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p-Hydroxyacetophenone Amides from *Cystobacter ferrugineus*, strain Cb G35

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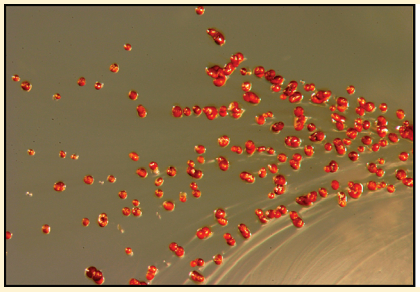
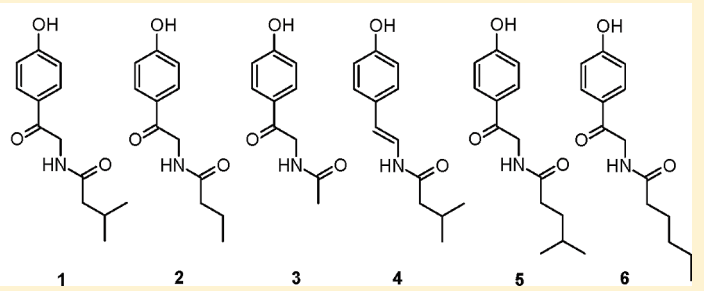
Supporting Information

ABSTRACT:





A family of six novel *p*-hydroxyacetophenone amides, **1**−**6**, was isolated from *Cystobacter ferrugineus,* strain Cb G35. Their structures were elucidated by ESI-TOF mass spectrometry and NMR spectroscopy. Feeding experiments with labeled [$^{13}C_9$,^{15}N]-tyrosine and [d_{10}]-leucine identified the biosynthetic precursors of **1**.

During the last three decades myxobacteria have been established as a versatile source of biologically active secondary metabolites.[1] Over 100 different basic structures with more than 500 derivatives have been reported. Of these, 67 metabolite types have been published, providing a diversity of unique structures as well as mode of actions.[2] In addition to strong antibiotic activities several metabolites interact with diverse and often new targets in eukaryotic cells such as tubulysins[3] and disorazols,[4] which inhibit the polymerization of tubulin, and argyrin,[5] a promising proteasome inhibitor. To date the most successful is a derivative of the myxobacterial metabolite epothilone that has been approved as an anticancer agent (Ixempra) against paclitaxel-resistant tumors.[6]

Most myxobacteria simultaneously synthesize more than one family of secondary metabolites. Strains of the genus *Cystobacter* are known to provide diverse secondary metabolites such as the cyrmenins, a group of antifungal peptides containing a nitrogen-linked β-methoxyacrylate terminus,[7] the bithiazole-type antibiotics cystothiazoles,[8] althiomycins,[9] and the tubulysins.[3,11] In the course of our ongoing screening for novel secondary metabolites from myxobacteria, *Cystobacter ferrugineus*, strain Cb G35, was found to produce a family of six *p*-hydroxyacetophenone amides and traces of myxalamide C.[10]

RESULTS AND DISCUSSION

C. ferrugineus, strain Cb G35, was isolated from a soil sample collected at the Franz Josef Glacier in New Zealand. The strain was transferred to liquid soy-flour medium supplemented with XAD-16 resin and fermented in a volume of 70 L for 7 days. The wet resin was recovered from the culture broth by sieving and was eluted with methanol. After evaporation of the organic solvent, the remaining aqueous mixture was extracted with ethyl acetate. In order to remove lipophilic byproducts, the raw extract was partitioned between methanol and *n*-heptane to yield 17.5 g of polar raw material. A 1.0 g amount was fractionated by silica gel flash chromatography and purified further by RP-HPLC to yield 59.6 mg of **1**, 7.1 mg of **2**, 3.3 mg of **3**, and 6.9 mg of **4**, respectively. In order to isolate the minor metabolites, 4.4 g of the polar material was processed similarly to yield after RP chromatography 0.8 mg of **5** and 1.1 mg of **6**.

The structures of the metabolites was unambiguously elucidated using a combination of high-resolution electrospray mass spectrometric (HRESIMS) and homo- and heteronuclear magnetic resonance (NMR) spectroscopic data. The main component **1** was crystallized from methanol as white needles and had a molecular formula of $C_{13}H_{17}NO_3$ according to HRESIMS analysis. The 2D ^{1}H,^{1}H-COSY and ^{1}H,^{13}C-HMBC data identified a *p*-hydroxybenzoyl and a 3-methylbutanoyl unit (Table 1 and Table S1 in the Supporting Information). One key fragment in the HRESIMS was found at *m*/*z* 152.0710 with an elemental composition of $C_8H_{10}NO_2$ indicating the presence of an amidic nitrogen linking the two subunits. This was confirmed by characteristic NMR chemical shifts of the attached carbon atoms and long-range ^{1}H,^{13}C-HMBC correlations shown in Figure 1.

