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Bioactive natural compounds of algae and invertebrates from the littoral of Cadiz

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ABSTRACT

The chemical study of an array of marine sponges and algae from the littoral of Cadiz (southwestern Spain) has led to the isolation and characterisation of several new natural products. The structures were elucidated by spectroscopic methods and, in certain instances, the absolute stereochemistries were determined using either the Mosher method or chemical interconversions. In particular, the new compounds isolated from the sponges *Reniera fulva* (Topsent, 1893), *Cacospongia scalaris* (Schmidt, 1862) and *Spongia officinalis* (Linnaeus, 1759) and from the alga *Dictyota dichotoma* (Hudson) Lamouroux, are presented and discussed. In order to anticipate a future pharmacological application, a cytotoxicity evaluation of the new compounds against several tumour cell lines was conducted.

Key words: Sponge, alga, natural product, furanoterpene, scalarane, dolabellane, cytotoxicity.

RESUMEN

Compuestos naturales bioactivos de algas e invertebrados del litoral de Cádiz

El estudio químico de varias esponjas y algas del litoral gaditano ha llevado al aislamiento y caracterización de varios nuevos productos naturales. Las estructuras se determinaron por métodos espectroscópicos y, en algunos casos, se establecieron las configuraciones absolutas usando el método de Mosher o correlaciones químicas. En particular, se presentarán y discutirán los nuevos compuestos aislados de las esponjas Reniera fulva *(Topsent, 1893),* Cacospongia scalaris *(Schmidt, 1862) y* Spongia officinalis *(Linnaeus, 1759) y el alga* Dictyota dichotoma *(Hudson) Lamouroux. Para anticipar una posible utilización farmacológica de estos nuevos productos naturales se ha relizado una evaluación de sus actividades citotóxicas frente a varios cultivos de células tumorales.*

***Palabras clave**: Esponja, alga, producto natural, furanoterpeno, escalarano, dolabellano, citotoxicidad.*

INTRODUCTION

Research into marine natural compounds has expanded rapidly over the last 20 years. Chemical compounds that are rare or unknown in the terrestrial realm are being characterised. The biomedical importance of these new compounds, along with developments in collection methodology, separation techniques and organic spectroscopy science, are responsible for this enhanced interest.

The projects carried out by our research group are focussed on the discovery of new cytotoxic metabolites from marine organisms found on the littoral of Cadiz (southwestern Spain). The biolog-

ical material includes invertebrates and algae, and after extraction and preliminary chemical analysis, a cytotoxicity evaluation is performed to detect activity in the crude extract. This initial evaluation enables us to decide which are the most promising organisms to be studied.

MATERIALS AND METHODS

The organic extract from marine specimens was fractionated using column chromatography. The fractions obtained were analysed using Thin-Layer Chromatography and ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy. Final separations using both normal and reversed phase High-Performance Liquid Chromatography (HPLC) enabled us to obtain pure compounds.

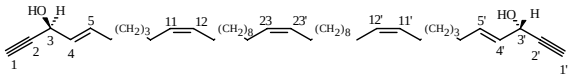
Identification and structural elucidation of pure compounds were performed using organic spectroscopy techniques, such as ^{1}H and ^{13}C NMR and Mass Spectrometry (RMS), as well as IR and UV spectroscopy. In particular, ^{1}H NMR and ^{13}C NMR spectra were made at 399.952 MHz and 100.577 MHz, respectively, using $CDCl_3$ as solvent. The resonance of residual chloroform at δ_H 7.26 and δ_C 77.00 was used as internal reference for ^{1}H and ^{13}C spectra, respectively. In High-Performance Liquid Chromatography separations, LiChrosorb silica 60 was used in normal phase mode and LiChrosorb RP-18 in reversed phase mode, using in both cases a differential refractometer and a UV detector. All solvents used were spectral grade or distilled from glass prior to use.

The tumour cell lines used in antitumour primary screening were P-388 (suspension culture of a lymphoid neoplasm from a DBA/2 mouse), A-549 (monolayer culture of a human lung carcinoma), HT-29 (monolayer culture of a human colon carcinoma) and MEL-28 (monolayer culture of a human melanoma). The results of these assays were used to generate dose-response curves from which the ED_{50} value was calculated (sample concentration that produces 50 % cell growth inhibition).

