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Xanthones from Marine-Derived Microorganisms: Isolation, Structure Elucidation, and Biological Activities

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1 INTRODUCTION

The marine ecosystem comprises more than 70% of the Earth's surface, representing 95% of the biosphere (Jimeno *et al*., 2004; Glaser and Mayer, 2009), being distinguished by an enormous biodiversity, mirrored by a variety of secondary metabolites found in animals, plants, and microorganisms (König *et al*., 2006). To date, approximately 22,500 products of marine origin have been described, and hundreds of new compounds are being discovered every year. A number of bioactive compounds have been isolated from marine invertebrates such as sponges, tunicates, mollusks, and bryozoans, in addition to algae and marine microorganisms, such as cyanobacteria and fungi (Kijjoa and Sawangwong, 2004; Saleem *et al*., 2007; Xu, 2011). Interestingly, these microorganisms are found to be in close contact with marine invertebrates, which are either symbiotic, that is, associated, or serve as food. Because many secondary metabolites isolated from marine invertebrates exhibited striking structural similarities to those from the microbial origin, it was suggested that microorganisms are at least involved in their biosynthesis or are in fact the true sources of these respective metabolites. Even though there are some evidences for this hypothesis, it is extremely difficult to definitively state the biosynthetic source of many marine natural products because of the complexity of associations in marine organisms (König *et al*., 2006).

Owing to their pharmacological potential, either directly as drugs or as lead structures for molecular modifications and/or drug synthesis especially in cancer research, marine natural products have aroused attention of many researchers (Blunt *et al*., 2009). Many marine-derived natural products have already undergone clinical or preclinical trials especially with regard to, not only their antitumor properties, but also anti-inflammatory and antiinfectious properties, among others (Kosta, Jain, and Tiwari, 2008). In fact, two marine natural products have already been approved in human therapeutics: ziconotide (Prialt®), a peptide first isolated from the venom of the cone snail *Conus magus*, an analgesic used for treatment

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of patients suffering from chronic pain, and trabectedin (Yondelis®), an alkaloid originally isolated from a marine tunicate and now obtained by semisynthesis, for the treatment of soft tissue sarcomas and ovarian cancer (Jimeno *et al*., 2004; Kijjoa and Sawangwong, 2004; Baker *et al*., 2007; Glaser and Mayer, 2009).

From microorganisms described as marine natural sources of the bioactive metabolites, fungi have shown great potential as suggested by the diversity of their isolated secondary metabolites. Fungi from the marine habitats are divided into obligate and facultative marine species, the former being restricted to the marine environment and the latter occurring also in freshwater or terrestrial localities or both (Bugni and Ireland, 2004). Isolation of a fungal strain from a marine sample does not prove that this fungus is actively living in the marine environment because it is possible to isolate a terrestrial fungus as being a contaminant in the marine habitat. Because most fungi isolated from marine samples are not proven to be obligate or facultative species, the more general expression "marine-derived fungi" is largely used (Bugni and Ireland, 2004). Marine-derived fungi have been recognized as a potential source of novel structures and biologically potent molecules, and a growing number of marine-derived fungi have been reported as sources of novel bioactive secondary metabolites (Bugni and Ireland, 2004; Saleem *et al*., 2007). Because of their particular living conditions, salinity, nutrition, higher pressure, temperature variations, and competition with bacteria, viruses, and other fungi, marine-derived fungi may have developed specific secondary metabolic pathways compared with their terrestrial counterparts (Bugni and Ireland, 2004; Saleem *et al*., 2007). In terms of the overall number of secondary metabolites, polyketides, prenylated polyketides (meroterpenes), peptides, and alkaloids are the most described. With no exception, xanthone derivatives that can be considered as originated from polyketide or prenylated polyketide have been largely isolated from marine-derived fungi and, to a lesser extent, from other microorganisms.

Xanthones, or 9*H*-xanthen-9-ones, encompass an important class of oxygenated heterocyclic compounds with the dibenzo-γ-pirone skeleton as a basic structure (Pinto, Sousa, and Nascimento, 2005) (Figure 1). The biological activities of these compounds are associated with their tricyclic scaffold but vary depending on the nature and/or position of the substituents. Natural xanthones can be subdivided, depending on the nature of the substituents in the dibenzo-γ-pirone scaffold, into simple oxygenated xanthones, glycosylated xanthones, prenylated xanthones and their derivatives, xanthone dimers, xanthonolignoids, and miscellaneous (Pinto, Sousa, and Nascimento, 2005; Vieira and Kijjoa, 2005; El-Seedi *et al*., 2009). Recently, a new classification based on structural characteristics of xanthones (monomers and dimers/heterodimers) and the level of oxidation of the xanthone C-ring (fully aromatic-, dihydro-, tetrahydro-, and hexahydroxanthone derivatives) was suggested by Masters and Bräse (2012).

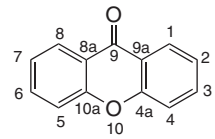

Figure 1 Xanthone's basic structure and its numbering system.

Xanthones can be described as "privileged structures" as they are molecular scaffolds that appear to be capable of binding to multiple targets and, consequently with appropriate structure modifications, could exhibit multiple activities (Pinto, Sousa, and Nascimento, 2005; Pinto and Castanheiro, 2009; Pouli and Marakos, 2009). This class of compounds can interact with a large variety of biological targets exhibiting important activities, for instance, cancer chemopreventive and antitumor, influence on several inflammatory mediators belonging to the arachidonic acid cascade, to achieve a variety of enzymes such as kinases, proteases, and monoaminoxidases (MAOs A and B). Moreover, they exhibit antimicrobial activity against a large number of human pathogenic microorganisms. Consequently, xanthonic molecules are a distinguished structural type of value to the discovery of new pharmaceutically interesting compounds (Pinto, Sousa, and Nascimento, 2005; Pinto and Castanheiro, 2009; Pouli and Marakos, 2009). Xanthones occur mainly in higher plants, fungi, and lichens. However, the number of xanthones isolated from marine sources, mainly from sponges- and algae-associated fungi, and form marine bacteria is not yet very high when compared with other classes of compounds.

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2 MARINE SOURCES OF XANTHONES

2.1 Marine-Derived Fungi

2.1.1 From the Genus Aspergillus (Trichocomaceae)

Marine invertebrate-associated microorganisms have recently attracted attention as an important source of novel, biologically active secondary metabolites. Among these, some *Aspergillus* spp. have been reported to produce a considerable number of cytotoxic compounds and other bioactive substances (Rateb and Ebel, 2011).

In the search for new and biologically active marine natural products, Wu *et al*. have isolated more than 300 fungal strains from marine mollusks and algae (Wu, Ouyang, and Tan, 2009). Screening of extracts from cultures of selected isolates led to the discovery of several strains that showed significant antimicrobial activity in agar diffusion assays. A new difuranoxanthone, asperxanthone (**1**) (Figure 2), along with other compounds, was then isolated from a selected marine fungus of the seawater, identified as *Aspergillus* sp. (MF-93), collected in the Quan-Zhou Gulf (Wu, Ouyang, and Tan, 2009; Rateb and Ebel, 2011; Masters and Bräse, 2012). Although asperxanthone (**1**) was the most active compound in inhibiting multiplication of tobacco mosaic virus (TMV) (inhibitory rates 62.9%), its activity was lower than that of the methanol extract from which it was isolated (inhibitory rates 81.2%). These results suggested that the inhibitory activity of the methanol extract was not primarily due to any of the isolated compounds (Wu, Ouyang, and Tan, 2009; Rateb and Ebel, 2011). Lee *et al*., in their continuing search for bioactive metabolites, have isolated another two xanthones: sterigmatocystin (**2**) and dihydrosterigmatocystin (**3**), along with other compounds, from the fungus *Aspergillus versicolor* isolated from a marine sponge *Petrosia* sp. (Petrosiidae) by bioactivity-guided fractionation (Lee *et al*., 2010). Sterigmatocystin (**2**) and dihydrosterigmatocystin (**3**) were evaluated for their cytotoxicity against five human tumor cell lines, A-549 (human lung adenocarcinoma), SK-OV-3 (human ovarian adenocarcinoma), SK-MEL-2 (human skin melanoma), XF-498 (central nervous system cancer), and HCT-15 (human colon adenocarcinoma). Sterigmatocystin (**2**) was found to exhibit significant cytotoxicity against all the cell lines tested, having IC_{50}

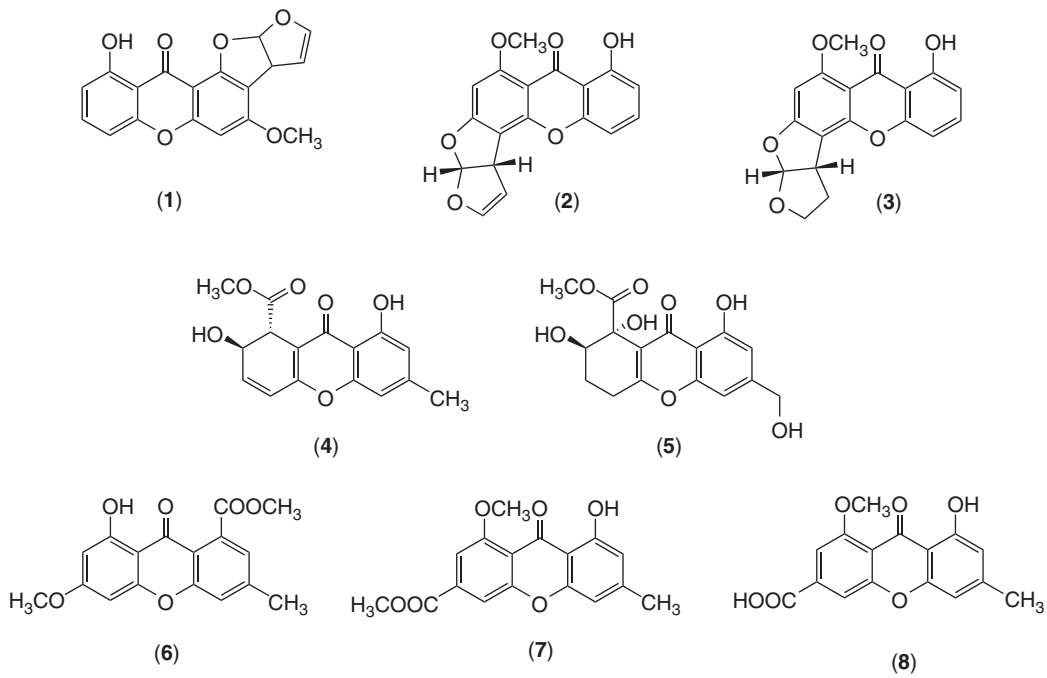

Figure 2 Xanthones of marine-derived fungi from the genus *Aspergillus*.

