文章编号:1001-6880(2016)Suppl-0179-05

海藻内生真菌 PT-20 次级代谢产物的分离与鉴定

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摘　要:从海萝中分离到内生真菌(TP-20),通过 18S rDNA 鉴定为杂色曲霉(*Aspergillus versicolor*)。通过正相硅胶柱层析、反相硅胶柱层析、Sephadex LH-20 层析、制备薄层层析等方法对该真菌的次级代谢产物进行分离纯化,获得 9 个化合物。通过波谱技术鉴定 9 个化合物为 eurothiocin A(**1**)、sterigmatocystin(**2**)、5-methoxysterigmatocystin(**3**)、anthraquinone aversin(**4**)、6,8-di-*O*-methyl averufin(**5**)、6,8-di-*O*-methyl versiconol(**6**)、brevianamide K(**7**)、brevianamides V(**8**)和 brevianamide R(**9**),其中化合物 **1**、**4**、**5** 是首次从杂色曲霉中分离得到。对于得到的化合物进行抗菌活性检测表明,化合物 **3** 表现对抗大肠杆菌、金黄色葡萄球菌、枯草芽孢杆菌有弱的抗菌活性,其最小抑菌浓度(MIC)分别为大于 1000 μg/mL、大于 1000 μg/mL 和 31.3 μg/mL。

关键词:杂色曲霉;次级代谢产物;结构鉴定;抗菌活性

中图分类号:Q939.5;O629.3　　**文献标识码**:A　　DOI:10.16333/j.1001-6880.2016.S.001

Isolation and Identification of the Secondary metabolites of Seaweed endophytic fungi PT-20

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Abstract: This paper reported isolation and identification of the secondary metabolites of the endophytic fungi TP-20, isolated from *Gloiopeltis furcata*. This fungus was identified as *Aspergillus versicolor* on the basis of 18S rDNA. The secondary metabolites were isolated and purified by using utilizing various chromatographic methods such as silica gel, reverse silica gel, Sephadex LH-20, preparacetate TLC. Their structures were identified by spectral technique as eurothiocin A(**1**), sterigmatocystin (**2**), **5**-methoxysterigmatocystin (**3**), anthraquinone aversin (**4**), 6,8-di-*O*-methyl averufin (**5**), 6,8-di-*O*-methyl versiconol (**6**), brevianamide K (**7**), brevianamides V (**8**) and brevianamide R (**9**). Compounds **1**, **4** and **5** were first isolated from *A. versicolor*. All compounds were tested for antibacterial activity, and compound **3** exhibited a weak activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. The Minimum inhibitory concentration (MIC) of compound 3 against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* was higher than 1000 μg/mL, 1000 μg/mL and 31.3 μg/mL.

Key words: *Aspergillus versicolor*; secondary metabolites; structure elucidation; antimicrobial

海洋微生物由于其生活在寡营养、弱碱性的高盐海洋环境中,形成了独特的耐饥、耐碱和耐盐等生理特征。具有独特的代谢机制,可产生有别于陆生微生物的次生代谢产物,成为天然产物研究的热点。其中海洋真菌由于其次生代谢产物的化学多样性丰富、产量高而成为海洋天然产物研究的一类重要生物资源。2014 年,从海洋的发酵产物中发现 1378 个新的次生代谢产物,其中海洋真菌分离次生代谢产物有 426 个,这些代谢产物表现出良好的抗肿瘤、抗菌、抗病毒等生物活性[1]。

本研究从采自烟台海岸潮间带的海萝(*Gloiopeltis furcata*)中分离得到杂色曲霉(PT-20)。对于发酵液的抗氧化、抗菌活性进行筛选证明其具有较好的生物活性,同时 HPLC 数据显示其具含量较为丰富。对次级代谢产物分离得到 9 个化合物,包括 1 个含硫苯并呋喃衍生物(**1**)、2 个吨酮类化合物(**2**、**3**)、3 个蒽醌类化合物(**4**、**5**、**6**)、3 个二酮哌嗪类化合物(**7**、**8**、**9**)。其中化合物 **1**、**4**、**5** 是首次从杂色曲霉中分离得到。

