COLLECTION AND SECONDARY METABOLITE INVESTIGATIONS OF MARINE ORGANISMS FROM THE TWO AZOREAN ISLANDS FAIAL AND SÃO JORGE

EVA GOCLIK, GABRIELE M. KÖNIG & ANTHONY D. WRIGHT

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In March and April of 1997 a total of 35 samples of marine organisms were collected from the Azorean Islands of Faial and São Jorge. These samples included 3 species of Chlorophyta, 7 species of Phaeophyta, 10 species of Rhodophyta, 2 species of Chordata, 3 species of Mollusca, and 7 species of Porifera. Of these samples *Laxosuberites rugosus* (Porifera), and *Pachymatisma johnstonia* (Porifera), are new records for the Azores. Secondary metabolite investigations of a number of these samples led to the isolation of *para*-hydroxybenzyl cyanide from the sponge *Laxosuberites rugosus*, and oxindol from the sponge *Tedania anhelans*. Both these compounds are reported here from the marine environment for the first time. These compounds were assessed for their human immunodeficiency virus type 1 reverse transcriptase (HIV-1-RT) and tyrosine kinase (TK) inhibition activities, and *para*-hydroxybenzyl cyanide found to inhibit the activity of TK to 64% at the 200 µg/ml level. This is the first report of the TK activity of *para*-hydroxybenzyl cyanide.

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Em Março e Abril de 1997 foram recolhidas, nas ilhas açoreanas do Faial e São Jorge, um total de 35 amostras de organismos marinhos. Estas amostras incluem 3 espécies de Chlorophyta, 7 espécies de Phaeophytas, 10 espécies de Rhodophytas, 2 espécies de Chordata, 3 espécies de Mollusca, e 7 espécies de Porifera. Destas amostras a *Laxosuberites rugosus* (Porifera), e a *Pachymatisma johnstonia* (Porifera), são novos registos para os Açores. Investigações de metabolitos secundários de um numero destas amostras levou ao isolamento do cianeto de *para*-hidroxibenzilo da esponja *Laxosuberites rugosus,* e do 2-hidroxindol da esponja *Tedania anhelans*. Ambos os compostos são pela primeira vez relatados para o ambiente marinho neste trabalho. Estes compostos estão associados à transcriptase reversa do vírus tipo 1 de imunodefeciencia humana (HIV-1-RT) da inibição da actividade da tirosina cinase (TK), e o cianeto de *para*-hidroxibenzilo que inibe a actividade do TK a 64% a um nível de 200 µg/m. Este é o primeiro relato da actividade do TK do cianeto de *para*-hidroxibenzilo.

Eva Goclik, Gabriele M. König & Anthony D. Wright, Institute for Pharmaceutical Biology, Technical University of Braunschweig, Mendelssohnstrasse 1, Braunschweig, DE-38106, Germany. Gabriele M. König & Anthony D. Wright Current address Institute for Pharmaceutical Biology, University of Bonn, Nussalee 6, Bonn 53115, Germany. e-mail: g.koenig@tu-bs.de

INTRODUCTION

The Azorean archipelago is located in the warm temperate region of the north-east Atlantic approximately 1500 km west of Lisbon, Portugal. The marine fauna and flora of this group of islands appears to be a mixture of species found in both the Atlantic and the Mediterranean. There have been a number of collecting trips to these islands which have concentrated on the sponges to be found there (MOSS 1992; REED & POMPONI 1992), but nothing has been published concerning the secondary metabolite chemistry of marine organisms coming from this group of islands. In 1997 we collected 35 marine samples including 3 species of Chlorophyta, 7 species of Phaeophyta, 10 species of Rhodophyta, 2 species of Chordata, 3 species of Mollusca, and 7 species of Porifera from the waters around the Islands of Faial and São Jorge (see Table 1). Of these samples, six have been examined for their secondary metabolite content [(CT197F, *Tedania anhelans* Lieberkühn, 1859 (Tedaniidae); CT197M, *Pachymatisma johnstonia* Bowerbank, 1864 (Geodiidae); CT197R, *Laxosuberites rugosus* (Schmidt, 1868) Topsent 1900 (Suberitidae); CT197II, *Mycale massa* Schmidt, 1862 (Mycalidae); CT197GG, *Aplysia punctata* Cuvier, 1803 (Aplysiidae); and CT197HH, *Platydoris argo* (Linnaeus, 1767) (Doridiidae); see Table 1)].

