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ASCIDIAN HALOGEN-CONTAINING SECONDARY METABOLITES FROM THE FAMILY DIDEMNIDAE[†]

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Abstract. *This review summarizes current knowledge concerning the isolation, structural elucidation, biological/pharmacological activities, and total synthesis of halogen-containing secondary metabolites isolated from ascidians of the family Didemnidae. Overall, 81 compounds are presented, displaying great structural diversity and possessing a number of significant biological/pharmacological properties. In addition to the most numerous brominated compounds, chlorinated and iodinated ones were also found. The most prolific genus in regard to the number of published papers and isolated molecules is the genus* Didemnum*, which was treated separately from the rest of the genera of the ascidian family. The structural complexity of the isolated metabolites prompted a number of synthetic endeavors that not only provided conclusive proof of the structure of the metabolites but also enabled the biological tests and the possible application of these metabolites.*

Key words: *marine natural products, halogenated secondary metabolites, ascidian, Didemnidae, Didemnum*

1. INTRODUCTION

More than 20 000 novel compounds have been found from marine sources, and that number is rising every year (Blunt et al., 2015). Marine invertebrates, together with ascidians, are responsible for the production of the major part of novel marine-derived bioactive compounds (Blunt et al., 2007). Studies of bioactive compounds from marine ascidians have started relatively recently; however, didemnin B, an ascidian metabolite, was the first marine natural product to be evaluated in a human clinical trial (Newman and Cragg, 2006).

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The oceans are the largest source of biogenic organohalogens, which are biosynthesized by seaweeds, corals, tunicates, and other marine life (Gribble, 2003). Marine organisms produce most of the 4000 identified organohalogens (Pauletti et al., 2010), this group comprises diverse compounds, ranging from peptides, polyketides, indoles, terpenes, and phenols to volatile halogenated hydrocarbons (Butler and Sandy, 2009). These compounds are of different biosynthetic origin, and bromine, rather than chlorine, is the most prevalent halogen found in marine-derived molecules. The sources of halogens are rather large amounts of these elements which exist in seawater in the form of their anions, the average concentrations of Cl^-, Br^-, and I^- are 19 000, 65, and 0.06 mg/L, respectively. It is surprising that bromine is the major covalently bound halogen, considering the fact that in seawater the concentration of chloride ions are 300 times higher than the concentration of bromide ions (Fenical, 1981). Simply saying, marine organisms can oxidize bromide ions more easily for incorporation into organic compounds.

Organohalogen natural products frequently display the highest level of biological activity. The onset of biological activity of many natural products is highly dependent on the presence of halogen atoms in the molecule; for example, consider the case of the chlorine-containing metabolite salinosporamide A vs. the non-halogenated salinosporamide B (Gribble, 2004; Macherla et al., 2005). Biological properties of halogenated metabolites have been investigated in the past decades, where antibacterial, antifungal, antiviral, anti-inflammatory, antiproliferative, antifeedant and cytotoxic activities were recognized (Blunt et al., 2009).

Ascidiacea is the main class of tunicates including about 3000 species distributed into three orders: Stolidobranchia, Phlebobranchia, and Aplousobranchia (Lambert, 2005). Within Aplousobranchia, the most diverse of the three orders, the family of Didemnidae, Giard, 1872, has been described as a vital source of diverse natural compounds with potent pharmacological properties with about 580 species (Shenkar and Swalla, 2011). This family comprises eight genera: *Atriolum* Kott, 1983; *Clitella* Kott, 2001; *Didemnum* Savigny, 1816; *Diplosoma* Macdonald, 1859; *Leptoclinides* Bjerkan, 1905; *Lissoclinum* Verrill, 1871; *Polysyncraton* Nott, 1892; and *Tridemnum* Della Valle, 1881. The first two genera account for only ten valid species, while most of the diversity is included in the other genera, especially *Didemnum* and *Polysyncraton* (da Silva Oliveira et al., 2017). *Didemnum* is a genus of colonial tunicates of the family Didemnidae. It is the most speciose genus in the didemnid family. Species from this genus often have small calcareous spicules embedded in the tunic and form irregular or lobed colonies. Some *Didemnum* species are considered invasive species.

Thousands of natural products have been isolated from ascidians; these mostly include alkaloids, cyclic peptides, and polyketides (Davidson, 1993; Chen et al., 2017). Most of these secondary metabolites have diverse bioactivities, such as antibacterial, antifungal, antitumor and anti-inflammatory activities (Palanisamy et al., 2017). However, it has remained unclear whether these bioactive products were produced by ascidians themselves, or by ascidian-associated microorganisms (Chen et al., 2018). Ascidians are recognized to have the ability to produce organohalogen compounds with a great variety of structural and biological activities. These marine invertebrates are renowned as a specifically unique source of peptides and alkaloids (Vera and Joullié, 2002). In many cases, isolated natural products display biological activities that are of either human therapeutic or ecological importance (Wang and Namikoshi, 2007). So far, 81 natural halogenated compounds were isolated from marine ascidians of the family Didemnidae (from the genera: *Didemnum* Savigny, 1816; *Diplosoma* Macdonald, 1859; *Leptoclinides* Bjerkan, 1905; *Lissoclinum* Verrill, 1871; and *Tridemnum* Della Valle, 1881) and these will be

discussed in this review in terms of their structural type, total synthesis and reported pharmacological/biological activities.

2. Structural Diversity of Halogen-Containing Secondary Metabolites of Ascidians Belonging to the Genus *Didemnum*

The following section describes the known halogenated compounds isolated from ascidians of the genus *Didemnum*. About 33 halogen-containing secondary metabolites have been isolated and identified from ascidians of the genus *Didemnum.* They display a wide spectrum of structural diversity and possess interesting and significant biological/ pharmacological activities.

2.1. Isolation and structure determination of brominated metabolites

Bromine-containing secondary metabolites were firstly isolated from the tunicate *Didemnum candidum* by Fahy and colleagues in 1991. Two specimens of this ascidian, collected from different environments in the southern Gulf of California, contained 6-bromotryptamine (**1**), 2,2-bis(6′-bromo-3′-indolyl)ethylamine (**2**), and 2,5-bis(6′-bromo-3′-indolyl)piperazine (dragmacidin C, **3**) (Fig. 1). Fahy and coworkers reported the first occurrence of 6-bromotryptamine as a natural product. The molecular formulae of **1**-**3** were established by high-resolution mass spectrometry, while the structures were elucidated by interpretation of ^{1}H and ^{13}C NMR spectra.

Schumacher and Davidson (1995) isolated two new brominated β-carboline–based metabolites, didemnolines A (**4**) and C (**5**) along with eudistomin O (**6**) and 2-(2′,4′-dibromophenoxy)-3,5-dibromophenol (**7**) (Fig. 2) from a species of the *Didemnum* genus, collected near the island of Rota, Northern Mariana Islands. Compounds **4** and **5** differ from most of the previously reported marine-derived β-carboline compounds - they are substituted at the N9 position rather than at the C1 position. The discovery of compound **7** in ascidians was somewhat surprising since these polybrominated diphenyl ethers were known to be produced by cyanobacteria, which are potential symbionts of these ascidians.

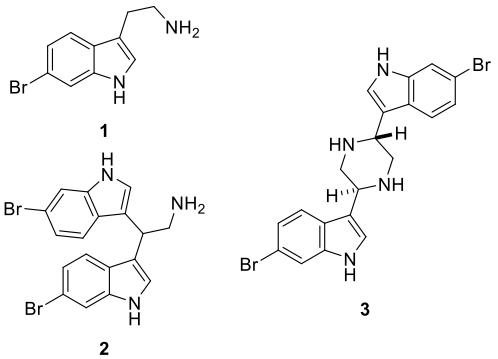

Fig. 1 Structures of 6-bromotryptamine (**1**), 2,2-bis(6′-bromo-3′-indolyl)ethylamine (**2**) and 2,5-bis(6′-bromo-3′-indolyl)piperazine (**3**).

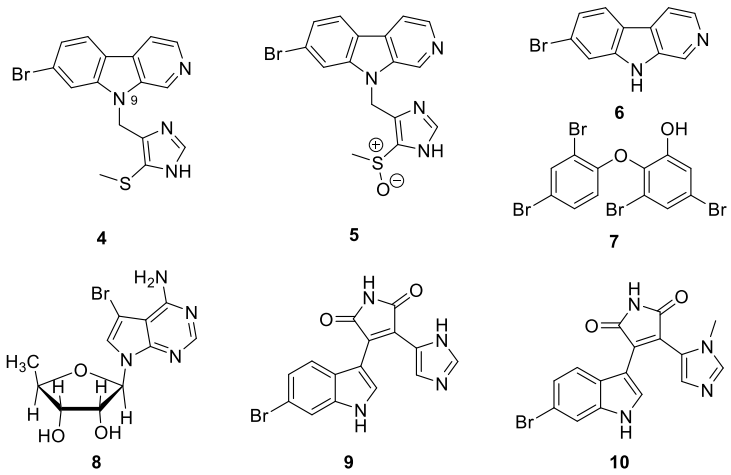

Fig. 2 Structures of didemnolines A (**4**), C (**5**), eudistomin O (**6**), 2-(2',4'-dibromophenoxy)-3,5-dibromophenol (**7**), 5′-deoxy-3-bromotubercidin (**8**), didemnimides B (**9**) and D (**10**).

Thus, the finding of **7** by Schumacher and Davidson (1995) could be brought into connection with algae associated with the ascidian.

In 1996, Mitchell and colleagues reported a new 7-deazapurine nucleoside (5′-deoxypyrrolo[2,3-*d*]pyrimidine), 5′-deoxy-3-bromotubercidin (**8**) (Fig. 2). It was isolated from the ascidian *Didemnum voeltzkowi* (Savigny, 1816), collected from Apo Reef rocks, the Philippines. The structure of **8** was determined by the interpretation of NMR spectral data, as well as HPLC – MS experiments.

A novel indol-maleimide-imidazole carbon skeleton was identified in 1997 from the Caribbean mangrove ascidian *D. conchyliatum* (Sluiter, 1898). Vervoort and coworkers (1997) isolated two brominated alkaloids, didemnimides B (**9**) and D (**10**) (Fig. 2), with this skeleton, among other non-halogen-containing compounds. The carbon skeleton of didemnimides was established by X-ray analysis of the non-brominated alkaloid didemnimide A, while the structures of compounds **9** and **10** were assigned using a combination of spectral techniques (UV, FTIR, and HRMS), and one- and two-dimensional NMR experiments. Didemnimide D was also identified from the methanol extract of the ascidian *D. granulatum* collected in Brazil (Berlinck et al., 1998). One year later (1999), Vervoort's group reinvestigated the ascidian *D. conchyliatum* and found compounds **9** and **10**.

Reinvestigation of the extract of the ascidian *D. granulatum* collected by scuba divers at several sites in Sao Sebastiao (Brazilian coastline) resulted in the discovery of new granulatimide alkaloid, 6-bromogranulatimide (**11**) (Fig. 3), along with the previously reported didemnimide D (**10**) (Britton et al., 2001). The structure of **11** was elucidated by spectroscopic methods; the carbon resonances of 6-bromogranulatimide were unavailable from standard ^{13}C NMR, due to the small amount of the substance, but were inferred from proton-detected 2D NMR (HMQC and HMBC).

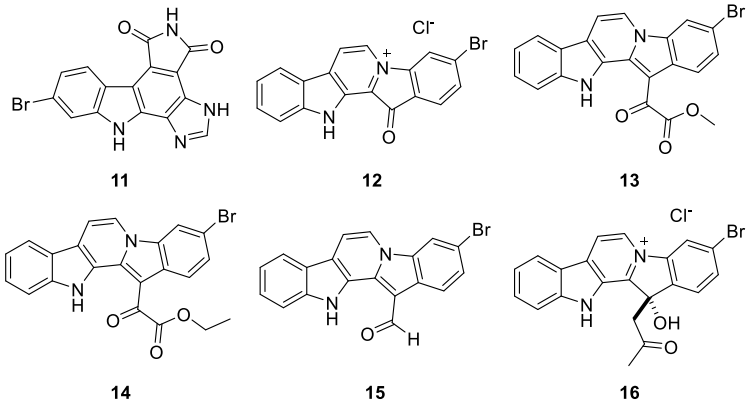

Fig. 3 Structures of 6-bromogranulatimide (**11**), 3-bromofascaplysin (**12**), 3-bromohomofascaplysin B (**13**), 3-bromohomofascaplysin B-1 (**14**), 3-bromohomofascaplysin C (**15**), 3-bromohomofascaplysin A (**16**).

In 2003 and 2004, Segraves and coworkers reported four new brominated fascaplysin-class compounds, 3-bromofascaplysin (**12**), 3-bromohomofascaplysin B (**13**), 3-bromohomofascaplysin B-1 (**14**), and 3-bromohomofascaplysin C (**15**) (Fig. 3). The compounds were isolated from two collections of an unidentified tunicate belonging to *Didemnum* sp., collected at 15-40 ft, inside the Northeast Pass at Chuuk Atoll, Federated States of Micronesia and near Northern Sulawesi, Indonesia. The structures of these compounds were elucidated by spectroscopic techniques including 2D NMR experiments (gHSQC, gHMBC, NOESY, and ^{1}H-^{1}H COSY), and high-resolution FAB mass measurements.

3-Bromohomofascaplysin A (**16**) (Fig. 3), a new fascaplysin analog, was isolated together with previously reported non-brominated derivatives from an unidentified Fijian *Didemnum* tunicate, collected by scuba divers from Pratt Reef, Fiji Islands (Lu et al., 2011). Their structures were elucidated by spectral methods. The absolute configuration of **16** was determined by comparison of the experimental ECD (electronic circular dichroism) spectrum with a simulated spectrum calculated by time-dependent density functional theory (TDDFT).