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values in the range of 1.22-4.61 μg mL^{-1}. Considering the only structural difference between sterigmatocystin (**2**) and dihydrosterigmatocystin (**3**), it is believed that the allylic ether moiety (a double bond at C-2′) could play an important role in the cytotoxicity of sterigmatocystin (**2**) (Lee *et al*., 2010). Trisuwan *et al*. reported isolation of two new hydrogenated xanthone derivatives, aspergillusones A (**4**) and B (**5**) (Figure 2), along with other known compounds, from the ethyl acetate extract of the culture broth of *Aspergillus sydowii* PSU-F154, isolated from a gorgonian sea fan of the genus *Annella* (Subergorgiidae), and neither of the isolated compounds showed antioxidant activity in the DPPH assay (Trisuwan *et al*., 2011). Sun *et al*., in their ongoing program to discover new bioactive compounds from algicolous fungi, reported isolation of three new xanthone derivatives, yicathin A (**6**), yicathin B (**7**), and yicathin C (**8**), from the endophytic fungus *Aspergillus wentii*, obtained from the inner tissue of the marine red alga *Gymnogongrus flabelliformis* Harvey (Phyllophoraceae). The structures of the three xanthones were unambiguously established by nuclear magnetic resonance (NMR) and mass spectroscopic methods, as well as by quantum chemical calculations (Sun *et al*., 2013). Xanthones **6–8** were evaluated for their antibacterial (against *Escherichia coli* and *Staphylococcus aureus*) and antifungal (against phytopathogens *Colletotrichum lagenarium* and *Fusarium oxysporum*) activities, using standard agar diffusion test at 10 μg/disk. Yicathin B (**7**) was active against *E. coli* (inhibition diameter 9 mm) and yicathin C (**8**) was found to be able to inhibit *E. coli* (12.0 mm), *S. aureus* (7.5 mm), and *C. lagenarium* (11.0 mm). In addition, all the three xanthones were shown to exhibit weak brine shrimp (*Artemia salina*) toxicity with IC_{50}'s of 0.20, 0.22, and 0.30 μmol mL^{-1}, respectively (Sun *et al*., 2013).

2.1.2 From the Genus Penicillium (Trichocomaceae)

Some *Penicillium* spp. are a rich source of secondary metabolites, and a number of xanthones have been reported from the members of this genus. Shao *et al*. described isolation of two new xanthones, 8-(methoxycarbonyl)-1-hydroxy-9-oxo-9*H*-xanthene-3-carboxylic acid (**9**) and dimethyl 8-methoxy-9-oxo-9*H*-xanthene-1, 6-dicarboxylate (**10**) along with the known xanthone methyl 8-hydroxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate (**11**) (Figure 3), from the culture broth of the mangrove endophytic fungus *Penicillium* sp. (ZZF 32#), isolated from the bark of *Acanthus ilicifolius* Linn. (Acanthaceae) collected from the South China Sea. Interestingly, the crude extract of the culture broth and the column fractions containing these xanthones were found to exhibit significant cytotoxicity against KB (human epidermoid carcinoma of the nasopharynx) and KBv200 (multidrug-resistant human epidermoid carcinoma of the nasopharynx) cells with IC_{50} values of 1.5 and 2.5 μg mL^{-1}, respectively (cisplatin was used as the positive control with IC_{50} value against KB and KBv200 of 0.56 and 0.78 μg mL^{-1}, respectively), whereas neither of the isolated compounds was active. Thus, the bioactivity demonstrated by the crude extract and its column fractions was not due to the individual xanthone constituents ($IC_{50} > 50$ μg mL^{-1}). Furthermore, antifungal activity assay revealed that xanthone **10** exhibited modest activity against *F. oxysporum* f. sp. *cubense* with the minimal inhibitory concentration (MIC) value of 12.5 μg mL^{-1} (Shao *et al*., 2008; Rateb and Ebel, 2011; Xu, 2011). Another endophytic fungus *Penicillium* sp., isolated from *Melia azedarach* L. (Meliaceae) growing in a Chinese mangrove habitat, also afforded 7-hydroxyjanthinone (**12**) (Figure 3), which was found to be devoid of cytotoxic activity (Rateb and Ebel, 2011). Recently, Khamthong *et al*. (2012a,b) reported isolation of three xanthone derivatives: sydowinin A (**13**), pinselin (**14**), and conioxanthone A (**15**) (Figure 3), from the fungus *Penicillium citrinum* PSU-F51, isolated from the gorgonian sea fan (*Annella* sp.). These xanthones were found to be inactive in the antifungal assay against *Microsporum gypseum* at a concentration of 200 μg mL^{-1}. Another endophytic fungus, *Penicillium sacculum*, isolated from the halophyte *Atriplex* sp. (Amaranthaceae), was recently found to afford a new xanthone whose structure was established as 1-hydroxy-3-methoxy-6-sulfo-8-methylxanthone (**16**) (Figure 3) (Liu *et al*., 2012a,b). During the study to evaluate the methods of isolation and growth of marine-derived fungal strains in artificial media for the production of secondary metabolites, Kossuga *et al*. (2012) have isolated norliquexanthone or 1,3,6-trihydroxy-8-methyl-9*H*-xanthen-9-one (**17**) from *Penicillium raistrikii*, which was isolated from

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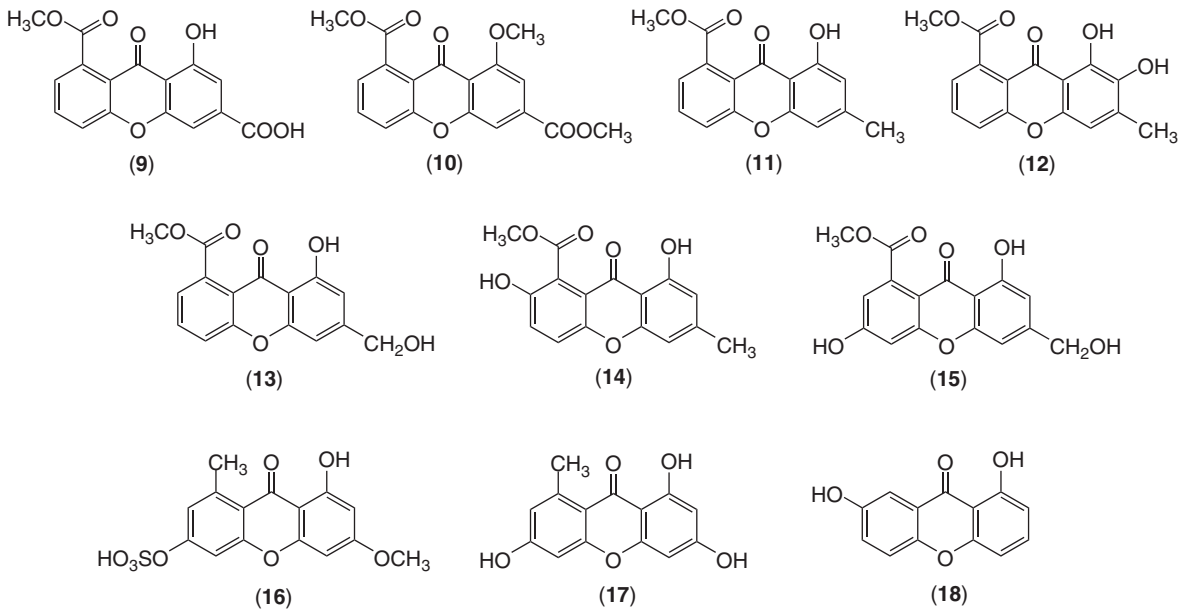

Figure 3 Xanthones of marine-derived fungi from the genus *Penicillium*.

the marine sponge *Axinella* cf. *corrugata* (Axinellidae) (Figure 3). Huang *et al*. reported isolation of 1,7-dihydroxy-9*H*-xanthen-9-one (**18**), from the mangrove endophytic fungus *Penicillium* sp. ZH16, isolated from the leaves of the mangrove tree *Avicennia* (Acanthaceae) from the South China Sea coast (Figure 3) (Huang *et al*., 2012).

2.1.3 From the Genus Monodictys (Dematiaceae)

The uncommon marine-derived fungus *Monodictys putredinis*, isolated from an unidentified green alga collected in Tenerife, Spain, was investigated for its secondary metabolites after cultivation on a solid biomalt medium (Pontius *et al*., 2008a,b). The separation steps of vacuum liquid chromatography (VLC) and high performance liquid chromatography (HPLC) resulted in isolation of two novel dimeric chromanones, monodictyochromone A (**19**) and monodictyochromone B (**20**) (Figure 4), whose structure consists of two unusually modified xanthone-derived subunits, being the only difference between the two compounds the site of connection of subunits I and II. Compounds **19** and **20** were also examined for their cancer chemopreventive potential and were found to inhibit cytochrome P450 1A activity with IC_{50} values of 5.3 and 7.5 μ*M*, respectively. In addition, both compounds were shown to display moderate activity as inducers of NAD(P)H: quinone reductase (QR) in cultured mouse Hepa 1c1c7 cells, with CD values (concentration required to double the specific activity of QR) of 22.1 and 24.8 μM, respectively. Furthermore, compound **19** was slightly less potent than compound **20** as aromatase inhibitor, showing IC_{50} values of 24.4 and 16.5 μ*M*, respectively (Pontius *et al*., 2008a,b). Krick *et al*. also reported isolation of, besides a benzophenone, four monomeric xanthones, monodictysin A–C (**21–23**) and monodictyxanthone (**24**) (Figure 4), from the extract of the same fungus. These compounds were subsequently tested in a series of *in vitro* bioassays relevant for the inhibition of carcinogenesis *in vivo* in order to evaluate their cancer chemopreventive potential (Krick *et al*., 2007; Rateb and Ebel, 2011). Xanthone **23** was shown to inhibit cytochrome P450 1A activity with an IC_{50} value of 3.0 μ*M*. Besides, xanthones **21–23** were also identified as inhibitors of the Cyp1A isoenzyme, which is involved in the metabolic conversion of procarcinogens into carcinogens. Xanthones **22** and **23** displayed moderate activity as inducers of NAD(P)H:QR, a carcinogen-detoxifying

