Anthranilamides: New Antimicroalgal Active Substances from a Marine *Streptomyces* sp.†

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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 extracts[1]. In the course of our biological screening, extracts of the marine streptomycete B7747 were shown to possess potent antimicroalgal activity.

Fermentation of this *Streptomyces* 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 daidzein[2]. The latter (4',7-dihydroxyisoflavone, 7-hydroxy-3-(4-hydroxyphenyl)-4*H*-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 **1e** as well as its analogues **1a**~**1d** and **1f**~**5a** 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 **1e** and analogues, as well as detailing their antimicroalgal activity.

Taxonomic Studies of Producing Strain

The *Streptomycete* 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

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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

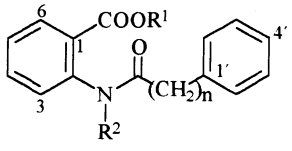
The slant culture of *Streptomyces* sp. B7747 was inoculated into Erlenmeyer flasks on malt extract/yeast extract/glucose in 50% synthetic sea water and incubated for 3 days at 28°C. Fermentation was carried out in a 20 litre jar fermentor under similar conditions. The ethyl acetate extract of the entire 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 ^{1}H NMR, EI-MS and confirmed by comparison with authentic samples.

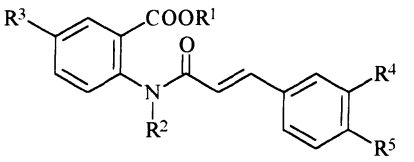
Results and Discussion

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 ^{1}H and ^{13}C 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, ^{1}H and ^{13}C 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**.

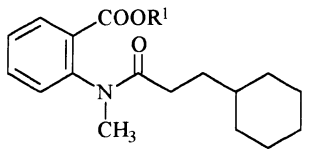
In order to study the structure-activity relationship, **1e**-analogues **1a**~**1d** and **1f**~**5a** were prepared. Variation in alkylation as well as saturation of the benzene



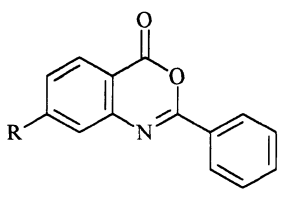
	R1	R2	n
1a	H	CH_3	0
1b	CH_3	CH_3	0
1c	H	CH_3	1
1d	CH_3	CH_3	1
1e	H	CH_3	2
1f	CH_3	CH_3	2
1g	H	H	2
1h	H	CH_3	3
1i	CH_3	CH_3	3



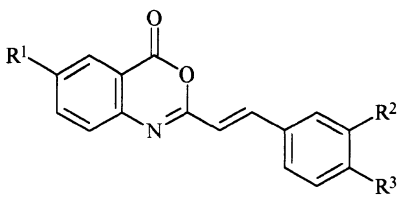
	R1	R2	R3	R4	R5
2a	H	CH_3	H	H	H
2b	CH_3	CH_3	H	H	H
2c	H	H	OH	H	OH
2d	H	H	OH	OCH_3	OH



3a = H
3b = CH_3



4a = H
4b = OH



	R1	R2	R3
5a	H	H	H
5b	OH	OCH_3	OH
5c	OH	H	OH

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.

Compound	Diameter of inhibition zone (mm)			
	C. vulgaris	*C. sorokiniana*	*C. salina*	*S. subspicatus*
Crude extract[a]	19	18	—	19
1a	10	12	—	10
1b	21	20	19	20
1c	0	0	—	0
1d	10	10	d	0
1e	13	11	d	12
1f	19	16	21	19
1h	13	13	—	12
1i	10	12	d	12
2a	13	13	—	12
2b	15	15	14	11
3a	0	0	—	0
3b	0	0	d	11
4a	14	13	—	12
5a	11	11	—	0
Staurosporine	29	29	d	29

[a] EtOAc extract of *Streptomyces* sp. B7747; d=diffuse inhibition zone; —=not tested.

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-4*H*-3,1-benzoxazin-4-one (**4a**) and 2-phenylethylenyl-4*H*-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 *Phytopthora 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.

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.

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.

Table 2. Antimicroalgal activity of esters **1b**, **1f** and **2b** by serial dilution method.