收稿日期:2016-06-28　　接受日期:2016-08-09

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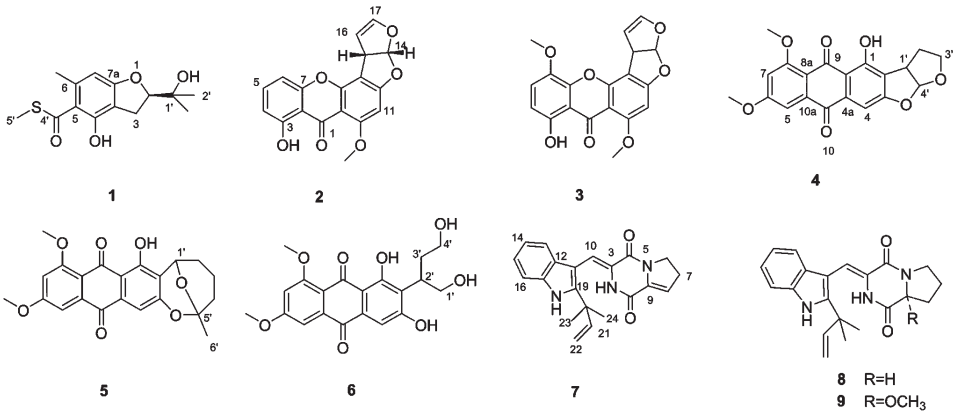

图 1 化合物 1 ~ 9 的化学结构

Fig. 1 Chemical structures of compounds **1-9**

1 材料与方法

1.1 材料

1.1.1 实验材料与供试菌株

原植物采自山东烟台海岸潮汐间带,经鉴定为海萝(*Gloiopeltis furcata*)。PT-20 是从海萝中分离的菌株,通过 18S rDNA 鉴定为杂色曲霉(*Aspergillus versicolor*)。大肠杆菌(*Escherichia coli*)、金黄色葡萄球菌(*Staphylococcus aureus*)、枯草芽孢杆菌(*Bacillus subtilis*)菌株等现均保藏于山东大学(威海)国际生物技术研发中心。

1.1.2 常用试剂与仪器

Bruker AVANCE 500 spectrometer 核磁共振谱仪;Thermo MSQ plus 质谱仪;三用紫外检测仪(上海康华生化仪器制造有限公司);旋转蒸发仪: Y-2000 型(上海亚荣生化仪器厂);分析天平: FA2004(上海民桥精密仪器有限公司);薄层层析板 GF_{254}(青岛海洋化工);柱层析硅胶 200 ~ 300 目、300 ~ 400 目(青岛海洋化工厂)。

1.2 发酵

发酵液为改良 PDA 培养基,其配方为马铃薯 300 g 去皮,切成边长为 1 cm 的方块,加 500 mL 纯水和 500 mL 陈海水,煮沸 20 min,过滤后用补足至 1 L,加入葡萄糖 20 g/L,蛋白胨 5.0 g/L,乙酸钠 1.66 g/L,硫酸镁 1.02 g/L,pH 自然。于 1000 mL 锥形瓶中加入培养基 300 mL,121 ℃下灭菌 30 min。将活化好的 PT-20 的菌株每个平板的八分之一加入到灭菌的锥形瓶中,共培养 30 L,室温下培养 42 d。

1.3 提取与分离

分离菌株 PT-20 发酵液的发酵液与菌膜,发酵液用同体积的乙酸乙酯萃取三次,所得乙酸乙酯相蒸干后得到发酵液浸膏。菌膜经组织匀浆机粉碎后,加入同体积的纯水和 50% 体积甲醇浸泡 24 小时,然后加入两倍体积的乙酸乙酯萃取三次。所得乙酸乙酯相蒸干后得到菌膜浸膏。合并两相浸膏即得到总浸膏的质量为 25.0 g。