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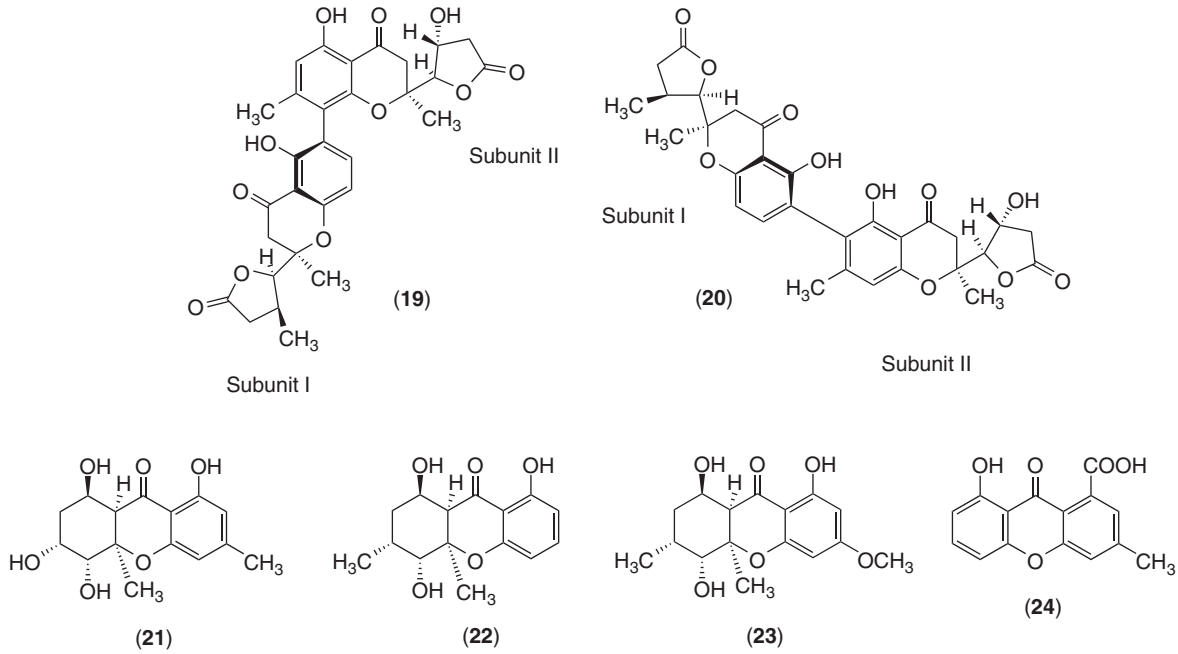

Figure 4 Xanthones of marine-derived fungi from the genus *Monodictys*.

enzyme, in cultured mouse Hepa 1c1c7 cells, with CD values of 12.0 and 12.8 μ*M*, respectively. Compound **23** was a weak inhibitor of aromatase activity essential for the biosynthesis of estrogens. Although the xanthone-carboxylic acid **24** showed a dose-dependent Cyp1A activity inhibition with an IC_{50} value of 34.8 ± 7.4 μ*M*, it did not induce QR activity. Overall, the substitution pattern of the core structure was found to strongly influence the biological effects of these compounds (Krick *et al*., 2007).

2.1.4 From the Genus Emericella (Trichocomaceae)

The genus *Emericella* is one of the *Aspergillus* anamorphs, and the members of this genus are found to biosynthesize a remarkable diversity of secondary metabolites with motivating biological properties and, thus, representing potential leads for the developing of new pharmaceutical agents (Figueroa *et al*., 2009). Thus, within the scope of a program aiming at the discovery of novel calmodulin (CaM)-inhibitors, useful as pesticide or drug leads, Figueroa *et al*. reported isolation and the CaM inhibitor properties of two new xanthones: 15-chlorotajixanthone hydrate (**25**) and 14-methoxytajixanthone (**26**), together with the known shamixanthone (**27**) and tajixanthone hydrate (**28**) (Figure 5) from *Emericella* sp. strain 25379, isolated from the surface of a coral species collected on the Mexican Pacific coast (Figueroa *et al*., 2009). The effect of xanthones **25–28** on CaM was initially assessed with the calmodulin-sensitive cAMP phosphodiesterase (PDE1) assay, which is commonly used to detect CaM antagonists; a human recombinant-CaM was used as the activator. The results showed that the activation of PDE1 was inhibited in the presence of **26** and **28** in a concentration-dependent manner. The effect of xanthones **26** ($IC_{50} = 5.54 \pm 1.28$ μ*M*) and **28** ($IC_{50} = 5.62 \pm 1.25$ μM) was comparable to that of chlorpromazine (CPZ; $IC_{50} = 7.26 \pm 1.60$ μ*M*), a well-known CaM inhibitor used as a positive control. A kinetic analysis using different amounts of CaM in the presence of different concentrations of **26** and **28** indicated that both xanthones acted as competitive antagonists of CaM, thus interfering with the formation of the CaM–PDE1 active complex. The estimated K_i (inhibition constant) values

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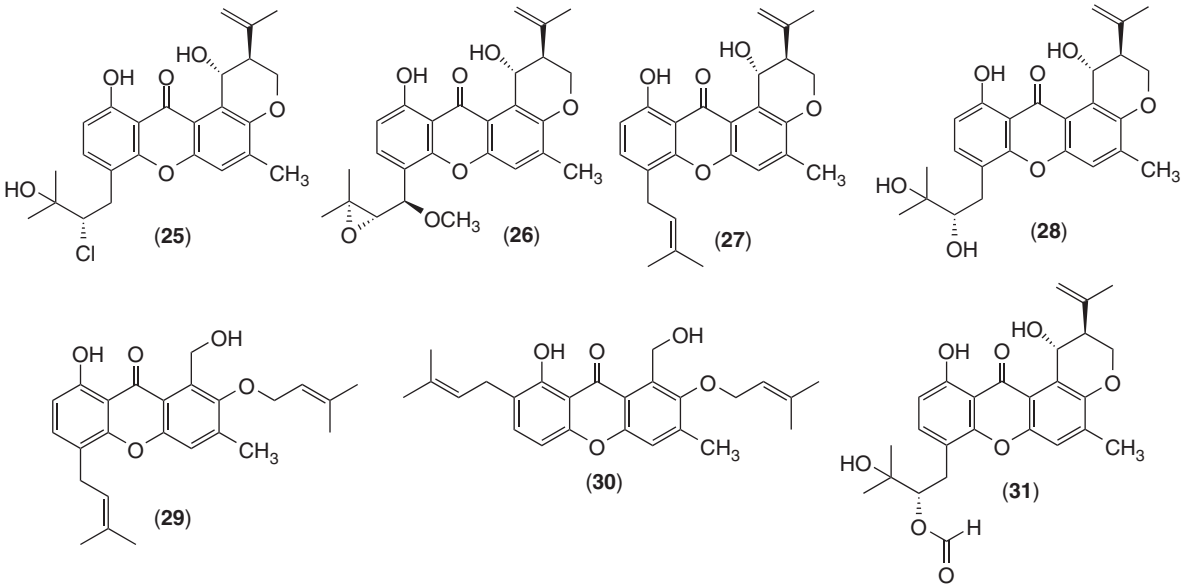

Figure 5 Xanthones of marine-derived fungi from the genus *Emericella*.

were 25.38 ± 2.26 and 13.92 ±2.29 μ*M*, respectively. AutoDock predictions suggested that these compounds interact with the protein at the same binding site of trifluoropiperazine (TFP), a recognized CaM inhibitor. The CaM antagonist effect of **28** might be related with its mild cytotoxic action and other pharmacological properties yet to be discovered (Figueroa *et al*., 2009). The results of their investigation led the authors to conclude that *Emericella* sp. contained novel type of competitive CaM inhibitors. The xanthones sterigmatocystin (**2**), shamixanthone (**27**), emericellin (**29**) (Figure 5), and the biosynthetically related benzoquinone derivatives were also isolated from the culture of *Emericella nidulans* var. *acristata*, an endophyte isolated from a Mediterranean green alga collected around Sardinia (Kralj *et al*., 2006). The effects of the crude extract and the pure compounds (except for sterigmatocystin) on tumor growth *in vitro* were investigated in a survival and proliferation assay using a panel of 36 human tumor cell lines representing 11 different tumor types. Antitumor activity was defined as test/control value smaller than 50% compared to the untreated control cells. The crude extract effected antitumor activity in all 36 cell lines (100%) at 50 μg mL^{-1}, in 31 of the 36 cell lines (86%) at 5 μg mL^{-1}, and in 2 of 36 (6%) cell lines at 0.5 μg mL^{-1}. This is indicative of a selective and concentration-dependent antitumor activity of the extract and one or more of its components. However, shamixanthone (**27**) and emericellin (**29**) showed either only marginal or no antitumor activity *in vitro* (Kralj *et al*., 2006). Bringmann *et al*. also reported isolation of shamixanthone (**27**) and isoemericellin (**30**) (Figure 5), along with other metabolites from *Emericella variecolor*, derived from the marine sponge *Haliclona valliculata* (Chalinidae). Malmstrøm *et al*. isolated, besides other metabolites, shamixanthone (**27**), tajixanthone hydrate (**28**), and varixanthone (**31**) (Figure 5), from the marine-derived strain M75-2 of *E. variecolor*. Varixanthone (**31**) was found to show no cytotoxic activity, at 1 μg mL^{-1}, against three tumor cell lines (P388, mouse lymphoma; A549, human lung carcinoma; and HT29, human colon carcinoma). However, it displayed antimicrobial activity against Gram-positive and Gram-negative bacteria, and it was also found to be active against *E. coli*, *Proteus* sp., *Bacillus subtillis*, and *S. aureus*, showing a MIC of 12.5 μg mL^{-1} in all these cases but showed lower potency against *Enterococcus faecalis* (MIC = 50 μg mL^{-1}) (Malmstrøm *et al*., 2002; Thomas, Kavlekar, and LokaBharathi, 2010; Bhatnagar and Kim, 2012; Masters and Bräse, 2012).

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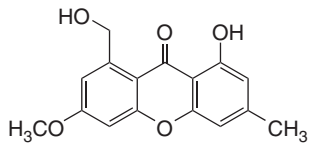
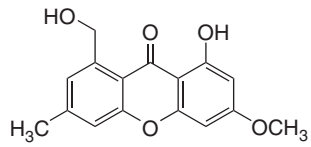
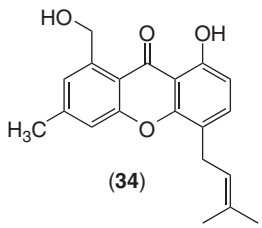


(**32**)



(**33**)

Figure 6 Xanthones of marine-derived fungi from the genus *Phoma*.



(**34**)

Figure 7 Xanthones of marine-derived fungi from the genus *Paecilomyces*.

2.1.5 From the Genus Phoma (Didymellaceae)

Pan *et al*. described isolation of two new xanthones: 1-hydroxy-8-(hydroxymethyl)-6-methoxy-3-methyl-9*H*-xanthen-9-one (**32**) and 1-hydroxy-8-(hydroxymethyl)-3-methoxy-6-methyl-9*H*-xanthen-9-one (**33**) (Figure 6), from a mangrove endophytic fungus *Phoma* sp. SK3RW1M., isolated from the roots of the mangrove tree *Avicennia marina* (Forsk.) Vierh., collected in Shankou mangrove in China (Pan *et al*., 2010; Rateb and Ebel, 2011). This was the first report on xanthone derivatives isolated as secondary metabolites from *Phoma* species [19]. Xanthones **32** and **33** were evaluated for their cytotoxic activity against KB and KBv200 cells, and preliminary results indicated that they were inactive (Pan *et al*., 2010; Rateb and Ebel, 2011).

