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封面图说: 涵养水源——在长白山南坡的峭壁上, 生长在坡面上的森林所涵养的水源还在汨汨地往下流个不停, 深红色的落叶掉在了苔藓上, 这里已经是长白山的深秋了。虽然雨季已经过去了很久, 但是林下厚厚的枯枝落叶层、腐殖质层、苔藓草本层所涵养的水分还在不间断地流淌, 细细的水线在壁下汇成了溪、汇成了河。涵养水源是森林的主要生态功能之一。

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美丽海绵提取物防污损作用

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摘要:从美丽海绵分离提取了 2-去氧-1-氢-1,2,4,三唑、环(脯氨酸-甘氨酸)、尿嘧啶、环(脯氨酸-丙氨酸)、6-氨基嘌呤、4-(1-苯乙基)苯酚、1-氢-1,2,4,三唑和胆甾-5-烯-3 β ,7 α -二醇等 8 种化合物,并研究了其对网纹藤壶金星幼虫的影响,其中 6-氨基嘌呤(7.1 $\mu\text{g}/\text{cm}^2$)和 4-(1-苯乙基)苯酚(5.9 $\mu\text{g}/\text{cm}^2$)对金星幼虫具有明显的毒杀作用。美丽海绵可能通过这些活性化合物的协同作用来防止它种生物附着污损。

关键词:海绵; 提取物; 藤壶; 幼虫

The antifouling activities of *Callyspongia* sponge extracts

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Abstract: Around the world, marine fouling can have serious impacts on human activities and other organisms. To survive in the highly competitive arena of the marine environment, many organisms have developed unique protection mechanisms against fouling, including tolerance, avoidance, low surface energy and the secretion of natural compounds. Some species of sponge and coral are rarely epiphytized because of the production of secondary metabolites against fouling. Unlike common man-made antifouling compounds, these natural chemicals are environmentally friendly in marine ecological systems.

The majority of marine fouling organisms are algae, coelenterates, polychaetes, bivalves, bryozoans and barnacles. Of them, the acorn barnacle *Balanus reticulatus* is one of the most important dominant species in the fouling communities of tropical and subtropical waters, particularly in the East and South China Sea. Thus it can be considered as an appropriate test representative for antifouling bioassays.

Barnacle nauplii were obtained by dissecting freshly collected adult barnacles from the aquaculture facilities at Daya Bay, Shenzhen, China. Barnacle larvae were reared in darkness, at 30°C and on a diet of the green algae *Platymonas subcordiformis*. Seawater was changed and more algae added as necessary. Cyprid larvae developed after around 5 days and were collected and stored at 4°C for subsequent use.

Sponges of the genus *Callyspongia* are widely distributed in the southern waters of China (e.g. coastal water of Fujian, Guangdong, Guangxi and Hainan Provinces). They are well known as sources of biologically active natural products, including polyacetylenes, peptides, alkaloids, fatty acids, polyketides, and sterols. Some of these compounds possess antimicrobial, anti-tumor, cytotoxicic, and HIV reverse transcriptase inhibition properties.

Freshly collected samples of *Callyspongia* were minced and extracts taken by washing with dilute ethanol three times.

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The aqueous ethanol extracts were concentrated under vacuum. The combined extracts were partitioned into ethyl acetate and water. The ethyl acetate soluble portion was separated using column chromatography. A silica gel column was eluted with petroleum ether / ethyl acetate and then ethyl acetate / acetone. Similar isolates were identified using thin layer chromatography (TLC). The structures of all isolates were determined using one dimensional and two dimensional nuclear resonance microscopy (NMR), mass spectroscopy (MS) analyses, and by comparison of the spectroscopic data with those reported in the literature. A total of eight compounds were isolated and purified: Cyclo-(S-Pro-Gly), Cyclo-Pro-Ala, 1-(2'-deoxy- β -D-erythro-pentofuranosyl)-1-H-1,2,4-triazole, uracil, 4-(1-phenylethyl) phenol, 6-NH₂-purine, 1-H-1,2,4-triazole and cholest-5-ene-3 β ,7 α -diol.