A new brominated indole-3-glyoxylamidospermidine analog, didemnidine B (**17**) (Fig. 4) was isolated from a New Zealand ascidian *Didemnum* sp. collected at Tiwai Point, Southland (Finlayson et al., 2011). However, didemnimides B (**9**) and D (**10**), were already known as they were previously isolated in the form of their trifluoroacetate salts (formed in the HPLC-purification steps), whereas the original isolation reported them as free base alkaloids (Vervoort et al., 1997). The occurrence of spermidine derivatives in marine organisms is limited and has been reported only several times, but compound **17** and its non-brominated analog were the only examples of the mono-N^{1}-substituted derivatives.

In order to identify new biologically active compounds, Liberio and colleagues (2014) created an ascidian extract library (143 samples) and screened their biological activities. The results of these studies were used to select an extract for fractionation. Analysis of 1D and 2D NMR, $[\alpha]_D$, CD and MS data for the isolated compound from *D. candidum*, collected from the Great Barrier Reef (Queensland, Australia), in combination with literature data, allowed the

reidentification of eusynstyelamide B (**18**), as the bis-trifluoroacetate salt, that is a previously reported bis-indole alkaloid (Fig. 4).

Recently, the chemical investigation by Hahn and coworkers (2015) of a colonial marine tunicate, *Didemnum* sp., collected by scuba divers near Haegeumgang, Geoje in the South Sea of Korea, led to the isolation of three indole alkaloids including a new, *N*-acetyl-*O*-(6-bromo-5-hydroxy-1*H*-indole-3-carbonyl)-D-tyrosine (**19**) – amino acid derivative, and bromoindole-containing compounds *N*-(6-bromo-1*H*-indole-3-carbonyl)-L-arginine (**20**) and (6-bromo-1*H*-indol-3-yl)oxoacetamide (**21**). The structures of compounds **19**-**21** were elucidated based on ^{1}H and ^{13}C NMR data, MS data and specific optical rotation (Fig. 4).

In 2018, 3,5-dibromotetramethyltyrosine (**22**) (Fig. 4) was identified from the extract of an ascidian *Didemnum* sp. from Algoa Bay, South Africa using LC-ICP-MS/ESI-MS technique (Bromley et al., 2018).

2.2. Isolation and structure determination of iodinated metabolites

In 1984, Sesin and Ireland reported the isolation of iodinated tyramine derivatives: 3,5-diiodo-4-methoxyphenethylamine (**23**) and its symmetrical urea derivative, 1,3-bis(3,5-diiodo-4-methoxyphenethyl)urea (**24**), (Fig. 5) as metabolites of an unidentified *Didemnum* species, collected on exposed rocks on the northwest end of Cocos Lagoon, Guam. Two research groups in 1997 identified **23** from an unidentified *Didemnum* sp. and *D. rubeum*, collected in Barrang Lompo, Indonesia and in the Lighthouse Channel, Republic of Palau, respectively (Smith et al., 1997); and *D. rubeum* collected near the island of Rota, Northern Mariana Islands (Ford and Davidson, 1997).

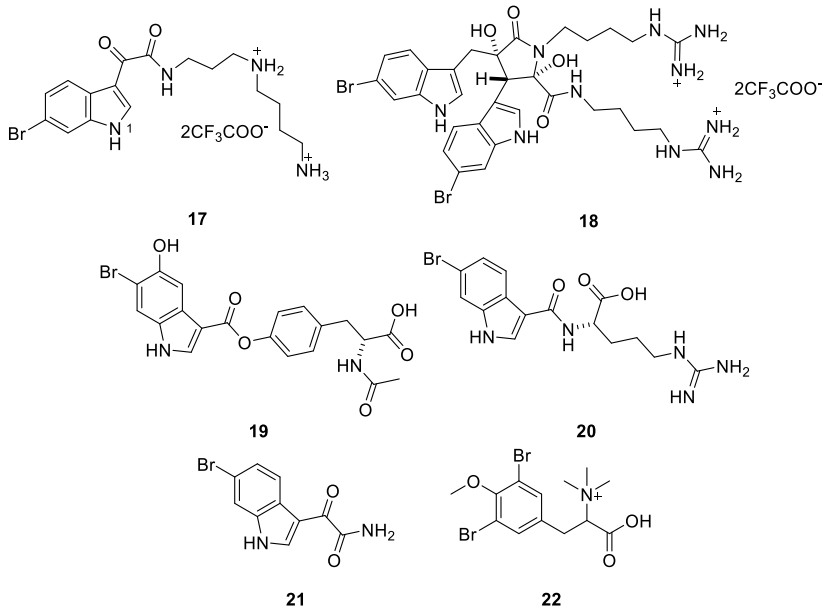

Fig. 4 Structures of didemnidine B (**17**), bis-trifluoroacetate salt of eusynstyelamide B (**18**), *N*-acetyl-*O*-(6-bromo-5-hydroxy-1*H*-indole-3-carbonyl)-D-tyrosine (**19**), *N*-(6-bromo-

1*H*-indole-3-carbonyl)-L-arginine (**20**), (6-bromo-1*H*-indol-3-yl)oxoacetamide (**21**) and 3,5-dibromotetramethyltyrosine (**22**).

Mitchell and coworkers (1996) purified two known anomers of 5′-deoxy-3-iodotubercidin (**25**, Fig. 5) from the methanol extract of the ascidian *D. voeltzkowi*, collected from isolated rocks scattered throughout shallow tide pools on Apo Reef, the Philippines (Fig. 5). The structure of the 7-deazapurine nucleoside **25** was elucidated by ^{1}H and ^{13}C NMR experiments and high- and low-resolution FABMS measurements.

Compound **23**, its protonated ammonium form - 2-(3,5-diiodo-4-methoxyphenyl) ethanaminium (**26**), and six new iodinated compounds (2-(3,5-diiodo-4-methoxyphenyl) ethanaminium benzoate (**27**), 2-(3,5-diiodo-4-methoxyphenyl)acetamide (**28**), *N*-[2-(3,5-diiodo-4-methoxyphenyl)ethyl] formamide (**29**), *N*-[2-(3,5-diiodo-4-methoxyphenyl)ethyl] benzamide (**30**), *N*,*N*′-bis[2-(3,5-diiodo-4-methoxyphenyl)ethyl] ethanediamide (**31**), 4-(2-aminoethyl)-2-iodophenol (**32**), Fig. 5) derived from **23** were isolated from an aqueous extract of the tunicate *D. rubeum*, collected by the Coral Reef Research Foundation at 10 m of depth in Chuuk Atoll (Solano et al., 2009). The structures of **23**, **26**-**32** were elucidated by NMR and MS analysis.

Very recently, Bromley and colleagues (2018) reported the presence of a new compound, 3-iodotetramethyltyrosine (**33**), in the extract an unidentified *Didemnum* species, collected by scuba divers at a depth of 18 m at White Sands Reef, Algoa Bay, South Africa (Fig. 5). They analyzed the extract by LC-ICP-MS/ESI-MS technique (in which a high-performance liquid chromatograph was coupled with inductively coupled plasma (ICP) and high-resolution electrospray ionization (ESI) mass spectrometers arranged in parallel); this afforded the rapid identification of known compounds and the discovery of new secondary metabolites. Compound **33** was the first example of the occurrence of iodinated metabolites in South African marine invertebrates.

2.3. Biological activities of brominated secondary metabolites

Didemnimides B (**9**) and D (**10**) were reported to be potent feeding deterrents against a natural assemblage of mangrove specific carnivorous fish (Vervoort et al., 1998). Field and laboratory experiments performed using whole animals, and their organic-solvent extracts indicated that the colonies were highly chemically defended against predation by carnivorous fish. Antipredatory activities of **9** and **10** depended on compound structure. Didemnimide D inhibited feeding in laboratory and field assays and deterred feeding at 0.035 mg/mL, and is among the most potent antipredatory chemical defenses.

3-Bromofascaplysin (**12**) was screened in a cell-based cytotoxicity assay and in the NCI 60 cell line panel. The cytotoxic properties of 3-bromofascaplysin were assessed against murine and human tumor cell lines using the disk diffusion soft agar assay. The presence of a bromine atom in compound **12** produced an increase in activity as compared to non-brominated derivative, but also caused inactivity in cell lines that are sensitive to non-brominated derivatives (Segraves et al., 2004). In 2010, Kuzmich and coworkers reported that 3-bromofascaplysin showed anticancer cytotoxic effects against seven different human cancer cell lines, HL-60, THP-1, HeLa, MDA-MB-231, DLD-1, SNU-C4, and SK-MEL-28. 3-Bromofascaplysin also exhibited a cancer-preventive effect at non-cytotoxic concentrations in JB6 Cl41 cells. These activities of **12** are mediated at least in part through the induction of caspase-3, caspase-8, or caspase-9-dependent apoptosis.

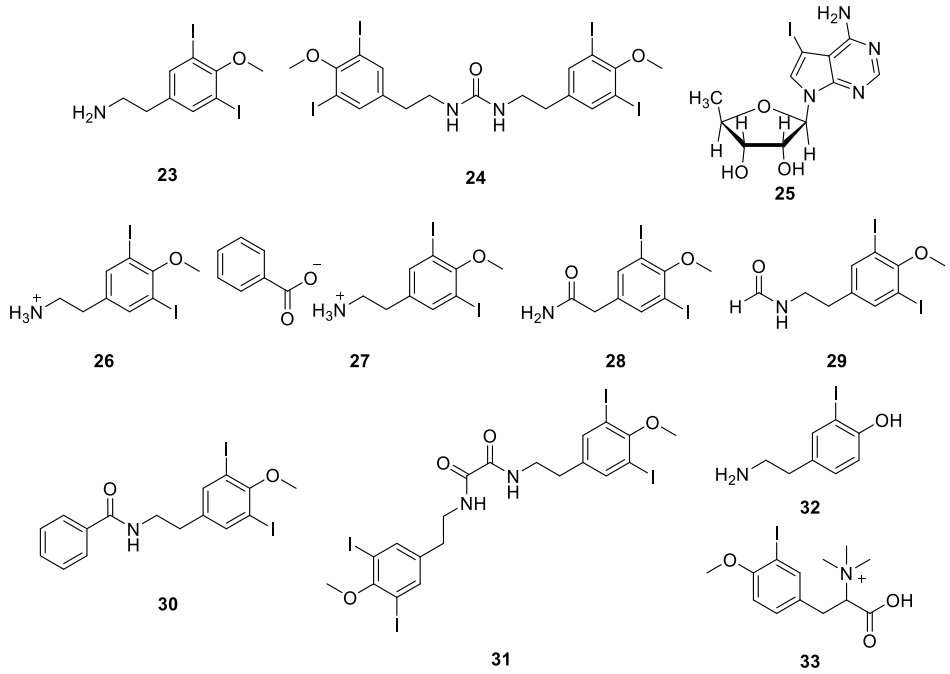

Fig. 5 Structures of 3,5-diiodo-4-methoxyphenethylamine (**23**), 1,3-bis(3,5-diiodo-4-methoxyphenethyl)urea, (**24**), 5′-deoxy-3-iodotubercidin (**25**), 2-(3,5-diiodo-4-methoxyphenyl)ethylaminium (**26**), 2-(3,5-diiodo-4-methoxyphenyl)ethylaminium benzoate (**27**), 2-(3,5-diiodo-4-methoxyphenyl) (**28**), *N*-[2-(3,5-diiodo-4-methoxyphenyl)ethyl] formamide (**29**), *N*-[2-(3,5-diiodo-4-methoxyphenyl)ethyl] benzamide (**30**), *N*,*N*′-bis[2-(3,5-diiodo-4-methoxyphenyl)ethyl] ethanediamide (**31**),4-(2-aminoethyl)-2-iodophenol (**32**), 3-iodotetramethyltyrosine (**33**).

Their results displayed that two of three main MAPK signaling pathways, JNKs and ERKs, might be involved in cell response to the treatment with **12**.

3-Bromofascaplysin exhibited the capacity to kill C6 glioma cells. The cytotoxic activity of this compound depends on its concentration and duration of exposure (Lyakhova et al., 2018). Lu and colleagues (2011) investigated differential activities of 3-bromohomofascaplysin A (**16**) against blood-borne life stages of the malaria pathogen *Plasmodium falciparum*. Compounds **16** displayed overall IC_{50} values comparable to the previously reported values against the strain K1.

Didemnidine B (**17**) and its synthetic intermediate were tested for bioactivity in a range of anti-inflammatory, antitumor, and neglected disease whole parasite assays. Compound **17** was found to be inactive to produce superoxide respiratory burst and in screening for phospholipase A_2 and farnesyltransferase enzyme inhibitors. Didemnidine B was mildly active toward the malaria parasite (IC_{50} 15 μM) and moderate cytotoxic towards the nonmalignant L6 cell line (Finlayson et al., 2011). Wang and coworkers, (2014) prepared and tested a library of analogs of didemnidine B, due to the identification of more active examples. Some analogs were identified as potent antimalarial agents, with good selectivity.

Eusynstyelamide B (**18**), bis-indole alkaloid displayed an IC_{50} of 5 μM in MDA-MB-231 cells. Moreover, this compound caused a strong cell cycle arrest in G2/M and induced apoptosis after 72 h treatment (Liberio et al., 2014). Pharmacological potentials as antibacterial agents and FXR antagonists of compounds **19**-**21** were investigated, but no significant activity was found (Hahn et al., 2015).