2.1.6 From the Genus Paecilomyces (Trichocomaceae)

In the course of an ongoing search for natural potent antitumor agents from marine mangrove fungi, Wen *et al*. have found that the extract of the fungus *Paecilomyces* sp., a metatrophic fungus Tree1-7 collected from the bark of a mangrove from the Taiwan Strait, exhibited good cytotoxicity. Chromatographic purification of the methanol extract of the mycelia led to isolation of a new xanthone, paeciloxanthone (**34**) (Figure 7), together with emodin and chrysophanol (Wen *et al*., 2008; Rateb and Ebel, 2011; Xu, 2011; Simpson, 2012). Paeciloxanthone (**34**) was found to exhibit an *in vitro* cytotoxicity against hepG2 (IC_{50} = 1.08 μg mL^{-1}), acetylcholine esterase (AChE) inhibitory (IC_{50} = 2.25 μg mL^{-1}) activity, and antimicrobial activity against *Curvularia lunata* (walker) Boedijn, *E. Coli*., and *Candida albicans*, affording inhibitory zones of 6, 12, and 10 mm, respectively (Wen *et al*., 2008; Rateb and Ebel, 2011; Xu, 2011).

2.1.7 From the Genus Chaetomium (Chaetomiaceae)

Investigations of the marine-derived fungus *Chaetomium* sp., by Pontius *et al*., led to the isolation of the new natural products chaetoxanthones A, B, and C (**35–37**) (Figure 8). Chaetoxanthones A (**35**) and B (**36**) are dioxane/tetrahydropyran substituted xanthones whose structures are rarely found in natural products, whereas chaetoxanthone B (**37**) is a tetrahydropyran-substituted chlorinated xanthone. Chaetoxanthones A, B, and C (**35–37**) were tested in a series of *in vitro* bioassays for their antiprotozoal and cytotoxic activities. Chaetoxanthone B (**36**) exhibited selective antiprotozoal activity toward *Plasmodium falciparum* with an IC_{50} value of 0.5 μg mL^{-1} (reference drug chloroquine, IC_{50} = 0.08 μg mL^{-1}) and no cytotoxic effects toward L6-cells (IC_{50} > 90 μg mL^{-1}) and 35 tumor cell lines (mean IC_{50} > 10 μg mL^{-1}) (Pontius *et al*., 2008a,b). These results let Pontius *et al*. to suggest that the xanthone scaffold was a suitable pharmacophore for antiplasmodial activity, and the nature and position of substituents clearly influence the biological

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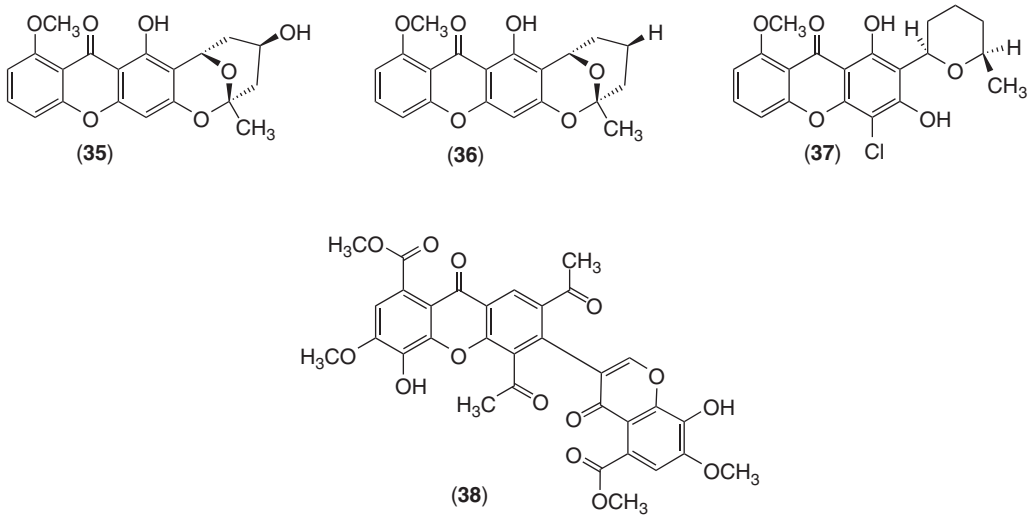

Figure 8 Xanthones of marine-derived fungi from the genus *Chaetomium*.

effect (Pontius *et al*., 2008a,b). Although chaetoxanthone B (**36**) did not reach the high antiplasmodial potency of some reported polyhydroxyxanthones or alkylamino-substituted xanthones with IC_{50} values below 0.1 μg mL^{-1}, it was more active than prenylated, nonnitrogenous xanthone derivatives. Consequently, they concluded that the heterocyclic substitution in chaetoxanthone B (**36**) did not cause a remarkable enhancement in activity. Interestingly, chaetoxanthone A (**35**), with an additional hydroxyl group at C-3′ compared to chaetoxanthone B (**36**), had a much weaker activity toward *P. falciparum* (IC_{50} = 3.5 μg mL^{-1}) (Pontius *et al*., 2008a,b). Conversely, chaetoxanthone C (**37**) was found to be moderately active against *Trypanosoma cruzi*, the causative pathogen of Chagas disease, with an IC_{50} value of 1.5 μg mL^{-1} (reference drug benznidazole, IC_{50} = 0.3 μg mL^{-1}) without having considerable cytotoxic effects on L6-cells (IC_{50} = 46.7 μg mL^{-1}). The antiprotozoal activity of chaetoxanthone C (**37**) is comparable to that reported for a series of synthetic chlorinated xanthones carrying aminoalkyl side chains. However, these compounds were tested only toward *P. falciparum*, and the best candidate showed an *in vitro* IC_{50} value of 1.4 μg mL^{-1}. Thus, the *in vitro* antiprotozoal activity of xanthones was further substantiated by this study. However, it can be inferred that substitution with a dioxane/tetrahydropyran moiety on the xanthone nucleus does not improve antiprotozoal activity (Pontius *et al*., 2008a,b). Another xanthone isolated from the culture broth of the marine-derived fungus *Chaetomium* sp., obtained from an undisclosed marine alga, was chaetocyclinone C (**38**) (Figure 8). This xanthone was found to be active toward selected phytopathogenic fungi but was not cytotoxic (Rateb and Ebel, 2011).

2.1.8 From the Genus Wardomyces (Microascaceae)

The culture of the marine fungal isolate *Wardomyces anomalus* Brooks and Hansford, isolated from the green alga *Enteromorpha* sp. (Ulvaceae) collected in the Baltic Sea, afforded two new xanthone derivatives, 2,3,6,8-tetrahydroxy-1-methylxanthone (**39**) and 2,3,4,6,8-pentahydroxy-1-methylxanthone (**40**), in addition to the known xanthone 3,6,8-trihydroxy-1-methylxanthone (**41**) (Figure 9) (Abdel-Lateff *et al*., 2003; Masters and Bräse, 2012). Xanthone **39** was found to have significant DPPH radical scavenging effects (94.7%, at 25.0 μg mL^{-1}) besides its capacity to inhibit peroxidation of linolenic acid (17.0%, at 7.4 μg mL^{-1}). Furthermore, the total extract and xanthones **39** and **41** were shown to be inhibitors of TK p56^{lck} tyrosine kinase but only minor antimicrobial activity was observed for xanthones **39–41** in agar diffusion assay (Abdel-Lateff *et al*., 2003).

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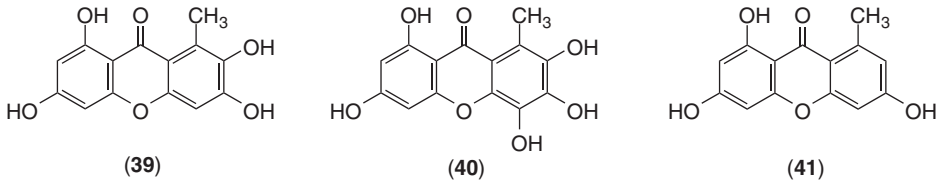

Figure 9 Xanthones of marine-derived fungi from the genus *Wardomyces*.

2.1.9 From Other Mangrove Endophytic Fungi

Marine fungi, especially mangrove endophytic fungi, have proved to be an abundant source for novel natural compounds. In the search for new metabolites from marine mangrove endophytic fungi, Huang *et al*. have reported isolation of two new xanthone derivatives, 1,7-dihydroxy-2-methoxy-3-(3-methylbut-2-enyl)-9*H*-xanthen-9-one (**42**) and 1-hydroxy-4,7-dimethoxy-6-(3-oxobutyl)-9*H*-xanthen-9-one (**43**) (Figure 10), from the fungus *Phomopsis* sp. (No. ZH19) (Diaporthaceae), isolated from the leaves of the mangrove tree *A. marina* collected from the South China Sea coast (Huang *et al*., 2010b). The same authors reported also the isolation of a new xanthone derivative, 3,5,8-trihydroxy-2,2-dimethyl-3,4,4-trihydro-2*H*, 6*H*-pyrano[3,2-*b*]xanthen-6-one (**44**), together with the known 5,8-dihydroxy-2,2-dimethyl-2*H*,6*H*-pyrano[3,2-*b*]xanthen-6-one (**45**) (Figure 10), from an unidentified species of the endophytic fungus ZSU-H16 from the mangrove tree *Avicennia* from the South China Sea coast (Huang *et al*., 2010a). Using the MTT cytotoxicity assay method, it was found that both xanthones **42** and **43** were able to inhibit the growth of KB cells (human epidermoid carcinoma of the nasopharynx) with IC_{50} values of 20 and 35 μmol mL^{-1}, and KB_V200 cells with IC_{50} values of 30 and 41 μmol mL^{-1}, respectively, whereas xanthone **44** exhibited weak cytotoxicity against KB and KB_V 200 cells having IC_{50} values greater than 50 μg mL^{-1} (Huang *et al*., 2010b). During a screening for novel structures from marine-derived mangrove endophytic fungus from the South China Sea, Zhu and Lin have isolated sterigmatocystin (**2**), dihydrosterigmatocystin (**3**), and secosterigmatocystin (**46**) from the unidentified species of the fungal isolate 1850 from a leaf of *Kandelia candel* (L.) Druce (Rhizophoraceae) from an estuarine mangrove in Hong Kong. A preliminary bioassay showed that sterigmatocystin (**2**) exhibited weak cytotoxic activity against tumor cell lines Bel-7402 (human hepatoma cell line) and NCI-H460 (human non small cell lung cancer cell line) with IC_{50} values of 96.53 and 72.52 μg mL^{-1}, respectively. All the three xanthones did not exhibit significant inhibitory activity against human DNA topoisomerase type (hTOP), showing IC_{50} values greater than 100 μg mL^{-1} (Zhu and Lin, 2007). Sterigmatocystin (**2**) was also isolated together with 5-methoxysterigmatocystin (**47**) (Figure 10) from another unidentified species of the mangrove endophytic fugal strain ZSUH-36, obtained from the mangrove tree *A. ilicifolius* (Shao *et al*., 2007; Xu, 2011).

