ANTHRANILAMIDES: NEW ANTIMICROALGAL ACTIVE SUBSTANCES FROM A MARINE Streptomyces sp.†

M.A. Farooq Biabani†, Matthias Baake†, Barbara Lovisetto†, Hartmut Laatsch†*, Elisabeth Helmke††, and Horst Weyland††

†Department of Organic Chemistry, University of Göttingen, Tammanstrasse 2, Göttingen D-37077, Germany

††Alfred-Wegener-Institute for Polar and Marine Research Am Handelshafen 12, D-27570 Bremerhaven, Germany

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2-[Methyl-(3-phenylpropionyl)amino]-benzoic acid (1e) was isolated from a culture of marine Streptomyces sp. strain B7747. Analogous compounds have potential importance as phytotoxic substances, hence compound 1e and the analogues 1a - 1d and 1f - 3a were synthesised. Antimicroalgal activity of the anthranilamide analogues showed that esters 1b, 1f and 2b were more active than the free acids. The minimum inhibitory concentration (MIC) against Chlorella vulgaris, Chlorella sorokiniana, Chlorella salina and Scenedesmus subspicatus ranged from 20 to 107 µg/ml. All anthranilamides were inactive against Staphylococcus aureus, Escherichia coli, and Mucor miehei.

The microbial diversity of the marine biosphere offers enormous scope for the discovery of novel natural products. Marine bacteria, fungi, cyanobacteria and symbiotic microorganisms have been particularly productive sources of bioactive natural products. Our efforts in this field have focussed upon screening for antimicroalgal, antibacterial and antifungal activity including chemical studies of biologically active marine bacterial extracts1). In the course of our biological screening, extracts of the marine streptomycete B7747 were shown to possess potent antimicroalgal activity.

Fermentation of this S. sp. and purification of extracts afforded a new anthranilamide, 2-[methyl-(3-phenylpropionyl)amino]-benzoic acid (1e) and the known metabolites N-methyl anthranilic acid and daidzein2). The latter (4',7-dihydroxyisoflavone, 7-hydroxy-3-(4-hydroxyphenyl)-4H-benzo[b]pyran-4-one, m/z = 254.24; C_{15}H_{10}O_{4}) is a widespread isoflavone in the Leguminosae plants and also marine streptomycetes. The anthranilamide 1e is

structurally related to three groups of phytoalexins i.e. dianthalexins\(^3\), avenalumins\(^4\) and avenanthramides\(^5\) which have been previously isolated from rose leaves, maize and oat, respectively. Anthranilamide \(1\text{e}\) as well as its analogues \(1\text{a}-1\text{d}\) and \(1\text{f}-5\text{a}\) were therefore synthesised due to their expected biological properties. This paper describes the taxonomic studies of the producing organism, the production, isolation and synthesis of compound \(1\text{e}\) and analogues, as well as detailing their antimicroalgal activity.

**Taxonomic studies of producing strain**

The *Streptomyces* strain B7747 has been derived from sediment of the Laguna de Terminos at the Gulf of Mexico, and was isolated on casein peptone medium containing 50 % natural sea water with incubation at 18 °C. The pure culture was maintained on yeast extract-malt extract agar\(^6\). The strain forms yellow-brown vegetative and white aerial mycelium, with spiral sporophores (*Spirales*). Spores are cylindrical with a smooth surface. Melanin pigment is produced neither on peptone-yeast extract-iron agar nor on tyrosine agar\(^7\). The temperature optimum is at approximately 30 °C. The strain does not grow at 45 °C or at 10 °C. Chitin, gelatine, and starch are degraded. Casein and esculin are not hydrolysed and hydrogen sulphide is not produced. The strain is catalase positive and nitrate reductase is not formed. The peptidoglycan cell wall of the strain contains major amounts of L-diaminopimelic acid (L-DAP) and glycine but no diagnostic sugars (cell wall chemotype I)\(^8\). Due to its chemical and morphological features the strain can be assigned to the genus *Streptomyces*. The strain is deposited in the culture collection of marine actinomycetes at the Alfred-Wegener-Institute for Polar and Marine Research, Bremerhaven, Germany.
Fermentation and Isolation

The slant culture of *S. sp. B7747* was inoculated into Erlenmeyer flasks on malt extract / yeast extract / glucose in 50 % synthetic sea water and incubated for 3 days at 28 °C. Fermentation was carried out in a 20 litre jar fermentor under similar conditions. The ethyl acetate extract of the entire culture was defatted with cyclohexane and subjected to Sephadex LH-20 column chromatography.

Activity screening of the fractions was done by agar diffusion tests using the algae *Chlorella vulgaris, Chlorella sorokiniana, Chlorella salina* and *Scenedesmus subspicatus*. The active fractions were re-chromatographed by silica gel column chromatography to afford 2-[methyl-(3-phenylpropionyl)amino]-benzoic acid (1e), along with known metabolites daidzein and N-methyl anthranilic acid. The latter were identified by 1H NMR, EI-MS and confirmed by comparison with authentic samples.