RESULTS

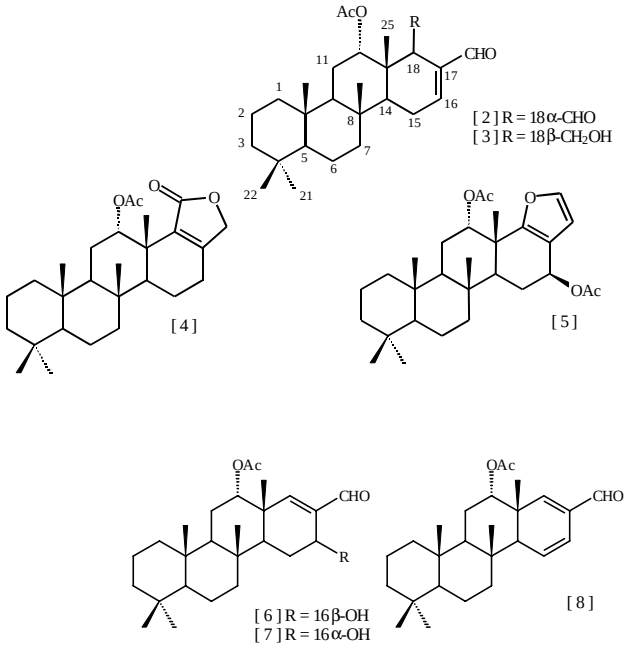
The orange sponge *Reniera fulva* (Topsent, 1893), from Algeciras Bay, contained, in addition to five acetylenic compounds described previously, a new long-chain acetylene named fulvinol [1]. Its highly symmetric structure was elucidated by interpretation of spectral data, and its absolute configuration was established using the Mosher method. Fulvinol [1] exhibited cytotoxicity against four tumour cell lines (ED_{50} = 1 μg/ml) (Ortega *et al.*, 1996).

The sponge *Cacospongia scalaris* (Schmidt, 1862), from Tarifa Island (Cadiz), contained



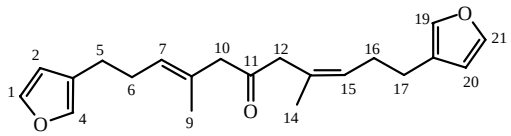
[1]

in addition to five known compounds, the new scalarane sesterterpenes 18-*epi*-scalaradial [2], 19-dihydroscalaradial [3], 12-*epi*-acetylscalarolide [4], and 16-*epi*-acetylfuroscalarol [5], together with three uncommon norscalaranes, norscalaral A [6], norscalaral B [7], and norscalaral C [8]. The structures were elucidated by interpretation of spectral data. 18-*epi*-scalaradial [2] represents the missing stereoisomer in structure-activity studies carried out with compounds of this series, and did not react with methylamine. The new compounds isolated from *C. scalaris* showed *in vitro* cytotoxicity against four tumour cell lines, with 18-*epi*-scalaradial showing the greatest activity (ED_{50} = 0.2 μg/ml) (Rueda *et al.*, 1997).

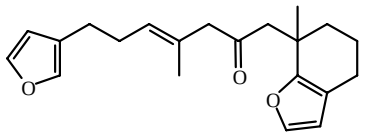


The sponge *Spongia officinalis* (Linnaeus, 1759), from La Caleta (Cadiz), contained two new C-21 fu-

ranoterpenes, furospongin-5 [9] and cyclofurospongin-2 [10], and the new furanosesterterpene demethylfurospongin-4 [11], in addition to several known terpenoids. The structures were established by interpretation of spectral data and chemical interconversions. Furospongin-5 [9] was weakly cytotoxic against the P-388 cell line (ED_{50} = 5 μg/ml) (Garrido *et al.*, 1997).