Secalonic acid D (SAD) (**48**) (Figure 10), one of the most prominent mycotoxins first characterized as a metabolite of *Penicillium oxalicum* in 1970, was found to induce cleft palate via inhibiting G1/S-phase-specific CDK2 activity. SAD was later reisolated from several sources, including the endophytic fungus *Paecilomyces* sp. (tree 1–7) and a mangrove-associated unidentified strain No. ZSU44. It was also considered as an acutely toxic and teratogenic fungal metabolite. SAD (**48**) also displayed extraordinarily significant cytotoxicity and induced apoptosis in leukemia cells HL60 and K562 with IC_{50} values of 0.38 and 0.43 μmol L^{-1}, respectively. Its inhibitory effect on human topoisomerase I was assessed with IC_{50} at 0.16 μmol mL^{-1}. The study of the mechanism of action showed that SAD (**48**) caused cell cycle arrest at G1, maintained via the GSK-3β/β-catenin/c-Myc pathway (Xu, 2011). Hong also reported isolation of SAD (**48**) from the fermentation broth of the marine lichen-derived fungus *Gliocladium* sp. T31 (Hypocreaceae), collected from marine sediments in South Pole, in addition to its ability to inhibit mammalian DNA topoisomerase I via *in vitro* plasmid supercoil relaxation assay and EMSA (Hong, 2011). SAD (**48**) was found to exhibit a considerable inhibition on DNA topoisomerase I in a dose-dependent manner with the MIC

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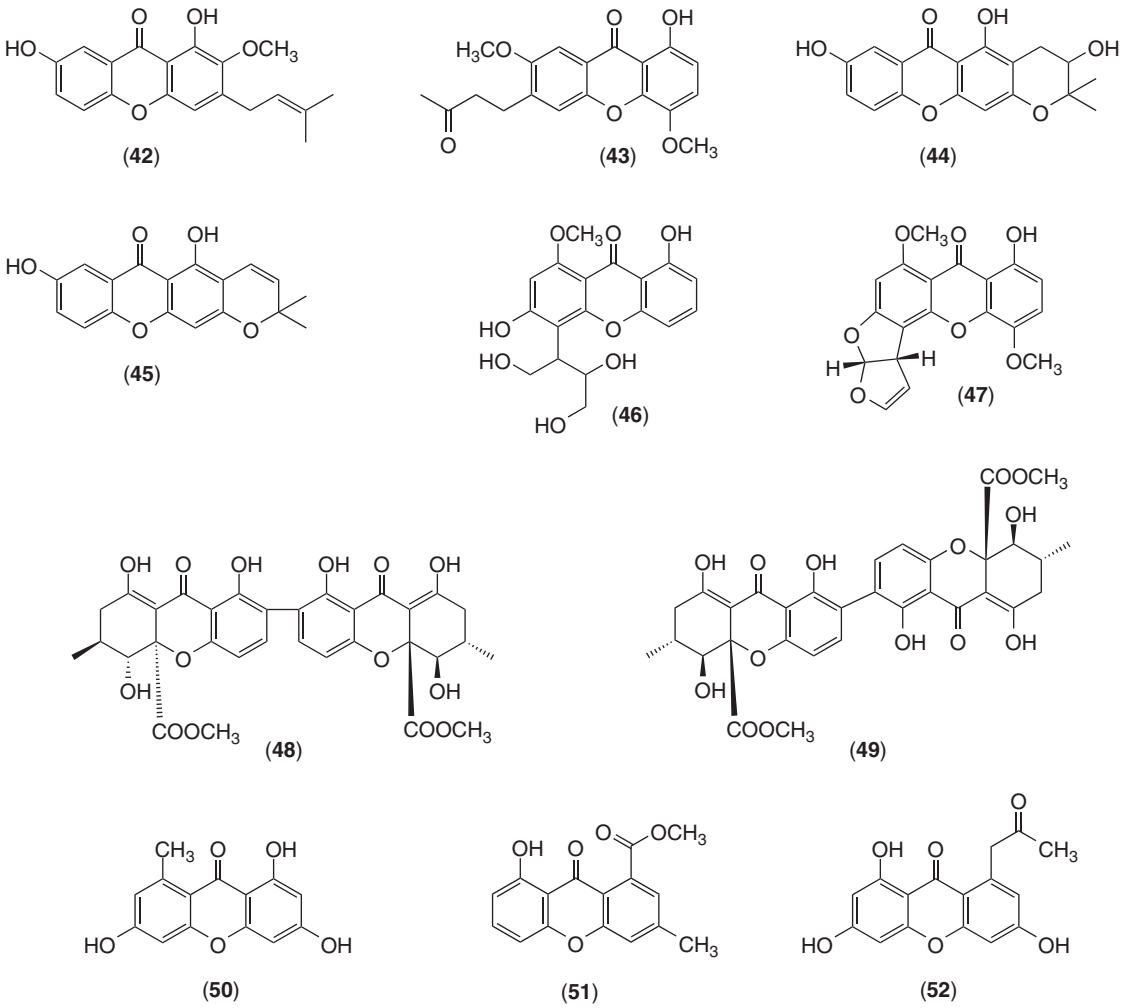

Figure 10 Xanthones from other mangrove endophytic fungi.

of 0.4 μ*M*. Unlike the prototypic DNA topoisomerase I poison camptothecin (CPT), SAD (**48**) inhibited the binding of topoisomerase I to DNA but did not induce the formation of topoisomerase I–DNA covalent complexes. This was the first study reporting the inhibitory effect of SAD (**48**) on mammalian DNA topoisomerase I. The ability of SAD (**48**) to inhibit the proliferation of several tumor cells and to interfere with DNA topoisomerase I suggested its potential as an anticancer candidate (Hong, 2011).

Liu *et al*. reported isolation of a xanthone dimer skyrin (**49**) and norlichexanthone (**50**) (Figure 10) from the extract of the endophytic fungus *Talaromyces* sp. ZH-154, from the mangrove tree *K. candel*. Both xanthones showed significant antimicrobial activity against *Pseudomonas aeruginosa* (Xu, 2011). Li *et al*. isolated a new xanthone derivative, 8-hydroxy-3-methyl-9-oxo-9*H*-xanthene-1-carboxylic acid methyl ether (**51**) (Figure 10), from the coculture broth of two mangrove fungi (strain No. K38 and E33) collected from the South China Sea coast. This xanthone was evaluated for its antifungal activity against five representative fungi: *Gloeasporium musae*, *Blumeria graminearum*, *F. oxysporum*, *Peronophthora cichoralearum*, and *Collectotrichum glocosporioides*) using the disk assay

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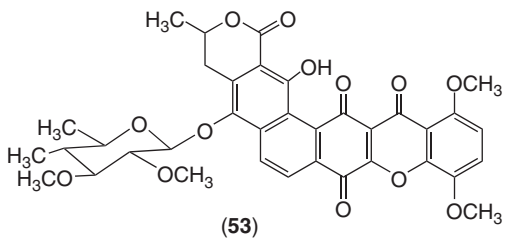
method. Xanthone **51** was found to exhibit broad inhibitory activity against these microorganisms, especially *G. musae* and *P. cichoralearum* (Li *et al*., 2011).

Another fungal genus that produces many types of bioactive metabolites is *Trichoderma* (Hypocreaceae). Khamthong *et al*., in their effort to search for biologically active compounds from a marine-derived fungi, reported isolation of trichodermaxanthone (**52**) from the broth extract of the marine-derived fungus *Trichoderma aureoviride* PSU-F95 (Figure 10) (Khamthong *et al*., 2012a,b).

2.2 From Marine-Derived Actinomycetes

2.2.1 From the Genus Actinomadura (Thermomonosporaceae)

Rodríguez *et al*. reported isolation of the new natural polycyclic xanthone IB-00208 (**53**) (Figure 11) from the fermentation broth of the actinomycete *Actinomadura* sp., isolated from the northern coast of Spain. IB-00208 (**53**) showed a potent cytotoxic activity against several tumor cell lines of both human: A-549 (human lung adenocarcinoma), HT-29 (human colon adenocarcinoma), and SK-MEL-28 (human melanoma); and murine: P-388 (murine leukemia). It also showed a good antibacterial activity against Gram-positive organisms (*S. aureus*, *B. subtilis*, *Micrococcus luteus*), but poor activity against Gram-negative bacteria (*E. coli*, *Klebsiella pneumonia*, *P. aeruginosa*) (Malet-Cascón *et al*., 2003; Rodríguez *et al*., 2003; Masters and Bräse, 2012).



(**53**)

Figure 11 The polycyclic xanthone IB-00208 (**53**) from *Actinomadura* sp.

2.2.2 From the Genus Streptomyces (Streptomycetaceae)

With the aim of discovering novel antibacterial natural products from marine bacteria, Liu *et al*. isolated four new polycyclic antibiotics: citreamicin θ A (**54**), citreamicin θ B (**55**), citreaglycon A (**56**), and dehydrocitreaglycon A (**57**) (Figure 12), from marine-derived *Streptomyces caelestis*, isolated from the coastal water of the Red Sea near Jeddah.

All four compounds were found to display antibacterial activity against *Staphylococcus haemolyticus*, *S. aureus*, and *B. subtillis*. Citreamicin θ A (**54**), citreamicin θ B (**55**), and citreaglycon A (**56**) also exhibited low MIC values of 0.25, 0.25, and 8.0 $\mu g\,mL^{-1}$, respectively, against methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300. Moreover, citreamicin θ A (**54**) and citreamicin θ B (**55**) were found to exhibit significant cytotoxic activity against HeLa cells (human cervical cancer) with IC_{50} values of 0.055 and 0.072 $\mu g\,mL^{-1}$, respectively (Liu *et al*., 2012a,b).

3 EXTRACTION, ISOLATION, AND PURIFICATION OF XANTHONES FROM MARINE-DERIVED MICROORGANISMS

3.1 Extraction of Xanthones from the Culture Media

The majority of xanthones have been isolated from the marine organisms-associated fungi and few of them were reported from the marine actinomycetes (Rodríguez *et al*., 2003; Liu *et al*., 2012a,b). Consequently, the methods of extraction of these compounds depended on the type of the culture media. When the fungi were cultured in solid media, the fungal biomass and media were first homogenized and extracted with organic solvents such as acetone or ethyl acetate. The group of König has used the solid media such as malt-yeast agar (MYA) medium (Pontius *et al*., 2008a,b) and biomalt medium (Krick *et al*., 2007; Kralj *et al*., 2006; Abdel-Lateff *et al*., 2003) to culture the algicolous fungi to produce the bioactive secondary metabolites. The fungal biomass and the media were then homogenized using Ultra-Turrax apparatus, and the mixture was extracted exhaustively with ethyl acetate to produce crude extracts. Ueda *et al*. used the solid medium containing brown