The lack of publication on the natural products chemistry of *L. rugosus* or *M. massa* prompted us to begin investigation of these organisms. In general, *Laxosuberites* is an under investigated genus with the most interesting report being that of a series of long-chain alkyl pyrrole derivatives (STIERLE & FAULKNER 1980). In contrast, there have been over sixty reports of natural products isolated from the genus *Mycale* (e. g., CORRIERO et al. 1989; KATO et al. 1985; ORTEGA et al. 1997a; PERRY et al. 1988). We were interested to determine whether an Azorean specimen would have chemistry related to that of other *Mycale* spp. *P. johnstonia* was selected for study because it has also received very little attention, there being only publications regarding its sterol content (BALLANTINE et al. 1979) and the compound pachymatismin (ZIDANE et al. 1996a; ZIDANE et al. 1996b). In an attempt to see if the secondary metabolite chemistry of an Azorean variety of a fairly well investigated sponge was similar to others of the same genus, *Tedania anhelans* (AIELLO et al. 1993; DILLMAN & CARDELLINA 1991; PARAMESWARAN et al. 1997; SCHMITZ et al. 1984) was selected for investigation. Due to their small sample size and the ease and speed with which they can be analysed, the nudibranch (*Platydoris argo*), and the sea hare (*Aplysia punctata*) were also selected for early secondary metabolite investigations. It should also be noted that no publications of the secondary metabolite content of the nudibranch species, *P. argo* have appeared and that the sea hare is known to contain interesting secondary metabolites (JIMÉNEZ et al. 1986; ORTEGA et al. 1997b; QUINOA et al. 1989).

MATERIALS AND METHODS

All samples were collected from the Islands of Faial and São Jorge during the months of March and April 1997 by divers using SCUBA (3-20 m), by snorkel diving (0-3 m), or by direct collection from shallow submerged rocky formations (0-1 m). Following collection all samples were frozen at -4°C until work up. Extracts and pure compounds were assayed for antibacterial, antifungal and antialgal activities in agar diffusion assays (SCHULZ et al. 1995). ELISA based assays (WESSELS et al. 1999) were used to test for HIV-1-RT and TK inhibition. The pure compounds were also tested for their antimalarial activity (ANGERHOFER et al. 1992). For detailed materials and methods see (WRIGHT et al. 1996).

RESULTS AND DISCUSSION

For a complete listing of all samples collected and the relevant collecting and taxonomic details see Table 1.

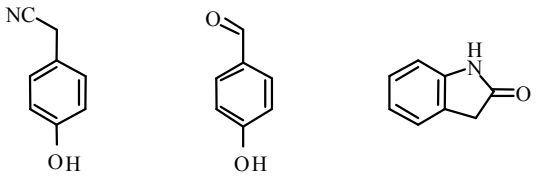
Table 1

Marine samples collected from the islands of Faial and São Jorge, Azores, during March and April 1997.