[9]



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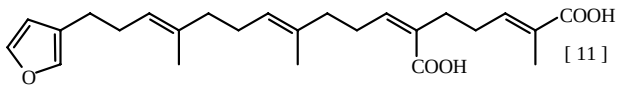
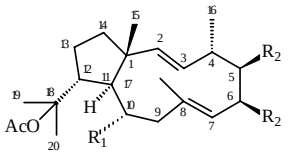
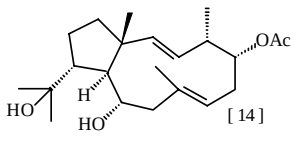


[11]

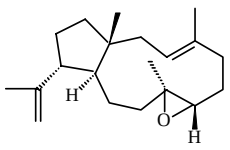
The brown alga *Dictyota dichotoma* (Hudson) Lamouroux, from Cortadura (Cadiz), also gave rise to 19 known diterpenes, five new dolabellanes [12-16] together with the new hydroazulene diterpenes isopachydictiol A [17] and dictyotatriol A [18]. The structures of the new compounds were elucidated by interpretation of spectroscopic data. The absolute configurations of compounds 14 and 18 were established using the Mosher method, and of compounds 12 and 13 by chemical interconversions. When the new compounds isolated from *D. dichotoma* were tested in bioassays directed to detect



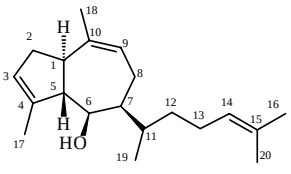
[12] R_1= OH; R_2= OAc
[13] R_1= OH; R_2= H
[16] R_1= R_2= H



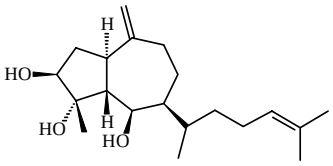
[14]



[15]



[17]



[18]

in vitro cytotoxicity, dolabellane 13 exhibited the greatest activity (Durán *et al.*, 1997).

DISCUSSION

In our ongoing efforts to discover biologically active compounds derived from marine organisms found on the southern coast of Spain, we obtained specimens of *R. fulva* collected in Algeciras Bay near the Strait of Gibraltar. Sponges of this genus have given rise to isoquinoline quinones, aryl carotenoids, sesquiterpene hydroquinones, pentacyclic alkaloids and diacetylene metabolites.

The sponge *R. fulva* was hand-collected by a scuba-diver, and immediately frozen. The sponge was extracted with acetone and concentrated to form an aqueous residue that was extracted with diethyl ether. Subsequent normal- and reversed-phase chromatography of the organic phase led to the isolation of fulvinol [1] as colourless crystals, mp 35-37 °C (0.035 % dry wt). FABMS showed a molecular ion peak [M+Na]+ at *m/z* 683. This finding, together with elemental analysis, indicated a molecular formula of $C_{46}H_{76}O_2$. The IR spectra indicated the presence of hydroxyl and double-bond functionalities in the structure of 1. Because both the ^{1}H- and the ^{13}C-NMR spectra contained a smaller number of signals than those expected from the molecular formula, it was concluded that fulvinol [1] possessed a highly symmetric structure, and each resonance of the spectrum was attributable to two magnetically equivalent nuclei. The plane structure of [1] was established on the basis of a careful analysis of ^{1}H-NMR, ^{13}C-NMR and FABMS data.

The absolute stereochemistry was determined using the Mosher method. Because the compound is optically active, only the (3*R*, 3'*R*) and (3*S*, 3'*S*) possibilities were considered. The (*R*)- and (*S*)-MTPA esters were prepared by treating fulvinol [1] with (*S*)- and (*R*)–α–methoxy–α–trifluoromethylphenylacetic chloride, respectively. The $\Delta(\delta_S-\delta_R)$, following the MTPA rules, indicated *S* configurations for both C-3 and C-3' and therefore an absolute configuration, as depicted in formula 1.

The presence of polyacetylenes in *R. fulva* represents the only example of this kind of compound in the genus *Reniera*. Phylogenetic studies using ribosomal RNA analyses have suggested that *R. fulva* is more closely related to the *Petrosia* genus than it is

to *Reniera mucosa*. Interestingly, fulvinol [1] closely resembles the long-chain acetylenes isolated from some *Petrosia* sponges.

