flow to the ESI-MS and 15–20% of the eluent flow to the ICP-MS. Elements detected were V (m/z 51), Mn (m/z 55) Fe (m/z 57), Co (m/z 63), Cu (m/z 66), Zn (m/z 75), As (m/z 77), Br (m/z 79), Mo (m/z 95) and I (m/z 127).

NMR spectra were acquired using standard pulse sequences on a Bruker Avance 600 MHz Avance II spectrometer. Chemical shifts are reported in ppm and referenced to residual solvent resonances (CDCl₃, δH 7.26, δC 77.0). Coupling constants are

Figure 1 Selected metabolites from marine invertebrates and algae
reported directly from the NMR spectra. Mass spectrometry of purified compound 12 was performed on a Waters API Q-TOF Ultima instrument using electrospray ionization in the positive ion mode (ESI+) at the University of Stellenbosch Central Analytical Facility.

2.2. Collection of Ascidian Material
The ascidian *Aplidium monile* Monniot, E., 2001*18* Aplusobranchia: Polyclinidae was collected by SCUBA from a depth of 12–15 m at Bell Buoy Reef, Algoa Bay, South Africa (33.9833°S, 25.6987°E), on 18 November 2011 and given the voucher code SAF2011-032. Polycitor sp. (suborder Aplusobranchia, family Polycitoridae) was collected by SCUBA at a depth of 21 m from Haarlem Reef, Algoa Bay, South Africa (33.9889°S, 25.6984°E), on 23 July 2004 and given the sample code SAF2004-068. Leptoclinides sp. was collected by SCUBA from the White Sands Reef, Algoa Bay, South Africa, (33.9961°S, 25.7072°E), from a depth of 21 m, on 13 July 2004 and given the sample code SAF2004-015. Didemnum sp.2 (suborder Aplusobranchia, family Didemnidae) was collected by SCUBA at a depth of 18 m at White Sands Reef, Algoa Bay, South Africa (33.9986°S, 25.7096°E), on 20 July 2004 and given the sample code SAF2004-61. After collection in the field all ascidian samples were carefully separated, cleaned of epibionts and frozen separately as whole specimens of individual species and kept at −20 °C until extracted.

2.3. Extraction of Frozen Ascidian Samples for LC-ICP-MS/ESI-MS Analysis
All glassware and laboratory equipment used in the preparation of extracts for LC-ICP-MS/ESI-MS screening were acid washed using 10 % HNO3 and subsequently rinsed using MilliPore® water. HPLC grade solvents were used and to prevent contamination. The mass spectrometry data revealed that the 127I and 79Br isotopes, together with the matching HRESI EICs of the 127I and 79Br isotopes, were present in these compounds. The loss of 17 atomic mass units (M+H−NH3) from the pseudomolecular ions in the ESI mass spectra of these compounds indicated the probable presence of a common amino functionality in these compounds.

3. Results and Discussion
Representative specimens of 13 ascidian species from five different families (Clavelinidae, Didemnidae, Holozoidae, Polyclinidae and Polycitoridae), belonging to the suborder Aplusobranchia, were collected by SCUBA from Algoa Bay over the period 2004–2011, carefully separated from any epibionts and frozen separately as whole specimens of individual species (~15 g wet mass) were made using HPLC grade MeOH and CH2Cl2. Extractions were carried out in the normal way except that all glassware and tools used were acid washed and an iced water bath was used when sonicating the material. The crude ascidian extracts were stored at −20 °C until LC-ICP-MS/ESI-MS screening. Aliquots of methanol solutions of the crude organic extracts were used for the LC-ICP-MS/ESI-MS speciation studies.
phenethylamine structures of 10 and 12 respectively.21,22 No compounds with the molecular formula C₉H₁₂ONBrI were identified in the literature suggesting that the proposed 3-bromo, 5-iido-4-methoxyphenethylamine structure proposed for 11 was unprecedented.

Partitioning of the A. monile extract between 70 % aqueous methanol and dichloromethane, followed by further fractionation of the methanol partition fraction on a C₁₈ SepPak® cartridge afforded 12 as the only metabolite in the 40 % aqueous acetonitrile fraction. The ¹H and ¹³C NMR data of 12 were consistent with published data for this compound. Further exhaustive semi-preparative HPLC of the C₁₈ SepPak® fractions failed to yield either 10 or 11 suggesting that these two compounds may be present in trace amounts in the ascidian. The detection of very low concentrations of 10 and 11 in the presence of the major metabolite 12 further highlights the sensitivity and value of the LC-ICP-MS/ESI-MS technique. Three decades ago Ireland and Sesin reported the isolation of 12 together with its urea derivative 13 from an unidentified species of Didemnum ascidian.23 This was the first isolation of these two compounds from a natural source. Compound 11 was later isolated as the major compound in two ascidians, an Indonesian Didemnum sp. and Palauan specimens of D. rubeum.22,23 More recently the chemistry of D. rubeum was revisited and an expanded series of iodinated tyramine derivatives were isolated from this asidians that included 12 and 13 and six new analogues 14–19.24 While we report here the first isolation of 12 from an African ascidian, an Axinella sponge collected off the coast of Ghana recently afforded the related iodotyramine analogue dakaramine (20).25

Compound 10 appears to be less common, in the marine environment, than its iodo congener. The only previously reported isolation of 10 was by Ireland and co-workers23 from the Indonesian ascidian Eudistoma sp.. However, related analogues of 10, e.g. the tetramethylated tyrosine analogue 21 and the monoiodotyrosine analogue 22 have been isolated from the sponge Verongula sp. and the ascidian Cnemidocarpa bicornuta, respectively.26–28 In a biological evaluation of a series of synthetic bromotyramines Schoenfeld et al. found 10 exhibited potent antifouling and cytotoxic properties.28