2.4. Biological activities of iodinated secondary metabolites

3,5-Diiodo-4-methoxyphenethylamine (**23**) and its symmetrical urea derivative (**24**) showed *in vitro* activity against *Candida albicans* and was mildly cytotoxic against tumor cell line L1210 with an IC_{50} of 20 μg/mL (Sesin and Ireland, 1984). 5′-Deoxy-3-iodotubercidin (**25**) was reported to cause potent muscle relaxation and hypothermia to mice. When tested on rats injection of 5′-deoxy-5-iodotubercidin reduced blood pressure and heart rate (Davies et al., 1986). *N*-[2-(3,5-Diiodo-4-methoxyphenyl)ethyl] benzamide (**30**), a natural product from *D. rubeum*, showed moderate activity against *Leishmania panamensis* and *Trypanosoma cruzi*, and high activity against *Plasmodium falciparum* (Restrepo et al., 2019). Compound **30** exerted strong toxicity towards brine shrimps (*Artemia salina*), the determined lethal concentration was lower than those of known toxicants (LD_{50}=20 μM). In the case of macrophage cultures, the tested tyramide was much less toxic but was found to have an effect on the functioning of these normal cells, most probably by interfering with the function of cell membranes and changing the reducing cellular capacity (Đorđević et al., 2019).

2.5. Total synthesis of brominated secondary metabolites

The first total synthesis of dragmacidin C (**3**) (Fig. 6) was accomplished by condensation of two indolylglycines followed by cyclization and reduction. Dragmacidin C was prepared by a sequence of reactions: Wittig olefination of 6-bromoindolin-3-one with an appropriate phosphonium ylide, azide reaction, reduction of azide ester with Ph_3P-H_2O, and *tert*-butyloxycarbonyl (Boc) protection provided the indolylglycine ester in 99% overall yield from 6-bromoindolin-3-one. Then, allyl deprotection of the obtained ester with $RhCl(PPh_3)_3$ in EtOH-H_2O gave the desired acid in 74% yield. The condensation of the obtained acid and indolylglycine using BOP (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate) and DIEA (*N*,*N*-diisopropylethylamine) proceeded diastereoselectively to give dipeptides (two diastereomers in 58% and 40% yields). Successive treatment of the appropriate diastereomer with HCO_2H and with NH_3 led to the deprotection and cyclization, and the final product dragmacidin C was obtained in the last reduction step with borane in 42% yield (Kawasaki et al., 2002).

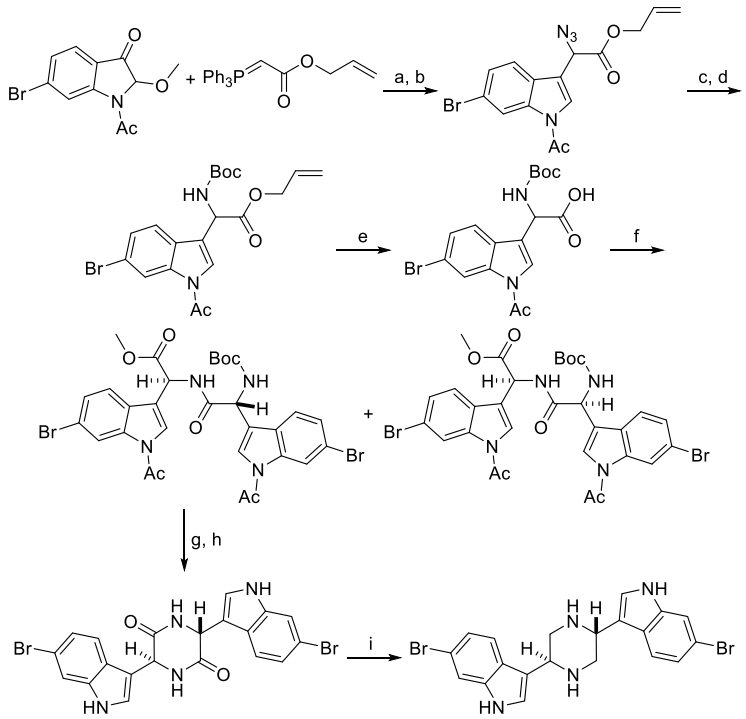

Fig. 6 Total synthesis of 2,5-bis(6′-bromo-3′-indolyl)piperazine (**3**, dragmacidin C). Reagents and conditions: a) C_6H_6, reflux; b) TMS-N_3, $MeSO_3H$, MS-4Å, CH_2Cl_2, 0 °C – rt; c) Ph_3P, H_2O, THF, rt; d) Boc_2O, $NaHCO_3$, rt; e) $RhCl(PPh_3)_3$, EtOH, H_2O, 70 °C; f) BOP, DIEA, indolylglycine, THF, 0 °C – rt; g) HCO_2H, rt; h) NH_3, MeOH, 0 °C – rt; i) BH_3·THF, 0 °C – rt.

The synthesis of didenmolines A (**4**) and C (**5**) enabled the unambiguous confirmation of the proposed structures and provided sufficient material for biological testing (Schumacher and Davidson, 1995) (Fig. 7). The synthesis of **4** and **5** involved the coupling of 1-[(benzyloxy)methyl]-4-chloromethyl-5-(thiomethyl)imidazole (**I**, which was prepared from 1-[(benzyloxy)methyl]-2,4,5-tribromoimidazole using an approach comprising sequential halogen-metal exchange) with 7-bromo-β-carboline (eudistomin O, **6**). In 1984, eudistomin O was prepared by Rinehart and coworkers in eight-step synthesis in a yield (7%). The reaction sequence was modified by Schumacher and Davidson (1999), but it also included 6-bromoindole and 1,2,3,4-tetrahydro-β-carboline as intermediates. Didemnoline C (**5**) could be generated from didemnoline A by treatment with $NaIO_4$ (Schumacher and Davidson, 1995).

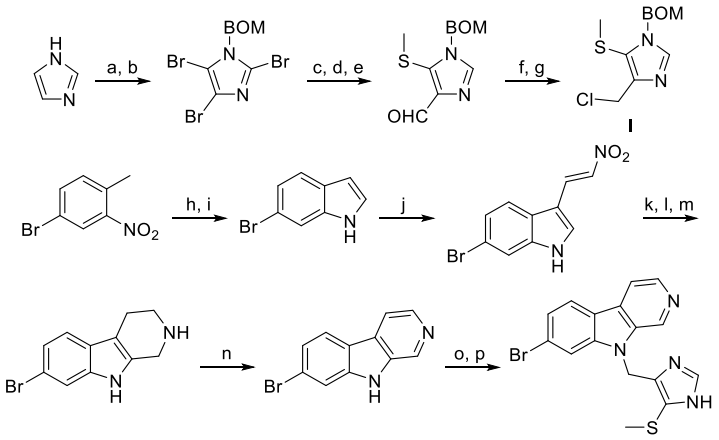

Fig. 7 Total synthesis of didemnolines A (**4**). Reagents and conditions: a) Br_2, AcOH; b) BOMCl, K_2CO_3, DMF; c) BuLi, -78 °C, TMSCl; d) BuLi, -78 °C, MeSSMe; e) BuLi, -78 °C, DMF; f) $NaBH_4$; g) $SOCl_2$; h) DMFDMA, pyrrolidine; i) $TiCl_3$; j) DMANE, TFA; k) $NaBH_4$, $BF_3{\cdot}OEt_2$; l) HO_2CCHO, KOH; m) HCl; n) S_8, xylene, Δ; o) KOH, DMF, I; p) HCl, 110 °C.

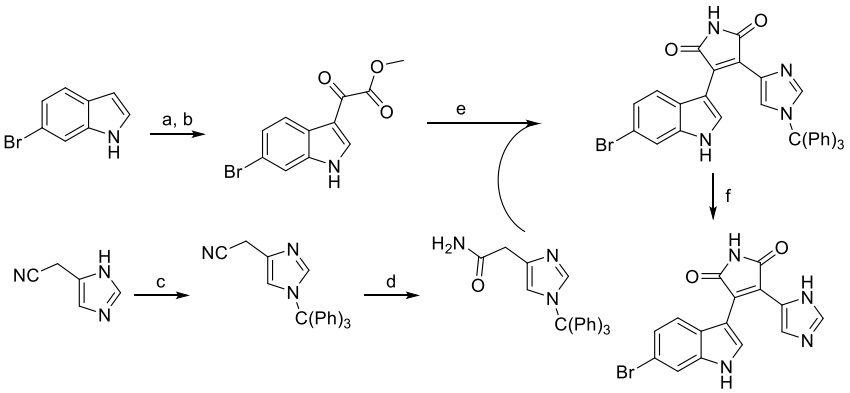

Fig. 8 Total synthesis of didemnimide B (**9**). Reagents and conditions: a) oxalyl chloride (1.1 equiv.), Et_2O, 0 °C to rt; b) MeOH (xs), rt, 24 h; c) trityl chloride (1.1 equiv.), CH_2Cl_2 (1.1 equiv.), Et_3N, rt, 24 h; d) powdered KOH (6 equiv.), *t*BuOH, reflux, 30 min; e) K_2CO_3 (7 equiv.), CETAB (0.07 equiv.), benzene (Dean-Stark), reflux, 48 h; f) TFA (8 equiv.), CH_2Cl_2, rt, 12 h.

The synthesis of didemnimide B (**9**) began with the preparation of methyl 6-bromoindolyl-3-glyoxylate (**72**) (Hughes and Cava, 1998; Fig. 8). 6-Bromoindole reacted with oxalyl chloride and the treatment of the corresponding acid chloride with methanol afforded this compound. Hughes and Cava used previously synthesized imidazole acetonitrile for the synthesis of the desired acetamide, which they condensed with methyl 6-bromoindolyl-3-glyoxylate, then the product gave didemnimide B after deprotection with TFA in dichloromethane.

Zhidkov and coworkers (2007) reported the first total synthesis of 3-bromofascaplysin (**12**) via a simple approach involving pyrido[1,2-*a*:3,4-*b′*]diindole formation (Fig. 9). The starting materials in their synthetic sequence were: tryptamine and (2,4-dibromophenyl) acetic acid (prepared in three steps from 3-bromo-4-methylaniline). The amide was prepared using *N*,*N′*-dicyclohexylcarbodiimide (DCC) as a condensing agent. Bischler-Napieralski cyclization was realized using $POCl_3$ in acetonitrile, and then the product was oxidized by MnO_2. Heating of β-carboline yielded the pyridodiindole quaternary salt, which was converted into compound **12** by treatment with dry HCl in MeOH.

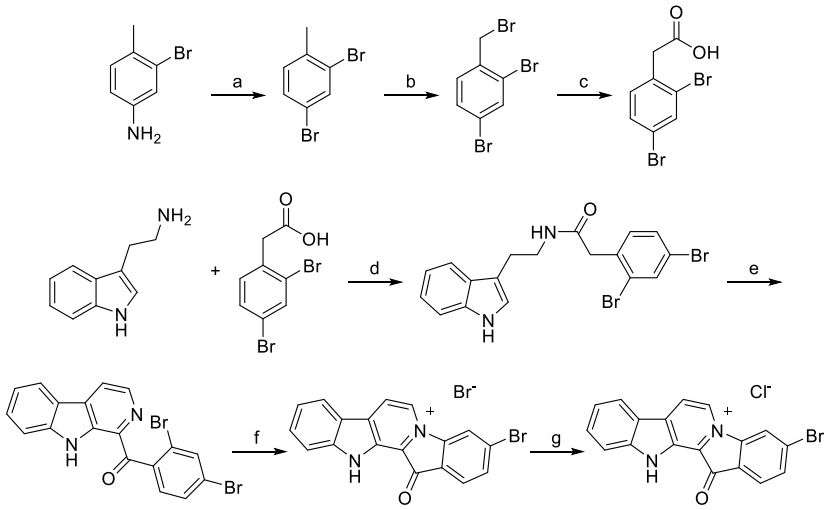

Fig. 9 Total synthesis of 3-bromofascaplysin (**12**). Reagents and conditions: a) $NaNO_2$, HBr (aq), 0 °C, then CuBr, HBr (aq), reflux, 40 min; b) NBS, AIBN, CCl_4, reflux, 1 h; c) NaCN, C_2H_5OH (aq), reflux, 1 h, then H_2SO_4 (aq), reflux, 12 h; d) DCC, CH_3CN, reflux, 30 min; e) $POCl_3$, CH_3CN, Ar, reflux, 40 min, then MnO_2, PhH, reflux, 3 h; f) 220 °C, 20 min; g) HCl (dry), CH_3OH.

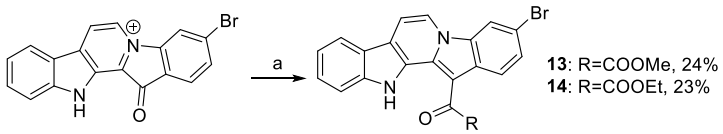

Fig. 10 Preparation of 3-bromohomofascaplysin B (**13**) and 3-bromohomofascaplysin B-1 (**14**) from 3-bromofascaplysin. Reagents and conditions: a) MeOOCCOOMe or EtOOCCOOEt (excess), autoclave, 200 °C, 30 min.

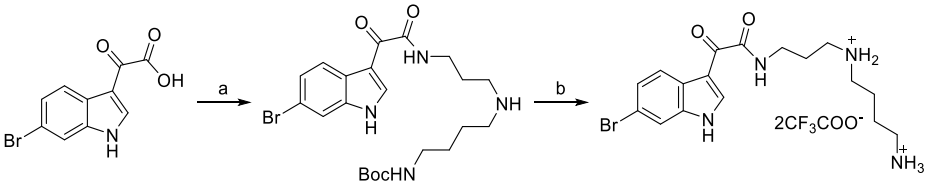

Fig. 11 Total synthesis of didemnidine B (**17**). Reagents and conditions: a) N^8-*tert*-butoxycarbonylspermidine, PyBOP, DMF; b) TFA, CH_2Cl_2.