Fulvinol [1] showed *in vitro* cytotoxicity against P-388 mouse lymphoma, A-549 human lung carcinoma, HT-29 human colon carcinoma, and MEL-28 human melanoma (ED_{50} = 1 μg/ml). These ED_{50} values are in the range of the other acetylenic compounds obtained from marine sources.

The tetracyclic sesterterpenes with scalarane skeleton have proved to be one of the most interesting groups of marine terpenoids. In particular, those bearing a 1,4-dialdehyde moiety have shown a remarkable antifeedant activity. Structure-activity studies have suggested that the key step involves the reaction between one aldehyde group of the terpenoid and the amine group of the receptor to form an imine. In addition, sponges of the *Cacospongia* genus have been reported to contain sesterterpenes, C-21 furanoterpenoids and xanthenes. This genus is an important source of scalarane sesterterpenes. This fact, together with the biological importance of these terpenoids, prompted us to study specimens of *Cacospongia scalaris* from Tarifa Island.

Chromatography of the acetone soluble material on silica gel, followed by final purification using HPLC, enabled us to isolate the new scalaranes 18-*epi*-scalaradial [2], 19-dihydroscalaradial [3]), 12-*epi*-acetylscalarolide [4], and 16-acetylfuroscalarol [5], three uncommon norscalaranes norscalaral A [6], norscalaral B [7] and norscalaral C [8], together with several known compounds. The structure of these compounds was established by interpretation of spectral data, in particular ^{1}H- and ^{13}C-NMR both mono and bidimensional and HRMS.

In vitro assays under biomimetic conditions have concluded that the stereochemistry at C-18 determines the ability of these 1,4-dialdehydes to react with methylamine. 18-*epi*-scalaradial [2] represents the missing stereoisomer in these structure-activity experiments, and therefore we tested the ability of 2 to react with methylamine. All our attempts were unfruitful, confirming that the larger distance between the axial aldehyde at C-18 and the aldehyde at C-17 blocks the reactions with primary amines. Furthermore, it has been reported that epimerisation at C-18 causes a significant loss in potency, as well as the ability to completely deactivate the bee venom PLA_2. Consequently, 18-*epi*-scalaradial (2) might be expected to be less active than other members of this series upon anti-inflammatory testing, though this point has yet to be demonstrated.

The new compounds [2-8] isolated from *C. scalaris* were tested against P-388 mouse lymphoma, A-549 human lung carcinoma, HT-29 human colon carcinoma, and MEL-28 human melanoma. The new compounds showed significant cytotoxicity toward these five tumour cell lines, with ED_{50} values between 1 and 5 μg/ml. 18-*epi*-scalaradial [2] showed the strongest, though unselective, cytotoxicity toward P-388, Schabel, A-549, and HT-29 tumour cell lines (ED_{50} = 0.2 μg/ml).

It is well known that sponges of the order Dictyoceratida are the source of a group of terpenoids characterised by possessing 21 carbon atoms and two terminal furan rings. These C-21 furanoterpenoids have been isolated from several genera of the Spongiidae and Thorectidae families and, occasionally, from nudibranchs that prey on them. Dyctioceratidae sponges of these two families and some of their predators have given rise, in addition, to linear furanosesterterpenes containing a single ring, an otherwise uncommon group of terpenoids.

We obtained a specimen of the sponge *S. officinalis* collected in the infralitoral zone of La Caleta. *S. officinalis* had been extensively studied, affording furanosesterterpenes and C-21 furanoterpene among its constituents. Our specimen contained two new C-21 furanoterpenes [9, 10] and a new linear furanosesterterpene [11], together with seven known C-21 furanoterpenes and a known furanosesterterpene.

The specimen of *S. officinalis* (62.2 g dry wt) was collected by hand and frozen immediately. The less polar material of an acetone extract was chromatographed on Si gel. Final purification of selected fractions using HPLC allowed isolation of the compounds.

The structures of the new C-21 furanoterpenes furospongin-5 [9] and cyclofurospongin-2 [10] were defined on the basis of H-RMS, ^{1}H- and ^{13}C-NMR analyses, as well as by nOe experiments. Although cyclic furospongins, such as 10, can be obtained from acyclic precursors through acid catalysed cyclisations, because the natural product isolated from *S. officinalis* is optically active it seems unlikely that 10 arose from an acyclic precursor through an acid-catalysed cyclisation during the isolation process.