Results and discussion

The crude compound 1e was obtained as an ochre coloured solid. The molecular formula of 1e was determined to be C_{17}H_{17}NO_{3} by HR-MS of the molecular ion at m/z 283. The 1H and 13C NMR spectra, the H,H-coupling pattern and the base peak at m/z 151 in the EI mass spectrum were similar to those of N-methyl anthranilic acid, pointing to an N-methyl anthranilic acid sub-structure. In addition to this, 1H and 13C NMR data further indicated the presence of an amide carbonyl, two methylenes and a phenyl group. Based on this information, the structure was determined as 2-[methyl-(3-phenylpropionyl)amino]-benzoic acid (1e) and finally confirmed by synthesis: The reaction of N-methyl anthranilic acid and phenyl propionyl chloride gave an amide which was identical to the natural compound 1e in every respect. Treatment of 1e with excess diazo methane or sulphuric acid/methanol afforded easily the methyl ester 1f.
In order to study the structure-activity relationship, 1e-analogues 1a-1d and 1f-5a were prepared. Variation in alkylation as well as saturation of the benzene ring afforded 2-(benzoyl-methylamino)benzoic acid (1a), 2-(methyl-phenylacetylamino)benzoic acid (1c), 2-[methyl-(4-phenylbutyryl)amino]-benzoic acid (1h) and 2-[(3-cyclohexylpropionyl)methylamino]-benzoic acid (3a). The phenylpropionic acid fragment was also modified by inserting a double bond to afford 2-(cinnamoyl-methylamino)benzoic acid (2a). Analogue 2a is structurally related to phytotoxins avenanthramides A (2c) and B (2d) which are reported to occur in oat groats and hulls.

Starting with anthranilic acid instead of N-methylanthranilic acid afforded the cyclisation products, 2-phenyl-4H-3,1-benzoxazin-4-one (4a) and 2-phenylethynyl-4H-3,1-benzoxazin-4-one (5a). These cyclised products are structurally related to phytoalexins, for example 4a is similar to dianthalexin (4b). Dianthalexin (4b) is a member of benzoxazinone phytoalexins previously isolated as a major component of carnation response to elicitation and infection by *Phytophthora parasitica*. Analogue 5a is related to phytoalexins avenalumins-1 (5b) and -2 (5c). Avenulamins are the phytoalexins of oat leaves produced on infection with oat crown crust (*Puccinia coronata sp. avenae*).

Treatment of compounds 1a, 1c, 1e, 1h, 2a, and 3a with an excess of ethereal diazomethane solution afforded the methyl esters 1b, 1d, 1f, 1i, 2b and 3b. These ester structures were confirmed by $^1$H NMR and combustion analysis.

** place formula block here **

** Antimicroalgal activity **

Antimicroalgal activities were determined using the agar diffusion method. Test organisms were incubated on agar plates for two days at 24-26 °C in daylight. Thereafter, paper discs containing the test substances at concentrations of 100 µg/disc were placed on pre-incubated
plates and further incubated for another 4 days at 24-26 °C in daylight. Table 1 shows the antimicroalgal activities of compound 1e and its respective analogues in comparison with staurosporine as a reference.

** place table 1 here **

The esters 1b, 1f and 2b showed stronger activity than the free acids in agar diffusion tests as well as in liquid test systems, using the dilution method. For MIC values, liquid medium was inoculated with test organisms and pre-incubated at 24-26 °C for one day in daylight. Test substances were added and results were recorded after 3 days (see Table 2). In addition, a known antibiotic, staurosporine showed strong antimicroalgal activity and hence this was tested as a reference. Compounds 1a-5a did not show activity against Staphylococcus aureus, Escherichia coli, and Mucor miehei at concentrations up to 200 µg/ml.

** place table 2 here **

**Experimental**

IR-spectra: Perkin Elmer, model 297 (KBr). – ¹H and ¹³C NMR-spectra: Varian VXR 200, VXR 500 (tetramethylsilane as internal standard). – Mass spectra: MAT 311 A (70 eV, high resolution with perfluorokerosene as reference). – Thin layer chromatography (TLC): TLC sheets Polygram SIL G/UV₂₅⁴ 4 × 8 cm (silica gel, Macherey-Nagel & Co, Düren, Germany). Column chromatography (CC): Silica gel 60 (0.05-0.2 mm and 0.04-0.063 mm / 230 - 400 mesh ASTM; Macherey-Nagel & CO). The columns were packed wet, all chromatographic zones are numbered in the sequence of decreasing \( R_f \) values. During fermentation Niax PPG 2025 (Union Carbide Belgium N.V., Zwindrecht) was used as anti foaming agent. – Assay discs: Ø 9 mm, Schleicher & Schuell, Dassel, Germany.  

