

Long-chain wax esters and diphenylamine in fire coral *Millepora dichotoma* and *Millepora platyphylla* from Saudi Red Sea Coast*

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SUMMARY: The characterization of the non-protein constituents of two species of fire corals *Millepora dichotoma* and *Millepora platyphylla*, exhibits very interesting results. The compounds were identified by gas chromatography-mass spectrometry (GCMS) and nuclear magnetic resonance (NMR) spectroscopy. The solvent extracts of the two species revealed four wax esters. The compounds were identified as C₃₀H₆₀O₂, C₃₂H₆₄O₂, C₃₄H₆₈O₂ and C₃₆H₇₂O₂, respectively. The presence of these compounds has been reported previously in different marine organisms as well as in marine samples. It is interesting that there were some variations in the number and nature of isomers of similar wax esters reported earlier. Long-chain wax esters are normally waxy in nature and their presence in fire coral plays a vital role in the nutrient transfer to the coral mass. They may also act as a protective coating of the nematocyst of dactylozooid. The coral species were also subjected to mild acidic hydrolysis, followed by neutralization and partitioning between water and ether. The organic phase was dried and purified by column chromatography and thin layer chromatography (TLC). Diphenylamine was revealed as the main product in one of the fractions. It is worth noting that diphenylamine is reported for the first time as a marine natural product. Diphenylamine is known to be toxic and causes allergic reactions to the skin, so it can be considered as responsible for the stinging property of fire coral.

Key words: long-chain wax esters, diphenylamine, solvent extraction materials, acid hydrolysate products, fire coral, *Millepora dichotoma*, *Millepora platyphylla*, Red Sea.

INTRODUCTION

Fire corals are the common inhabitants of fringing reefs. The name comes from the burning sensation inflicted by the nematocyst of the dactylozooid or defensive polyp. These corals have long been known to possess a stinging property. Divers have experienced intense pains and rashes on the face and

the skin when coming in contact with such corals. The fire corals were identified as belonging to the genus *Millepora* (Darwin, 1851). Although nine species have been identified world-wide (Lewis, 1989), only two of these species have been observed along the coast of the Saudi Red Sea, i.e. *Millepora dichotoma* and *Millepora platyphylla*. The symbiotic algae were found to be *Gleodinium viscum* (Banazek *et al.*, 1993). As the main constructive element of the coral reefs, corals are capable of com-

*Received November 20, 2000. Accepted September 13, 2001.

plex systems fostered by their close symbiotic relationship with phototrophic zooxanthellae. The interrelationship between the algae and coral determines the mode of feeding. Algae in the coral are responsible for oxygen production and some nutrient transfer (Schlinchter *et al.*, 1995). Considerable amount of research work has been done on the biological aspects of these corals (Wittle *et al.*, 1971, 1974; Frank *et al.*, 1994; Hoegh *et al.*, 1987; Mueller *et al.*, 1983). The chemical characterisation of the protein constituents was carried out by a Japanese group of workers (Shiomi *et al.*, 1989). They studied only the protein part of the fire corals. Recently, the lipid content and particularly the wax esters of the corals from Okinawa, has been reported (Yamashiro *et al.*, 1999). Moreover, the lipid composition of the reef building coral eggs was studied by Arai *et al.*, (1993). They found that the majority of the lipid content of eggs from *Acropora millepora*, *A. tenuis* and *Montipora digitata* was wax esters (69-81%) and ranged from C_{14:0} to C_{22:0}. Wax esters, however, are present widely in many marine phyla and species (Sargent, 1978). Sargent found that this type of lipid is abundant in organisms that experience short periods of food plenty followed by long periods of food shortage. Wakeham and Frew (1982) have also reported the presence of wax esters from marine particulate matter. They found a mixture of saturated and unsaturated wax esters over the range C₂₈-C₄₂. Also, several studies have identified individual saturated wax esters in different marine organisms. For example, hexadecanyl-hexadecanoate (C₃₂) has been identified in soft coral *Sinularia microclavate* from China (Zhujin *et al.*, 1990), while hexadecanyl-octadecanoate (C₃₄) has also been found in soft coral *Sarcophyton* from China (Rosenheng *et al.*, 1982). Bandara *et al.* (1987) found a saturated C₃₆ wax ester from a marine organism from Sri-Lanka. Identification of wax ester structures using different mass spectrometry methods was established (e.g. Aasen *et al.*, 1971; Wakeham and Frew, 1982). These studies concluded that each peak is composed of a number of wax esters differing in alkyl and acyl moieties but having the same total carbon number (Wakeham and Frew, 1982).