Zhidkov and colleagues (2018) elaborated a method for a one-step conversion of the marine alkaloid 3-bromofascaplysin into 3-bromohomofascaplysin B (**13**) and 3-bromohomofascaplysin B-1 (**14**) (Fig. 10). The unusual reductive acylation at the C-13 position of 3-bromofascaplysin provided compounds **13** and **14**.

The structure of the natural product didemnidine B (**17**) was confirmed by synthesis. 2-(6-Bromoindol-3-yl)-glyoxylic acid was coupled with N^8-*tert*-butoxycarbonylspermidine and in this way N^1-(indolyl-3-glyoxamido)-N^8-*tert*-butoxycarbonylspermidine was obtained in 47% yield (Fig. 11). Deprotection of the *tert*-butoxycarbonyl-protected intermediate yielded **17** as the bistrifluoroacetate salt (Finlayson et al., 2011).

2.6. Total synthesis of iodinated secondary metabolites

Two syntheses of *N*-[2-(3,5-diiodo-4-methoxyphenyl)ethyl] benzamide (**30**) have been published in the same year (Đorđević et al., 2019, Restrepo et al., 2019). The first synthesis of the natural compound **30** was commenced with a carbodiimide-facilitated coupling procedure utilizing commercially available 2-(4-methoxyphenyl)ethanamine and benzoic acid. The resulting amide was subjected to iodination in two stages. The first stage afforded intermediary organomercury compound, which involved mercuration of the amide with mercury(II) acetate. In the second stage, following an exchange of the acetate ligands with chlorides, Hg-C bond was cleaved by the action of elemental iodine resulting in the replacement of the –HgCl group with an iodine atom (Đorđević et al., 2019; Fig. 12). The second synthesis of **30** (Fig. 13), which was accomplished by Restrepo and coworkers, started with the protection of the amino group in the tyramine. The iodination of the aromatic ring was accomplished with *N*-iodosuccinimide and acid hydrolysis of this product gave the iodinated amine. The amide synthesis was done by DIC-coupling of the desired amine with benzoic acid (Restrepo et al., 2019).

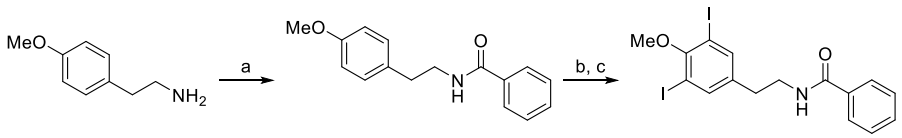

Fig. 12 Synthesis of compound *N*-[2-(3,5-diiodo-4-methoxyphenyl)ethyl] benzamide (**30**). Reagents and conditions: a) benzoic acid (1.5 equiv.), DCC (1.5 equiv.), 3–10 mol% DMAP, CH_2Cl_2, rt, 5 h; b) $Hg(OAc)_2$, 70% $HClO_4$, glacial CH_3COOH, rt, 48 h; c) NaCl, H_2O, 30 min; I_2, CH_2Cl_2 (dry), 16 h.

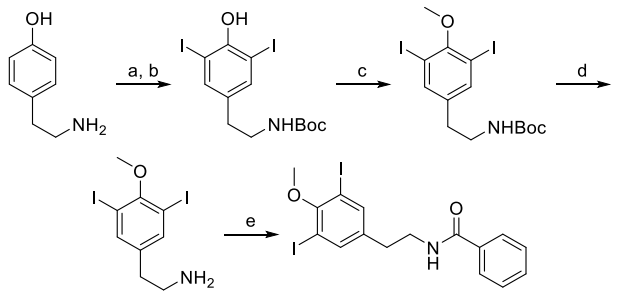

Fig. 13 Synthesis of compound **30**. Reagents and conditions: a) Boc_2O, TEA, MeOH; b) NIS, MeOH; c) MeI, K_2CO_3, acetone; d) HCl, EtOAc; e) benzoic acid, DIC, DMAP, DMF.

3. Structural Diversity of Halogen-Containing Secondary Metabolites of Ascidians Belonging to other Genera of the Family Didemnidae

The following section presents the known halogenated compounds isolated from ascidians of the genera *Lissoclinum*, *Tridemnum*, *Leptoclinides*, and *Diplosoma*, and also from unidentified/unknown ascidians of the family Didemnidae. Assessment of bioactivity and especially further medical application of halogen-contained secondary metabolites from the mentioned genera of Didemnidae is usually hampered by the isolation of only minute amounts of these compounds from natural sources. Such valuable data and potential utilization are only possible via synthesis. A dozen papers focusing on the synthesis of the abovementioned compounds have been published until now.

3.1. Isolation and structure determination of chlorinated metabolites

In 2004, Teruya and coworkers isolated haterumalide NA (**34**) and two new polyketides biselides A (**35**) and B (**36**) from the Okinawan Didemnidae ascidian collected at Bise and determined their structures by detailed spectroscopic analyses (Fig. 14). The following year, they reported the isolation of three more analogs, biselides C (**37**), D (**38**), and E (**39**) from the same organism (Teruya et al., 2005). Structurally, the haterumalides and biselides are all 14-membered macrolactones containing a trisubstituted 3-hydroxytetrahydrofuran moiety, with the exception of biselide E which is a linear analog (Fig. 14). Halogenated marine macrolides are a rare, unique class of compounds (Ueda and Hu, 1999).

Novel serinolipid derivative, shishididemniol B (**40**) was isolated in 2007 from the extract of a Didemnidae taxon (collected by scuba divers at depths of 5-15 m off Kushizaki in Shishijima of the Amakusa Islands) by Kobayashi and coworkers (Fig. 15). Further investigation of an antibacterial *n*BuOH fraction of the extract led to the identification of a new compound, shishididemniol D (**41**) (Kobayashi et al., 2007b). Shishididemniols possess C_{28}-polyketide acid in the central part of the molecule, two serinol, and oxygenated tyramine moieties. Their structures were elucidated by an interpretation of NMR and MS data, whereas the absolute stereochemistry was determined by chemical conversions.

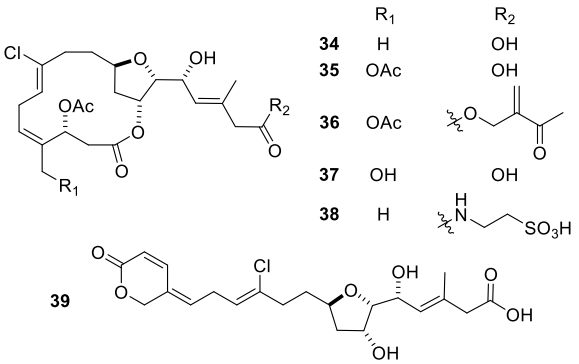

Fig. 14 Structures of haterumalide NA (**34**) and biselides A – E (**35**-**39**).

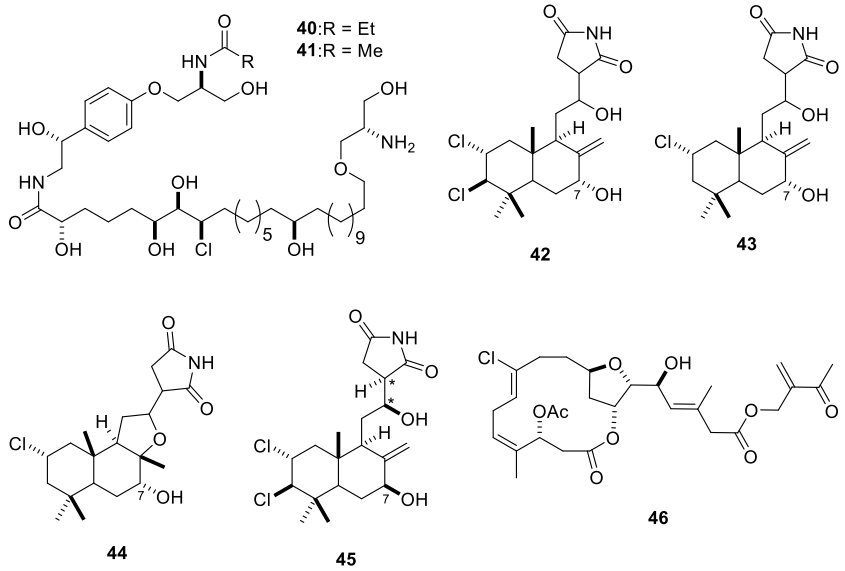

Fig. 15 Structures of shishididemniols B (**40**), D (**41**), the initially wrongly assigned structures **42**-**44**, the corrected structure of dichlorolissoclimide **45** (configuration of centers marked by an asterisk were determined by Könst and coworkers, 2017), and haterumalide B (**46**).

In 1991, Malochet-Grivois and colleagues isolated a new labdane substance, dichlorolissoclimide from the extract of *Lissoclinum voeltzkowi* Michaelson, collected in 1988 on Platier du Mont Dore, Caledonia (Fig. 15). Isolation was performed by liquid/liquid purification, HPLC and monitored by cytotoxic bioassay using SESAME mathematical analysis (Pouchus et al., 1989). The structure of dichlorolissoclimide was initially elucidated by spectroscopic analysis and was assigned to be **42**. Naturally occurring succinimides are very rare, and only one other succinimide compound has been

isolated from a marine organism; dichlorolissoclimide was the first labdane and the first chlorinated substance isolated from the subphylum Urochordata.

Three years later, in 1994, the same research group reported on a monochlorinated diterpene, chlorolissoclimide, isolated from the same ascidian collected from the same location. The structures **43** and **44** of chlorolissoclimide and a hemi-synthetic by-product of chlorolissoclimide were proposed by a comparative analysis of their spectral data (mass and NMR spectra) to that of the previously ascribed structure **42** of dichlorolissoclimide (Fig. 15). However, a study that dealt with the absolute stereochemistry of dichlorolissoclimide, by means of X-ray analysis (Toupet et al., 1996), finally resulted in the unambiguous assignment of the configuration. The previous assignment of the stereochemistry of the hydroxyl group at C7 by Malochet-Grivoisa and colleagues was incorrect and it was revised to an equatorial position as shown in compound **45**, (Fig. 15).

Lissoclinum voeltzkowi is known to be in symbiosis with the prokaryotic algae genus *Prochloron*. In such cases, it is not easy to determine the origin of the isolated metabolites, i.e. whether chlorolissoclimide and dichlorolissoclimide originate from the ascidian itself or from its symbionts. Their results showed that the biosynthesis of lissoclimides appears to be more pronounced in *Prochloron*, but it was not possible to completely exclude an ascidian origin for these compounds.

In 1999, Ueda and Hu reported the isolation and structural elucidation of haterumalide B (**46**), a new cytotoxic macrolide, which was obtained from the marine ascidian *Lissoclinum* sp. collected from dead corals off Hateruma Island, Okinawa (Fig. 15). Bio-assay-guided fractionation of the extract led to the isolation of this new chlorinated macrolide. The stereochemistry of **46** was elucidated on the basis of different NOESY experiments.

Five new dichlorolissoclimide-type diterpenoids, haterumaimides A–E (**47**-**51**), were isolated from an ascidian, *Lissoclinum* sp., collected off the coast of Hateruma Island, Okinawa, Japan (Uddin et al., 2001a) (Fig. 16). The absolute stereostructures of the haterumaimides A–D (**47**-**50**) and the relative stereostructure of haterumaimide E (**51**) were determined by spectroscopic and chemical analyses. The structures were elucidated by an analysis of their ^{1}H, ^{13}C and 2D NMR and high-resolution mass spectra. In addition, Uemura′s research group (in 2001 and 2002) isolated six new compounds from the same extract. They reported the isolation, structure elucidation, and absolute stereostructures of haterumaimides F-K (**52**-**57**, Fig. 16) together with two known compounds, dichlorolissoclimide (**45**) and chlorolissoclimide. Further bioassay-guided fractionation of the same extract led to the isolation of three new cytotoxic labdane alkaloids, haterumaimides N–P (**58**-**60**, Fig. 16), and haterumaimides J (**56**) and K (**57**) (Uddin et al., 2006).

In 2013, the investigation of the tunicate *Trididemnum solidum* (collected from Little Cayman Island at a depth of 10 m) resulted in the isolation of two new chlorinated didemnim-class compounds (**61**, **62**) in addition to two known compounds (Ankisetty et al., 2013). The structural determination of the compounds was based on extensive NMR and mass spectroscopic analysis (Fig. 17).