For antifouling tests, each compound was dissolved in 1ml methanol (or chloroform) and transferred into Petri dishes. Petri dishes were dried on a shaker prior to the addition of filtered seawater and larvae. Control dishes, using 1 ml of solvent (pre dried as above) and filtered seawater only were also prepared. The mean values of larval survival rate were compared among samples using least significant difference tests.

Six compounds had no influence on the behavior and survival rate of larvae ($P>0.05$): 1-(2'-deoxy- β -D-erythro-pentofuranosyl)-1-H-1,2,4-triazole, Cyclo-(S-Pro-Gly), Cyclo-Pro-Ala, uracil, 1-H-1,2,4-triazole and cholest-5-ene-3 β ,7 α -diol. The other two compounds 6-NH₂-purine (7.1 $\mu\text{g}/\text{cm}^2$) and 4-(1-phenylethyl) phenol (5.9 $\mu\text{g}/\text{cm}^2$) had significant toxic effects on the cyprid larvae of the acorn barnacle *Balanus reticulatus* ($P<0.05$). The synergistic effect of these compounds may play an important role in the antifouling activities of the sponge.

Key Words: sponge; compounds; barnacle; larvae

海洋环境中各物种之间对空间的竞争十分激烈,任何大型动、植物都是营固着或附着生活种类的潜在附着污损对象,为避免其他生物附着可能产生的危害,海洋中一些生物在长期的进化过程中,形成了许多独特的自我保护机制来保持自身体表的洁净^[1]。

其中,许多海洋生物可通过产生对环境无危害的、具有防污活性的次生代谢产物以防止其他生物污损附着,利于自身的生存。这些天然防污化合物是一类非常理想的防污剂,其通过驱赶而不是毒杀作用实现防污目的,这不仅具有极好的防污效果,而且也不会对环境产生危害作用。因此,开展海洋化学生态学研究,将其应用于海洋污损生物防除领域,不仅有利于弄清海洋污损生物的防除作用机制,还可以提供大量新型、无毒、环保、高效的天然防污化合物,在基础研究和生产实践中均具有重要意义。

海绵动物是一类体表多孔,营水中固着生活,体呈不对称或辐射对称,具两胚层和水沟系,无消化腔和神经系统的最原始的多细胞动物,分布范围广泛,栖息环境多样,体内含有大量生理活性物质,其中某些次级代谢产物还被证实对花石莼 *Ulva conglobata*、海鞘 *Herdmania curvata*、致密藤壶 *Balanus improvisus*、线管虫 *Salmacina tribranchiata* 等常见污损生物幼虫和孢子的附着萌发具有抑制作用^[2-5]。

美丽属海绵 (Callyspongia) 隶属寻常海绵纲 (Demospongiae) 间骨海绵目 (Haplosclerida) 蜂海绵科 (Halicionidae),在我国南方海域(如福建、广东、广西及海南沿岸)均有较丰富的分布^[6-7]。自 20 世纪 70 年代开始研究该属海绵以来,已从中分离获得多肽、肽类、生物碱、脂肪酸、聚酮类及甾体等生物活性物质,且显示出较好的抗菌、抗肿瘤、细胞毒性及抑制 HIV 逆转录酶活性等作用^[6]。

在热带海域,污损生物种类繁多,生长迅速,附着量大。其中,网纹藤壶 (*Balanus reticulatus*) 不仅是我国华南沿海污损生物群落中的绝对优势种,也是世界性热带和亚热带广布种^[8]。目前虽然已对美丽属海绵代谢产物的化学组成及其活性开展了研究,但尚未见这些化合物防污损研究的报道。本文初步探讨了源自美丽海绵的 8 种化合物对网纹藤壶金星幼虫的影响,以期为进一步工作奠定基础。

1 材料与方法

1.1 化合物的分离提取

将新采集的美丽海绵用 95% 的工业酒精室温浸泡、提取(5 d $\times$ 3),合并浸提液,减压浓缩,再用乙酸乙酯

萃取,浓缩萃取液得浸膏。干燥后进行硅胶柱层析分离,先用石油醚/乙酸乙酯体系进行梯度洗脱,然后改换乙酸乙酯/丙酮体系,通过薄层色谱(TLC)追踪,合并相同组分。将分离提取的化合物进行核磁共振¹H谱、¹³C谱、二维谱、质谱分析,并参照已有文献资料确定其结构。