This study aimed to investigate the chemical constituents of the two species of *Millepora* from the Saudi Red Sea, firstly by extracting the constituents by normal solvent extraction and secondly by subjecting the coral to mild acid hydrolysis. The extracts and acid hydrolysate were fractionated and purified using chromatography techniques and

examined by gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy.

MATERIALS AND METHODS

The coral species were collected from out side Sharm Obhur, 35 km north of Jeddah, in order to avoid the suspicion of contamination. The two coral species were collected from the fringing reef of Jeddah coast at a depth of about 10 m (Fig. 1). The collection was made during the months of September-December 1999; the average temperature of the surface seawater was 29°C, salinity 39.6‰ and pH 8.16. The specimens were washed with seawater and ground in a mortar to about 1 mm pieces. Lipid was extracted from the two species using chloroform and methanol (1:1). The samples of the two species were soaked in the mixture and kept overnight in a shaker. The supernatant was filtered and the solvent was removed using a rotary evaporator under vacuum at 40°C. The residue of the organic matter was dried under a stream of nitrogen and finally weighed using a microbalance. In another

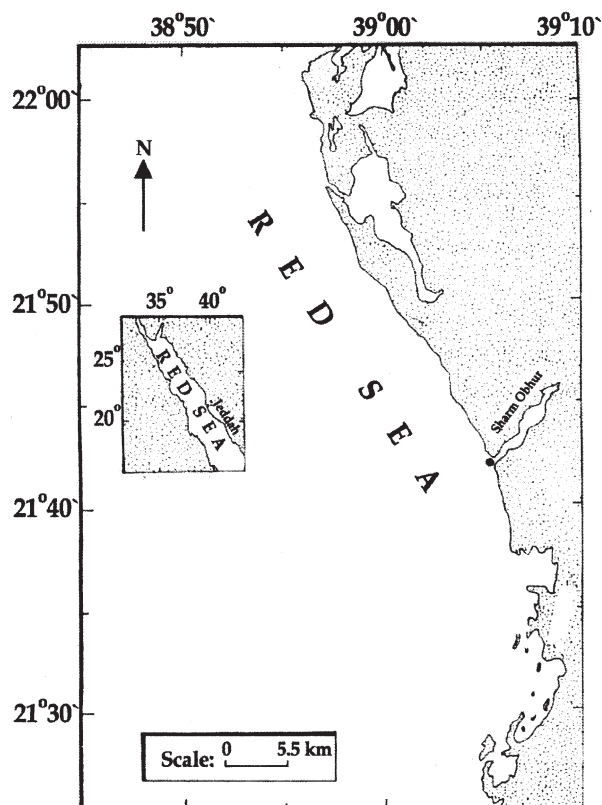


FIG. 1. – Map of the sample collection from the Saudi Red Sea Coast.

er experiment, the residue of the fire coral was dried, weighed and then subjected to acid hydrolyses. An aliquot of the fire coral residue was treated with 2 N hydrochloric acid. The acid was gradually added at intervals till the effervescence of carbon dioxide ceased. The solution was heated on a hot plate at 80°C for 30 minutes. It was cooled and neutralised. Then, it was partitioned with ether (3 x 200 ml), and the ether extract was dried over anhydrous sodium sulphate and evaporated under vacuum at 40°C to a crude gummy material. The concentrate of solvent extraction and the acid hydrolysate were separately chromatographed with TLC on silica gel using benzene: ethyl acetate, (9:11) as the solvent system. The spots were distinctly visible under ultra-violet (UV) light. The top spot was scratched from the TLC plate, and dissolved in chloroform/methanol and then filtered. The filtrate was evaporated under a nitrogen atmosphere. The fraction was further chromatographed on TLC using hexane : chloroform (85:15) as the solvent system. Again the top spot was scratched from the TLC plate and the compounds were extracted and then analysed using GC-MS. The GC-MS analyses were made on a Shimadzu GC-17A gas chromatograph