浸膏经过正相硅胶石油醚/乙酸乙酯(10∶1→0∶1)分离得到 4 个粗组分(Fr. 1-Fr. 4)。F1 经石油醚/丙酮分离后,得到化合物 **1**(10.8 mg)、**2**(37.3 mg)。F2 经氯仿/甲醇分离后,得到四个亚组分(F2a-F2d)。F2a 经过凝胶(氯仿/甲醇,1∶1)纯化得到化合物 **5**(38.0 mg)。F2b 经二氯甲烷/丙酮(200∶1 ~ 0∶1)体系梯度层析后,用凝胶、反相柱层析(甲醇/水)分离得到化合物 **3**(36.5 mg)、**4**(7 mg)、**9**(36 mg)。F2c、F2d 经由薄层层析得到化合物 **7**(30.8 mg)。F3 经由氯仿/甲醇(100∶1 ~ 0∶1)梯度分离后,经凝胶、正相纯化得到化合物 **8**(15.4 mg)。F4 化合物通过凝胶柱层析分离得到化合物 **6**(12.0 mg)。

1.4 抗菌实验

1.4.1 纸片扩散法(K-B 法)

通过纸片法扩散法[2]测定分离化合物对大肠杆菌、枯草芽孢杆菌、金黄色葡萄球菌的抗菌活性。将大肠杆菌、枯草芽孢杆菌、金黄色葡萄球菌活化培养 24 h 后,将三种指示菌均匀涂布于固体培养基。以氯仿/甲醇(1∶1)作为溶剂将单体化合物溶解,浓度为 10 mg/mL,30 μg/片,将滤纸片均匀放置于培养基上。以 10 μg/片氨苄西林钠盐为阳性对照。平板 37 ℃孵育 24 h,观察测量抑菌圈的大小,所得实验结果均为三次重复实验。

1.4.2　微量肉汤稀释法抗菌实验

参照 CLSI-2012-M07-A9 方法[3]。通过微量肉汤稀释法测定分离化合物对大肠杆菌、枯草芽孢杆菌、金黄色葡萄球菌的最低抑菌浓度。配置样品初始浓度 10 mg/mL,取无菌试管 12 支稀释样品,第 1 管加入 1.6 mL MH 肉汤,其余每管加入 MH 肉汤 1 mL。取样品溶液 0.4 mL 于第 1 管中混匀,吸取 1 mL 至第 2 管,混匀后再吸取 1 mL 至第 3 管,如此连续倍比稀释至第 11 管,并从第 11 管中吸取 1 mL 弃去。将倍比稀释后不同浓度的样品溶液 100 μL 分别加到灭菌的 96 孔聚苯乙烯板中,第 12 孔为不加样品溶液的对照组。制备浓度相当于 0.5 麦氏浊度的三株菌株的菌悬液,经 MH 肉汤 1∶100 稀释后,向每孔中加 100 μL,将样品与菌悬液混合均匀。密封后 37 ℃孵育 20 h。将 96 孔板置于酶标仪下,602 nm 测定各孔的吸光度值。阳性对照为氨苄西林钠盐,所得实验结果均为三次重复实验。

2　结果与分析

2.1　结构鉴定

化合物 1　黄色油状($CHCl_3$);^{1}H NMR ($CDCl_3$,400 MHz) δ: 11.84 (1H, s, OH-4), 6.23 (1H, s, H-7), 4.69 (1H, dd, J = 9.6, 8.3 Hz, H-2), 3.07 (2H, qd, J = 15.6, 8.9 Hz, H-3), 2.66 (3H, s, H-6′), 2.44 (3H, s, H-5′), 1.31 (3H, s, H-2′), 1.20 (3H, s, H-3′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 197.82 (C-4′), 164.39 (C-7a), 158.27 (C-4), 141.91 (C-6), 116.13 (C-5), 111.58 (C-3a), 105.80 (C-7), 91.44 (C-2), 72.06 (C-1′), 25.89 (C-2′), 25.18 (C-6′), 23.91 (C-3′), 13.20 (C-5′); + ESI-MS m/z: 283 $[M+H]^+$。以上数据均与文献报道数据[4]一致,故鉴定为 eurothiocin A。

