Antimicrobial Activity of the Marine Alkaloids Haminol and Pulo'upone and Related Compounds

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The marine alkaloids haminol A, haminol B and pulo'upone as well as 17 related compounds (twelve 2-substituted pyridine derivatives, four 3-substituted ones and one analogue of the bicyclic terminus of pulo'upone) were tested for antimicrobial activity against a panel of six microbes (*Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus epidermidis, Candida albicans* and *Saccharomyces cerevisiae*) using the paper disc agar diffusion method. Six compounds were tested also against the mold *Aspergillus niger*. Some of the compounds displayed noteworthy antimicrobial activity, only one congener being completely devoid of activity. Nearly all compounds had activity against *B. cereus* and *S. epidermidis*. The growth of *E. coli*, *C. albicans* and *S. cerevisiae* was also distinctly inhibited by many compounds. In contrast, most compounds were inactive or had minimal activity against *P. aeruginosa*. Interestingly, most of the compounds tested against the opportunistic pathogen *A. niger* were active, one of them having noteworthy inhibitory potency.

Introduction

Pulo'upone (compound **12**, Fig. 1) is a 2-substituted pyridine derivative that was isolated from a Hawaiian opisthobranch mollusk (*Philinopsis speciosa*) in 1985 (Coval and Scheuer, 1985). Haminol A (**3**) and its acetate ester haminol B (**4**) in turn are 3-substituted pyridine derivatives isolated from some Mediterranean cephalaspideans (Cimino and Sodano, 1989; Cimino *et al.*, 1991; Spinella *et al.,* 1993).

The defensive strategies of such organisms have been subject to many chemical studies (Spinella *et al*., 1993), and we considered it possible that compounds of the pulo'upone and haminol types might have also antimicrobial activities. Therefore, we have now screened twenty such compounds against a panel of several types of microbes (bacteria, yeasts and one mold). Interesting activity was observed with some of these synthetic compounds.

Experimental

Compounds studied

The synthesis of compounds **1–4** (Matikainen *et al*., 1995a), **5–11** (Matikainen *et al*., 1995b), **12** and **14–17** (Matikainen *et al*., 1993), **13** (Kaltia *et al*., 1991) and **19** (Kaltia *et al.* , 2000) have been published. Compounds **3** and **4** were in the form of pure optical isomers (Matikainen *et al*., 1995b). Compounds **12** and **19** each consisted of a pair of enantiomers, only one enantiomer being shown in Fig. 1.

Compound **18** was synthesized from compound **14** and compound **20** from compound **6** analogously with the published synthesis of 8-(3-pyridyl)-2(*E*),7(*E*)-octadienal (Matikainen *et al*., 1995a).

Microbial strains and culture conditions

A panel of seven microbes was employed in the study. The microbial strains studied, their origin, the media employed and the growth temperatures used are shown in Table I. Facilities for the culture

Table I. The microbial strains used, their origins, the media employed and growth temperatures used.

Microbe	Source and strain code	Liquid medium	Solid medium	Temperature [°C]
Aspergillus niger	ATCC[a] 11414	Sabouraud Dextrose Broth (Difco)	Sabouraud Dextrose Agar (Difco)	30
Bacillus cereus	ATCC 10987	Luria-Bertani Broth[b]	Antibiotic Medium 3 Agar[d]	30
Candida albicans	ATCC 10231	Sabouraud Dextrose Broth (Difco)	Sabouraud Dextrose Agar (Difco)	37
Escherichia coli	ATCC 11303	Luria-Bertani Broth [b]	Luria-Bertani Agar[b]	37
Pseudomonas aeruginosa	ATCC 10145	Luria-Bertani Broth [b]	Antibiotic Medium 3 Agar[d]	37
Saccharomyces cerevisiae	Alko, Helsinki, Finland	YPD Broth [c]	Malt Agar (Biokar Diagnostics, Beavais, France)	30
Staphylococcus epidermidis	Kansanterveys-laitos (KTL), Helsinki, Finland	Luria-Bertani Broth [b]	Antibiotic Medium 3 Agar[d]	37

[a] American Type Culture Collection.
[b] Luria-Bertani broth contained 10.0 g peptone from casein (E. Merck, Darmstadt, Germany), 5.0 g yeast exract (E. Merck), 5.0 g NaCl and 10.0 g glucose per litre, and the corresponding agar contained in addition 16.0 g agar (Ph. Eur.).
[c] YPD broth contained 20.0 g peptone from casein (E. Merck), 10.0 g yeast extract (E. Merck) and 20.0 g glucose per litre.
[d] 17.5 g antibiotic medium 3 (Difco) plus 16.0 g agar per litre.

of *Aspergillus niger* were not available when compounds **1–12** and **19–20** were tested but were available for the other compounds (**13–18**).

All strains were preserved deep-frozen (*ca.* −20 °C) in Bacto® Skim Milk (Difco Laboratories, Detroit, MI, USA). This medium was prepared from the dehydrated powder and autoclaved according to the instructions of the manufacturer.