Test organism	MIC (μg/ml)			
	Staurosporine	**1b**	**1f**	**2b**
Chlorella vulgaris	10	20	34	36
Chlorella sorokiniana	0.64	70	107	36
Chlorella salina	0.51	23	88	62
Scenedesmus subspicatus	1.3	74	107	36

Experimental

IR-spectra: Perkin Elmer, model 297 (KBr). ^{1}H and ^{13}C 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/UV_{254} 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 Rf values. During fermentation Niax PPG 2025 (Union Carbide Belgium N.V., Zwindrecht) was used as anti foaming agent. Assay discs: i.d. 9 mm, Schleicher & Schuell, Dassel, Germany.

Artificial Sea Water

To prepare 20 litres sea water, dissolve iron citrate 2 g (powdered), NaCl 389 g, $MgCl_2 \cdot 6H_2O$ 176 g, Na_2SO_4 64.8 g (dissolve separately in 2 litres water), $CaCl_2$ 36 g, Na_2HPO_4 0.16 g, SiO_2 0.3 g, trace element stock soln. 20 ml, stock soln. 200 ml in 18 litres demin. water.

Trace Elements Stock Solution for Artificial Sea Water

To prepare trace elements stock solution, dissolve H_3BO_3 0.611 g, $MnCl_2$ 0.389 g, $CuSO_4$ 0.056 g, $ZnSO_4 \cdot 7H_2O$ 0.056 g, $Al_2(SO_4)_3 \cdot 18H_2O$ 0.056 g, $NiSO_4 \cdot 6H_2O$ 0.056 g, $Co(NO_3)_3 \cdot 6H_2O$ 0.056 g, TiO_2 0.056 g, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.056 g, LiCl 0.028 g, $SnCl_2$ 0.028 g, KI 0.028 g in 1 litre demin. water.

Stock Solution for Artificial Sea Water

To prepare stock solution, dissolve KCl 110 g, $NaHCO_3$ 32 g, KBr 16 g, $SrCl_2 \cdot 6H_2O$ 6.8 g (dissolve separately), H_3BO_3 4.4 g, NaF 0.48 g, NH_4NO_3 0.32 g in 2 litres demin. water.

Table 3. Bold's basal medium (BBM).

	Stock solution (g/100 ml)	Nutrient solution (ml of stock solution)
$NaNO_3$	25.0	10.0
KH_2PO_4	17.5	10.0
K_2HPO_4	7.5	10.0
$MgSO_4 \cdot 7H_2O$	7.5	10.0
NaCl	2.5	10.0
$CaCl_2 \cdot H_2O$	2.5	10.0
Fe-EDTA	—	1.0
Trace element solution		1.0
H_2O (distilled)		made up to 1 litre

Trace Element Stock Solution for Algae

$MnSO_4 \cdot H_2O$ (169 mg), $Na_2MoO_4 \cdot 2H_2O$ (130 mg), $Co(NO_3)_2 \cdot 6H_2O$ (100 mg), $CuSO_4 \cdot 5H_2O$ (50 mg), H_3BO_3 (100 mg), $ZnSO_4 \cdot 7H_2O$ (100 mg) are each dissolved in 100 ml of distilled water to obtain trace elements stock solution.

Fe-EDTA Stock Solution

$FeSO_4 \cdot 7H_2O$ (0.7 g) and 0.93 g EDTA (Titriplex III) are dissolved in 80 ml H_2O 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 hour 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

Table 4. Composition of sea water medium-5[10] for *Chlorella salina*.

	Stock solution (g/litre)	Nutrient solution (ml of stock solution)
KNO_3	1.0	20
K_2HPO_4	0.1	20
$MgSO_4 \cdot 7H_2O$	0.1	20
Soil extract		30
Trace element solution		5
Filtered synthetic sea water		made up to 1 litre

the indicated volumes of stock solutions (see Table 3) and sterilised for 1 hour 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 minutes at 120°C.