Division / Family	Species	Sample Code Number	Date of Collection	Depth	Location
Chlorophyta / Bryopsidaceae	*Bryopsis* sp.	CT197B	28/3/97	0-1 m	Faial, Baía entre Montes*
Chlorophyta / Siphonocladaceae	*Valonia utricularis* (Roth) C. Agardh	CT197G	28/3/97	0-1 m	Faial, Baía entre Montes
Chlorophyta / Ulvaceae	*Enteromorpha* sp.	CT197K	28/3/97	0-1 m	Faial, Baía entre Montes
Phaeophyta / Cystoseiraceae	*Cystoseira abies-marina* (S. G. Gmel.) C. Agardh	CT197V	4/4/97	0-2 m	Faial, Feteira, from the top of the rock platform
Phaeophyta / Cystoseiraceae	*Cystoseira foeniculaceus* (L.) Grev.	CT197U	4/4/97	0-2 m	Faial, Varadouro, from sea water swimming pool
Phaeophyta / Cystoseiraceae	*Cystoseira foeniculaceus* (L.) Grev.	CT197W	4/4/97	0-2 m	Faial, Feteira, from the top of the rock platform
Phaeophyta / Dictyotaceae	*Dictyota dichotoma* (Huds.) J. V. Lamour *Dictyota* sp.	CT197Z	5/4/97	13-15 m	Faial, Monte de Guia, location T6 on the map of MOSS (1992)
Phaeophyta / Dictyotaceae	*Padina povonica* (L.) J. V. Lamour	CT197S	1/4/97	0-3 m	São Jorge, Santo Cristo lagoon
Phaeophyta / Fucaceae	*Fucus spiralis* L.	CT197X	4/4/97	0-2 m	Faial, Feteira, from the top of the rock platform
Phaeophyta / Sargassaceae	*Sargassum* sp.	CT197T	4/4/97	0-2 m	Faial, Varadouro, from sea water swimming pool
Phaeophyta / Scytosiphonaceae	*Hydroclathrus clathrus* (Bory) Howe	CT197Q	1/4/97	0-3 m	São Jorge, Santo Cristo lagoon
Rhodophyta / Bonnemaisoniaceae	*Asparagopsis taxiformis* (Delile) Trevis	CT197BB	6/4/97	0-3 m	Faial, Baía entre Montes
Rhodophyta / Champiaceae	*Lomentaria articulata* (Huds.) Lyngbye	CT197H	28/3/97	0-1 m	Faial, Baía entre Montes
Rhodophyta / Corallinaceae	*Corallina elongata* Ellis et Sol. and *Haliptilon* sp.	CT197E	28/3/97	0-1 m	Faial, Baía entre Montes
Rhodophyta / Gelidiaceae	*Gelidium microdon* Kützing	CT197J	28/3/97	0-1 m	Faial, Baía entre Montes
Rhodophyta / Gelidiaceae	*Pterocladia capillacea* (S. Gmelin) Bornet et Thuret	CT197D	28/3/97	0-1 m	Faial, Baía entre Montes
Rhodophyta / Plocamiaceae	*Plocamium cartilageneum* (L.) P. Dixon	CT197C	28/3/97	0-1 m	Faial, Baía entre Montes
Rhodophyta / Rhodomelaceae	*Osmundia pinnatifide* (Hudson) Stakhouse**	CT197A	28/3/97	0-1 m	Faial, Baía entre Montes
Rhodophyta / Rhodomelaceae	*Polysiphonia cf. Fucoides* (Hudson) Greville***	CT197L	4/4/97	0-2 m	Faial, Varadouro, from sea water swimming pool
Rhodophyta / Rhodymeniaceae	*Rhodymenia* sp.	CT197I	28/3/97	0-1 m	Faial, Baía entre Montes
Chordata / Didemnidae	*Lissoclinum* sp	CT197P	1/4/97	0-3 m	São Jorge, Santo Cristo lagoon
Chordata / Polycitoridae	*Distaplia corolla* Monniot (F.) 1974 (Orange variety)	CT197Y	5/4/97	13-15 m	Faial, Monte de Guia, location T6 on the map of MOSS (1992)
Chordata / Polycitoridae	*Distaplia corolla* Monniot (F.) 1974 (Purple variety)	CT197EE	8/4/97	0-2 m	Faial, moorings in Horta harbour
Mollusca / Pleurobranchidea	*Berthellina engeli* Gardiner, 1936****	CT197AA	1/4/97-5/4/97	15-20 m	Faial, Monte de Guia, seamount, sandy bottom between T6 and T8, see MOSS (1992) about 50-80 m from the land
Mollusca / Pleurobranchidea	*Berthellina engeli* Gardiner, 1936****	CT197O	1/4/97	0-3 m	São Jorge, Santo Cristo lagoon
Mollusca / Aplysiidae	*Aplysia punctata* Cuvier, 1803	CT197GG	1/4/97-5/4/97	15-20 m	Faial, Monte de Guia, seamount, sandy bottom between T6 and T8, see MOSS (1992) about 50-80 m from the land
Mollusca / Doridiidae	*Platydoris argo* (Linnaeus, 1767)	CT197HH	1/4/97-5/4/97	15-20 m	Faial, Monte de Guia, seamount, sandy bottom between T6 and T8, see MOSS (1992) about 50-80 m from the land
Porifera / Chalinidae	*Haliclona* sp.	CT197DD	8/4/97	0-2 m	Faial, moorings in Horta harbour
Porifera / Geodiidae	*Pachymatisma johnstonia* Bowerbank, 1864	CT197M	1/4/97	0-3 m	São Jorge, Santo Cristo lagoon
Porifera / Mycalidae	*Mycale massa* Schmidt, 1862	CT197II	1/4/97	0-3 m	São Jorge, Santo Cristo lagoon
Porifera / Tedaniidae	*Tedania anhelans* Lieberkühn, 1859	CT197F	28/3/97	0-2 m	Faial, Baía entre Montes
Porifera / Suberitidae	*Laxosuberites ferrerhernandezi* Boury-Esnault and Lopez, 1985	CT197FF	8/4/97	0-2 m	Faial, rock wall Horta harbour to left of the yatch club
Porifera / Suberitidae	*Laxosuberites rugosus* (Schmidt, 1868), Topsent 1900	CT197R	1/4/97	0-3 m	São Jorge, Santo Cristo lagoon
Porifera / Suberitidae	*Suberites carnosus* Johnston, 1842	CT197CC	8/4/97	0-2 m	Faial, moorings in Horta harbour