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Artificial Sea Water
To prepare 20 ℓ sea water, dissolve iron citrate 2 g (powdered), NaCl 389 g, MgCl₂ · 6 H₂O 176 g, Na₂SO₄ 64.8 g (dissolve separately in 2 ℓ water), CaCl₂ 36 g, Na₂HPO₄ 0.16 g, SiO₂ 0.3 g, trace element stock soln. 20 ml, stock soln. 200 ml in 18 ℓ demin. water.

Trace Elements Stock Solution for Artificial Sea Water
To prepare trace elements stock solution, dissolve H₃BO₃ 0.611 g, MnCl₂ 0.389 g, CuSO₄ 0.056 g, ZnSO₄ · 7 H₂O 0.056 g, Al₂(SO₄)₃ · 18 H₂O 0.056 g, NiSO₄ · 6 H₂O 0.056 g, Co(NO₃)₃ · 6 H₂O 0.056 g, TiO₂ 0.056 g, (NH₄)₆Mo₇O₂₄ · 4 H₂O 0.056 g, LiCl 0.028 g, SnCl₂ 0.028 g, KI 0.028 g in 1 ℓ demin. water.

Stock Solution for Artificial Sea Water
To prepare stock solution, dissolve KCl 110 g, NaHCO₃ 32 g, KBr 16 g, SrCl₂ · 6 H₂O 6.8 g (dissolve separately), H₃BO₃ 4.4 g, NaF 0.48 g, NH₄NO₃ 0.32 g in 2 ℓ demin. water.

Trace Element Stock Solution for Algae
MnSO₄ · H₂O (169 mg), Na₂MoO₄ · 2 H₂O (130 mg), Co(NO₃)₂ · 6 H₂O (100 mg), CuSO₄ · 5 H₂O (50 mg), H₃BO₃ (100 mg), ZnSO₄ · 7 H₂O (100 mg) are each dissolved in 100 ml of distilled water to obtain trace elements stock solution.

Fe-EDTA Stock Solution
FeSO₄ · 7 H₂O (0.7 g) and 0.93 g EDTA (Titriplex III) are dissolved in 80 ml H₂O under warming, then diluted to 100 ml.

Soil Extract
Garden soil (10 g) is heated on a steamer with 200 ml distilled water for 1 h at 100 °C, filtered and diluted to make a 2 litre stock soil solution. The solution can be kept for 1 week at 4 °C.
Microalgae Media

Bold’s Basal Medium (BBM-medium)\(^9\) for *Chlorella vulgaris*, *Chlorella sorokiniana*, and *Scenedesmus subspicatus*. The nutrient solution was obtained by mixing the indicated volumes of stock solutions (see Table 3) and sterilised for 1 h at 120 °C.

Sea water medium-5\(^{10}\) for *Chlorella salina* was obtained in a similar way corresponding to Table 4 and sterilised for 40 min at 120 °C.

** place table 3 and table 4 here **

Antimicroalgal Tests

Microalgae strains of *Chlorella vulgaris* SAG-211-11b, *Chlorella sorokiniana* SAG-211-8K, *Chlorella salina* SAG-8.86 and *Scenedesmus subspicatus* SAG-86-81 were obtained from the microalgae collection, University of Göttingen, Göttingen, Germany. Cultures were maintained on slant agar of BBM medium (*C. vulgaris*, *C. sorokiniana*, and *S. subspicatus*) or liquid sea water medium-5 (*C. salina*).

BBM medium (200 ml) in 500 ml conical flasks was inoculated from agar slant cultures of *C. vulgaris*, *C. sorokiniana*, or *S. subspicatus* and placed in daylight at 24-26 °C for one week. Under sterile conditions, 3 ml of test algae suspension (10\(^7\) cell/µl) were mixed uniformly with 6 ml of soft agar (0.5 % of agar-agar in the corresponding medium) at not more than 40 °C. This suspension was poured as a second layer on top of BBM medium plates containing 1.5 % agar. Assay discs containing 100 µg/disc test substance were placed on the surface of these inoculated agar plates and incubated for 4 days in daylight at 24-26 °C.

For MIC determination, test compounds were dissolved in H\(_2\)O:MeOH 80:20 to obtain a stock solution. Correspondingly diluted solutions were added to test tubes each containing a suspension of 200 µl of test organisms in 1 ml liquid BBM-medium (sea water medium-5 for *C. salina*). The tubes were incubated in daylight at 24-26 °C and results were recorded after 3
days. Tubes inoculated in an identical manner but devoid of the active substances served as controls.