3.2. Isolation and structure determination of brominated metabolites

Two new dibromo proaporphine alkaloids, saldedines A (**63**) and B (**64**), were isolated from an unidentified tunicate of the family Didemnidae, collected at Salary Bay, ca. 100 km north of Tulear, Madagascar, in January 2007 (Sorek et al., 2009). This work was the

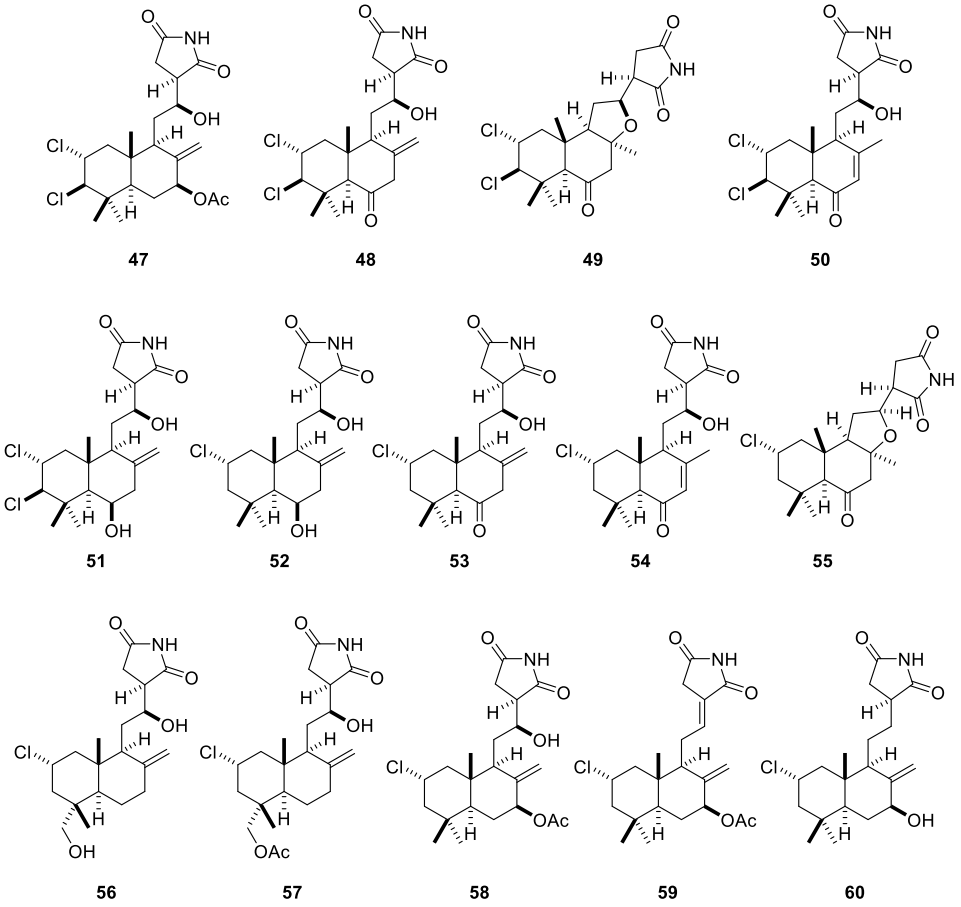

Fig. 16 Structures of haterumaimides A–K (**47**-**57**), N–P (**58**-**60**).

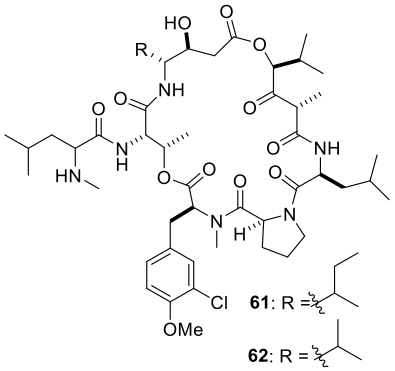

Fig. 17 Structures of chlorinated didemnims.

first report of proaporphine alkaloids from a marine source (Fig. 18), and for the first time of bromoproaporphines. Their structures were elucidated by extensive spectroscopic analysis, and the structure of **63** was confirmed by single-crystal X-ray diffraction analysis.

A new indole alkaloid, lissoclin C (**65**), was isolated along with the known 6-bromotryptamine (**1**) from the blue-white colonial ascidian *Lissoclinum* sp. from the Great Barrier Reef, collected in May 1991 (Searle and Molinski, 1994). Lissoclin C (**65**) was identified along with its putative precursor, 6-bromotryptamine (**1**) that probably engaged a suitable aldehyde or β-keto acid in a Pictet-Spengler condensation to give a tetrahydro-β-carboline ring system. From biosynthetic considerations, **65** is obtained by the condensation of **1** with 4-imidazolylpyruvic acid at C2 rather than C1, which was probably derived by transamination from histidine, followed by the loss of carbon Cl (Fig. 18).

In 1987, Bloor and Schmitz reported on the isolation and structure elucidation of a novel pentacyclic aromatic alkaloid 2-bromoleptoclinidinone from an ascidian tentatively identified as *Leptoclinides* sp. collected in Chuuk Lagoon, Federated States of Micronesia. The structure of 2-bromoleptoclinidinone was initially inferred from long-range $^{1}H/^{13}C$ COSY and nOe experiments along with other conventional spectroscopic techniques. After other possible structures were eliminated, structure **66** was first assigned to 2-bromoleptoclinidinone (Fig. 18).

In 1989, de Guzman and Schmitz re-examined the structure of **66** in the light of the newly isolated alkaloid ascididemin (Kobayashi et al., 1988), which exhibits the same pentacyclic skeleton (Fig. 18).

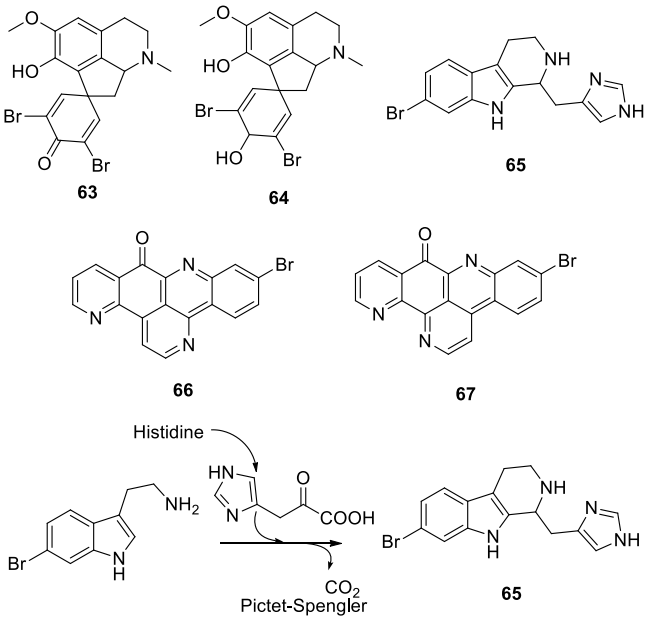

Fig. 18 Structures of saldedines A (**63**) and B (**64**), lissoclin C (**65**), 2-bromoleptoclinidinone (the initially wrongly assigned structure **66**, and the corrected structure **67**), proposed biosynthesis of lissoclin C (**65**).

The crucial long-range $^{1}H/^{13}C$ couplings were re-evaluated using the more sensitive and definitive INAPT experiment (the selective version of INEPT, the experiment is used for detecting long-range interactions and provides a good method for assigning quaternary carbon nuclei) in $CDCl_3$ as the solvent. On the basis of these results, the structure of 2-bromoleptoclinidinone was reassigned to that of **67**. The structure of 2-bromoleptoclinidinone (**67**) was further confirmed by single-crystal X-ray crystallographic analysis (Lindsay et al., 1998).

García and colleagues (1996) described the isolation of simple bromine-containing peptides from the polar cytotoxic extract (100% inhibition of KB cells at 10 µg/mL and 80% inhibition of P-388 cells at 10 µg/mL) of the ascidian *Leptoclinides dubius* (Sluiter, 1909) collected by hand (scuba diving, -5 to -10 m) in Woodin Canal, New Caledonia. *N*-(6-Bromo-1*H*-indolyl-3-carbonyl)-R, where R is L-Arg (**20**), L-His (**68**), and the very rare amino acid L-enduracididine (**69**), have been isolated and identified by spectroscopic data, hydrolysis, and comparison with authentic samples (Fig. 19). This was the first time the amino acid enduracididine has been found in a marine organism.

In 2009, Carroll and Avery isolated leptoclinidamine C (**70**) from a specimen of *Leptoclinides durus*, Kott, 2001 (collected by scuba divers (-18 m) off Heron Island, Central Queensland, Australia, in August 1996). Compound **70** contains the naturally rare 1,3-dimethyl-5-(methylthio)histidine attached to 6-bromoindole-3-carboxylic acid (Fig. 19).

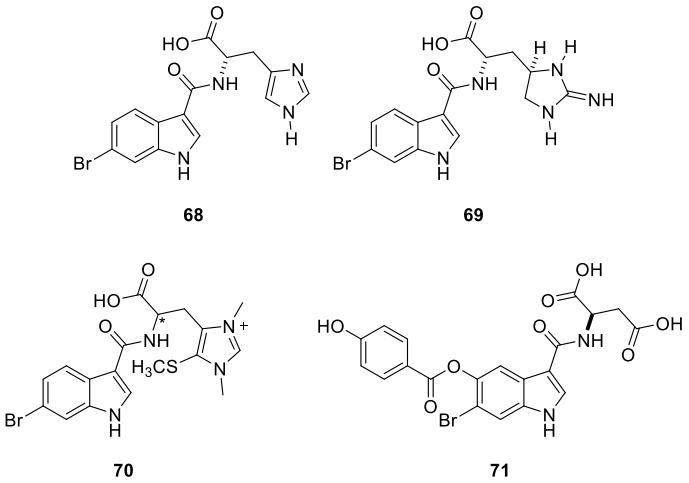

Fig. 19 Structures of *N*-(6-bromo-1*H*-indolyl-3-carbonyl)-R, where R is L-His (**68**), and L-enduracididine (**69**), leptoclinidamine C (**70**, configuration of center marked by an asterisk not determined), kingamide A (**71**).

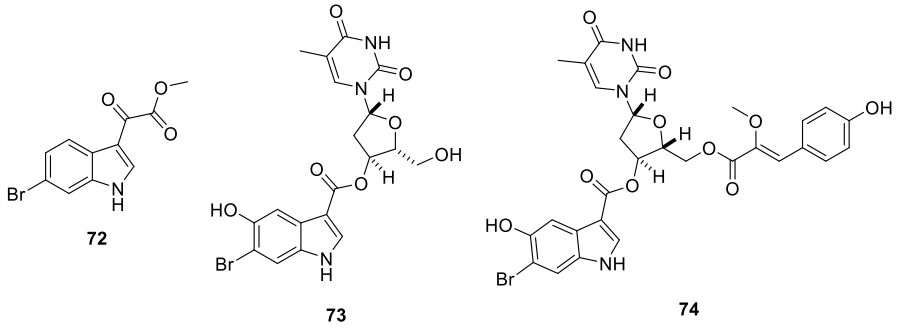

Fig. 20 Structures of 6-bromo-1*H*-indolo-3-yl-oxoacetic acid methyl ester (**72**), leptoclinidine A (**73**), and leptoclinidine B (**74**).

Chemical investigation of the extract of the tunicate *Leptoclinides kingi* Michaelsen 1930 (collected by scuba divers at a depth of 26.7 m between Hook and Hardy Reefs in Queensland, Australia, during June 1999) led to the isolation of kingamide A (**71**) (Fig.19), a new brominated indole alkaloid (Liberio et al., 2011). The structure of **71** was elucidated by an interpretation of 1D/2D NMR and MS data, and the absolute configuration was determined using Marfey's method.

In 2013, Carroll and colleagues recollected the colonial ascidian *Leptoclinides durus* from the Swain Reefs region of the Great Barrier Reef and isolated 6 new bromine-containing alkaloids along with compound **70** (previously reported from *L. durus* (Carroll and Avery, 2009)) and 6-bromo-1*H*-indolo-3-yl-oxoacetic acid methyl ester (**72**) that was purified from a Korean sponge. NMR and MS analysis of the 6 new compounds revealed that they belonged to three different structure classes that the authors named leptoclinidines (leptoclinidine A (**73**) and leptoclinidine B (**74**)), durabetaines (durabetaine B (**75**)), leptoclinidamines (leptoclinidamine D-F (**76**-**78**)). Compounds **73** and **74** are the first 3′-indole-3-carboxylic acid ester derivatives of thymidine, while **75** is the first glyceryl-3-(*O*-carboxyhydroxymethylcholine) alkaloid reported from an animal source and are also the only known derivatives from this class to be acylated with aromatic carboxylic acids. The new leptoclinidamines (**77**, **78**) are benzamide derivatives of histamine in which the imidazole is substituted by 5-methylsulfanyl and 1,3-dimethyl groups (Figs 20-21).

Shermilamine A (**79**), a new tunicate-derived thiazinone-containing pentacyclic alkaloid, was isolated from the leathery purple colonial tunicate, *Tridemnum* sp., collected in Pago Bay, Guam (Cooray et al., 1988). The structure of **79** was determined using NMR, HREIMS, IR, UV data and confirmed by X-ray diffraction analysis (Fig. 22).

Bioassay-guided fractionation of the toxic extract (from the small brown tunicate *Diplosoma* sp. collected at low tide from the coast of Hateruma Island, Okinawa, Japan) by a series of chromatographic steps yielded 4-bromo-3-pentylphenol (**80**) (Rob et al., 2011). The structure of **80** was determined by spectroscopic analysis and corroborated by synthesis (Fig. 22).

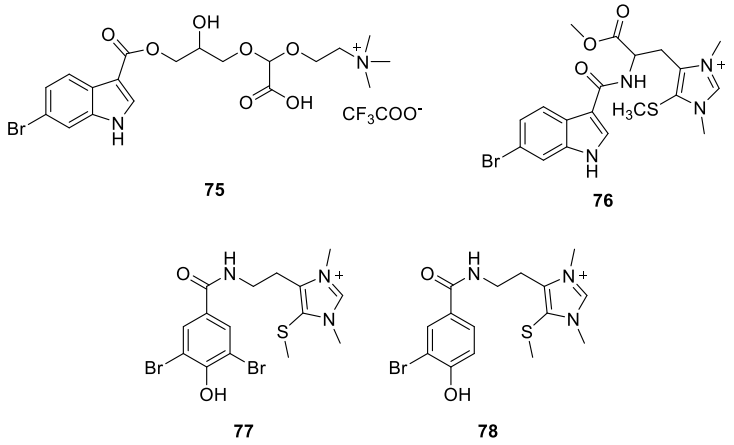

Fig. 21 Structures of durabetaine B (**75**), leptoclinidamine D-F (**76**-**78**).