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_2O : 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 litre sea water, and 1 litre tap water, adjusted to pH 7.8 and sterilised for 1 hour 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 litres/minute) 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 $CHCl_3$/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, Rf = 0.2 in cyclohexane/EtOAc 1 : 1). Silica gel column chromatography ($CHCl_3$: MeOH, 9 : 1) of fraction 2 afforded 20 mg of 2-[methyl-(3-phenylpropionyl)-amino]-benzoic acid (**1e**) (Rf = 0.3, $CHCl_3$: MeOH, 9 : 1). The highly polar fraction 3 was extracted with $NaHCO_3$ and obtained from the water phase by acidifying and extraction with diethyl ether to give blue fluorescent *N*-methylanthranilic acid (11 mg, Rf = 0.2, $CHCl_3$/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 minutes under an argon atmosphere at 0°C. The reaction mixture was stirred for 2 hours 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 Na_2SO_4 and evaporated to dryness to afford a viscous raw product.

2-(Benzoyl-methylamino)benzoic Acid[11,12] (**1a**)

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, mp: 152°C (lit.[12] 162°C). ^{1}H NMR ($CDCl_3$, 200 MHz): δ=9.78 (s br, 1H, COOH), 7.90 (d, ^3J=8 Hz, 1H, 6-H), 7.30~7.06 (m, 8H, 3-, 4-, 5-H and 2′ to 6′-H), 3.47 (s, 3H, NCH_3). 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**)

^{1}H NMR ($CDCl_3$, 200 MHz): δ=7.89 (d, ^3J=8 Hz, 1H, 6-H), 7.30~7.06 (m, 8H, 3-, 4-, 5-H and 2′ to 6′-H), 3.90 (s, 3H, $COOCH_3$), 3.45 (s, 3H, NCH_3).

2-(Methyl-phenylacetylamino)benzoic Acid (**1c**)

Freshly prepared phenylacetyl chloride (1.36 g, 10 mmol) was reacted with *N*-methylanthranilic acid. Re-crystallisation of reaction product with diethyl ether afforded 673 mg (50%) of **1c** as colourless crystals, mp: 169°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ=9.10 (s br, 1H, COOH), 8.13 (d, ^3J=7 Hz, 1H, 6-H), 7.62~7.46 (m, 2H, 4-H, 5-H), 7.19~7.01 (m, 6H, 3-H, 2′ to 6′-H), 3.37, 3.47 (AB, ^2J=12 Hz, 2H, CH_2), 3.26 (s, 3H, N–CH_3). ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ=171.97 (COOH), 168.17 (O=C–N), 143.37 (C-1), 134.82 (C-2), 133.88, 132.58 (2C), 130.07, 129.13 (2C), 128.67, 128.36, 128.21, 126.55 (C-3 to C-6, and C-1′ to C-6′), 41.20 (CH_3), 37.67 (CH_2). 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 $C_{16}H_{15}NO_3$: C 71.36, H 5.61.
Found: C 71.15, H 5.79.

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

^{1}H NMR ($CDCl_3$, 200 MHz): δ=8.02 (d, ^3J=7 Hz, 1H, 6-H), 7.51~7.46 (m, 2H, 4-H, 5-H), 7.19~6.96 (m, 6H, 3-H, 2′ to 6′-H), 3.37, 3.47 (AB, ^2J=12 Hz, 2H, CH_2), 3.83 (s, 3H, $COOCH_3$), 3.26 (s, 3H, N–CH_3).

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

Following the general procedure, 1.67 g (10.0 mmol) phenylpropionyl chloride was added to *N*-methylanthranilic acid. The crude product was chromatographed on silica gel (100 g, cyclohexane/EtOAc, 3 : 7) to afford **1e** (877 mg, 62%) with mp 107°C after crystallisation from diethyl ether. ^{1}H NMR ($CDCl_3$, 200 MHz): δ=10.50 (s, 1H, COOH, D_2O exchangeable), 8.12 (dd, $^3J_1$, $^3J_2$=8 Hz, ^4J=2 Hz, 6-H), 7.50 (m, 2H, 4-H, 5-H), 7.18~7.00 ($2m_C$, 6H, 3-H, 2′-6′-H), 3.26 (s, 3H, NCH_3), 2.90 (m, 2H, 8-H_2), 2.35 (m, 2H, 7-H_2). ^{13}C NMR (C_6D_6, 50.3 MHz): δ=174.42 (COOH), 176.02 (O=C–N), 143.22 (C-1), 141.62 (C-2), 132.98, 132.12, 130.17, 129.22 (2C), 128.66 (2C), 128.31 (2C), 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).