Fermentation of the Streptomyces sp. B7747
The strain B7747 was grown for 3 days at 95 rpm and 28 °C in ten 1 litre Erlenmeyer flasks shake cultures, each containing 200 ml of liquid medium. Medium consisted of malt extract 20 g, yeast extract 8 g, glucose 8 g, 1 ℓ sea water, and 1 ℓ tap water, adjusted to pH 7.8 and sterilised for 1 h at 120 °C. The shake culture was transferred into a 20 litre jar fermentor, containing 18 litres of the same culture medium as above. Incubation was carried out at 28 °C for 3 days with automatic addition of 2 N NaOH to maintain the pH between 5 - 6. If needed, Niaxs was added to control foaming, sterile air was supplied (5 ℓ /min) and agitation was at 120 rpm.

The entire culture broth was filtered on celite, and the culture filtrate and mycelium were extracted three times with 5 litres of ethyl acetate. Evaporation to dryness of the combined organic layers gave 1.6 g of crude extract. The latter was defatted with cyclohexane and the insoluble part was subjected to Sephadex LH-20 column chromatography (3 × 65 cm) with methanol. The phytotoxic fractions were re-chromatographed on silica gel with CHCl₃/MeOH (7 : 3), to afford four UV absorbing fractions. Less polar fraction 1 was further purified on silica gel (cyclohexane/EtOAc, 1:1) to yield daidzein (16 mg, R_f = 0.2 in cyclohexane/EtOAc 1:1). Silica gel column chromatography (CHCl₃:MeOH, 9:1) of fraction 2 afforded 20 mg of 2-[methyl-(3-phenylpropionyl)amino]-benzoic acid (1e) (R_f = 0.3, CHCl₃:MeOH, 9:1). The highly polar fraction 3 was extracted with NaHCO₃ and obtained from the water phase by acidifying and extraction with diethyl ether to give blue fluorescent N-methylantranilic acid (11 mg, R_f = 0.2, CHCl₃/MeOH 9:1).
Synthesis of Anthranilamides

General Procedure

To a solution of N-methyl anthranilic acid (0.755 g, 5.00 mmol) in 30 ml of dry pyridine, acid chloride (10 mmol) was added dropwise over a period of 30 min under an argon atmosphere at 0 °C. The reaction mixture was stirred for 2 h at ca. 20 °C and then poured into ice water (200 ml) and acidified with conc. HCl (5.0 ml). The precipitate was filtered off and the filtrate was extracted with diethyl ether (3 × 70 ml). The combined solid and organic phases were dried with Na₂SO₄ and evaporated to dryness to afford a viscous raw product.

2-[Benzoyl-methylamino]benzoic Acid

Benzoyl chloride (1.41 g, 10.0 mmol) was allowed to react with N-methylanthranilic acid. Re-crystallisation of the raw product with diethyl ether afforded 714 mg (56 %) (1a) as colourless crystals, m.p: 152 °C (lit.12) 162 °C). – 1H NMR (CDCl₃, 200 MHz): δ = 9.78 (s br, 1 H, COOH), 7.90 (d, 3 J = 8 Hz, 1 H, 6-H), 7.30-7.06 (m, 8 H, 3-, 4-, 5-H and 2’ to 6’-H), 3.47 (s, 3 H, NCH₃). – MS (70 eV): m/z (%) = 255 [M⁺] (30), 210 (12), 150 (16), 133 (46), 105 (100), 77 (46).

2-[Benzoyl-methylamino]benzoic Acid Methyl Ester (1b)

1H NMR (CDCl₃, 200 MHz): δ = 7.89 (d, 3 J = 8 Hz, 1 H, 6-H), 7.30-7.06 (m, 8 H, 3-, 4-, 5-H and 2’ to 6’-H), 3.90 (s, 3 H, COOCH₃), 3.45 (s, 3 H, NCH₃).
2-(Methyl-phenylacetylamino)benzoic Acid (1c)

Freshly prepared phenylacetyl chloride (1.36 g, 10 mmol) was reacted with N-methylan-
trianilic acid. Re-crystallisation of reaction product with diethyl ether afforded 673 mg (50 %) of 1c as colourless crystals, m.p: 169 °C. – 1H NMR (CDCl3, 200 MHz): δ = 9.10 (s br, 1 H, COOH), 8.13 (d, 3J = 7 Hz, 1 H, 6-H), 7.62-7.46 (m, 2 H, 4-H, 5-H), 7.19-7.01 (m, 6 H, 3-
H, 2’to 6’-H), 3.37, 3.47 (AB, 2J = 12 Hz, 2 H, CH2), 3.26 (s, 3 H, N-CH3). – 13C NMR
(CDCl3, 50.3 MHz): δ = 171.97 (COOH), 168.17 (O=C-N), 143.37 (C-1), 134.82 (C-2),
133.88, 132.58 (2 C), 130.07, 129.13 (2 C), 128.67, 128.36, 128.21, 126.55 (C-3 to C-6, and
C-1’ to C-6’), 41.20 (CH3), 37.67 (CH2). – MS (70 eV): m/z (%) = 269 [M+] (4), 251 (2), 178
(20), 151 (100), 133 (16), 105 (24), 91 (38), 65 (10).