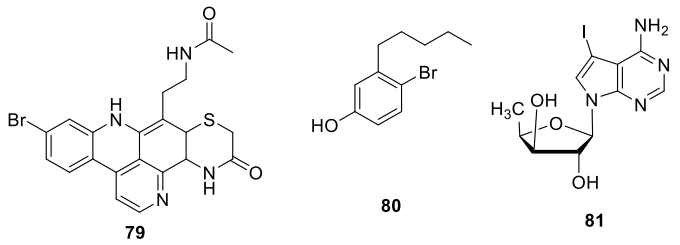

Fig. 22 Structures of shermilamine A (**79**), 4-bromo-3-pentylphenol (**80**), 4-amino-7-(5′-deoxy-β-D-xylofuranosyl)-5-iodopyrrolo[2,3-*d*]pyrimidine (**81**).

3.3. Isolation and structure determination of iodinated metabolites

In 2008, Margiastuti and colleagues isolated an unusual nucleoside, 4-amino-7-(5′-deoxy-β-D-xylofuranosyl)-5-iodopyrrolo[2,3-*d*]pyrimidine (**81**), from a green encrusting ascidian, *Diplosoma* sp., overgrown on dead coral collected by hand from the coast of Hateruma island, Okinawa (Fig. 22). The structure of the marine metabolite **81** was determined by 1D and 2D NMR spectra. The relative stereochemistry of the sugar moiety was determined by NOE experiments and chemical transformation (attempted acetonide formation). The mentioned research group synthesized a dibenzoate derivative (by treatment of **81** with 4-bromobenzoyl chloride) to confirm the absolute stereostructure of the 5-deoxy-β-xylofuranose unit of **81** by the analysis of CD spectrum.

3.4. Biological activities of chlorinated secondary metabolites

Haterumalide NA (**34**) and biselides A (**35**) and C (**37**) have been reported to possess a variety of biological effects. Haterumalide NA exhibited cytotoxicity against P388 leukemia cancer cells, with IC_{50} of 0.32 μg/mL, and moderate acute toxicity in mice (Takada et al., 1999). In 2005, Kigoshi group reported on the cytotoxicity of **34**, **35** and **37**. Among the tested compounds haterumalide NA methyl ester was the most cytotoxic

derivative, while biselide A was more active than biselide C. Biselide A showed the strongest cytotoxicity against human colon cancer cells DLD-1, among the tested cell lines. Compound **34** displayed strong toxicity against brine shrimps (LD_{50}=0.6 μg/mL), while **35** and **37** showed no acute toxicity towards *A. salina* even at 50 μg/mL (Teruya et al., 2005). Ueda and coworkers (2009) investigated the structure-cytotoxicity relationships of haterumalides. Their results imply that a specific combination of the lactone and side-chain parts is essential for the onset of strong cytotoxicity of haterumalides.

Shishididemniols A (**40**) and D (**41**) showed antibacterial activity in a disk agar diffusion assay against the fish pathogenic bacterium *Vibrio anguillarum* (Kobayashi et al., 2007a, 2007b). Strong cytotoxic activity of **45** was reported on human carcinoma KB cells (IC_{50} = 14 ng/mL) and P388 leukemia cells (IC_{50} = 1 ng/mL) (Malochet-Grivois et al., 1991). Chlorolissoclimide proved to be an effective cytotoxic agent against four tumor cell-lines: NSCLC, KB, P388, and doxorubicin-resistant P388, while its hemi-synthetic by-product was about 1000 times less active (Biard et al., 1994). It is likely that the cytotoxicity is mediated by oxidation of the hydroxyl group to the reactive α,β-unsaturated ketone. In New Caledonia, the cytotoxicity of lissoclimides may be the cause of food poisoning related to oyster consumption, as the oysters are known to contain the ascidian *Lissoclinum voeitzkmi* in their shells (Biard et al., 1994).

Haterumalide B (**46**), a chlorinated 14-membered ring macrolide, displayed a complete inhibition of the first cleavage of fertilized sea urchin eggs at a concentration of 0.01 μg/mL (Ueda and Hu, 1999). Similarly, haterumaimides A, B, and D (**47**, **48** and **50**) inhibited the cleavage of fertilized sea urchin eggs, and compounds **47**-**51** exhibited potent cytotoxicity against mouse lymphocytic leukemia cells (P388) (Uddin et al., 2001a). Haterumaimides F (**52**), G (**53**), and H (**54**) also completely inhibited the first cleavage of fertilized sea urchin eggs. Haterumaimides F-I (**52**-**55**) exhibited potent to weak cytotoxicity against mouse lymphocytic leukemia cells (P388). Haterumaimide G (**53**) and haterumaimide I (**55**) showed weak cytotoxicity compared to haterumaimides F (**52**) and H (**54**) (Uddin et al., 2001b). Accordingly, the secondary hydroxyl groups at C-6 and C-12 seem to be very important for the observed *in vitro* cytotoxicity. Haterumaimides J (**56**) and K (**57**) inhibited the first cleavage of the cell division of fertilized sea urchin eggs at 3 ppm, and showed potent cytotoxicity against P388 cells with IC_{50} values of 0.23 ng/mL and 0.45 ng/mL, respectively (Uddin et al., 2002). In 2006, Uddin and colleagues investigated the structure-activity relationships (SAR) of **47**-**60**, dichlorolissoclimide, and chlorolissoclimide, (Figs 15-16). The SAR results suggested that the presence of hydroxyl groups at C-6, C-7, C-12, and C-18, a chlorine atom at C-2, and an imido NH in ring C, are very important for their cytotoxicity.

Compounds **61** and **62** were tested for their anti-inflammatory activity using *in vitro* assays for inhibition of inducible nitric oxide synthase (iNOS) and nuclear factor-kappa B (NF-κB) activity. Chlorinated didemnins showed strong activity against iNOS and NF-κB in LPS-induced macrophages and PMA-induced chondrocytes. The isolated metabolites were also evaluated for their cytotoxicity towards four human solid-tumor cell lines (SK-MEL: melanoma; KB: epidermal carcinoma; BT549: breast carcinoma and SK-OV-3: ovarian carcinoma) and non-cancer kidney cells (Vero: monkey kidney fibroblasts); they inhibited cell proliferation of all four cancer cell lines and were less toxic towards noncancerous cells in comparison to the control drug (Ankisetty et al., 2013).

3.5. Biological activities of brominated secondary metabolites

Saldedines A (**63**) and B (**64**) were tested for toxicity to brine shrimps (*Artemia salina*) and were found to be moderately active. Compound **63** showed a greater potency with an LD_{50} value of 4.4 μM, while **64** displayed an LD_{50} value of 10.9 μM (Sorek et al., 2009).

2-Bromoleptoclinidone (**67**) was reported to exhibit cytotoxicity to the cultures of murine leukemia cells (P388) at 0.4 μg/mL (Bloor and Schmitz, 1987). Although complexation of **67** in a standard colorimetric test was not observed, its complex with ruthenium intercalates into DNA and causes photo-induced double-strand cleavage (Goulle et al., 1991). Leptoclinidamine C (**70**) was tested for bioactivity against chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) strains of the malarial parasite *Plasmodium falciparum*, trypanosomal activity against *Trypanosoma brucei*, cytotoxicity against the cancerous cell line HeLa, and cytotoxicity to noncancerous HEK 293 cells. Compound **70** was inactive in all bioassays when tested up to 40 μM in the antimalarial and cytotoxicity assays and 250 μM in the antitrypanosomal assay (Carroll and Avery, 2009). Kingamide A (**71**) showed no cytotoxicity at 10 μM after 24 h against the breast cancer cell line MDA-MB-231, and a panel of seven prostate cancer cell lines (DU-145, PC3 ATCC, PC3-DsRed, LNCaP and derivatives C4, C4-2 and C4-2B). Compound **71** displayed no parasitic growth inhibition at 10 μM after 24 h against the chloroquine-sensitive *P. falciparum* 3D7 line (Liberio et al., 2011). Compounds **73-78** showed no cytotoxicity at 10 μM after 72 hours against the cell line MDA-MB-231 or the prostate cancer cell line LNCaP; and no antimicrobial activity was observed after 18 hours against bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* at concentrations up to 330 μM (Rudolph et al., 2013). Compound **80** completely inhibited the first cleavage of fertilized sea urchin eggs at 1 ppm (Rob et al., 2011).

3.6. Biological activities of iodinated secondary metabolites

Compound **81** caused complete inhibition of cell division in fertilized sea urchin eggs at a concentration of 1 mg/mL (Margiastuti et al., 2008). The iodinated nucleoside **81** showed strong cytotoxic activity against HCT116, A431 and A549 cancer cell lines, with IC_{50} values of 1.8, 3.1 and 3.5 μg/mL, respectively (Ogi et al., 2009).

3.7. Total synthesis of chlorinated secondary metabolites

The first report on the synthesis of haterumalides came from the Kigoshi group. They reported an enantioselective synthesis of *ent*-haterumalide NA methyl ester (Kigoshi et al., 2003) by the use of an intramolecular Reformatsky reaction as a key step to macrocyclization at C2-C3. Gu and Snider used an intermolecular Stille coupling reaction at C5-C6 followed by a Yamaguchi macrolactonization (Gu and Snider, 2003). Hoye and Wang (2005) reported the first total synthesis of the correct enantiomer of haterumalide NA. Their synthetic route involved a key intramolecular cyclization at C6-C7 based on a Pd-mediated alkyne haloallylation reaction (Kaneda reaction). Roulland (2008) and the Kigoshi group, in two separate reports, described the use of variants of Suzuki-Miyaura cross-coupling along with macrolactonization to achieve the synthesis of haterumalide NA (Hayakawa et al., 2008).

Hayakawa and coworkers reported an efficient method for the second-generation total synthesis of haterumalides and biselides. Synthesis of the common intermediate **I** (Fig.

23) started from a known glycal (int.1); the hydroxyl group of the glycal was protected, then the protected compound was converted to a hemiacetal by an oxymercuration reaction. The Wittig reaction, oxy-Michael cyclization, and reduction by $LiAlH_4$ gave the appropriate alcohol. The alcohol was converted to the terminal olefin, the desired compound for *B*-alkyl Suzuki–Miyaura coupling. The desired chloroolefin was prepared from alkenylsilane by NCS in DMF at 50 °C in the presence of K_2CO_3 as the base. Then, the treatment of the chloroolefin with PPTS provided a triol, the 1,2-diol group of which was reprotected as an acetonide group to afford the common intermediate (**I**) for haterumalides and biselides.

Oxidation of **I** afforded an aldehyde, which was converted into the *Z*-conjugated ester. The DIBAL reduction of the ester gave an allylic alcohol, which was oxidized to the conjugated aldehyde, and then the aldol reaction with the lithium enolate of isopropyl acetate provided a β-hydroxy ester (Fig. 24). The hydroxyl group was protected as a TBS ether, and the acetonide group in this ether was removed by using CSA to give a diol. Oxidative cleavage of the diol by $NaIO_4$ followed by a reductive workup afforded an alcohol with the protected hydroxyl group as a trityl group, and the DMPM group was removed in an additional step. The macrolactonization was accomplished by the Yamaguchi conditions to give the desired lactone, then the TBS group was removed by TBAF, and the final intermediate (**II**) for haterumalides was obtained by a procedure reported by Snider and Gu after oxidation and Luche reduction.

To convert **II** into haterumalide NA (**34**), they followed their first-generation synthesis (Kigoshi et al., 2003), thus Nozaki-Hiyama-Kishi coupling with the appropriate 2,4-dimethoxybenzyl derivative afforded the coupling product (Fig. 25). The 2,4-dimethoxybenzyl ester in coupling compound was cleaved with TFA and anisole to afford haterumalide NA (**34**) (Hayakawa et al., 2008).

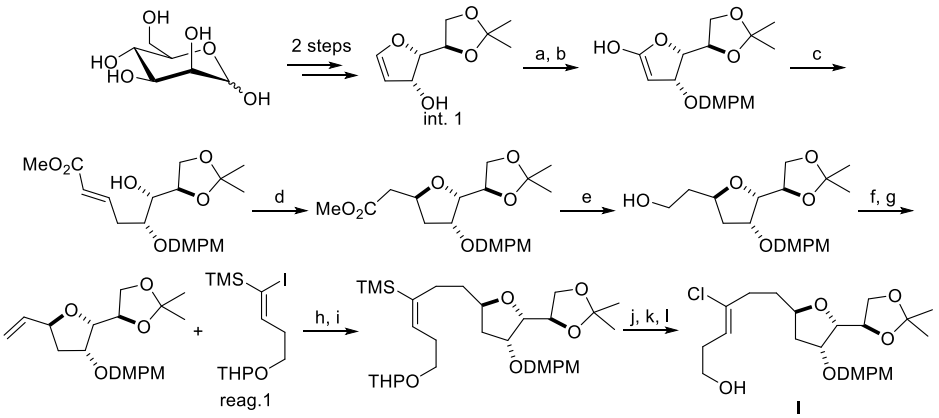

Fig. 23 Synthesis of the common intermediate **I** of haterumalides and biselides. Reagents and conditions: a) DMPMCl, NaH, DMF, rt, quant; b) $Hg(OAc)_2$, then KI, $NaBH_4$, THF-H_2O, rt; c) Ph_3PCHCO_2Me, benzene, reflux; d) Triton B, MeOH, rt; e) $LiAlH_4$, Et_2O, reflux, 97%; f) *o*-$NO_2C_6H_4SeCN$, Bu_3P, THF, rt, quant; g) H_2O_2, pyridine, THF, rt, 95%; h) 9-BBN dimer, THF, rt; i) reag.1, $PdCl_2$(dppf), Cs_2CO_3 aq, dioxane, rt; j) NCS, K_2CO_3, DMF, 50 °C, 58%; k) PPTS, MeOH, rt; l) PPTS, acetone, rt, 96%, in 2 steps.