化合物 2　无色粉末($CHCl_3$);^{1}H NMR ($CDCl_3$,400 MHz) δ: 13.22 (1H, s, OH-3); 7.48 (1H, t, J = 8.2 Hz, H-5); 6.81 (1H, t, J = 8.2 Hz, H-6); 6.79 (1H, d, J = 7.2 Hz, H-14), 6.74 (1H, d, J = 8.2 Hz, H-4), 6.50 (1H, m, H-17), 6.41 (1H, s, H-11); 5.44 (1H, t, J = 2.6 Hz, H-16); 4.77 (1H, dt, J = 2.6, 7.2 Hz, H-15), 3.99 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 181.25 (C-1); 164.54 (C-10); 163.24 (C-12); 162.27 (C-3); 154.90 (C-8); 153.95 (C-7); 145.34 (C-17); 135.61 (C-5); 113.26 (C-14); 111.18 (C-4); 108.92 (C-2); 106.51 (C-9); 105.86 (C-6); 105.86 (C-13); 102.50 (C-16); 90.48 (C-11); 56.75 (C-18); 48.02 (C-15); + ESI-MS m/z: 325 $[M+H]^+$。以上数据均与文献报道[5,6]一致,故鉴定为 sterigmatocystin。

化合物 3　黄色粉末($CHCl_3$);^{1}H NMR ($CDCl_3$, 500 MHz) δ: 12.60 (1H, s, OH-3), 7.16 (1H, d, J = 8.9 Hz, H-6), 6.81 (1H, d, J = 7.1 Hz, H-14), 6.66 (1H, d, J = 8.9 Hz, H-4), 6.49 (1H, m, H-17), 6.40 (1H, s, H-11), 5.50 (1H, t, J = 2.5 Hz, H-16), 4.82 (1H, d, J = 7.1 Hz, H-15), 3.98 (3H, s, OCH_3-12), 3.91 (3H, s, OCH_3-6); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 181.37 (C-1), 164.59 (C-10), 163.32 (C-12), 163.34 (C-3), 154.98 (C-7), 154.06 (C-17), 145.38 (C-8), 135.68 (C-6), 113.25 (C-5), 111.26 (C-14), 109.01 (C-2), 106.52 (C-4), 106.00 (C-9), 105.89 (C-13), 102.51 (C-16), 90.53 (C-11), 56.80 (OCH_3-6) 56.80 (OCH_3-12), 48.08 (C-15); + ESI-MS: 355 $[M+H]^+$。以上数据均与文献报道[7]一致,故鉴定为 5-methoxysterigmatocystin。

化合物 4　黄色油状($CHCl_3$);^{1}H NMR ($CDCl_3$, 500 MHz) δ: 13.53 (1H, s, OH-1), 7.48 (1H, d, J = 2.5 Hz, H-5), 7.25 (1H, s, H-4), 6.82 (1H, d, J = 2.5 Hz, H-7), 6.48 (1H, d, J = 5.7 Hz, H-4), 4.15 (1H, m, H-1′), 4.05 (3H, s, OCH_3-8), 4.01 (3H, s, OCH_3-6), 2.38 (2H, m, H-3′), 2.29 (2H, m, H-2′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 187.11 (C-9), 182.48 (C-10), 165.10 (C-3), 165.02 (C-6), 162.92 (C-8), 160.28 (C-1), 137.55 (C-10a), 134.94 (C-4a), 120.11 (C-2), 115.08 (C-8a), 112.92 (C-4′), 112.62 (C-9a), 104.89 (C-7), 104.16 (C-5), 101.13 (C-4), 67.71 (C-3′), 56.99 (OCH_3-6), 56.63 (OCH_3-08), 44.46 (C-1′), 30.77 (C-2′); + ESI-MS: 369 $[M+H]^+$。以上数据均与文献报道[7]一致,故鉴定为 anthraquinone aversin。