The Table is organised according to Phyla; Chlorophyta, Phaeophyta, Rhodophyta, Chordata, Mollusca, Porifera; and then alphabetically according to family. When there are two or more species from the same family they are then listed alphabetically according to species; *Baía entre Montes; as such this site no longer exists. At the time of collections extensive engineering work was underway to extend the Horta harbour facility. This construction work included building seawalls out to Baía entre Montes to create a triangular inland sea which was eventually filled in making Baía entre Montes the outer most corner of the reclaimed land on which the new harbour facilities have now been constructed. It is thus likely that due to loss of habitat a number of the samples collected from this location will never be found in this region again; **Formerly known as *Laurencia pinnatifida*; ***Formerly known as *Polysiphonia nigrescens*; ****This is possibly a synonym of the Indo-West Pacific species *Berthellina citrina* (Ruppell & Leuckart, 1828).

L. rugosus was collected in April, 1997, from a depth of 1-3 m at the Santo Cristo lagoon, São Jorge (The voucher specimen, voucher number CT197R, is stored at the Museum d'Histoire Naturelle, Geneva, Switzerland). The material obtained was frozen at -4°C and then freeze dried prior to extraction. Dry tissue (398 g) was extracted exhaustively with dichloromethane (CH_2Cl_2, 2.5 l), followed by acetone ($(CH_3)_2CO$, 2.5 l), and finally methanol (MeOH, 2.5 l). TLC, ^1H NMR, and biological activity assessment of this extract indicated it to be of further interest with respect to its secondary metabolite chemistry. The $(CH_3)_2CO$ and MeOH extracts were partitioned between CH_2Cl_2 and H_2O, and the CH_2Cl_2 solubles combined with the CH_2Cl_2 extract to yield 27.9 g (7.0 %) of CH_2Cl_2 soluble material. Fractionation of the CH_2Cl_2 solubles by vacuum liquid chromatography (VLC, silica gel, gradient elution from CH_2Cl_2 to MeOH), followed by HPLC (normal phase silica, cyclohexane: $(CH_3)_2CO$:H_2O 6:4:0.1) yielded *para*-hydroxybenzyl cyanide (Fig. 1. A) (4.5 mg, 0.00001 %), and *para*-hydroxybenzaldehyde (Fig. 1. B) (1.0 mg, 0.000003 %). Both structures were deduced on the basis of their spectroscopic data, predominantly ^1H and ^{13}C-NMR, and EIMS. This is the first report of *para*-hydroxybenzyl cyanide (Fig. 1. A) as a marine natural product. It has been isolated previously from the fungus *Aspergillus fumigatus* (PACKTER & COLLINS 1974). The production of *para*-hydroxybenzyl cyanide by *A. fumigatus* may also suggest that the *para*-hydroxybenzyl cyanide isolated in this study may have been produced by sponge symbionts and not by the sponge itself. *Para*-hydroxybenzaldehyde (Fig. 1. B), in contrast, is a fairly common constituent of marine organisms (WRIGHT et al. 1990), but may also be symbiont derived. The proposed symbiont origin of *para*-hydroxybenzyl cyanide and *para*-hydroxybenzaldehyde is clearly only speculation at this stage as it is also clear that such biosynthetically simple compounds often can evolve in multiple organisms, it is also possible due to their very low concentrations that they may also be from the animals diet or transient associates/contaminants.