Anal Calcd. for C16H15NO3 C 71.36 H 5.61
found C 71.15 H 5.79

2-(Methyl-phenylacetylamino)benzoic Acid Methyl Ester (1d)

1H NMR (CDCl3, 200 MHz): δ = 8.02 (d, 3J = 7 Hz, 1 H, 6-H), 7.51-7.46 (m, 2 H, 4-H, 5-
H), 7.19-6.96 (m, 6 H, 3-H, 2’ to 6’-H), 3.37, 3.47 (AB, 2J = 12 Hz, 2 H, CH2), 3.83 (s, 3 H,
COOCH3), 3.26 (s, 3 H, N-CH3)

2-[Methyl-(3-phenylpropionyl)amino]-benzoic Acid (1e)

Following the general procedure, 1.67 g (10.0 mmol) phenylpropionyl chloride was added to
N-methylantranilic acid. The crude product was chromatographed on silica gel (100 g,
cyclohexane / EtOAc, 3:7) to afford 1e (877 mg, 62 %) with m.p. 107 °C after crystallisation
from diethyl ether. – 1H NMR (CDCl3, 200 MHz): δ = 10.50 (s, 1 H, COOH, D2O exchange-
able), 8.12 (dd, 3J1,3J2 = 8 Hz, 4J = 2 Hz, 6-H), 7.50 (m, 2 H, 4-H, 5-H), 7.18-7.00 (2 m,c, 6
H, 3-H, 2’ - 6’-H), 3.26 (s, 3 H, NCH3), 2.90 (m, 2 H, 8-H2), 2.35 (m, 2 H, 7-H2). 13C NMR
(C$_6$D$_6$, 50.3 MHz): $\delta = 174.42$ (COOH), 176.02 (O=C-N), 143.22 (C-1), 141.62 (C-2), 132.98, 132.12, 130.17, 129.22 (2 C), 128.66 (2 C), 128.31 (2 C), 126.16, 37.50 (NCH$_3$), 36.56 (C-8), 32.06 (C-7). – MS (70 eV): $m/z$ (%) = 283 [M$^+$] (40), 265 (16), 238 (10), 207 (4), 151 (100), 133 (24), 105 (44), 91 (42), 83 (36), 77 (18), 60 (12), 43 (16).

$\text{Anal Calcd. for } C_{17}H_{17}NO_3$  
found  
C 72.61  H 6.42  
C 72.56  H 6.24

2-[[Methyl-(3-phenylpropionyl)amino]-benzoic Acid Methyl Ester (1f)]

A solution of 1e (0.57 g, 2.0 mmol) and 2 drops of conc. sulphuric acid in 10 ml methanol was refluxed for 6 h under an argon atmosphere. Methanol was distilled off, the residue poured into cold water (100 ml) and the mixture extracted with diethyl ether (3 $\times$ 30 ml). The combined organic phases were washed with NaHCO$_3$ solution and dried with Na$_2$SO$_4$. Silica gel chromatography (column 3 $\times$ 30 cm) afforded 400 mg of 1f (67 %) as viscous colourless oil. – $^1$H NMR (CDCl$_3$, 200 MHz): $\delta = 7.97$ (dd, $^3J = 7.6$ Hz, $^4J = 2.0$ Hz, 1 H, 6-H), 7.52 (ddd, $^3J_1, ^3J_2 = 7.4$ Hz, $^4J = 1.6$ Hz, 1 H, 4- or 5-H), 7.41 (ddd, $^3J_1, ^3J_2 = 7.6$ Hz, $^4J = 1.6$ Hz, 1 H, 5- or 4-H), 7.30-6.99 (m, 6 H, 3-H, 2'- 6'-H), 3.83 (s, 3 H, COOCH$_3$), 3.18 (s, 1 H, NCH$_3$), 2.90 (m, 2 H, 8-H$_2$), 2.24 (t, $^3J = 7.9$ Hz, 1 H, 7-H$_2$). – $^{13}$C NMR (CDCl$_3$, 50.3 MHz): $\delta = 171.78$ (COOCH$_3$), 165.79 (O=C-N), 143.41 (C-1), 141.27 (C-2), 133.64, 131.85, 129.84, 128.51, 128.37, 128.29, 128.24, 125.86 (C-3 - C-6 and C-1' - C-6'), 52.57 (OCH$_3$), 36.95 (NCH$_3$), 35.94 (C-8), 31.41 (C-7). – MS (70 eV): $m/z$ (%) = 297 [M$^+$] (68), 266 (10), 238 (18), 207 (4), 165 (100), 132 (29), 105 (38), 91 (38), 77 (15).