The major and most polar component isolated from *S. officinalis* was the furanosesterterpene demethylfurospongin-4 [11]. The structure of [11] was defined by HRMS, ^{1}H- NMR and, in particular, ^{13}C-NMR analysis.

In general, the compounds isolated from *S. officinalis* exhibited low cytotoxicity against the aforesaid tumour cells with ED_{50} values over 10 μg/ml in nearly all cases, with the exception of furospongin-5 [9], which showed a mild cytotoxicity against the P-388 cell line (ED_{50} = 5 μg/ml).

Marine algae have been the group of organisms that has received the most attention from marine natural-product chemists over that last 25 years. However, since many of the algal metabolites were described before the current pharmacological bioassays became available, the potential of most of these metabolites remains unexplored.

This prompted us to collect specimens of *D. dichotoma* in Cortadura. Our specimens contained five new dolabellane diterpenes [12-16], two new hydroazulenoid diterpenes [17, 18] together with 19 known diterpenes.

Specimens of *D. dichotoma* (62 g dry wt) were collected by hand and frozen immediately. The less polar material of an acetone extract was chromatographed on silica gel. Final purification of selected fractions using normal phase HPLC made it possible to isolate the following compounds: (1*R*, 2*E*, 4*S*, 5*R*, 6*S*, 7*E*, 10*S*, 11*S*, 12*R*)-5,6,18-triacetoxy-10-hydroxy-2,7-dolabelladiene (12, 0.140 % dry wt), (1*R*, 2*E*, 4*R*, 7*E*, 10*S*, 11*S*, 12*R*)-18-acetoxy-10-hydroxy-2,7-dolabelladiene (13, 0.018 % dry wt), (1*R*, 2*E*, 4*S*, 5*R*, 7*E*, 10*S*, 11*S*, 12*R*)-5-acetoxy-10,18-dihydroxy-2,7-dolabelladiene (14, 0.021 % dry wt), (1*R**, 3*E*, 7*S** ,8*S**, 11*S**, 12*R**)-7,8-epoxy-3,18-dolabelladiene (15, 0.009 % dry wt), (1*R**, 2*E*,4*R**, 7*E*, 11*S**, 12*R**) -18-acetoxy-2,7-dolabelladiene (16, 0.016 % dry wt), isopachydictyol A (17, 0.010 % dry wt), and dictyotatriol A (18, 0.031 % dry wt), along with 19 known compounds.

The structures of the new compounds were elucidated by interpretation of spectral data, with special emphasis in COSY and nOe experiments.

The absolute stereochemistry of dolabellane 14 was defined using the Mosher method. The $\Delta\delta$ (δ_S-δ_R) values for selected proton signals of the (*R*)- and (*S*)-MTPA esters indicated an *S* configuration for C-10, and therefore an absolute configuration, as depicted in formula [14]. Following the same method and a similar rationale, the absolute stereochemistry of dictyotatriol A [7] was defined as shown in formula [18].

Chemical interconversions were used to establish the absolute configurations of dolabellanes [12] and [13]. The reduction with lithium aluminium hydride of [12] to afford a tetrol whose absolute stereochemistry had been defined using Horeau's method, and of [13] to give a diol whose absolute stereochemistry was established using the Mosher method, indicated an absolute stereochemistry for dolabellanes [12] and [13] as depicted in the formulae.

In general, the new diterpenes isolated from *D. dichotoma* showed a mild activity in bioassays aimed at detecting *in vitro* cytotoxicity against the aforesaid tumour cell lines. However, dolabellane [12] and dictyotatriol A [18] were inactive in these tests. Compounds [14-17] were mildly active with ED_{50} values of 5 μg/ml in all cases. Dolabellane [13] exhibited the greatest activity with ED_{50} = 1.2 μg/ml against P-388 and A-549 tumour cell lines, and ED_{50} = 2.5 μg/ml against HT-29 and MEL-28 tumour cell lines.

ACKNOWLEDGEMENTS

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