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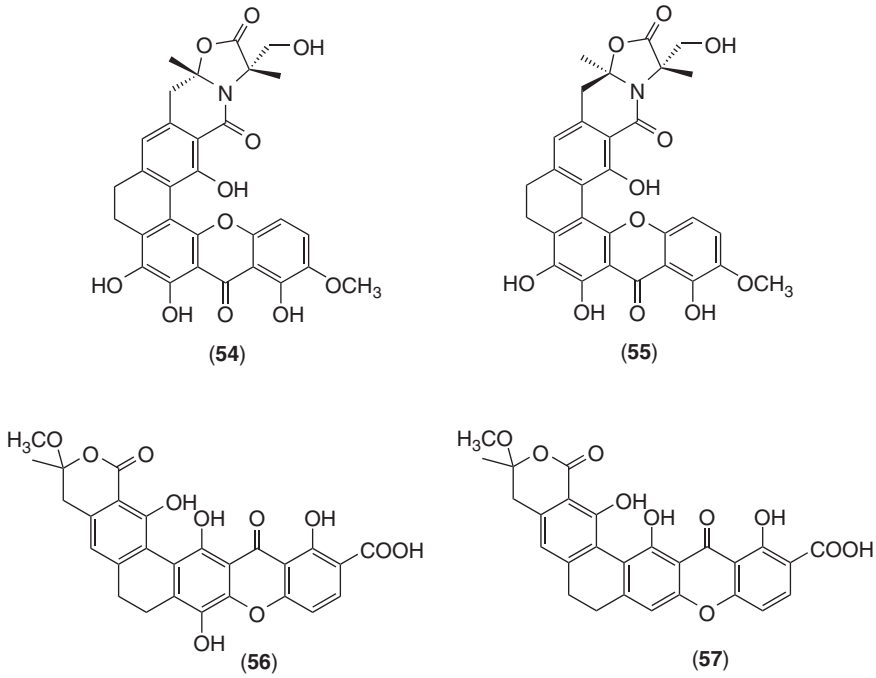

Figure 12 Xanthones from the marine-derived actinomycetes *Streptomyces caelestis*.

rice, bacto-yeast extract, sodium tartrate, potassium hydrogen phosphate, and water, for a static culture of the marine sponge-derived fungus *Tritirachium* sp. SpB081112MEf2. The solid culture was then extracted with 80% aqueous acetone, and after concentration *in vacuo*, the aqueous concentrate was extracted with ethyl acetate to give a crude ethyl acetate extract (Ueda, Takagi, and Shin-ya, 2010). Malmstrøm *et al*. used the yeast extract sucrose (YES) medium to culture the marine-derived strain of the fungus *E. variecolor* to investigate its secondary metabolites (Malmstrøm *et al*., 2002). Then, mycelium and agar were harvested and extracted with a mixture of ethyl acetate, chloroform, and methanol (3 : 2 : 1) containing 1% of formic acid in a Stomacher bag. After evaporation of the solvent, the dried extract was split into two fractions by partition between aqueous methanol solution and hexane to give the hexane and methanol crude extracts.

On the other hand, when the fungi were cultured in liquid media, the mycelia were first separated from the culture broth by filtration before extraction by organic solvents. Bringmann *et al*. used the WSA liquid medium to culture the fungus *E. variecolor* and isolated from the marine sponge *H. valliculata* (Bringmann *et al*., 2003). After separation from the culture medium, the fungus was extracted exhaustively with a 1 : 1 mixture of dichloromethane and methanol, whereas the medium was extracted three times with ethyl acetate. Both extracts were then dried *in vacuo* and partitioned between aqueous methanol and petroleum ether. The methanol fraction of the medium extract was desalted by partitioning between water and ethyl acetate to give the ethyl acetate crude extract. Zhu and Li used the liquid medium containing glucose, peptone, yeast extract, and sodium chloride, to grow the marine-derived mangrove endophytic fungus (stain 1850). After incubation at 30 °C for 35 to 40 days, the mycelia were separated from the culture broth by filtration through cheese cloth. The mycelia were dried by air and extracted with methanol to give crude mycelial extract. The culture filtrate was then concentrated below 50 °C and extracted by shaking with an equal volume of ethyl acetate to give crude broth extract (Zhu and Lin, 2007). Shao *et al*. also used the same type of medium (glucose, peptone, yeast extract, and sodium chloride) and extraction process to isolate two new xanthone derivatives from the endophytic fungus *Penicillium* sp. (ZZF32#), isolated from the

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bark of the mangrove tree *A. ilicifolius* from the South China Sea (Shao *et al*., 2008). Wen *et al*. used the same type of liquid medium to culture the fungus *Paecilomyces* sp. (Tree1–7), isolated from mangrove saprophytic bark from the Taiwan Strait. After 30 days of incubation at 28°C, the culture was filtered through cheese cloth to separate mycelia from the culture broth. After air drying, the mycelia were dipped in methanol, and the filtrate was concentrated *in vacuo* below 55°C and extracted five times by shaking with equal volume of ethyl acetate (Wen *et al*., 2008). Figueroa *et al*. cultured the fungus *Emericella* sp. strain 25379, isolated from the surface of a coral collected at Marietas Islands (Mexico), in a liquid medium composed of Czapek concentrate, potassium hydrogen phosphate, powdered yeast extract, and sucrose. The culture broth and the mycelia were then separated by filtration through cheese cloth. Both the culture broth and the mycelia were extracted with dichloromethane (Figueroa *et al*., 2009). The Chinese groups have also used similar liquid media and methods of extraction to study xanthone derivatives from the marine-derived fungi. Huang *et al*. used the liquid GPY medium (glucose, peptone, yeast extract, and sodium chloride) as a culture medium for the endophytic fungi, isolated from the mangrove tree *A. marina* from South China Sea. After incubation for 30 days at room temperature, the cultures were separated into mycelia and filtrate by filtration through the cheese cloth (Huang *et al*., 2010a, 2010b). The filtrate was then concentrated below 50 °C and extracted by shaking with equal volume of ethyl acetate. Collection and evaporation of ethyl acetate *in vacuo* yielded the crude extract. Pan *et al*. used not only the same liquid medium (GPY) to culture the mangrove endophytic fungus, *Phoma* sp. SK3RW1M, but also a similar method for extraction of xanthones from the culture medium (Pan *et al*., 2010). Li *et al*. also used the liquid GPY medium to coculture the marine fungi (strain Nos. E33 and K 38) and the same method of extraction of xanthones from the filtrate (Li *et al*., 2011). Trisuwan *et al*. cultured the marine-derived fungus *A. sydowii* PSU-F154, isolated from a sea fan *Annella* sp., collected from the coastal area of Southern Thailand in the potato dextrose broth at room temperature for 4 weeks. After filtration, the filtrate and mycelia were extracted with ethyl acetate to afford broth and mycelial ethyl acetate crude extracts (Trisuwan *et al*., 2011). Using the same methods of culture and extraction, Khamthong *et al*. were able to isolate several xanthones from the culture of the marine-derived fungus *P. citrinum* PSU-F51, isolated from the gorgonian sea fan *Annella* sp., collected from the Similan islands in Southern Thailand (Khamthong *et al*., 2012a,b). Liu *et al*. cultivated the fungus *P. sacculum*, separated from the halophyte *Atriplex* sp., by shaking at 150 rpm and at 24°C in flasks containing the liquid medium composed of potato extract, peptone, yeast extract, glucose, and seawater. After 17 days, the fermented broth was filtered through cheese cloth and separated into supernatant and the mycelia. The mycelia were extracted with acetone to afford the crude extract (Liu *et al*., 2012a,b).

Besides the marine-derived fungi, only few marine-derived actinomycetes were found to produce xanthones. Rodríguez *et al*. cultured *Actinomadura* sp. (strain BL-42-PO13-046), an actinomycete isolated from the Northern coast of Spain in a shake flasks containing liquid medium composed of glucose, tryptone, calcium carbonate, sodium chloride, monobasic potassium phosphate, and distilled water at pH 7. After completion of cultivation, whole harvested broth was filtered with diatomaceous earth. The mycelial cake was then extracted with a 2 : 1 : 1 mixture of chloroform, methanol, and water. After filtration, the organic layer was concentrated under reduced pressure to give a brownish oily residue (Rodríguez *et al*., 2003). Recently, Liu *et al*. used a liquid medium containing starch, peptone, yeast extract, and sea salt to culture *S. caelestis*, a marine-derived actinomycete, collected from the coastal water of the Red Sea. The culture was maintained at 23°C for 5 days after which was filtered with eight layers of cheese cloth. The broth was then extracted with ethyl acetate, and the mycelia were extracted with acetone and methanol (1 : 2 v/v). The extracts from the broth and the mycelia were combined and partitioned between water and hexane. The resulting aqueous residue was further extracted with ethyl acetate to give the crude ethyl acetate extract (Liu *et al*., 2012a,b).

More often than not, the broth and mycelial organic extracts are directly applied on column chromatography for fractionation procedure. However, when the crude extracts are to be directly separated by HPLC, they should be cleaned up or fractionated by solid-phase extraction (SPE). SPE is a very popular technique currently available for rapid and selective sample preparation. The versatility of SPE allows use of this technique for many purposes, such as

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purification, trace enrichment, desalting, derivatization, and class fractionation. The principle of SPE involves partitioning between a liquid (sample matrix or solvent with analytes) and a solid (sorbent) phase. SPE uses the same type of stationary phases as in liquid chromatography columns. Reversed phase (RP) involves a polar or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase. The analyte of interest is typically mid- to nonpolar. Normal phase (NP) involves a polar analyte, a mid- to nonpolar matrix (e.g., acetone, chlorinated solvents, and hexane) and a polar stationary phase (Żwir-Ferenc and Biziuk, 2006). Abdel-Lateff *et al*. used a Bakerbond RP octadecyl C-18 SPE and a gradient elution from water to methanol to fractionate the ethyl acetate extract of the algicolous marine fungus *W. anomalus*, before purification by RPs HPLC to isolate 2,3,6,8-tetrahydroxy-1-methylxanthone (**39**), 2,3,4,6,8-pentahydroxy-1-methylxanthone (**40**), and 3,6,8-trihydroxy-1-methylxanthone (**41**) (Abdel-Lateff *et al*., 2003).