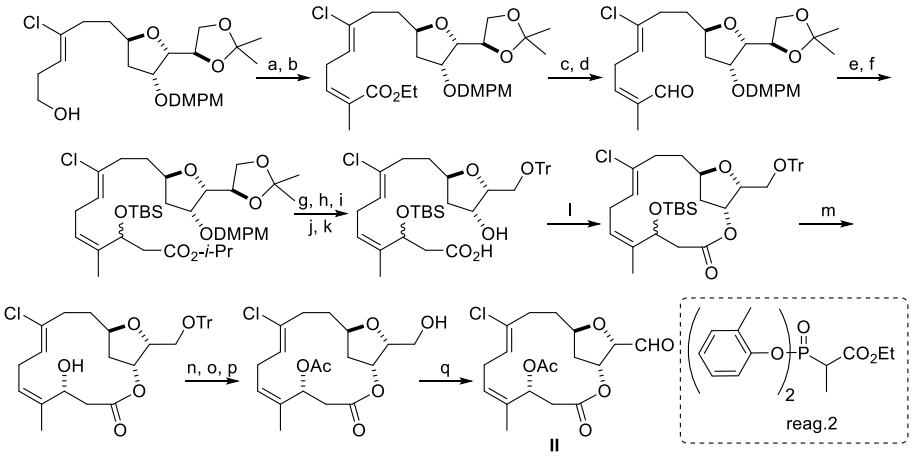

Fig. 24 Synthesis of common intermediate **II** of haterumalides. Reagents and conditions: a) Dess-Martin periodinane, CH_2Cl_2, rt; b) reag.2, NaH, THF, -78 °C, 79% in 2 steps; c) DIBAL, toluene, -78 °C; d) Dess-Martin periodinane, CH_2Cl_2, rt; e) *i*PrOAc, LiHMDS, THF, -78 °C, 76% in 3 steps; f) TBSCl, imidazole, DMF, rt, 91%; g) CSA, ethylene glycol, *i*PrOH, rt, 79%; h) $NaIO_4$, then $NaBH_4$, dioxane-H_2O, rt, 94%; i) TrCl, DMAP, pyridine, CH_2Cl_2, rt, 98%; j) DDQ, pH 6.6 phosphate buffer, *t*BuOH-CH_2Cl_2, rt, 93%; k) 3 M LiOH (aq), THF-MeOH, rt, 77%; l) 2,4,6-$Cl_3C_6H_2COCl$, Et_3N, THF, rt; then DMAP, toluene, rt, 61% + 6% dimer; m) TBAF, THF, rt; separation; n) Dess-Martin periodinane, CH_2Cl_2, rt, 94%; o) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, EtOH, -30 °C, 94%; p) AcCl, DMAP, pyridine, rt; 80% AcOH, rt, 91 % in 2 steps; q) Dess-Martin periodinane, CH_2Cl_2, rt.

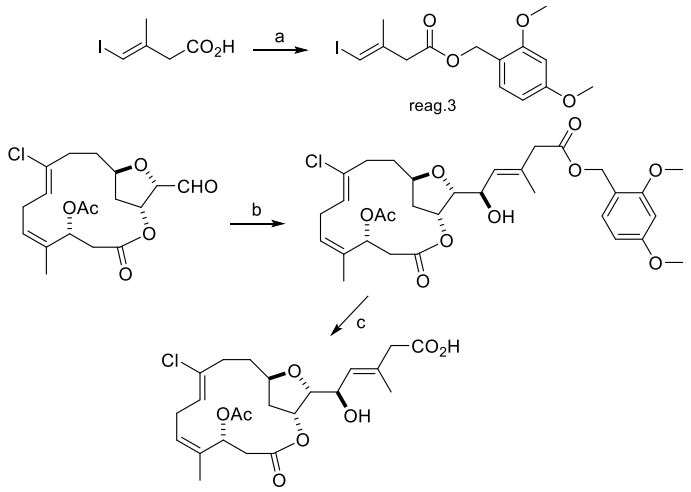

Fig. 25 Synthesis of haterumalide NA (**34**). Reagents and conditions: a) 2,4-dimethoxybenzyl alcohol, PyBOP, DMAP, CH_2Cl_2, rt, 54%; b) reag.3, 0.5% $NiCl_2 \cdot CrCl_2$, DMSO, rt, 44% in 2 steps; c) TFA, anisole, CH_2Cl_2, rt, 66%.

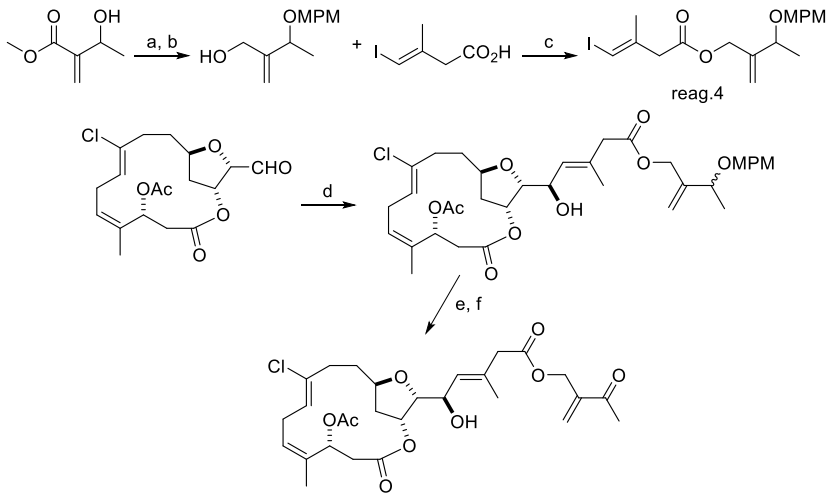

Fig. 26 Synthesis of haterumalide B (**46**). Reagents and conditions: a) MPMOC(=NH)CCl_3, PPTS, CH_2Cl_2, rt, 65%; b) DIBALH, CH_2Cl_2, -78 °C, 85%; c) DCC, DMAP, CH_2Cl_2, rt, 90%; d) reag.4, 1% $NiCl_2 \cdot CrCl_2$, DMSO, rt, 48%; e) DDQ, pH 6.6 phosphate buffer, *t*BuOH-CH_2Cl_2, 0 °C, quant; f) MnO_2, CH_2Cl_2, rt, 73%.

The Nozaki-Hiyama-Kishi coupling reaction of the aldehyde (**II**) and the iodide (reag.4) afforded the coupling product (Fig. 26). Removal of the MPM group and subsequent selective oxidation of the allylic alcohol with MnO_2 afforded haterumalide B (**46**).

Satoh and coworkers (2012a,b) reported the synthesis of the macrolactone part of biselides A by using regioselective allylic oxidation as the key step. The total synthesis of biselide A (Fig. 27) based on the strategy of synthesizing haterumalides by Hayakawa and colleagues (2008) was also published (Hayakawa et al., 2017a). The hydroxy group in **I** was converted to an iodide, which was transformed into a triphenylphosphonium salt. A subsequent Wittig reaction afforded a diacetate as a precursor from regioselective enzymatic hydrolysis. When the reaction was carried out at 27 °C with PPL type II in acetone-phosphate buffer (pH 7.0) the desired allylic alcohol was obtained in the highest yield. The allylic alcohol was converted into (*Z*)-α,β-unsaturated aldehyde. An asymmetric aldol reaction was attempted between (*Z*)-α,β-unsaturated aldehyde, and 3-acetylthiazolidine thione. Removal of the chiral auxiliary of the desired aldol and silylation of the secondary hydroxyl group gave a TBS ether. To convert this ether into biselide A (**35**), they followed their previous total synthesis of haterumalides.

Synthesis of biselide E (**39**) started from the allylic alcohol employed in total synthesis of biselide A, that was oxidized to an aldehyde, subsequently subjected to the aldol reaction of with the enolate of isopropyl acetate and in the end afforded a β-hydroxy ester (Fig. 28). The β-hydroxy ester was transformed into a dihydroxycarboxylic acid by the removal of the TBDPS group and hydrolysis of the isopropyl ester. Lactonization of the dihydroxycarboxylic acid with EDC and DMAP gave a β-hydroxy-δ-lactone. Removal of the acetonide group and the oxidative cleavage of the diol group afforded the corresponding aldehyde. Ni/Cr-mediated coupling between this aldehyde and vinyl iodide and the elimination of the TBS groups gave a triol as a precursor for the β-elimination reaction.

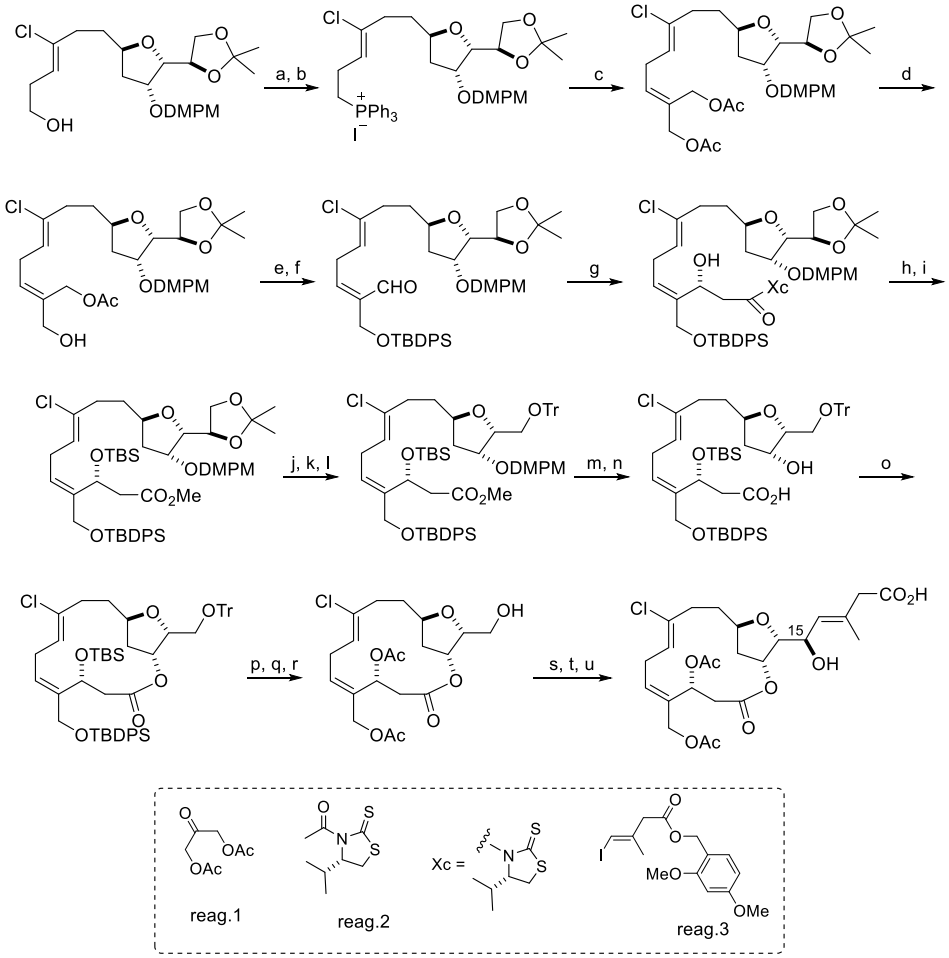

Fig. 27 Total synthesis of biselide A (**35**). Reagents and conditions: a) I_2, imidazole, CH_2Cl_2, rt, quant; b) PPh_3, toluene, 90 °C, quant; c) reag.1, LHMDS, MS 3Å, THF-HMPA, -78 °C, 89%; d) PPL type II, acetone/PB (pH 7.0) = 1:2, 27 °C, 2 h; e) TBDPSCl, imidazole, DMF, rt, K_2CO_3, MeOH; f) Dess-Martin periodinane, CH_2Cl_2, rt; g) $Sn(OTf)_2$, *N*-ethylpiperidine, reag.2; CH_2Cl_2, -40 °C to -78 °C, 96%, 2 steps; h) K_2CO_3, MeOH, rt; i) TBSCl, imidazole, DMF, rt, quant; j) CSA, ethylene glycol, *i*PrOH, rt, 97%; k) $NaIO_4$, 1,4-dioxane, H_2O, then $NaBH_4$, rt, 94%; l) TrCl, pyridine, DMAP, CH_2Cl_2, rt; m) DDQ, PB (pH 6.6), *t*BuOH, CH_2Cl_2, n) 3 M LiOH (aq), THF, MeOH, rt, 81%; o) 2,4,6-$Cl_3C_6H_2COCl$, Et_3N, DMAP, toluene, rt, 61%; p) *n*Bu_4NF, THF, rt; q) Ac_2O, Et_3N, DMAP, CH_2Cl_2, rt, 83%; r) HCO_2H, Et_2O, rt, 89%; s) Dess-Martin periodinane, CH_2Cl_2, rt; t) reag.3, 1% $NiCl_2/CrCl_2$, DMSO, rt, 55% desired/ undesired = 3:1; u) TFA, anisole, CH_2Cl_2, 0 °C, HPLC separation, 50% for biselide A, 16% for C15-*epi* biselide A.