equipped with a split/splitless injector, and a DB fused silica column (25 m x 0.3 mm i.d., 0.17 µm DB-1% phenyl/ 99% methyl-silicone) using helium carrier gas. The GC conditions were 40-300°C at 5°C min⁻¹ then isothermal for 5 min., and the injector temperature was 250°C. The end of the GC column was introduced into the electron impact (EI) source of a Shimadzu QP-5000 Quadrupole mass spectrometer. Typical mass spectrometer operating conditions were as follows: transfer line 230°C, ion source 250°C, electron energy 70 eV. All samples were analysed in full data acquisition (SCAN) mode by scanning from 50-500 daltons at 1 cycle/s. Identification of the compounds was based on comparison of retention indices and mass spectra of the analytes with literature data (e.g. Hites, 1992; Aasen *et al.*, 1971; Wakeham and Frew, 1982) and in certain cases by co-injection with authentic compounds. The high-resolution mass spectrometry and NMR analyses of the fatty acid esters were performed at the H.E.J. Research Institute of Chemistry, University of Karachi, Pakistan. The conditions of the NMR (Bruker-500 MHz) were MI=0.5 cm, MAX=1000 cm, pc=1.0, and the solvent used was CDCl₃

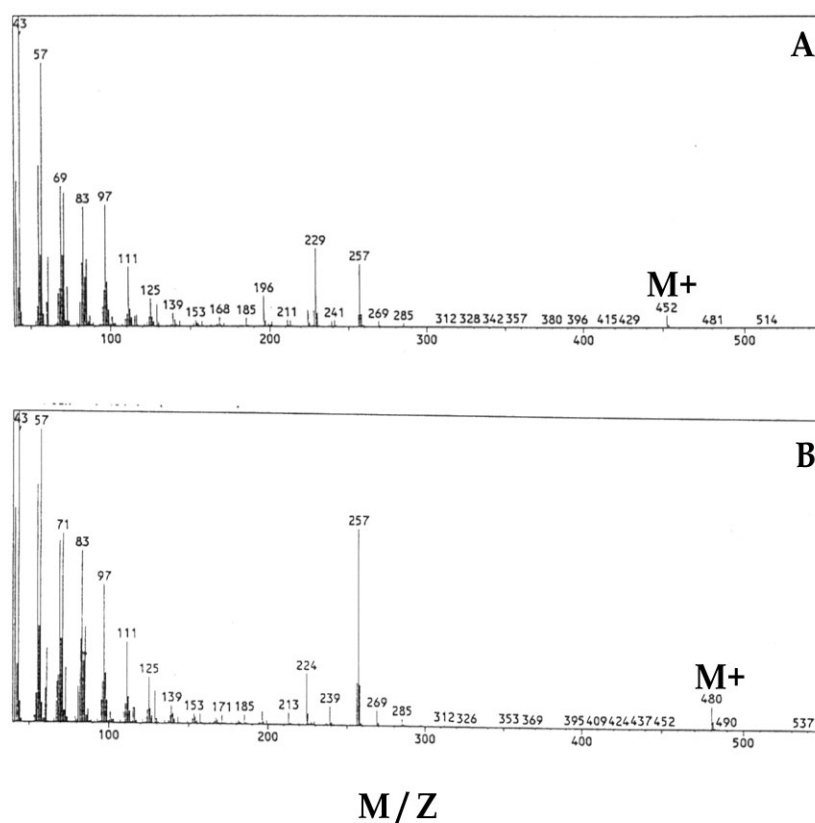


FIG. 2. – Mass spectra (background corrected) of the wax esters isolated from the two species of fire coral from Saudi Red Sea Coast. (A) a mixture of 14/16 and 12/18 (B) 16/16.