1.2 网纹藤壶幼虫培养

从大亚湾水产养殖设施上采集网纹藤壶成熟个体,解剖取出成熟受精卵块,放入盛有消毒海水的500 mL烧杯中,数分钟后无节幼虫即可孵化出来。采集活泼健壮的无节幼虫,转移到1000 mL烧杯中并加入适量海水,培养时幼虫密度为1—2个/mL。以亚心形扁藻(*Platymonas subcordiformis*)作为饵料,投放量约 2.5×10^5 — 3.0×10^5 个细胞/mL。放入温度控制在30℃左右的恒温培养箱中于黑暗环境中培养。每天早晨和傍晚取出,置于阳光下0.5—1 h,并视具体情况添加少许饵料。5 d左右金星幼虫就可大量出现,收集后置4℃冰箱中储存备用。

1.3 化合物活性测试

分别用甲醇(或氯仿)溶解上述8种化合物。实验组均各取1 mL化合物溶液加入直径为6 cm的培养皿中,并使其均匀覆盖培养皿底部。待溶剂挥发完全后,加入13 mL海水。另设空白组和对照组,其中对照组只加1 mL溶剂,待溶剂挥发完全后再加入海水;空白组只加海水。所有实验组、对照组和空白组均设5个平行样,每个培养皿加入约30个网纹藤壶金星幼虫。置于30℃培养箱内于黑暗环境中培养。每24 h观察1次,共观察72 h。采用最小显著差数法进行差异显著性分析。

2 实验结果

2.1 化合物提取分离

将石油醚-乙酸乙酯(0:10)部分上正相硅胶(45—75 μ),把乙酸乙酯-丙酮(1:1)洗脱的流分再上正相硅胶(45—75 μ),用氯仿-甲醇(9:1)洗脱,得环(脯氨酸-甘氨酸)。反复上Sephadex LH-20(氯仿-甲醇,1:9)洗脱,再经正相硅胶(45—75 μ),氯仿-甲醇(9:1)洗脱,最后再上正相硅胶(45—75 μ),氯仿-甲醇(98:2)洗脱得化合物环(脯氨酸-丙氨酸)。

石油醚-乙酸乙酯(1:9)部分上正相硅胶(45—75 μ),将石油醚-乙酸乙酯(5:5)洗脱组分再上正相硅胶(45—75 μ),石油醚-丙酮(5:5)洗脱,再反复Sephadex LH-20(氯仿-甲醇,2:8),最后再上正相硅胶(45—75 μ)以氯仿-甲醇(9:1)洗脱,得尿嘧啶。

石油醚-乙酸乙酯(1:1)部分上正相硅胶(45—75 μ),将石油醚-丙酮(梯度洗脱)的流分再上Sephadex LH-20,氯仿-甲醇(2:8)洗脱,得4-(1-苯乙基)苯酚。

纯丙酮冲柱部分上正相硅胶(45—75 μ),氯仿-甲醇(9:1)洗脱的流分再反复上Sephadex LH-20,以氯仿-甲醇(2:8)洗脱,得6-氨基嘌呤。

将乙酸乙酯-丙酮(1:1)部分上正相硅胶(45—75 μ),把氯仿-甲醇(9:1)洗脱的流分反复过Sephadex LH-20(氯仿-甲醇,2:8),先后得2-去氧-1-氢-1,2,4,三唑和1-氢-1,2,4,三唑。

乙酸乙酯-丙酮(1:1)部分上正相硅胶柱(10—50 μ),经等梯度洗脱(氯仿-甲醇,20:1)得到了11个流份,流份2用正相硅胶柱(10—50 μ),经等梯度洗脱(氯仿-甲醇,20:1)得到化合物胆甾-5-烯-3β,7α-二醇。