$\text{Anal Calcd. for } C_{18}H_{19}NO_3$  
found  
C 72.71  H 6.44  
C 72.66  H 6.34
2-(3-Phenyl-propionylamino)benzoic Acid (1g)

Phenylpropionyl chloride (1.09 g, 6 mmol) was added to a solution of anthranilic acid (0.41 g, 3.0 mmol) in 30 ml pyridine. The reaction mixture was stirred for 2 h at 20 °C and then transferred into cold water (200 ml). The precipitate was washed with cold water (3 × 70 ml) and re-crystallised from ethanol to afford 399 mg of 1g (51 %), m.p: 132 °C. – 1H NMR (CDCl₃, 200 MHz): δ = 10.92 (br s, 1 H, COOH), 8.78 (d, \(3J = 8\) Hz, 1 H, 3-H), 8.25 (d, \(3J = 8\) Hz, 1 H, 6-H), 7.62 (dd, \(3J = 8\) Hz, 1 H, 5-H), 7.30-7.14 (m, 6 H, 4-H, 2’-H - 6’-H), 3.08 (dd, \(3J_1, 3J_2 = 8\) Hz, 2 H, 8-H₂), 2.78 (dd, \(3J_1, 3J_2 = 8\) Hz, 2 H, 7-H₂). – 13C NMR (CDCl₃, 50.3 MHz): δ = 172.38 (COOH), 171.56 (O=C-N), 141.77 (C-1), 140.35 (C-2), 135.64, 131.74, 128.54 (2 C, C-2’, C-6’ or C-3’, C-5’), 128.31 (2 C, C-3’, C-5’ or C-2’, C-6’), 126.31, 126.29, 122.80, 120.62, 113.97 (5 arom. C), 40.30 (C-8), 31.45 (C-7). – MS (70 eV): m/z (%) = 269 [M⁺] (57), 251 (4), 146 (6), 137 (100), 119 (44), 105 (16), 91 (40), 65 (8), 51 (4).

**Anal Calcd. for C₁₁H₁₁NO₃ C 71.36 H 5.61 found C 71.42 H 5.55**

2-(Methyl-4-phenylbutyrylamino)benzoic Acid (1h)

A mixture of 2.97 g of SOCl₂ (25.0 mmol) and 2.46 g of phenylbutyric acid (15.0 mmol) was refluxed for 2 h under an argon atmosphere. Excess SOCl₂ was distilled off under vacuum. The crude acid chloride residue was then allowed to react with 0.755 g N-methylanthranilic acid (5.00 mmol) to afford 512 mg of 1h (48 %) as colourless needles, m.p. 95 °C. – 1H NMR (CDCl₃, 200 MHz): δ = 9.34 (s br, 1 H, COOH), 8.10 (dd, \(3J = 7.6\) Hz, \(4J = 1.2\) Hz, 1 H, 6-H), 7.58 (ddd, \(3J_1, 3J_2 = 7.4\) Hz, \(4J = 1.6\) Hz, 1 H, 4- or. 5-H), 7.46 (dd, \(3J_1, 3J_2 = 7.4\) Hz, 1 H, 5-H or 4-H), 7.22-7.13 (m, 6 H, 3-H, 1- to 6-H), 3.23 (s, 1 H, NCH₃), 2.50 (d, \(3J = 8\) Hz, 2 H, CH₂ terminal), 2.10 (m, 2 H, CH₂ terminal), 203 (m, 2 H, 8-H₂). – 13C NMR (CDCl₃, 50.3 MHz): δ = 174.00 (COOH), 167.87 (O=C-N), 143.44 (C-1), 141.71 (C-2), 133.87, 132.63, 129.53, 128.51 (2 C), 128.33 (2 C), 128.16 (2 C), 125.65, 37.32 (NCH₃), 35.09 (C-7), 33.45
2-(Cinnamoyl-methylamino)benzoic Acid (2a)

A mixture of 2.96 g of trans-cinnamic acid (20.0 mmol) and SOCl$_2$ (3.57 g 30 mmol) was refluxed under an argon atmosphere for 2 h. Excess SOCl$_2$ was distilled off and the acid chloride residue was allowed to react with N-methyl anthranilic acid to afford 2a (1.07 g, 76%). Compound 2a crystallised from diethyl ether as colourless needles, m. p. 176 °C. – $^1$H NMR (CDCl$_3$, 200 MHz): $\delta$ = 9.15 (s br, 1 H, COOH), 8.17 (d, $J$ = 8 Hz, 6-H), 7.72 (d, $^3J$ = 16 Hz, 1 H, 8-H), 7.65-7.45 (m, 8 H, arom. H), 6.53 (d, $J$ = 16 Hz, 1 H, 7-H), 3.36 (s, 3 H, NCH$_3$). – $^{13}$C NMR (CDCl$_3$, 50.3 MHz): $\delta$ = 167.78 (COOH), 167.22 (O=C-N), 142.92, 134.84, 133.78, 132.66, 129.68, 129.52, 129.03, 128.60, 128.51 (2 C), 127.88 (2 C), 117.65, 37.79 (NCH$_3$). – MS (70 eV): $m/z$ = 281 [M$^+$] (40), 236 (10), 193 (4), 151 (84), 131 (100), 103 (40), 91 (4), 77 (24), 51 (6).