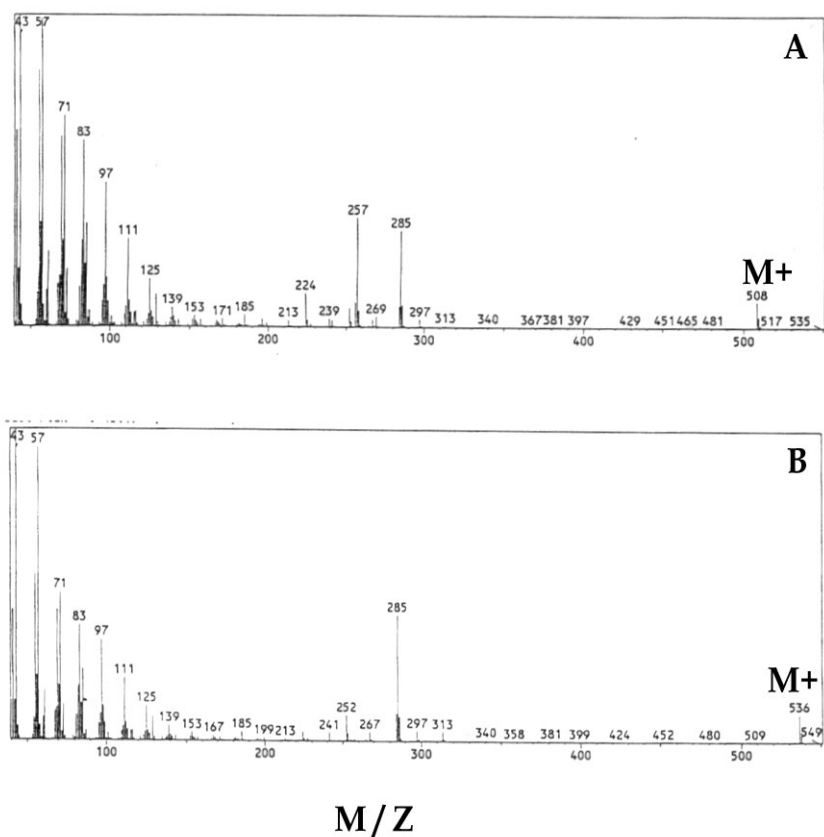


FIG. 3. – Mass spectra (background corrected) of the wax esters isolated from the two species of fire coral from Saudi Red Sea Coast. (A) a mixture of 16/18 and 18/16 (B) 18/18.

RESULTS AND DISCUSSION

The extractable organic matter of the two species of fire coral was fractionated using TLC and three spots were distinctly visible under the UV light. The top layer (high R_f) of the second TLC plate was analysed using GC-MS. Four compounds were identified as long-chain wax esters (Figs. 2 and 3). The structural identification was confirmed by nuclear magnetic resonance analyses. It would be very inter-

esting to compare and contrast the results of our studies with those of other groups workers (Aasen, 1971; Wakeham and Frew, 1982). The latter group have isolated all these four wax esters, but from particulate matter, and have stated that each one of these existed in three isomeric forms. In the present study, the GC-quadrupole spectra were carefully examined to calculate the diagnostic ions, R' being the alkyl group in the alcohol moiety and R being the alkyl group in the acid moiety. The relative

TABLE 1. – Intensities of characteristic ions containing the alcohol moiety in mass spectra of saturated wax esters of fire coral from Saudi Red Sea Coast