2.2 网纹藤壶金星幼虫测试

2.2.1 环(脯氨酸-甘氨酸)等4种化合物对藤壶幼虫的影响

图1为环(脯氨酸-甘氨酸) $5.3 \mu\text{g}/\text{cm}^2$ 、4-(1-苯乙基)苯酚 $5.9 \mu\text{g}/\text{cm}^2$ 、2-去氧-1-氢-1,2,4,三唑 $8.3 \mu\text{g}/\text{cm}^2$ 和尿嘧啶 $8.8 \mu\text{g}/\text{cm}^2$ 等4种化合物对网纹藤壶幼虫存活率影响的测试结果。结果表明,培养72 h后空白组和对照组的幼虫存活率分别为99.0%和98.4% ($P>0.05$);环(脯氨酸-甘氨酸)、2-去氧-1-氢-1,2,4,三唑和尿嘧啶三组的幼虫存活率分别为98.4%、95.0%和95.0%,与对照组无显著差异($P>0.05$);而4-(1-苯乙基)苯酚组幼虫存活率为0,与对照组的差异显著($P<0.05$)。因此,在 $5.9 \mu\text{g}/\text{cm}^2$ 的作用剂量下,4-(1-苯乙基)苯酚对藤壶幼虫有明显的毒杀作用。

2.2.2 6-氨基嘌呤等4种化合物对藤壶幼虫的影响

图2为6-氨基嘌呤 $7.1 \mu\text{g}/\text{cm}^2$ 、环(脯氨酸-丙氨酸) $5.3 \mu\text{g}/\text{cm}^2$ 、1-氢-1,2,4,三唑 $5.9 \mu\text{g}/\text{cm}^2$ 和胆甾-5-烯-3 β ,7 α -二醇 $8.3 \mu\text{g}/\text{cm}^2$ 等4种化合物对网纹藤壶幼虫存活率影响的测试结果。结果表明,72h后空白组和对照组的幼虫存活率均为100%,无显著差异($P>0.05$);环(脯氨酸-丙氨酸)、1-氢-1,2,4,三唑和胆甾-5-烯-3 β ,7 α -二醇3组的幼虫存活率分别为99.0%、100%、和100%,与对照组均无显著差异($P>0.05$);而6-氨基嘌呤组幼虫存活率为93.6%,与对照组的差异显著($P<0.05$)。因此,在 $7.1 \mu\text{g}/\text{cm}^2$ 的作用剂量下,6-氨基嘌呤对藤壶幼虫表现出一定的毒性作用。

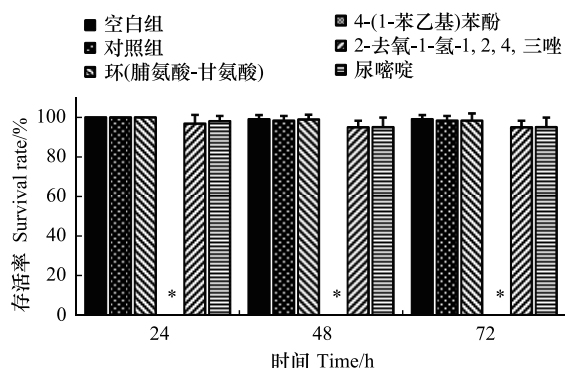


图1 环(脯氨酸-甘氨酸)、4-(1-苯乙基)苯酚、2-去氧-1-氢-1,2,4,三唑和尿嘧啶对藤壶幼虫的影响

Fig. 1 The effect of four compounds (cyclo-(S-Pro-Gly), 4-(1-phenylethyl) phenol, 1-(2'-deoxy- β -D-erythro-pentofuranosyl)-1-H-1,2,4-triazole, uracil) on the larvae of *Balanus reticulatus*

“*”表示该实验组与对照组差异显著($P<0.05$)

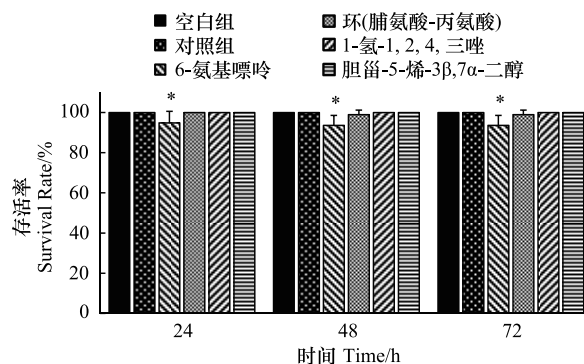


图2 6-氨基嘌呤、环(脯氨酸-丙氨酸)、1-氢-1,2,4,三唑和胆甾-5-烯-3 β ,7 α -二醇对藤壶幼虫的影响

Fig. 2 The effect of four compounds (6-NH₂-purine, Cyclo-Pro-Ala, 1-H-1,2,4, triazole, cholest-5-ene-3 β ,7 α -diol) on the larvae of *Balanus reticulatus*