3.2 Isolation and Purification of Xanthones from Crude Extracts

The first step of isolation of secondary metabolites involves fractionation of the crude extract to allow the compounds of similar polarities to be eluted together. Several types of column chromatography are commonly used to fulfill this purpose. The factors determining the method of choice include sample capacity and cost. Low pressure liquid chromatography (LPLC) such as column chromatography is still a major tool for fractionation of the crude fungal extracts to isolate xanthones. In this method, the mobile phase flows down through the stationary phase at an atmospheric pressure without any additional forces either by vacuum or pressure. Several types of stationary phases can be used in LPLC; however, silica gel is the most popular as it is available in relatively low cost and it can be applied to a wide range of compound classes. Malmstrøm *et al*. used column chromatography of silica gel to isolate shamixanthone (**27**) from the less polar fraction and varixanthone (**31**) and tajixanthone hydrate (**28**) from the most polar fraction of the crude extract of *E. variecolor* (Malmstrøm *et al*., 2002). Zhu and Li also used silica gel column with gradient elution from petroleum ether to ethyl acetate as a fractionation process to isolate the xanthones sterigmatocystin (**2**), dihydrosterigmatocystin (**3**), and secosterigmatocystin (**46**) from the ethyl acetate crude extract of a marine-derived mangrove endophytic fungus (Zhu and Lin, 2007). Similarly, Shao *et al*., reported isolation of 8-(methoxycarbonyl)-1-hydroxy-9-oxo-9*H*-xanthene-3-carboxylic acid (**9**), dimethyl 8-methoxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate (**10**), and methyl 8-hydroxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate (**11**), using silica gel column chromatography, with gradient elution from petroleum to ethyl acetate, for fractionation of the ethyl acetate crude extract of the fungus *Penicillium* sp. (ZZF32#), followed by preparative separation on silica gel thin layer chromatography (TLC) (Shao *et al*., 2008). Wen *et al*. also used silica gel column chromatography, with gradient elution from petroleum ether to ethyl acetate, to isolate paeciloxanthone (**34**), emodin, and chrysophanol, from the crude ethyl acetate extract of the marine mangrove fungus *Paecilomyces* sp. (Tree1-7) (Wen *et al*., 2008). In their search for CaM inhibitors, Figueroa *et al*. have first fractionated the dichloromethane crude extract of the fungus *Emericella* sp. by silica gel open column with hexane-dichloromethane-ethyl acetate gradient, followed by further fractionation by another silica gel column or Sephadex LH-20 column (eluted with methanol) before final purification by crystallization, preparative TLC of silica gel or by RP-HPLC, to isolate four xanthones: 15-chlorotajixanthone hydrate (**25**), 14-methoxytajixanthone (**26**), shamixanthone (**27**), and tajixanthone hydrate (**28**) (Figueroa *et al*., 2009). Huang *et al*. (2010b) reported isolation of 1,7-dihydroxy-2-methoxy-3-(3-methylbut-2-enyl)-9*H*-xanthen-9-one (**42**) and 1-hydroxy-4,7-dimethoxy-6-(3-oxobutyl)-9*H*-xanthen-9-one (**43**) from the mangrove endophytic fungus No. ZH19 and 3,5,8-trihydroxy-2,2-dimethyl-3,4,4-trihydro-2*H*, 6*H*-pyrano[3,2-b]xanthen-6-one (**44**) and 5,8-dihydroxy-2,2-dimethyl-2*H*,6*H*-pyrano[3,2-b] xanthen-6-one (**45**) from the mangrove endophytic fungus No. ZSU-H16 (Huang *et al*., 2010a), using column chromatography of silica gel and gradient elution from petroleum ether to ethyl acetate to fractionate the ethyl acetate crude extracts before purification by preparative TLC of silica gel. Pan *et al*. and Li *et al*. used the same method of fractionation of crude extracts to isolate various xanthones from the mangrove endophytic fungus *Phoma* sp. and from the coculture broth of two marine fungi, respectively (Pan *et al*., 2010; Li *et al*., 2011). Liu *et al*. also used the silica gel column chromatography with

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gradient elution from chloroform to methanol for the initial fractionation of the crude mycelial acetone extract of the marine-derived fungus *P. sacculum*. The obtained fractions were further fractionated by Sephadex- LH 20 and then purified by HPLC, to give 1-hydroxy-3-methoxy-6-sulfo-8-methylxanthone (**16**) (Liu *et al*., 2012a,b).

Another useful stationary phase is the hydroxylpropylated dextran gel Sephadex LH-20®, which has been widely used in separation of natural products, including xanthones. Owing to its relatively high cost, this gel is not normally used for fractionation of the crude extracts but instead for purification of fractions first obtained from the silica gel column chromatography. However, Trisuwan *et al*. and Khamthong *et al*. used the column chromatography of Sephadex LH-20, with methanol as a mobile phase, to fractionate crude extracts of the fungi *P. citrinum* and *A. sydowii* (Trisuwan *et al*., 2011; Khamthong *et al*., 2012a, 2012b).

Medium pressure liquid chromatography (MPLC) is also commonly used for preliminary fractionation of crude fungal extracts because of its lower cost and higher throughput. Ueda *et al*. used NP-MPLC, with a stepwise solvent system of *n*-hexane–ethyl acetate (3 : 1) and chloroform–methanol (49 : 1), for fractionation of the ethyl acetate crude extract of the marine sponge-derived fungus *Tritirachium* sp., before purification of the new xanthones by NP-TLC and RP-HPLC (Ueda, Takagi, and Shin-ya, 2010). Lee *et al*., in their investigation on the bioactive metabolites from the sponge-derived fungus *A. versicolor*, used a stepped-gradient (50% to 100% methanol elution) RP-MPLC for the preliminary fractionation of the crude ethyl acetate extract, followed by purification of the obtained fractions by a RP-HPLC to yield the xanthones sterigmatocystin (**2**) and dihydrosterigmatocystin (**3**), besides anthraquinone derivatives (Lee *et al*., 2010).

Vacuum liquid chromatography (VLC) is another widely used method for fractionation of crude extracts. VLC is a column chromatography to which the vacuum is applied to increase flow rate and, thus, speed up the fractionation procedure. Another advantage of VLC is that application of the extract and eluents is easily achieved owing to the open end of the column. Besides, both NP and RP silica gel can be used as stationary phase. Pontius *et al*. used NP-VLC (Merck silica gel 60, 63–200 µm, and petroleum ether–acetone–methanol gradient elution) to fractionate the crude ethyl acetate extract of the fungal culture of *Chaetomium* sp. and the RP-VLC with methanol–water gradient to further fractionate the fractions obtained from the first column, before final purification of chaetoxanthones (**35–37**) by RP(-18)-HPLC (Pontius *et al*., 2008a, 2008b). Krick *et al*. also used VLC of silica gel 60 (63–200 µm) and a gradient elution from petroleum ether to ethyl acetate, acetone, and methanol to fractionate the ethyl acetate crude extract of the culture of the marine algicolous fungus *M. putredinis*, before purification with NP- and RP-HPLC to obtain monodictysins A, B, and C (**21–23**), monodictyxanthone (**24**), and monodictyphenone (Krick *et al*., 2007). In the same way, Kralj *et al*. used the VLC of silica gel 60 of 63–200 µm particle size and eluting with a gradient of dichloromethane–ethyl acetate and methanol for preliminary fractionation of the crude ethyl acetate extract of another algicolous fungus *E. nidulans* var. *acristata*, followed by further fractionation of the obtained fractions with Sephadex LH-20 column, to isolate several secondary metabolites including prenylated xanthones [shamixanthone (**27**) and emericellin (**29**)] (Kralj *et al*., 2006). Another type of column chromatography used for preliminary fractionation of crude extracts and coarsely separated fractions is flash chromatography (FC). In this method, nitrogen or compressed air are applied on top of the column to force the mobile phase to flush through the tightly packed stationary phase. Similar to VLC, both NP and RP silica gel can be used as stationary phase for FC. Besides being a rapid method of fractionation, the increasing peak resolution is normally obtained as it can use the stationary phase of the smaller particle size (e.g., 40 µm silica gel). Using vacuum flash chromatography (VFC) eluted with a stepwise gradient of ethyl acetate–methanol, followed by purification with LPLC of silica gel and eluted with mixtures of chloroform–methanol, Rodríguez *et al*. have isolated a cytotoxic polycyclic xanthone from the crude extract of a marine-derived *Actinomadura* (Rodríguez *et al*., 2003). Liu *et al*. used a RP C-18 FC and eluted with a gradient of water to methanol, for fractionation of the ethyl acetate fraction of the marine-derived actinomycetes *S. caelestis*. The eluted fractions from the FC were then further fractionated by a LPLC of Sephadex LH-20, using methanol as eluent before final purification by RP semipreparative HPLC, to give the antibacterial xanthone derivatives citreamicin θ A (**54**), citreamicin θ B (**55**), citreaglycon A (**56**), and dehydrocitreaglycon A (**57**) (Liu *et al*., 2012a,b).

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After fractionation of crude extracts by either of the methods described above (LPLC, MPLC, VLC, and FC), these coarsely purified fractions can be further fractionated by column chromatography of either silica gel or Sephadex LH-20, to yield less complex fractions, which will be ultimately purified by crystallization, preparative TLC or HPLC, depending on the amount and nature of the target compounds in the fractions. Because many xanthones are crystalline or solid, crystallization in an appropriate mixture of organic solvents can be achieved very easily. This method is not only less costly but also yields a final product with higher degree of purity. On the contrary, if the xanthone does not exist in solid form, preparative TLC of silica gel is a method of choice as it is rapid and less expensive. As most xanthones can be detected by a ultraviolet (UV) lamp at 254 nm, it is easy to isolate them from the matrix on the TLC plate, and consequently, many marine-derived xanthones were purified by this method (Zhu and Lin, 2007; Shao *et al*., 2008; Figueroa *et al*., 2009; Huang *et al*., 2010b; Ueda, Takagi, and Shin-ya, 2010; Khamthong *et al*., 2012a,b). However, it should bear in mind that the impurities in the silica gel used for preparative TLC can compromise the purity of the final product, especially when the desirable compounds exist in a very small concentration. In this case, HPLC should be considered as a preferred method for purification and both NP- and RP-HPLC have been widely used to purify the marine-derived xanthones (Abdel-Lateff *et al*., 2003; Bringmann *et al*., 2003; Krick *et al*., 2007; Pontius *et al*., 2008a,b; Lee *et al*., 2010; Ueda, Takagi, and Shin-ya, 2010; Liu *et al*., 2012a,b).

4 STRUCTURE ELUCIDATION OF XANTHONES FROM MARINE-DERIVED MICROORGANISMS

In relation to xanthones isolated from terrestrial organisms (Vieira and Kijjoa, 2005), the number of xanthones isolated from marine organisms is comparatively small and almost all of them were isolated from marine organisms-associated or endophytic mangrove fungi. Both simple oxygenated and prenylated xanthones have been reported from the marine sources. Similar to their terrestrial counterparts, the structure elucidation of the marine-derived xanthones is based, mainly, on 1D (^{1}H, ^{13}C NMR, and DEPT experiments) and 2D (COSY, HSQC, HMBC, and NOESY) NMR spectral analysis. Silva and Pinto have summarized the use of the NMR techniques for structure assignment of xanthones and the typical proton and carbon chemical shifts of simple and prenylated xanthones (Silva and Pinto, 2005).

Generally, with the ^{1}H and ^{13}C NMR spectra, one can decide if the compound under study is a simple oxygenated or prenylated xanthone. For simple oxygenated xanthones, their ^{1}H NMR spectra normally exhibit the signals of aromatic protons about δ6.2–9.0, depending on the number, nature, and positions of the substituents on the benzene rings. The number of the aromatic protons exhibited in the ^{1}H NMR spectrum can also determine the degree of substitution. The multiplicity and coupling constants of the aromatic protons can be useful for determination of the substitution pattern on rings A and B, and this can be confirmed by cross peaks observed in the COSY spectrum. In addition, the chemical shift values of the hydroxyl protons can also be indicative of its position. The hydrogen bonding hydroxyl group in the *peri* position adjacent to the carbonyl carbon (C-9) normally appears as a singlet around δ12–14, whereas the hydroxyl group at other positions appears as broad singlet around δ9–10.