M^+		Wax		Ester		$[R' - 1]^+$		CO_2R^+		$CH_2=C^+ \begin{matrix} OH \\ O-R' \end{matrix}$		$CH_2=C^+ \begin{matrix} OH_2 \\ O-R' \end{matrix}$	
Mass	Int.	Alcohol	Acid	Mass	Int.	Mass	Int.	Mass	Int.	Mass	Int.	Mass	Int.
452	4.1	14	16	196	10.0	241	2.0	256	3.9	257	20.6		
		12	18	168	3.1	213	1.8	228	5.3	229	25.7		
480	6.49	16	16	224	14.9	269	4.47	284	0.45	285	1.88		
508	9.5	16	18	224	11.6	269	4.03	284	8.0	285	34.9		
		18	16	252	6.9	297	3.1	312	0.44	313	0.68		
536	9.1	18	18	252	8.5	297	3.3	312	0.67	313	3.04		

TABLE 2. – Intensities of characteristic ions containing the acyl moiety in mass spectra of saturated wax esters of fire coral from Saudi Red Sea Coast.

M+ Mass	Int.	Wax Alcohol	Ester Acid	RCO ₂ H ⁺ -propyl		RCO ⁺		RCO ₂ H ⁺		RCO ₂ H ₂ ⁺	
				Mass	Int.	Mass	Int.	Mass	Int.	Mass	Int.
452	4.1	14	16	213	1.8	239	1.7	256	3.9	257	20.6
		12	18	241	2.0	267	0.1	284	0.59	285	1.1
480	6.49	16	16	213	3.4	239	4.8	256	12.5	257	58.3
508	9.5	16	18	241	2.4	267	2.5	284	8.0	285	34.9
		18	16	213	2.4	239	0.48	256	9.14	257	39.5
536	9.1	18	18	241	2.6	267	2.8	284	9.1	285	40.7

intensities of [R'-H]⁺; CO₂R'⁺; CH=COH-O-R' and CH=COH₂-O-R' were determined (Table 1). Similarly the intensities of (RCO₂H-propyl)⁺; (RCO)⁺; (RCO₂H)⁺ and (RCO₂H₂)⁺ were calculated (Table 2). From these tables (1 and 2) it can be concluded that for ester 30:0 two isomers were possible, 14:0/16:0 and 12:0/18:0, while for the 32:0 wax ester one structure was confirmed, 16:0/16:0. However, two isomers were observed for 34:0 and were 16:0/18:0 and 18:0/16:0. The last compound, 36:0, existed in 18:0/18:0 form. The other isomeric wax esters mentioned by Wakeham and Frew (1982) were not detected in the spectra of the present study. It is very

likely that the lack of these isomers in the coral may be attributed to the specific metabolic pathways. It is noteworthy that the number and nature of isomers of wax esters of the fire coral extract show some variations compared with similar wax esters reported earlier. The NMR spectrum supported the proposed structures. The spectrum was taken in CDCl₃, and 500 MHz indicated the following signals 0.86 (t, CH₃), 1.24 (m, CH₂), 2.27 (t, -CH₂-CO) and 4.04 (t, -OCH₂). The presence of wax esters in marine organisms has been reported by several groups of workers (e.g. Sergeant, 1978; Wakeham and Frew, 1982; Arai *et al.*, 1993).