A **B** **C**

Fig. 1 A-C. Chemical structures of secondary metabolites derived from the sponges *Laxosuberites rugosus* (A and B) and *Tedania anhelans* (C).

T. anhelans was collected in March, 1997, from 0-1 m depth at Baía entre Montes, Faial (The voucher specimen, voucher number CT197F, is stored at the Museum d'Histoire Naturelle, Geneva, Switzerland). The material obtained was frozen (-4°C) and then freeze dried prior to extraction. Dry tissue (30.5 g) was extracted exhaustively with CH_2Cl_2, (1 l), to yield 1.33 g (4.4 %) of extract. TLC, ^1H NMR, and biological activity assessment of this extract indicated it to be of further interest with respect to its secondary metabolite chemistry. Fractionation of this extract by vacuum liquid chromatography (VLC, silica gel, gradient elution from hexane to ethyl acetate (EtOAc) to MeOH), followed by column chromatography (normal phase silica, CH_2Cl_2 followed by EtOAc) yielded oxindol (Fig. 1. C) (2.5 mg, 0.0082 %). The structure of oxindol (Fig. 1. C, indolin-2-one) was deduced on the basis of its spectroscopic data, predominantly ^1H and ^{13}C-NMR, and EIMS. This is the first report of oxindol from the marine environment. It has previously been isolated from tea (KAWAKAMI et al. 1995), wild rice (WITHYCOMBE et al. 1978), and many other terrestrial plants (e. g., NGADJUI et al. 1995). Prior chemical investigations of *T. anhelans* from other locations yielded pyrazole acids (AIELLO et al. 1993). Other sponges in the genus *Tedania* afforded compounds similar to oxindol (DILLMAN & CARDELLINA 1991), but not oxindol itself.

Compounds Fig. 1. A and Fig. 1. C were assessed for their biological activities (antimalarial, antibacterial, antifungal, antialgal, HIV-1-RT inhibition and tyrosine kinase (TK) inhibition). Compound Fig. 1. A was found to inhibit the activity of TK to 64% at the 200

μg/ml level. Compound Fig. 1. B is already known for its antibacterial properties (ORTEGA et al. 1997b).

Samples of *Pachymatisma johnstonia* were collected in April, 1997, from a depth of 0-3 m at the Santo Cristo lagoon, São Jorge (The voucher specimen, voucher number CT197M, is stored at the Museum d'Histoire Naturelle, Geneva, Switzerland). The material obtained was frozen (-4°C) and freeze dried prior to extraction. Dry tissue (7.3 g) was extracted exhaustively with CH_2Cl_2 (150 ml), followed by MeOH (150 ml), to yield 0.13 g (1.8 %) of organic extract. The lack of biological activity together with the TLC and ^{1}H NMR information indicated the main components of the extract to be ubiquitous lipids and sterols and therefore no additional chemical purification was carried out.

Mycale massa was collected in April, 1997, from a depth of 0-3 m at the Santo Cristo lagoon, São Jorge (The voucher specimen, voucher number CT197II, is stored at the Museum d'Histoire Naturelle, Geneva, Switzerland). The material obtained was frozen at -4°C and freeze dried prior to extraction. Dry tissue (12.3 g) was extracted exhaustively with CH_2Cl_2, (200 ml) and MeOH (200 ml), to yield 0.42 g (3.4%) of CH_2Cl_2 soluble materials. TLC, ^{1}H NMR, and biological activity assessment of this extract indicated it to be of no further interest with respect to its secondary metabolite chemistry. The TLC and ^{1}H NMR investigation indicated the main components of the extract to be ubiquitous lipids and sterols.