Considering the xanthone scaffold (Figure 1), it is obvious that the ^{13}C NMR spectrum is very useful to determine if the compound under investigation is a xanthone derivative because the xanthone nucleus contains one carbonyl carbon (C-9) and twelve sp^2 hybridized carbons, two of which are oxygen bearing (C-4a and C-10a). Together with DEPT 90° experiment, it is possible to deduce the degree of substitution as this spectrum exhibits only the signals of the methine sp^2 carbons (CH). The ^{13}C NMR chemical shift values are also helpful to establish the position of the substituents. The carbonyl carbon (C-9) of xanthones with a hydroxyl group in the *peri* position adjacent to it appears at δ80–185, whereas the carbonyl carbon without the hydroxyl group in this position normally resonates around δ174–175 (Kijjoa *et al*., 2000a,b). However, it is not easy to predict the chemical shifts of the oxygen bearing quaternary sp^2 carbons of the xanthone nucleus (C-4a and C-10a), which are found to resonate at δ144–160, depending on the nature and position of substituents on each ring. On the contrary, the carbon chemical shift of the methoxyl substituent is very useful to determine its position. The sterically compressed

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methoxyl groups, that is, when they are flanked by two oxygenated substituents or one oxygenated substituent and a carbonyl group, are found to have resonance at δ61–63; otherwise, they are found at δ55–57 (Kijjoa *et al*., 1998). Although the proton and carbon chemical shifts from the ^{1}H and ^{13}CNMR spectra were important in deducing the structures of myriad of xanthones, 2D NMR (COSY, HSQC, HMBC, and NOESY) have been used to elucidate unambiguously not only simple oxygenated and prenylated xanthones but also xanthones with complex structures. Through the diagonal cross peaks in the COSY spectrum, it is possible to observe the proton coupling systems which, in turn, allow us to identify the substitution pattern on the benzene rings of the xanthone scaffold. On the other hand, the HSQC cross peaks enable us to pinpoint the proton directly bonded to a particular carbon. Therefore, a combination of COSY and HSQC spectra could be used to determine the structures of fragments of molecule composed entirely of protonated carbons; however, the different fragments could not be assembled into a full structure when these fragments are separated by nonprotonated carbons or heteroatoms (Breton and Reynolds, 2013). Thus, the HMBC spectrum, whose cross peaks allow us to observe the connectivity of the proton and the carbons separated by two or three bonds, is fundamental to solve this problem. The cross peaks between the aromatic, hydroxyl, methoxyl protons and the aromatic and carbonyl carbons in the substituted xanthones can be used to establish the position of the hydroxyl and methoxyl groups on the ring. In addition, the HMBC cross peaks can be also useful to determine the position of the prenyl and modified prenyl substituents (Figueroa *et al*., 2009). NOESY experiment, which based on the nuclear Overhauser enhancement between the protons in close proximity, is sometimes very important to determine the positions of the substituents on the xanthone nucleus (Figueroa *et al*., 2009), in addition to the relative configuration of the stereogenic carbons of the substituents of the xanthone derivatives. Liu *et al*. used the NOESY cross peaks between the methyl protons on C-3 (CH_3-21) and C-18 methylene protons to determine the relative configuration of C-3 and C-19 of citreamicin θ B (**55**) (Liu *et al*., 2012a,b). In order to determine the absolute configuration of the stereogenic carbons of the substituents of xanthone derivatives by NMR, a modified Mosher method has been used (Ohtani *et al*., 1991). This method is based on the differences between the proton chemical shifts in the (*R*)-MPA and in the (*S*)-MPA esters. Pontius *et al*. applied this method to determine the absolute configuration of C-3′ of the tetrahydropyran moiety of chaetoxanthone A (**35**) (Pontius *et al*., 2008a,b), whereas Figueroa *et al*. applied the advanced Mosher's methodology to determine the absolute configuration at C-20 and C-25 of 14-methoxytajixanthone (**26**) (Figueroa *et al*., 2009). Besides the NMR methods, the positive and negative Cotton effects observed in the circular dichroism (CD) spectrum is very useful to prove the configuration of the stereogenic carbons. Liu *et al*. used the CD curve to observe the opposite Cotton effects of citreamicin θ A (**54**) and citreamicin θ B (**55**), which led to the conclusion that they were diastereoisomers (Liu *et al*., 2012a,b). On the other hand, Pontius *et al*., by comparison of the CD curves of chaetoxanthone A (**35**) and chaetoxanthone B (**36**), have concluded that the latter was isolated as a racemate (Pontius *et al*., 2008a,b).

Electron impact (EI) and chemical ionization (CI) low resolution mass spectra (LRMS) had been widely used to determine the molecular weight of xanthone derivatives. The limitation of these techniques is that they do not provide an accurate mass of a molecule and, hence, its molecular formula. On the contrary, high resolution mass spectrum (HRMS) gives an accurate molecular mass and, thus, provides the molecular formula of the compounds. Consequently, HRMS has proved to be vital for structure elucidation of all classes of secondary metabolites, and therefore there is no exception for xanthone derivatives, especially those isolated from marine organisms as they sometimes contain chlorine, bromine, nitrogen, and sulfur atoms (Figueroa *et al*., 2009; Liu *et al*., 2012a,b). From the molecular formula obtained from HRMS, one can determine the degree of unsaturation of the compound, and because the xanthone nucleus has 10° of unsaturation, the total degree of unsaturation of the molecule can allow us to determine the nature of the substituents, for example, cyclic/noncyclic or saturated/unsaturated. There are a few HRMS techniques used to determine the molecular mass/molecular formula of small molecules such as xanthones, depending mainly on the type of the ion source. HR-EIMS, which uses a hard source (incident energy 70 eV) for ionization, gives the *m*/*z* value of a molecular ion [M^+]. Although electron impact causes extensive fragmentation of a molecule, it normally gives a strong

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molecular ion peak [M^+] for simple xanthones (Kijjoa *et al*., 2000a,b). On the other hand, fast atom bombardment (FAB), which uses high energy argon or xenon atoms to bombard molecules in the ion source, has been used to determine the molecular mass of unstable large and small molecules. $^+$FAB-HRMS, which gives a strong $[M+H]^+$ ion, has been used to determine the molecular formula of many xanthone derivatives. Recently, electrospray ionization (ESI) technique, which uses soft ionization method to obtain stable molecular ion, was developed. HR-ESIMS is now widely used to determine an accurate mass of the $[M+H^+]$ ion and molecular formula of large and small thermally fragile molecules. Liu *et al*. used HR-ESIMS to determine the molecular formula of polycyclic xanthones isolated from the marine-derived actinomycetes *S. caelestis* (Liu *et al*., 2012a,b).

Even though infrared (IR) and UV spectra are not currently the first choice for structure elucidation of secondary metabolites, including xanthones, they can also give some valuable structural information. Xanthone derivatives exhibit characteristic absorptions of the conjugated carbonyl (C=O stretching) at 1700–1720 cm^{-1} or the hydrogen-bonded carbonyl (with the hydroxyl group on C-1 or C-8) at 1650–1660 cm^{-1} and aromatic at 3000–3100 cm^{-1} (C–H stretching) and 1450–1600 cm^{-1} (C=C stretching) in the IR spectra. Xanthones with a hydroxyl substituent also exhibit absorption band characteristic of the hydroxyl group at 3300–3500 cm^{-1} (O–H stretching). Xanthones also exhibit characteristic absorptions of extended conjugated aromatic system in the UV spectrum at 200–400 nm, and the patterns of absorption are dependent on the number and positions of the hydroxyl and methoxyl groups, but in general, there is a strong absorption above 340 nm (Dean, 1963). Kijjoa *et al*. used the effects of shift reagents on the UV absorption maxima to determine the position of the hydroxyl substituents (Kijjoa *et al*., 2000a,b). Similar to flavonoids, xanthones containing hydroxyl group adjacent to the carbonyl group, that is, on C-1 or C-8, form acid stable complexes with $AlCl_3$. Furthermore, Kijjoa *et al*., after being unable to distinguish between the structures of 3,8-dihydroxy-1,2,4-trimethoxyxanthone and 2,8-dihydroxy-1,3,4-trimethoxyxanthone by analysis of HMBC correlations, used the UV spectrum with shift reagent to solve the structural problem. Because the UV spectrum of the compound exhibited a pronounced bathochromic shift on addition of sodium acetate solution, which is characteristic of a phenolic hydroxyl group *para* to the carbonyl carbon, they have concluded that the structure of the compound was 3,8-dihydroxy-1,2,4-trimethoxyxanthone (Kijjoa *et al*., 2000a).

Another important technique for structure elucidation is X-ray crystallography. This technique is useful not only to elucidate conclusively the structure of the compounds but also to determine the absolute configuration of the stereogenic carbons. Because the xanthone scaffold does not have stereogenic carbon, the single crystal X-ray diffraction is not widely used to elucidate the structure of xanthone derivatives. However, this technique can be valuable for xanthone derivatives with complex structure, especially those having stereogenic carbons. Kijjoa *et al*. obtained the X-ray crystal structure of 7-hydroxy-1,2,3,8-tetramethoxyxanthone, which confirmed the structure elucidated by NMR spectral analysis (Kijjoa *et al*., 1998). Gales and Damas have reviewed the X-ray diffraction data of 47 xanthone derivatives (Gales and Damas, 2005). Krick *et al*. obtained the X-ray crystal structure of the xanthone derivative monodictysin A (**21**), which allowed them to confirm its relative configuration found on the basis of NMR data but could not determine its absolute configuration reliably (Krick *et al*., 2007). Shao *et al*. was able to obtain the X-ray crystal structure of methyl 8-hydroxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate (**11**), which was used to confirm its structure elucidated by NMR methods (Shao *et al*., 2008). Pan *et al*. also used the X-ray crystal structure to confirm the structure of 1-hydroxy-8-(hydroxymethyl)-3-methoxy-6-methyl-9*H*-xanthen-9-one (**33**), isolated from a mangrove endophytic fungus *Phoma* sp. (Pan *et al*., 2010).

5 CONCLUSION

Xanthones are a class of secondary metabolites, with interesting biological and pharmacological activities, normally found in higher plants and fungi; however, they are not frequently mentioned as marine natural products. This was due to the fact that a vast majority of marine natural products have been traditionally derived from marine macroorganisms such as invertebrates and algae. Only recently, attention has been focused on the marine microorganisms as sources

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of secondary metabolites with unique structure and interesting biological and pharmacological activities. For this reason, marine-derived fungi have emerged as a potential source of marine natural products as they are capable of producing a variety of interesting secondary metabolites. Because marine fungi are normally associated with other marine macroorganisms, it is also believed that they are true producers of the compounds isolated thereof. Similar to their terrestrial counterparts, marine-derived fungi also produce xanthones. Although many common xanthones have been isolated from both marine-derived and terrestrial fungi, some xanthones with complex structure and rare substituents have been found only in the marine-derived fungi and actinomycetes.

Therefore, the chemical diversity allied with a myriad of biological and pharmacological activities of marine-derived xanthones can make the research in this field more challenging. Furthermore, this class of compounds could provide the core scaffolds for the development of new drugs. While the marine world offers an extremely rich resource for novel compounds, it also represents a large field of research that requires inputs from various scientific areas to bring the marine chemical diversity up to its therapeutic potential.

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