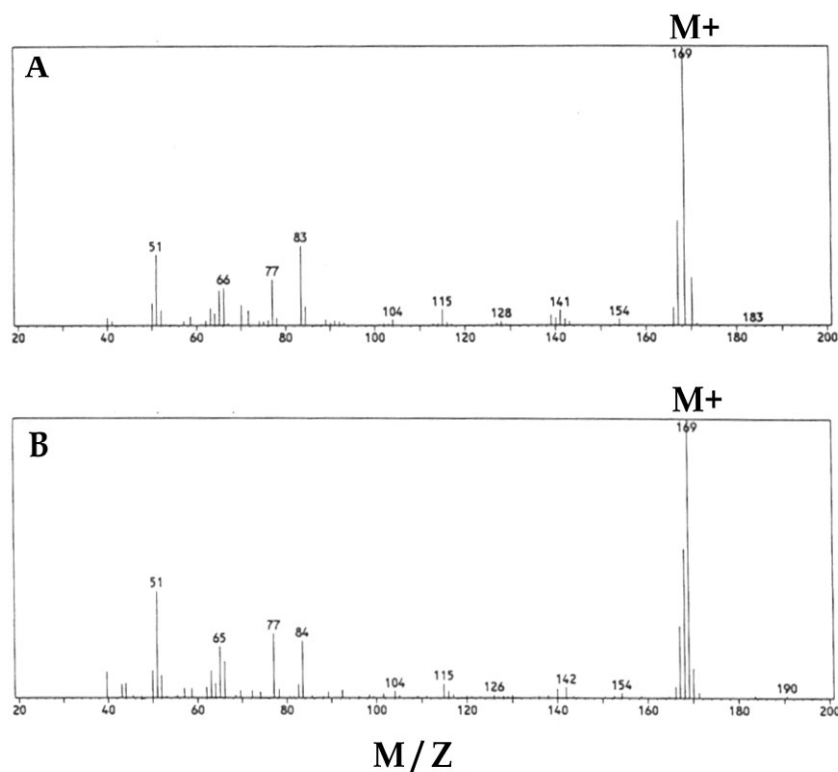


FIG. 4. – (A) Mass spectrum (background corrected) of the diphenylamine isolated from the two species of fire coral from Saudi Red Sea Coast. (B) mass spectrum of the authentic diphenylamine.

Many corrosive compounds are phenolic in nature (Harborne, 1984). Therefore *M. dichotoma* and *M. platyphylla* were subjected to acidic hydrolysis to isolate any phenolic compounds if present. It was observed that three fractions were obtained when it was subjected to acidic hydrolysis. The top fraction was separated by preparative layer chromatography. It was further resolved by running in a different solvent system (hexane : chloroform 85:15). A spot was scratched from the TLC plate that had a similar R_f value to that of authentic diphenylamine (Sigma Chemical Ltd.). There was no change in the R_f value when authentic diphenylamine was mixed with the one isolated from coral in two different solvent systems on TLC (hexane: chloroform, 85:15, R_f 0.35) and with (benzene: methanol, 60:40, R_f 0.81). It was also noticed that the mass spectrum pattern was similar to that of the authentic diphenylamine (Fig. 4). The extract was co-injected with the authentic diphenylamine to yield a single peak. Moreover, the mass spectra patterns of both diphenylamines (isolated from fire coral and authentic) were identical to those described in the literature (Hites, 1992). The mass spectrum of diphenylamine exhibited a molecular ion peak, a base peak at non-even m/z 169, and the presence of a moderate peak at m/z 168 (relative intensity 61%), confirming the loss of hydrogen from the amino group. The relatively abundant fragment at m/z 142 is an important feature that is derived from M-HCN. In addition, the mass spectrum showed a series of peaks m/z 51, 65 and 77 indicating a phenyl group. It is interesting to note that this compound has not yet been reported from other marine organisms (MarinLit Database, 1996). It is to be noted that diphenylamine is harmful to skin and causes rashes and a burning sensation. It was found during the toxicity test of the extract in guinea pigs that LD_{50} was 30 mg/kg of body weight, indicating a high sensitivity to this compound (Leng, 1987).

CONCLUSIONS

Four long-chain wax esters with a total number of 32-36 carbon atoms were observed in fire coral extract. All these have been previously reported from other sources. The isomeric compounds of these wax esters were to some extent present in a different distribution to those reported previously. It is normally known that wax esters are waxy in

nature and in *M. dichotoma* and *M. platyphylla* they act as an energy reserve for nutrient transfer and also may act as a protective coating of the defense system (nematocyst polyps) of this type of coral. However, much work is needed to study the biochemical pathways of these compounds and also to verify, if possible, the position of these materials in fire coral.

The presence of diphenylamine in acid hydrolysate of the two species of fire coral could be considered as one of the mechanisms used by fire coral to possess its stinging property.

ACKNOWLEDGEMENTS

The authors are thankful to King Abdulaziz City for Science and Technology for financial support (Grant No. MS-83/2). Many thanks are also due to the technicians at the Marine Chemistry Department for their assistance in fieldwork and laboratory analyses.

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Scient. ed.: P.J. Wangersky