化合物 5　黄色油状($CHCl_3$);^{1}H NMR ($CDCl_3$, 400 MHz) δ: 13.57 (1H, s, H-6′), 7.46 (1H, d, J = 2.5 Hz, H-5), 7.22 (1H, s, H-4), 6.79 (1H, d, J = 2.5 Hz, H-7), 5.40 (1H, dd, J = 4.6, 1.8 Hz, H-1′), 4.03 (3H, s, OCH_3-8), 3.99 (3H, s, OCH_3-6), 2.10 (1H, m, H-2′), 2.07 (1H, m, H-4′), 2.05 (1H, m, H-2′), 1.92 (1H, m, H-4′), 1.86 (1H, m, H-3′), 1.81 (1H, m, H-3′), 1.27 (3H, s, H-5′);

^{13}C NMR ($CDCl_3$, 100 MHz) δ: 186.77 (C-9), 182.58 (C-10), 164.93 (C-6), 162.79 (C-8), 159.90 (C-3), 159.50 (C-1), 137.57 (C-10a), 132.50 (C-4a), 116.80 (C-2), 115.28 (C-8a), 110.01 (C-4), 106.99 (C-7), 104.83 (C-5), 103.95 (C-9a), 100.84 (C-5′), 67.07 (C-1′), 56.61 (OCH_3-8), 55.99 (OCH_3-6), 35.95 (C-4′), 27.86 (C-6′), 27.45 (C-2′), 16.00 (C-3′); +ESI-MS: 397[M+H]$^+$。以上数据均与文献报道[8]一致,故鉴定为6,8-di-*O*-methyl averufin。

化合物6 黄色粉末($CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 14.06 (1H, s, OH-1), 7.26 (1H, s, H-5), 6.99 (1H, s, H-7), 7.10 (1H, s, H-4), 3.95 (3H, s, OCH_3-8), 3.94 (3H, s, OCH_3-6), 3.75 (2H, m, H-1′), 3.69 (1H, m, H-2′), 3.43 (1H, m, H-4′), 3.31 (1H, m, H-4′), 1.93 (2H, q, J = 6.9 Hz, H-3′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 186.24 (C-9), 182.51 (C-10), 164.97 (C-6), 164.96 (C-8), 163.69 (C-1), 163.30 (C-3), 137.00 (C-10a), 131.54 (C-4a), 123.74 (C-2), 114.60 (C-8a), 109.47 (C-9a), 107.62 (C-4), 105.03 (C-7), 104.84 (C-5), 63.38 (C-1′), 60.53 (C-4′), 57.06 (OCH_3-6) 56.51 (OCH_3-8) 39.50 (C-2′), 33.10 (C-3′); +ESI-MS: 389 [M+H]$^+$。以上数据均与文献报道[7]一致,故鉴定为6,8-di-O-methyl versiconol。

化合物7 黄色油状($CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 8.59 (1H, s, NH-2), 7.62 (1H, s, H-10), 7.37 (1H, d, J = 7.8 Hz, H-13), 7.20 (3H, m, H-14, H-15, H-16), 6.25 (1H, t, J = 3.0 Hz, H-8), 6.07 (1H, dd, J = 17.4, 10.6 Hz, H-21), 5.19 (2H, m, H-22), 4.21 (2H, t, J = 9.2 Hz, H-6), 2.89 (2H, td, J = 9.2, 3.0 Hz, H-7), 1.53 (6H, s, H-23, H-24); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 155.17 (C-4), 154.36 (C-1), 144.39 (C-21), 144.06 (C-19), 134.53 (C-17), 133.90 (C-9), 126.18 (C-12), 126.05 (C-3), 122.39 (C-15), 121.12 (C-14), 119.90 (C-13), 118.96 (C-8), 113.30 (C-22), 111.44 (C-10), 111.42 (C-16), 103.24 (C-11), 45.90 (C-6), 39.32 (C-20), 28.28 (C-7), 27.48 (C-23, C-24); +ESI-MS: 348[M+H]$^+$。以上数据均与文献报道[9]一致,故鉴定为 brevianamide K。