“*”表示该实验组与对照组差异显著($P<0.05$)

3 讨论与分析

在海洋环境中,海绵动物之所以能保持体表洁净,应该是通过其体表的天然防污化合物(活性代谢产物)的作用来实现,且与单位面积内活性代谢产物的作用剂量密切相关,这与防污漆的作用方式相一致。因此,本研究借鉴了 de Nys 等人的方法^[9],将分离提取的各组分用有机溶剂溶解,使之均匀覆盖于培养皿底部,着重从单位面积角度探讨化合物对藤壶幼虫的影响。这样既可以避免有机溶剂可能对测试结果产生的干扰,而且应能更好的反映海洋环境中海绵体表的化合物对网纹藤壶金星幼虫的影响。

本研究直接利用分离提取的化合物进行网纹藤壶金星幼虫测试实验,由于各类化合物在海绵体内的含量不同,某些组分的量极少,而且测试实验的目的是首先要定性分析哪些组分可对幼虫产生影响,再以此为基础来进一步分析测试各化合物的防污损活性,故在实验方案中暂时不考虑各化合物在同一作用剂量条件下以及同一化合物在不同作用剂量下对藤壶幼虫的影响。从实验结果来看,本研究的初步目标已然达到。

目前人们除了从海绵提取到嘌呤碱、神经酰胺、呋喃萜类和吡喃类化合物等具备防污活性的组分外^[10-12],Sera 等人还分别从 *Dysidea herbacea*、*Lendenfeldia chondrodes*、*Haliclona* sp. 等数种海绵中提取到对紫贻贝具有驱避作用的倍半萜^[13]、过氧化甾醇^[14]、螯合肽^[15]和六肽^[16]等化合物。

测试中6-氨基嘌呤的作用剂量为 $7.1 \mu\text{g}/\text{cm}^2$,此时网纹藤壶金星幼虫存活率低于对照组,表明该化合物对网纹藤壶金星幼虫具有一定的毒杀作用。在脊椎动物方面,Akintonwa 等人发现高剂量腺嘌呤可使小白鼠产生进行性的运动失调、肌无力和呼吸困难等症状,甚至会造成死亡^[17]。而6-氨基嘌呤通过何种方式对以藤壶为代表的无脊椎动物幼虫产生影响还需深入分析研究。

酚类化合物是芳烃的含羟基衍生物,其毒性以苯酚为最大。高浓度的酚能凝固蛋白质,低浓度酚则有较

强的渗透性,可透至组织深部而引起严重的后果^[18];有关研究还发现苯酚致使鱼类中毒死亡的原因和机理与微核出现、染色体断裂有关^[19]。在剂量为 $5.9 \mu\text{g}/\text{cm}^2$ 的 4-(1-苯乙基)苯酚的作用下,网纹藤壶幼虫 24 h 内即全部死亡,其对网纹藤壶金星幼虫产生影响的作用机制尚需进一步探讨。

为了在竞争激烈的海洋环境中得以生存,许多生物形成了独特的自我保护机制,通过忍耐、躲避等被动防御途径或借助物理、化学和机械作用等主动出击方式来保持自身体表的洁净,其中更以化学手段最为常见。美丽海绵可能正是通过这些活性化合物的协同作用,使其自身具备广谱抗污损能力,抵御藻类孢子和动物幼虫的附着萌发,减轻(或避免)海洋污损生物的危害。

可以说,海洋生物化学自我防护作用机制及过程十分复杂,海绵动物作为珊瑚礁生态系统中重要组成,在长期进化过程中形成的天然化学防污损作用机制是现有防污涂料技术革新的理想研究模式。今后的工作重点除了进一步分离筛选新的天然活性防污化合物外,还需确定生物体表单位面积内相关活性化合物的含量及其之间的最佳比例,从而为研制经济、高效、环保的第二代污损生物防除涂料提供理论依据和奠定物质基础。

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