The C₃ monohalogenation and C₃, C₅ dihalogenation of the phenyl ring in halogenated marine tyramine and tyrosine analogues appears ubiquitous, and without exception (Fig. 1), thus suggesting that other possible regioisomers within this cohort of halogenated natural products are unlikely. Biosynthetic arguments were therefore used to support the C₃, C₅ dihalogenation pattern proposed for 10 and 11. Interestingly, albeit speculative from high-resolution mass data alone, this is the first report of an ascidian yielding 10 and 12 and the previously unreported 11. Similar hyphenated LC-ICP-MS/ESI-MS examination of the extracts of the Polyctor sp. and Leptoclinides sp. (Fig. 3b,c) also revealed the presence of 12 in these extracts suggesting that this metabolite is relatively common in Algoa Bay ascidian species with 25 % of the small cohort of 13 ascidians screened in this study containing this compound. Although the LC-ICP-MS/ESI-MS mass data suggested the presence of further iodo- and bromohalogenated metabolites in both the Polyctor and Leptoclinides extracts, the structures of these compounds could not be resolved from the mass data. The paucity of ascidian material in hand prevented their isolation and identification by other spectroscopic techniques.

The ICP-MS EICs, selected for the ¹²⁷I and ⁷⁹Br isotopes, together with the matching HRESI EICs and the corresponding mass spectra of selected peaks from LC-ICP-MS/ESI-MS of the methanolic extract from Didemnum sp 2 are presented in Fig. 5. The 1:2:1 isotopic ratio of the molecular ion (M⁺) peak in the ESI mass spectrum of the compound with TR 10.6 min (m/z 393.9650; 395.9629; 397.9607) was indicative of dibromination in this
compound. The closest fit molecular formula simulation\textsuperscript{29} for a molecular mass of m/z 395.9629 suggested a molecular formula of C\textsubscript{13}H\textsubscript{18}O\textsubscript{3}NBr\textsubscript{2} (D\textsubscript{mmu 0.2). A literature search\textsuperscript{21} revealed only one compound from a marine source, 3,5-dibromotetramethyl-tyrosine (\textsuperscript{21}) with this molecular formula and comparable molecular mass. Compound \textsuperscript{21} was isolated by Ciminiello \textit{et al.} from the Caribbean Verongida sponge \textit{Aiolochroia crassa} (synonymous with \textit{Pseudoceratina crassa})\textsuperscript{26} and was found to be inactive in both anti-fouling and anti-parasitic bioassays.\textsuperscript{30,31} The iodine (\textsuperscript{127}I) ICP-MS EIC (Fig. 5a) revealed two major peaks (TR 8.64 and 13.99 min). Unfortunately, no ESI mass spectrum was observed at TR 13.99 min and the source of this peak in the \textsuperscript{127}I EIC is unknown. A molecular formula of C\textsubscript{13}H\textsubscript{19}O\textsubscript{3}NI (M\textsuperscript{+} m/z 364.041 \textsubscript{D}mmu –0.3) emerged for \textsuperscript{23} from the closest fit molecular formula simulation.\textsuperscript{29} With the putative structure of \textsuperscript{21} in hand the 3-iodotetramethyltyrosine structure for \textsuperscript{23} was proposed. Further mass spectroscopic evidence in support of the chemical structure of \textsuperscript{23} (Fig. 5b) emerged from the fragment ion (m/z 237.0949, M\textsuperscript{+}127) in the HRESI mass spectrum and the product ions from tandem mass spectrometry (MSMS) of the

**Figure 4** Halogenated tyramine analogues detected in the \textit{A. monile} extract by LC-ICP-MS/ESI-MS. (a) Selected ICP-MS (\textsuperscript{127}I – blue trace; \textsuperscript{79}Br – red trace) and ESI-MS extracted ion chromatograms; (b) ESI mass spectra and proposed structures of \textsuperscript{10}, TR 7.06 min; \textsuperscript{11}, TR 7.46 min; and \textsuperscript{12}, TR 8.09 min.
M+ precursor ion (m/z 304.9661, M+-N(CH$_3$)$_3$; m/z 258.9614, M+-HCOOH-N(CH$_3$)$_3$; m/z 178.0624, M+-I-N(CH$_3$)$_3$). A search of the chemical literature revealed that 23 has not been previously reported from nature. Regrettably, the paucity of Didemnum sp. 2 available precluded the chromatographic isolation of 21 and 23 for further spectroscopic analysis.

4. Conclusion
This preliminary survey of the distribution of halogenated metabolites in a small subset of the ascidian fauna in Algoa Bay South Africa suggests that iodinated and brominated tyrosines and tyramines may be relatively common in aplousobranch ascidians. The potential of the LC-ICP-MS/ESI-MS technique to detect these metabolites in trace amounts is clearly apparent.

Acknowledgements
The authors would like to thank Professor Bill Fenical of the Scripps Institution of Oceanography, San Diego, U.S.A., for the...
generous donation of funding which enabled the first large-scale SCUBA collection of marine ascidians in Algoa Bay, South Africa, in 2004. C.B., M.D.C. and M.J. acknowledge the support of a Royal Society international collaborative grant which enabled travel between universities in South Africa and the U.K. Financial support from Rhodes University and the South African National Research Foundation through the SeaChange Programme is gratefully acknowledged.

References and Notes


21 MarinLit http://pubs.rsc.org/marinlit/

22 SciFinder Scholar http://www.cas.org/products/scifinder


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