2-(Cinnamoyl-methylamino)benzoic Acid Methyl Ester (2b)

$^1$H NMR (CDCl$_3$, 200 MHz): $\delta$ = 8.00 (d, $J$ = 8 Hz, 6-H), 7.65 (d, $^3J$ = 16 Hz, 1 H, 8-H), 7.54-7.45 (m, 8 H, arom. H), 6.53 (d, $J$ = 16 Hz, 1 H, 7-H), 3.89 (s, 3 H, COOCH$_3$), 3.36 (s, 3 H, NCH$_3$).
**2-[(3-Cyclohexylpropionyl)methylamino]-benzoic Acid (3a)**

In an analogous synthesis, cyclohexylpropionic acid (2.34 g, 15.5 mmol) was heated with SOCl₂ (2.97 g, 25.0 mmol) for 2 h under an argon atmosphere. The acid chloride was allowed to react with N-methylanthranilic acid (0.755 g, 5.00 mmol) which afforded accordingly 3a. Re-crystallisation of the crude product in diethyl ether gave 3a (318 mg, 22 %), m.p. 91 °C –

1H NMR (CDCl₃, 200 MHz): δ = 10.10 (s br, 1 H, COOH), 8.17 (d, 3J = 7 Hz, 1 H, 6-H), 7.63 (dd, 3J = 7 Hz, 4-H or 5-H), 7.51 (dd, 3J₁, 3J₂ = 7 Hz, 5-H or 4-H), 7.30 (m, 1 H, 3-H), 3.23 (s, 3 H, NCH₃), 2.00 (m, 2 H, 7-H₂), 1.65, 1.05, 0.80 (3 m, 13 H, 8-H₂, 1’- to 6’-H). –

13C NMR (C₆D₆, 50.3 MHz): δ = 175.71 (COOH), 166.88 (O=C-N), 143.57 (C-1), 130.54 (C-2), 132.91, 132.28, 129.21, 128.30 (C-3 to C-6), 37.63 (NCH₃), 37.39 (CH), 33.37, 33.22, 32.12, 26.86, 26.54 (5 CH₂). – MS (70 eV): m/z = 289 [M⁺] (2), 244 (4), 206 (4), 193 (30), 151 (100), 133 (20), 105 (22), 77 (8), 55 (10).

Anal. Calcd. for C₁₁H₂₃NO₃  C 70.71 H 7.74
found C 70.53 H 7.90

**2-[(3-Cyclohexylpropionyl)methylamino]-benzoic Acid Methyl Ester (3b)**

1H NMR (CDCl₃, 200 MHz): δ = 8.15 (d, 3J = 7 Hz, 1 H, 6-H), 7.70 (dd, 3J = 7 Hz, 4-H or 5-H), 7.50 (dd, 3J₁, 3J₂ = 7 Hz, 5-H or 4-H), 7.30 (m, 1 H, 3-H), 3.90 (s, 3 H, COOCH₃), 3.23 (s, 3 H, NCH₃), 2.00 (m, 2 H, 7-H₂), 1.80, 1.05, 0.80 (3 m, 13 H, 8-H₂, 1’- to 6’-H).

**2-Phenyl-4H-3,1-benzoxazin-4-one (4a)**

Benzoyl chloride (1.40 g, 10.0 mmol) was added to a solution of anthranilic acid (0.69 g, 5.0 mmol) in 30 ml pyridine. The reaction mixture was stirred for 2 h at 20 °C and the product obtained, precipitated by addition of cold water (200 ml). The precipitate was washed with cold water (3 × 70 ml) and re-crystallised from ethanol to afford 4a (0.87 g, 78 %), m.p. 122 °C. – 1H NMR (CDCl₃, 200 MHz): δ = 8.30 (dd, 3J = 7.5 Hz, 4J = 1.6 Hz, 2 H, 6-H, 3-H), 8.23 (dd, 3J = 7.8 Hz, 4J = 1.4 Hz, 1 H, 4-H or 5-H), 7.82 (ddd, 3J = 7.5 Hz, 4J = 1.6 Hz, 1 H, 6-
5-H or 4-H), 7.69 (dd, $^3J = 8$ Hz, $^4J = 1$ Hz, 1 H, 4'-H), 7.58-7.40 (m, 4 H, 2'-H, 3'-H, 5'-H, 6'-H). – $^{13}$C NMR (CDCl$_3$, 50.3 MHz): $\delta = 159.46$ (COO), 157.00 (O=C-N), 146.87 (C-1), 136.47 (C-2), 132.54, 139.13, 128.66, 128.51 (2 C), 128.23, 128.17 (2 C), 127.14, 116.93. – MS (70 eV): $m/z$ (%) = 223 [M$^+$] (100), 179 (50), 146 (20), 105 (50), 90 (10), 77 (38), 63 (4), 51 (10).