Twice a year, the strains were transferred twice consecutively onto the agar plates indicated below in Table I, after which single colonies (in the case of *Aspergillus niger*, however, hyphae) were transferred into aliquots of the skim milk, cultivated for 24 h at either 30 °C or 37 °C (as indicated in Table I), cooled and deep-frozen.

For routine testing of antimicrobial activities, single colonies (or hyphae) from agar plates were grown at 30 °C or 37 °C in several 5 ml aliquots of the liquid media indicated in Table I.

All cultivations were carried out aerobically (air at ambient pressure), and in the case of cultivations in liquid media, orbital shaking (120 rotations per min) was invariably employed.

For all media and agars, water was purified using the the Milli-RO 12 plus system (Millipore Corporation, Molsheim, France). All media were sterilized by autoclaving at 121 °C.

Antimicrobial activity measurements

The paper disc agar diffusion method was employed, using discs of 6 mm diameter (Antibiotica-testblättchen, Schleicher & Schuell, Dassel, Germany). Liquid cultures of the bacteria and yeasts from over-night cultivations (5 ml) were centrifuged aseptically in 1 ml aliquots. The microbial pellets obtained were washed and individually centrifuged again. Each pellet obtained was resuspended into a volume of 400 µl, and 200 µl of this suspension were inoculated onto each plate (diameter 14 cm, volume of agar approximately 50 ml). Then, the discs were put on the plates. A volume of 10 µl of a test solution was then pipetted onto each disc.

All compounds studied were dissolved in dimethyl sulfoxide. Sterilization of these solutions was found to be unnecessary and was not performed.

In the case of *Aspergillus niger*, hyphae were taken from freshly pre-cultivated agar plates with the aid of sterile Pasteur pipettes and were inocu-

lated into molten Sabouraud Dextrose Agar. The agar was plated into empty plates and was allowed to solidify. Paper discs were then put onto the agar, and 10 µl of test solution were pipetted onto each disc.

Control discs (dimethyl sulfoxide alone as negative control and antibiotics as positive controls) were employed on each plate. Dimethyl sulfoxide alone never gave any inhibitory zone over 7 mm (*i.e.*, about 0.5 mm from each side of the paper disc), and usually gave none. The antibiotic used in the case of *E. coli* was ampicilline (A-PEN, Orion, Espoo, Finland), in the case of other bacteria doxycycline (Doximycin, Orion, Espoo, Finland) and in the case of all fungi amphotericin B (Fungizone, Bristol-Myers Squibb, Bromma, Sweden).