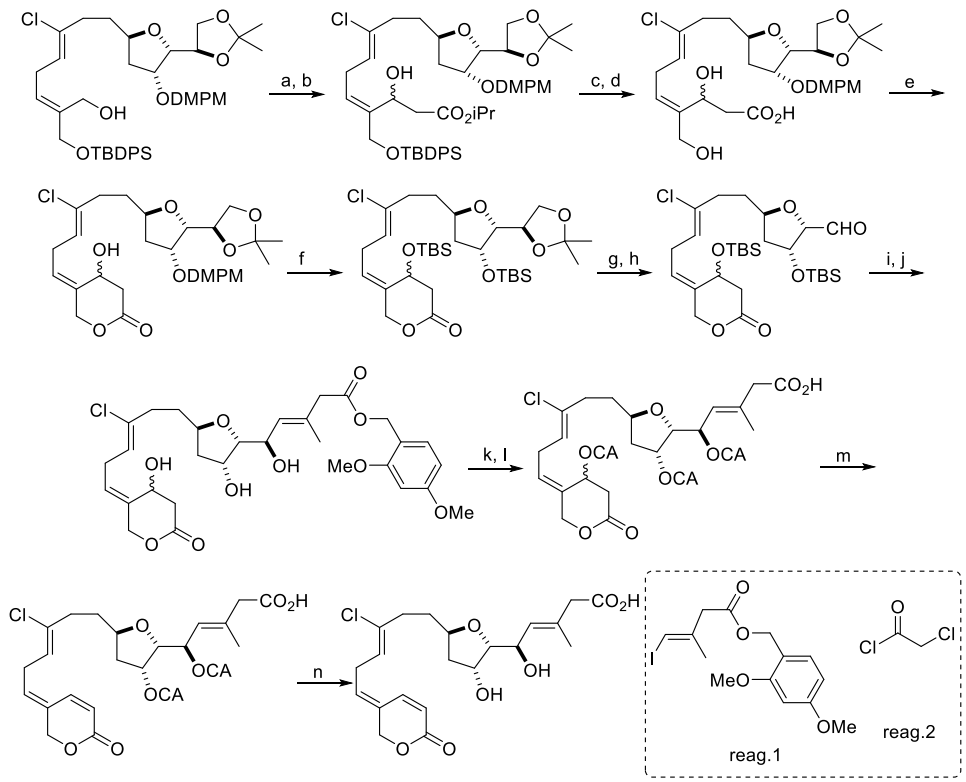

Fig. 28 Synthesis of biselide E (**39**). a) Dess-Martin periodinane (1.5 equiv.), CH_2Cl_2, rt; b) AcO*i*Pr (12 equiv.), LHMDS (12 equiv.), THF, -78 °C, 3.5 h; c) *n*Bu_4NF (5.3 equiv.), AcOH (5.3 equiv.), THF, rt, 1.5 h, quant; d) 3 M LiOH (aq), THF, *i*PrOH, rt, 14 h; e) EDC·HCl, DMAP, CH_2Cl_2, rt, 3 h; f) DDQ, PB (pH 6.6), *t*BuOH, CH_2Cl_2, rt, 50 min; TBSCl, imidazole, DMF, rt, 3 h; g) $BF_3{\cdot}OEt_2$, 1,3-propanedithiol, -78 °C, 1.5 h, 88%; h) $NaIO_4$, 1,4-dioxane-H_2O, rt, 4 h; i) reag.1, $NiCl_2$, $CrCl_2$, DMSO, rt, 33 h; j) *n*Bu_4NF, AcOH, THF, rt, 30 h, 77%; k) reag.2, py, CH_2Cl_2, -5 °C, 10 min; l) TFA, anisole, CH_2Cl_2, -5 °C, 1 h, 63%; m) *i*Pr_2NEt, CH_2Cl_2, rt, 1 h; n) $Zn(OAc)_2$, MeOH, rt, 20 h.

Introduction of the chloroacetyl groups at all hydroxy groups in the triol and the removal of the 2,4-DMPM esters gave the desired carboxylic acid, which underwent an *i*Pr_2NEt-caused β-elimination of the chloroacetoxy group to afford the desired six-membered α,β,γ,δ-doubly unsaturated lactone. The two chloroacetyl groups were removed by the use of $Zn(OAc)_2$ in MeOH (Hayakawa et al., 2017b).

3.8. Total synthesis of brominated secondary metabolites

2-Bromoleptoclinidinone (**67**) was synthesized in four steps from the quinolinequinone by Bracher in 1990. The reaction of the quinolinequinone with the appropriate aniline in the

presence of $CeCl_3$ yielded the corresponding product, which was subjected to ring closure using conc. H_2SO_4 (Fig. 29). Formation of the last ring, to afford 2-bromoleptoclinidinone, was carried out by reacting the synthetic intermediate with *N*,*N*-dimethylformamide diethyl acetal (DMF-DEA), while the ring was closed with ammonium chloride.

The synthesis of 4-bromo-3-pentylphenol (**80**) started with the Grignard reaction (Fig. 30) of methyl 2-bromo-5-methoxybenzoate with an excess of butylmagnesium chloride which gave a low yield of an alcohol that is an apparent product of the addition of 1 equiv. of the organometallic reagent, while the other acted as a reducing agent of the intermediate ketone, probably due to steric congestion (Rob et al., 2011). Mesylation of the obtained alcohol, followed by hydrogenolysis of the mesylate with $NaBH_4$, gave 4-bromo-3-pentylanisole. Cleavage of the ether with phenyltrimethylsilane/iodine yielded the target **80**.

3.9. Total synthesis of iodinated secondary metabolites

Crystalline 1,2-*O*-isopropylidene-α-D-xylofuranose was prepared by sulfuric acid-catalyzed acetalation of D-xylose (Fig. 31), followed by partial hydrolysis with aqueous sodium carbonate. Then 5-OH was selectively tosylated with *p*-toluenesulfonyl chloride and triethylamine in THF to afford a monotosylate, and then the tosylate was reduced to a methyl group with 2 equiv. of $LiAlH_4$ in anhydrous THF.

After benzoylation, the acetonide was transformed to the diacetate glycosylation acceptor. The nucleobase was prepared by chlorination of pyrrolo[2,3-*d*]pyrimidin-4-ol with phosphoryl chloride. The reaction of this product with 1.05 equiv. of NIS in DMF at room temperature afforded the iodinated base in 95% yield. 5-Iodo-7*H*-pyrrolo[2,3-*d*]pyrimidine was treated with BSA, followed by 1.2 equiv. of 5′-deoxy-1,2,3-tri-*O*-acetyl-β-D-xylofuranose and 2 equiv. of TMSOTf as a Lewis acid at room temperature, and then the mixture was heated to 80 °C for 12 h. The product was suspended in a saturated solution of ammonia in methanol and was heated at 130 °C in an autoclave for 12 h to remove the protecting ester groups and substitute the 4-Cl by 4-NH_2 on the base (Sun et al., 2012).

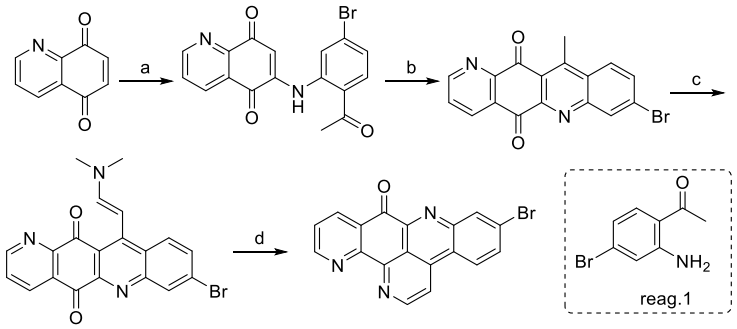

Fig. 29 Synthesis of 2-bromoleptoclinidinone (**67**). Reagents and conditions: a) reag.1, $CeCl_3$; b) conc. H_2SO_4; c) DMF-DEA; d) NH_4Cl.

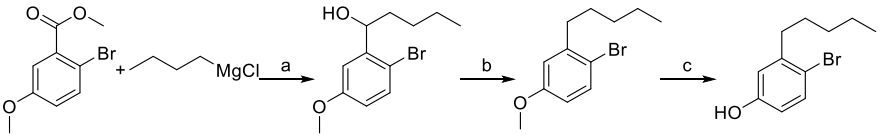

Fig. 30 Synthesis of 4-bromo-3-pentylphenol (**80**). Reagents and conditions: a) THF, rt, 2 h, 24.5%; b) MsCl, Et_3N, CH_2Cl_2, rt, 2 h, and then $NaBH_4$, DMSO, rt, 5 h, 23%; c) Me_3PhSi, I_2, 115 °C, 2 h, 46%.

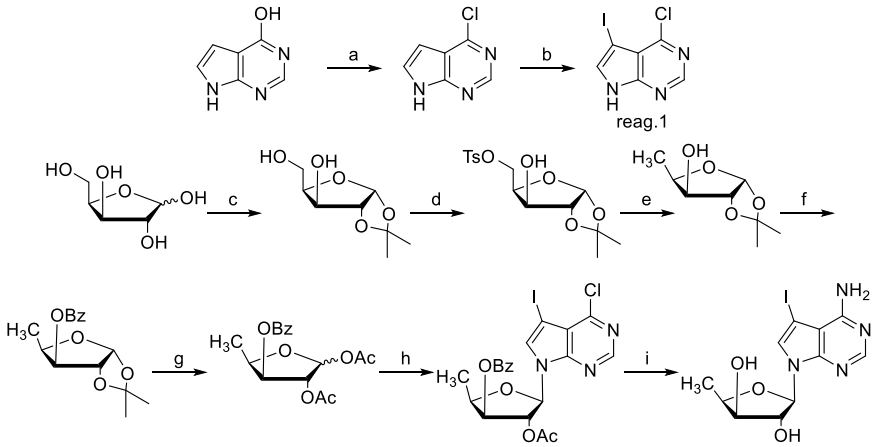

Fig. 31 Total synthesis of pyrrolo[2,3-*d*]pyrimidine nucleoside (**81**). Reagents and conditions: a) $POCl_3$ (excess), reflux, 3 h, 98%; b) NIS (1.01 equiv.), DMF, 0 °C, 2 h, 98%; c) conc. H_2SO_4, acetone, 0 °C, 3 h; then 5% Na_2CO_3 (aq), 30 min, 87%; d) TsCl (1.1 equiv.), THF, Et_3N, 0 °C, overnight, 92%; e) $LiAlH_4$ (0.5 equiv.), THF, reflux, 6 h, 95%; f) BzCl (1.1 equiv.), CH_2Cl_2, Et_3N, 2 h, 98%; g) conc. H_2SO_4, Ac_2O, 26 h, 78%; h) reag.1 (1 equiv.), BSA (1.2 equiv.), TMSOTf (1 equiv.), CH_3CN, rt – 80 °C, 12 h 56%; i) sat. NH_3 in MeOH (excess), 130 °C, 12 h, 82%.

Abbreviations

AIBN	Azobisisobutyronitrile
9-BBN	9-Borabicyclo[3.3.1]nonane
Boc_2O	Di-*tert*-butyl dicarbonate
BOMCl	Benzyl chloromethyl ether
BOP	Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
BSA	Bis(trimethylsilyl)acetamide
BzCl	Benzoyl chloride
CSA	Camphorsulfonic acid
CTAB	Hexadecyltrimethylammonium bromide
DCC	*N*,*N*′-Dicyclohexylcarbodiimide
DDQ	2,3-Dichloro-5,6-dicyano-*p*-benzoquinone
DIBAL	Diisobutylaluminum hydride
DIC	*N*,*N*′-Diisopropylcarbodiimide

DIEA	*N*,*N*-diisopropylethylamine
DMAE	Dimethylethanolamine
DMANE	1-Dimethylamino-2-nitroethylene
DMAP	4-(Dimethylamino)pyridine
DMF	*N*,*N*-Dimethylformamide
DMFDMA	Dimethylformamid-dimethylacetal
DMPMCl	3,4-Dimethoxybenzyl chloride
DMSO	Dimethyl sulfoxide
DTBMP	2,6-Di-*tert*-butyl-4-methylpyridine
EDC	*N*-(3-(Dimethylamino)propyl)-*N*′-ethylcarbodiimide
HMPA	Hexamethylphosphoramide
LHMDS	Lithium bis(trimethylsilyl)amide
MeOTf	Methyl trifluoromethanesulfonate
MPMCl	*p*-Methoxybenzyl chloride
MsCl	Methanesulfonyl chloride (Mesyl chloride)
NBS	*N*-Bromosuccinimide
NCS	*N*-Chlorosuccinimide
NIS	*N*-Iodosuccinimide
PB	Phosphate buffer
$PdCl_2$(dppf)	1,1′-Bis(diphenylphosphino)ferrocene)palladium(II) dichloride
PPL type II	Lipase from porcine pancreas Type II
PPTS	Pyridinium *p*-toluenesulfonate
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
TBAF	Tetrabutylammonium fluoride
TBAI	Tetrabutylammonium iodide
TBDPSCl	*tert*-Butyl(chloro)diphenylsilane
TBSCl	*tert*-Butyl(chloro)dimethylsilane
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TMSCl	Chlorotrimethylsilane
TMS-N_3	Trimethylsilyl azide
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
TrCl	Triphenylmethyl chloride
TsCl	4-Toluenesulfonyl chloride (Tosyl chloride)

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HALOGENOVANI SEKUNDARNI METABOLITI ASCIDIJANA PORODICE DIDEMNIDAE

U ovom radu sumirani su rezultati dosadašnjih istraživanja halogenovanih sekundarnih metabolita izolovanih iz ascidijana porodice Didemnidae (određivanje strukture, biološke/ farmakološke aktivnosti i njihove totalne sinteze). Ukupno je predstavljeno 81 jedinjenje, koja ilustruju veliku strukturnu raznolikost i pokazuju značajna biološka/farmakološka svojstva. Pored najzastupljenijih, bromovanih, pronađena su i hlorovana i jodovana jedinjenja. Najispitivaniji rod u pogledu broja objavljenih radova i izolovanih molekula, rod Didemnum*, razmatran je odvojeno od ostalih rodova ove porodice. Složenost struktura izolovanih metabolita podstakla je veliki broj sintetskih studija koje ne samo da su pružile uverljiv dokaz o strukturi metabolita, već su omogućile biološka testiranja, kao i potencijalnu primenu ovih metabolita.*

Ključne reči: *morski prirodni proizvodi, halogenovani sekundarni metaboliti, ascidijan, Didemnidae, Didemnum*