Anal Calcd for $C_{17}H_{17}NO_3$: C 72.61, H 6.42.
Found: 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 hours under an argon atmosphere. Methanol was distilled off, the residue poured into cold water (100 ml) and the mixture extracted with diethyl ether (3×30 ml). The combined organic phases were washed with $NaHCO_3$ solution and dried with Na_2SO_4. Silica gel chromatography (column 3×30 cm) afforded 400 mg of **1f** (67%) as viscous colourless oil. ^{1}H NMR ($CDCl_3$, 200 MHz): δ=7.97 (dd, ^3J=7.6 Hz, ^4J=2.0 Hz, 1H, 6-H), 7.52 (ddd, $^3J_1$, $^3J_2$=7.4 Hz, ^4J=1.6 Hz, 1H, 4- or 5-H), 7.41 (ddd, $^3J_1$, $^3J_2$=7.6 Hz, ^4J=1.6 Hz, 1H, 5- or 4-H), 7.30~6.99 (m, 6H, 3-H, 2′-6′-H), 3.83 (s, 3H, $COOCH_3$), 3.18 (s, 1H, NCH_3), 2.90 (m, 2H, 8-H_2), 2.24 (t, ^3J=7.9 Hz, 1H, 7-H_2). ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ=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).

Anal Calcd for $C_{18}H_{19}NO_3$: C 72.71, H 6.44.
Found: 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 hours 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%), mp: 132°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ=10.92 (br s, 1H, COOH), 8.78 (d, ^3J=8 Hz, 1H, 3-H), 8.25 (d, ^3J=8 Hz, 1H, 6-H), 7.62 (dd, ^3J=8 Hz, ^4J=1.4 Hz, 1H, 5-H), 7.30~7.14 (m, 6H, 4-H, 2′-H-6′-H), 3.08 (dd, $^3J_1$, $^3J_2$=8 Hz, 2H, 8-H_2), 2.78 (dd,

$^3J_1$, $^3J_2$=8 Hz, 2H, 7-H_2). ^{13}C NMR ($CDCl_3$, 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 (2C, C-2', C-6' or C-3', C-5'), 128.31 (2C, 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 $C_{16}H_{15}NO_3$: 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 $SOCl_2$ (25.0 mmol) and 2.46 g of phenylbutyric acid (15.0 mmol) was refluxed for 2 hours under an argon atmosphere. Excess $SOCl_2$ 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, mp 95°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ=9.34 (s br, 1H, COOH), 8.10 (dd, ^3J=7.6 Hz, ^4J=1.2 Hz, 1H, 6-H), 7.58 (ddd, $^3J_1$, $^3J_2$= 7.4 Hz, ^4J=1.6 Hz, 1H, 4- or 5-H), 7.46 (dd, $^3J_1$, $^3J_2$= 7.4 Hz, 1H, 5-H or 4-H), 7.22~7.13 (m, 6H, 3-H, 1- to 6-H), 3.23 (s, 1H, NCH_3), 2.50 (d, ^3J=8 Hz, 2H, CH_2 terminal), 2.10 (m, 2H, CH_2 terminal), 203 (m, 2H, 8-H_2). ^{13}C NMR ($CDCl_3$, 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 (2C), 128.33 (2C), 128.16 (2C), 125.65, 37.32 (NCH_3), 35.09 (C-7), 33.45 (C-8), 26.68 (C-9). MS (70 eV): *m/z* (%)=297 [M^+] (38), 193 (44), 151 (100), 148 (60), 133 (18), 105 (40), 91 (46), 77 (18), 65 (10), 55 (8).

Anal Calcd for $C_{18}H_{19}NO_3$: C 72.71, H 6.44.
Found: C 72.78, H 6.24.