2-Phenylethyleryl-4H-3,1-benzoxazin-4-on$^{13}$ (5a)
Cinnamoyl chloride (1.00 g, 6.0 mmol) was added to a solution of 0.41 g (3.0 mmol) anthranilic acid in 30 ml pyridine. The reaction mixture was further stirred for 2 h at 20 °C and poured into cold water (200 ml). The precipitate was filtered, washed with cold water (3 × 70 ml) and re-crystallised from ethanol to afford pure 5a (0.49 g, 66 %), m.p. 151 °C. – $^1$H NMR (CDCl$_3$, 200 MHz): $\delta = 8.22$ (d, $^3J = 8$ Hz, 1 H, 6-H), 7.83 (d, $^3J = 16$ Hz, 1 H, 8-H), 7.85-7.39 (m, 9 H, 9 Ar-H), 6.81 (d, $^3J = 16$ Hz, 1 H, 7-H). – $^{13}$C NMR (CDCl$_3$, 50.3 MHz): $\delta = 159.15$ (COO), 157.13 (O=C-N), 146.95 (C-1), 141.85 (C-2), 136.40, 134.45, 130.16, 128.84, 128.47, 120.01, 127.84, 126.74, 118.66, 116.77 (12 C, arom. C, C=C). – MS (70 eV): $m/z$ (%) = 249 [M$^+$] (84), 248 [M$^+$-H] (100), 220 (24), 204 (16), 193 (8), 146 (6), 131 (25), 119 (24), 103 (25), 90 (8), 77 (15).

Acknowledgments

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References

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Table 1. Antimicroalgal activity of compound 1e and analogues 1a-1d and 1f-5a in an agar plate diffusion assay at concentrations of 100 µg/disc

<table>
<thead>
<tr>
<th>Compound</th>
<th>C. vulgaris</th>
<th>C. sorokiniana</th>
<th>C. salina</th>
<th>S. subspicatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude extracta)</td>
<td>19</td>
<td>18</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>1a</td>
<td>10</td>
<td>12</td>
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<td>10</td>
</tr>
<tr>
<td>1b</td>
<td>21</td>
<td>20</td>
<td>19</td>
<td>20</td>
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<tr>
<td>1c</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>1d</td>
<td>10</td>
<td>10</td>
<td>d</td>
<td>0</td>
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<tr>
<td>1e</td>
<td>13</td>
<td>11</td>
<td>d</td>
<td>12</td>
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<td>19</td>
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<tr>
<td>1h</td>
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<td>13</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>1i</td>
<td>10</td>
<td>12</td>
<td>d</td>
<td>12</td>
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<tr>
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<td>11</td>
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<tr>
<td>4a</td>
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<td>12</td>
</tr>
<tr>
<td>5a</td>
<td>11</td>
<td>11</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>29</td>
<td>29</td>
<td>d</td>
<td>29</td>
</tr>
</tbody>
</table>

a) EtOAc extract of S. sp. B7747; d = diffuse inhibition zone; - = not tested

Table 2. Antimicroalgal activity of esters 1b, 1f and 2b by serial dilution method; MIC (µg /ml)

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Staurosporine</th>
<th>1b</th>
<th>1f</th>
<th>2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella vulgaris</td>
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<td>34</td>
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<td>Chlorella sorokiniana</td>
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<td>Chlorella salina</td>
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<td>88</td>
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<tr>
<td>Scenedesmus subspicatus</td>
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<td>74</td>
<td>107</td>
<td>36</td>
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</table>
Table 3. Bold’s Basal Medium (BBM)

<table>
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<tr>
<th>Stock solution (g/100 ml)</th>
<th>Nutrient solution (ml of stock solution)</th>
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</thead>
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<tr>
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<tr>
<td>KH₂PO₄</td>
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<td>K₂HPO₄</td>
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</tr>
<tr>
<td>MgSO₄ · 7 H₂O</td>
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</tr>
<tr>
<td>NaCl</td>
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<td>CaCl₂ · H₂O</td>
<td>2.5</td>
</tr>
<tr>
<td>Fe-EDTA</td>
<td>-</td>
</tr>
<tr>
<td>Trace element solution</td>
<td></td>
</tr>
<tr>
<td>H₂O (distilled)</td>
<td>made up to 1 Litre</td>
</tr>
</tbody>
</table>

Table 4. Composition of sea water medium-5¹⁰ for *Chlorella salina*

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<th>Stock solution (g/l)</th>
<th>Nutrient solution (ml of stock solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>1.0</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.1</td>
</tr>
<tr>
<td>MgSO₄ · 7 H₂O</td>
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<tr>
<td>Soil extract</td>
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</tr>
<tr>
<td>Trace element solution</td>
<td>5</td>
</tr>
<tr>
<td>Filtered synthetic sea water</td>
<td>made up to 1 Litre</td>
</tr>
</tbody>
</table>