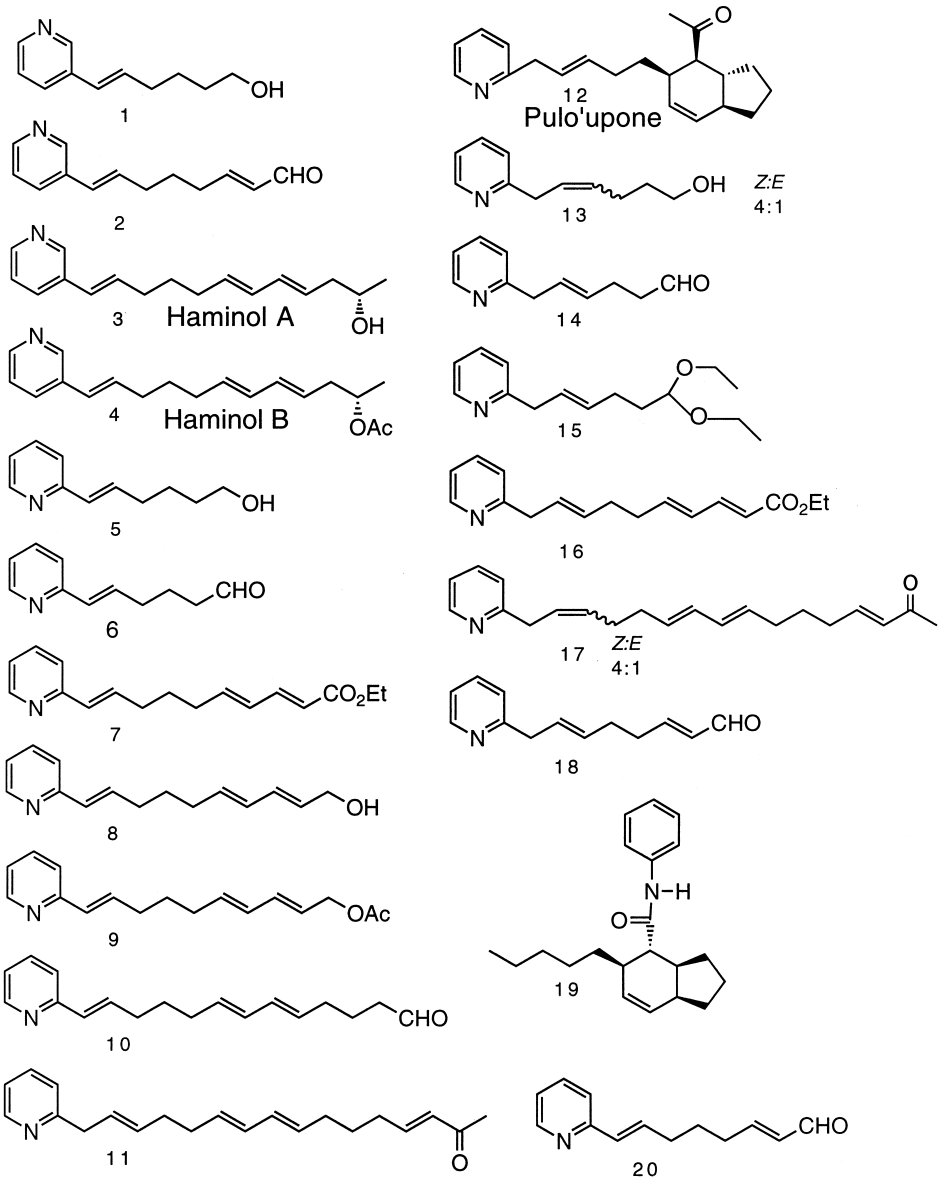

Fig. 1. Structures of the compounds studied.

Results and Discussion

The compounds studied are shown in Fig. 1 and the results obtained are shown in Table II. Most of the compounds had distinct activity against some or even all of the microbes studied, only compound **5** being totally devoid of detectable activity. Especially compounds **2**, **3**, **10**, **11**, **13** and **18** had noteworthy antimicrobial activity. In many cases, also lower concentrations than those shown in Table II were tested but inhibitory zones (if any) had diameters smaller than 8 mm. Compounds **1**, **5** and **7** (each at 5 and 40 mg/ml) and **14**, **17** and **19** (at 5 and 20 mg/ml) never gave inhibitory zones of 8 mm or greater and have been omitted from Table II.

In general, highest activity was observed against *Staphylococcus epidermidis* and *Bacillus cereus,* the largest inhibitory zone having a diameter of 17 mm (compound **2** against as *S. epidermidis* at 40 mg/ml). One compound (**13**) also had considerable activity against *Aspergillus niger* (diameter of inhibitory zone 16 mm at 20 mg/ml). Several compounds had some activity against the yeasts studied (*Candida albicans* and *Saccharomyces cerevisiae*) and/or against *Escherichia coli*. In contrast, the compounds studied had minimal or, mostly, no detectable activity against *Pseudomonas aeruginosa*. Any clear-cut structure-activity relationships are difficult to define at this stage.

Because of the rapidly increasing importance of the opportunistic pathogen *A. niger*, it is interesting that three out of the six compounds tested against it gave a distinct inhibitory zone (10 mm or more at 20 mg/ml), that of compound **13** being as broad as 16 mm. Unfortunately, facilities for the culture of *A. niger* were not available when the first part of the study (testing of compounds

Table II. Results of growth inhibition tests.

Compound	Concentration[b] [mM]	Mean diameter of inhibitory zone [mm][a] *B. cereus*	*E. coli*	*P. aeruginosa*	*S. epidermidis*	*C. albicans*	*S. cerevisiae*	*A. niger*
2	195	ND[c]	9		17	12	ND	ND
3	156	10			14		8	ND
4	17[e]				9		ND	ND
4	134	8			11			ND
6	229	8	9				12	ND
8	176	10	8			8	12	ND
9	148	8	8			8		ND
10	19[e]	8			10			ND
10	149	9			13	8		ND
11	16[e]	11			8			ND
11	129	15			15	9	9	ND
12	16[e]	8						ND
12	129	11			9			ND
13	113[f]	9	9			13	11	16
15	79[f]	9						
16	19[e]							9
16	74[f]	9			10		9	12
18	98[f]	14	11		14	11	10	10
20	195	12	10–13		RU[d]	11	11	

[a] Diameter of filter paper disc = 6 mm. The diameters given include the disc diameter. For each contration of each compound, three filter discs were employed, and for each disc the inhibitory zone diameter was measured in at least three directions using a standard ruler, whose smallest division was 1 mm. For each disc, the mean of the individual measurements was calculated. In most cases, the same result was obtained for all three discs, and if not, the range of the results was usually 1 mm (maximally 3 mm). – Diameters smaller than 8 mm have been omitted for clarity of presentation (blank spaces are shown instead).

[b] Concentration of test substance in DMSO. 10 µl of this solution were pipetted onto each paper disc. If not otherwise noted, the concentrations shown are equivalent to 40 mg/ml.

[c] ND = not determined.

[d] RU = results uncertain (variable and unclear zones).

[e] Equivalent to 5 mg/ml.

[f] Equivalent to 20 mg/ml.

1–12) was performed, and also not when the newest compounds (**19** and **20**) became available.

As a conclusion, the class of substances now tested deserves further microbiological studies. It would be interesting to find out the mechanism(s) of action of the compounds. The reasons for the differences between the different compounds also remain unclear at present. Whether more active congeners could be synthesized also remains to be studied.

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