2-(Cinnamoyl-methylamino)benzoic Acid[13] (**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 hours. 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, mp 176°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ=9.15 (s br, 1H, COOH), 8.17 (d, J=8 Hz, 6-H), 7.72 (d, ^3J=16 Hz, 1H, 8-H), 7.65~7.45 (m, 8H, arom. H), 6.53 (d, J=16 Hz, 1H, 7-H), 3.36 (s, 3H, NCH_3). ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ=167.78 (COOH), 167.22 (O=C–N), 142.92, 134.84, 133.78, 132.66, 129.68, 129.52, 129.10, 129.03, 128.60, 128.51 (2C), 127.88 (2C), 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).

Anal Calcd for $C_{17}H_{15}NO_3$: C 72.58, H 5.37.
Found: C 72.44, H 5.22.

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

^{1}H NMR ($CDCl_3$, 200 MHz): δ= 8.00 (d, J=8 Hz, 6-H), 7.65 (d, ^3J=16 Hz, 1H, 8-H), 7.54~7.45 (m, 8H, arom. H), 6.53 (d, J=16 Hz, 1H, 7-H), 3.89 (s, 3H, $COOCH_3$), 3.36 (s, 3H, NCH_3).

2-[(3-Cyclohexylpropionyl)methylamino]-benzoic Acid (**3a**)

In an analogous synthesis, cyclohexylpropionic acid (2.34 g, 15.5 mmol) was heated with $SOCl_2$ (2.97 g, 25.0 mmol) for 2 hours under an argon atmosphere. The acid chloride was allowed to react with *N*-methyl-anthranilic 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%), mp 91°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ=10.10 (s br, 1H, COOH), 8.17 (d, ^3J=7 Hz, 1H, 6-H), 7.63 (dd, ^3J=7 Hz, 4-H or 5-H), 7.51 (dd, $^3J_1$, $^3J_2$=7 Hz, 5-H or 4-H), 7.30 (m, 1H, 3-H), 3.23 (s, 3H, NCH_3), 2.00 (m, 2H, 7-H_2), 1.65, 1.05, 0.80 (3m_C, 13H, 8-H_2, 1'- to 6'-H). ^{13}C NMR (C_6D_6, 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 (NCH_3), 37.39 (CH), 33.37, 33.22, 32.12, 26.86, 26.54 (5CH_2). 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 $C_{17}H_{23}NO_3$: C 70.71, H 7.74.
Found: C 70.53, H 7.90.

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

^{1}H NMR ($CDCl_3$, 200 MHz): δ=8.15 (d, ^3J=7 Hz, 1H, 6-H), 7.70 (dd, ^3J=7 Hz, 4-H or 5-H), 7.50 (dd, $^3J_1$, $^3J_2$=7 Hz, 5-H or 4-H), 7.30 (m, 1H, 3-H), 3.90 (s, 3H, $COOCH_3$), 3.23 (s, 3H, NCH_3), 2.00 (m, 2H, 7-H_2), 1.80, 1.05, 0.80 (3m_C, 13H, 8-H_2, 1'- to 6'-H).

2-Phenyl-4*H*-3,1-benzoxazin-4-one[12] (**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 hours 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%), mp 122°C.

^{1}H NMR ($CDCl_3$, 200 MHz): δ=8.30 (dd, ^3J=7.5 Hz, ^4J=1.6 Hz, 2H, 6-H, 3-H), 8.23 (dd, ^3J=7.8 Hz, ^4J=1.4 Hz, 1H, 4-H or 5-H), 7.82 (ddd, ^3J=7.5 Hz, ^4J=1.6 Hz, 1H, 5-H or 4-H), 7.69 (dd, ^3J=8 Hz, ^4J=1 Hz, 1H, 4'-H), 7.58~7.40 (m, 4H, 2'-H, 3'-H, 5'-H, 6'-H). ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ=159.46 (COO), 157.00 (O=C–N), 146.87 (C-1), 136.47 (C-2), 132.54, 139.13, 128.66, 128.51 (2C), 128.23, 128.17 (2C), 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-Phenylethylenyl-4*H*-3,1-benzoxazin-4-one[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 hours 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%), mp 151°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ=8.22 (d, ^3J=8 Hz, 1H, 6-H), 7.83 (d, ^3J=16 Hz, 1H, 8-H), 7.85~7.39 (m, 9H, 9Ar-H), 6.81 (d, ^3J=16 Hz, 1H, 7-H). ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ=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 (12C, 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).

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