化合物8 白色油状($CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 8.69 (1H, s, H-18), 7.45 (1H, s, H-2), 7.36 (1H, m, H-16), 7.30 (1H, m, H-13), 7.23 (1H, s, H-10), 7.18 (1H, m, H-15), 7.14 (1H, m, H-14), 6.06 (1H, dd, J = 17.4, 10.6 Hz, H-21), 4.32 (1H, dd, J = 10.3, 6.4 Hz, H-9), 3.88 (1H, m, H-6), 3.66 (1H, ddd, J = 12.5, 9.4, 3.1 Hz, H-6), 2.46 (1H, m, H-8), 2.04 (3H, m, H-7, H-8), 1.53 (6H, s, H-23, H-24); ^{13}C NMR ($CDCl_3$, 500 MHz) δ: 165.31 (C-4), 158.10 (C-1), 144.45 (C-19), 143.90 (C-21), 134.55 (C-17), 126.16 (C-3), 126.30 (C-12), 122.21 (C-15), 120.50 (C-14), 118.97 (C-13), 111.69 (C-10), 111.37 (C-16), 113.15 (C-22), 103.32 (C-11), 59.45 (C-9), 45.52 (C-6), 39.28 (C-20), 29.16 (C-8), 27.55 (C-24), 27.41 (C-23), 21.90 (C-7); +ESI-MS: 391 [M+ACN+H]$^+$。以上数据均与文献报道[10]一致,故鉴定为 brevianamides V。

化合物9 黄色油状($CHCl_3$); ^{1}H NMR (CDCl, 400 MHz) δ: 8.63 (1H, s, NH-18), 7.53 (1H, s, NH-2), 7.38 (1H, d, J = 7.9 Hz, H-16), 7.32 (1H, s, H-10), 7.27 (1H, m, H-13), 7.16 (2H, dt, J = 14.8, 7.2 Hz, H-14, H-15), 6.06 (1H, dd, J = 17.3, 10.6 Hz, H-21), 5.18 (2H, m, H-22), 3.93 (1H, m, H-6), 3.76 (1H, m, H-6), 3.36 (3H, s, OCH_3-9), 2.17 (1H, m, H-7), 2.07 (3H, m, H-7, H-8), 1.53 (6H, s, H-22, H-23); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 162.50 (C-1), 158.75 (C-4), 144.23 (C-21), 144.05 (C-19), 134.43 (C-17), 126.08 (C-12), 125.60 (C-3), 122.32 (C-15), 121.04 (C-14), 118.71 (C-13), 113.25 (C-22), 111.40 (C-16), 103.09 (C-11), 103.09 (C-10), 91.65 (C-9), 51.45 (OCH_3-9), 45.29 (C-6), 39.19 (C-20), 34.55 (C-8), 27.45 (C-23), 27.28 (C-24), 19.34 (C-7); +ESI-MS: 380 [M+H]$^+$。以上数据均与文献报道[11]一致,故鉴定为 brevianamide R。

2.2 抗菌活性检测

化合物**3**对金黄色葡萄球菌、大肠杆菌、枯草芽孢杆菌表现出较弱的抗菌活性,其他化合物未表现出抗菌活性。其对枯草芽孢杆菌的最低抑菌浓度为31.1 μg/mL,最大浓度下化合物对大肠杆菌、金黄色葡萄球菌未表现明显抑菌活性。阳性对照氨苄西林钠对大肠杆菌、金黄色葡萄球菌、枯草芽孢杆菌最低抑菌浓度分别为2.2、0.3、1.1 μg/mL。

3 分析与讨论

海洋内生真菌由于其次级代谢产物丰富,结构独特,具有抗肿瘤、抗菌、降血糖等广泛的药理活性,受到国内外天然产物化学研究者的关注。本报道中研究的海洋杂色曲霉中分离得到 9 个化合物,分别是含硫苯并呋喃衍生物、二酮哌嗪化合物、蒽醌类化合物、吨酮类化合物,研究表明这些化合物都具备一定的生物活性。研究表明化合物 **1** 为 α-糖苷酶抑制剂[3],对于 2 型糖尿病具有治疗作用。二酮哌嗪类化合物(**7**、**9**)具有抗氧化作用[11,12],且化合物无细胞毒性。因此,内生真菌 PT-20 具有一定的应用价值。

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