The samples of *Aplysia punctata* (2 individuals) were collected in April, 1997, from a depth of 15-20 m at Monte de Guia, Faial, sandy bottom between T6 and T8, see MOSS (1992) ca. 50-80 m offshore (The voucher specimen, voucher number C203756, is stored at the Australian Museum, Sydney, NSW, Australia). The material obtained was frozen (-4°C) and then freeze dried prior to extraction. Dry tissue (1.53 g) was extracted exhaustively with a 1:1 mixture of CH_2Cl_2, and MeOH (100 ml), to yield 0.45 g (29.4%) of a brown gum. TLC and ^{1}H NMR indicated the extract to contain a number of compounds, possibly terpenes, with both conjugated and unconjugated double bonds and oxygen and/or halogen containing funtionalities. These analyses also showed the extract to contain relatively high concentrations of ubiquitous lipids, sterols and salt. Biological activity assessment of the extract indicated it to be weakly antimicrobial. Unfortunately, the TLC and ^{1}H NMR data also indicated that the compounds of interest were present in such low concentrations (< 1 mg) that no further analyses were undertaken.

The samples of *Platydoris argo* (3 individuals) were collected in April, 1997, from a depth of 15-20 m at Monte de Guia, sandy bottom between T6 and T8, see MOSS (1992) ca. 50-80 m offshore (The voucher specimen, voucher number C203757, is stored at the Australian Museum, Sydney, NSW, Australia). The material obtained was frozen (-4°C) and then freeze dried prior to extraction. Dry tissue (0.62 g) was extracted exhaustively with a 1:1 mixture of CH_2Cl_2, and MeOH (100 ml), to yield 0.2 g (32.3 %) of a brown gum. TLC, ^{1}H NMR, and biological activity assessment of this extract indicated it to be of further interest with respect to its secondary metabolite chemistry. For the same reasons as those outlined for the extract of *Aplysia punctata*, particularly the TLC and ^{1}H NMR data and the low concentrations of compounds, no further analyses were undertaken of the extract of *Platydoris argo*.

From the data obtained with the six samples studied to date it is evident that samples from the Azores are likely to have quite different secondary metabolite chemistry from the same or similar species located in other parts of the world. The remaining 29 samples will be investigated in a similar fashion to those described here, and the results of those investigations will be presented at a later date.

SPECTROSCOPIC DATA

Information in detail on the work-up procedure and copies of the original spectra are obtainable from Prof. G. M. König, Institute for Pharmaceutical Biology, Technical University of Braunschweig, Mendelssohnstrasse 1, Braunschweig, D-38106, Germany.

The data presented below for compounds Fig. 1. A and Fig. 1. C is not to be found in the primary literature.

Para-hydroxybenzyl cyanide (Fig. 1. A): ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.65 (2H, s, H_2-7), 6.85 (2H, m, H-2 and H-6), 7.18 (2H, m, H-3 and H-5); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 22.9 (t, C-7), 116.1 (2 x d, C-2, C-6), 118.2 (s, C-8), 121.9 (s, C-4), 129.3 (2 x d, C-3, C-5), 155.5 (s, C-1); EIMS: *m/z* (rel. int.) = 134 $[M+H]^+$ (10), 133 $[M]^+$ (100), 132 $[M-H]^+$ (50), 107 (8), 106 (22), 105 (20), 104 (16), 78 (22).

Oxindol (Fig. 1. C): ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.53 (2H, s, H_2-3), 6.85 (1H, d, *J* = 7.9 Hz, H-8), 7.01 (1H, t, *J* = 7.2, 7.6 Hz, H-6), 7.22 (1H, t, *J* = 7.6, 7.9 Hz, H-7), 7.23 (1H, d, *J* = 7.2 Hz, H-5), 7.85 (1H, brs, H-1); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 36.1 (t, C-3), 109.5 (d, C-8), 122.4 (d, C-6), 124.8 (d, C-5), 125.3 (s, C-4), 128.0 (d, C-7), 142.3 (s, C-9), 177.0 (s, C-2); EIMS: *m/z* (rel. int.) = 134 $[M+H]^+$ (10), 133 $[M]^+$ (100), 105 (35), 104 (80), 78 (35).

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