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Table 1. NMR Data of *p*-Hydroxyacetophenone Amides 1−6[a]

	1		2		3	
position	δ_C, mult	δ_H, mult (J in Hz)	δ_C, mult	δ_H, mult (J in Hz)	δ_C, mult	δ_H, mult (J in Hz)
1	164.2, qC		164.2, qC		164.4, qC	
2, 6	116.4, CH	6.90, d (8.7)	116.4, CH	6.90, d (8.7)	116.6, CH	6.86, d (8.8)
3, 5	131.6, CH	7.94, d (8.7)	131.6, CH	7.93, d (8.7)	131.7, CH	7.90, d (8.8)
4	128.2, qC		128.1, qC		128.2, qC	
7	194.6, qC		194.6, qC		194.7, qC	
8	46.6, CH_2	4.66, s	46.6, CH_2	4.66, s	46.9, CH_2	4.62, s
9	176.0, qC		176.5, qC		173.8, qC	
10	46.2, CH_2	2.21, d (7.1)	38.8, CH_2	2.33, t (7.4)	22.5, CH_3	2.05, s
11	27.4, CH	2.14, m	20.3, CH_2	1.72, tq (7.4, 7.4)		
12, 13	22.8, CH_3	1.03, d (6.6)	14.0, CH_3	1.03, t (7.4)		
	4		**5**		**6**	
position	δ_C, mult	δ_H, mult (J in Hz)	δ_C, mult	δ_H, mult (J in Hz)	δ_C, mult	δ_H, mult (J in Hz)
1	157.6, qC		164.5, qC		164.4, qC	
2, 6	116.5, CH	6.75, d (8.7)	116.6, CH	6.91, d (8.8)	116.6, CH	6.86, d (8.8)
3, 5	127.7, CH	7.19, d (8.7)	131.7, CH	7.95, d (8.8)	131.7, CH	7.90, d (8.8)
4	129.2, qC		128.2, qC		128.3, qC	
7	114.8, CH	6.18, d (14.5)	194.8, qC		194.4, qC	
8	121.5, CH	7.31, d (14.5)	46.8, CH_2	4.66, s	46.8, CH_2	4.62, s
9	172.9, qC		177.0, qC		176.9, qC	
10	46.2, CH_2	2.18, m	35.2, CH_2	2.38, m	37.1, CH_2	2.31, t (7.5)
11	27.5, CH	2.16, m	36.1, CH_2	1.60, m	26.8, CH_2	1.66, quin (7.5)
12	22.7, CH_3	1.02, d (6.6)	29.1, CH	1.66, m	32.7, CH_2	1.39, m
13	22.7, CH_3	1.02, d (6.6)	22.9, CH_3	0.99, d (6.6)	23.6, CH_2	1.38, m
14			22.9, CH_3	0.99, d (6.6)	14.4, CH_3	0.93, t (6.6)

[a] Additional NMR data are provided in the Supporting Information.

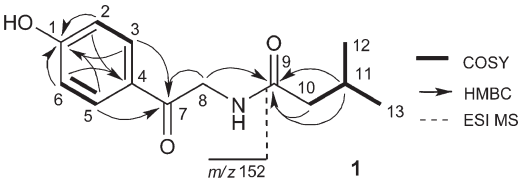

Figure 1. ^{1}H,^{1}H-COSY sequences, selected ^{1}H,^{13}C-HMBC correlations, and the key fragment in the ESI mass spectrum of **1**.

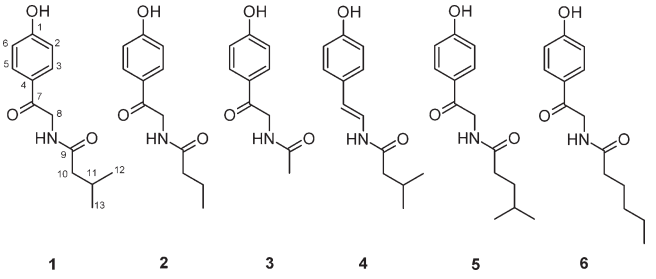

Figure 2. *p*-Hydroxyacetophenone amides isolated from *Cystobacter ferrugineus*, strain Cb G35.

Of particular importance, the position of the carbonyl group in the aromatic subunit was also unambiguously evident from the ^{1}H,^{13}C-HMBC correlations of 3,5H and 8H as well as from the characteristic chemical shift of the aromatic ketone at δ_C = 194.6 ppm. Compatible observations were reported for hibispeptin A (**7**), a cyclic peptide containing an analogous aromatic ketone unit.[12]

In addition to **1**, five further secondary metabolites of this family were isolated from the crude extract of strain Cb G35, and their structures shown in Figure 2 were established using the same approach as for **1**. In compound **2** (HRESIMS m/z 222.1125, $C_{13}H_{17}NO_3$), an *n*-propyl residue replaced the isobutyl side chain of **1** and the smallest metabolite **3** (m/z 194.0809, $C_{10}H_{11}NO_3$) showed an acetyl group with the characteristic methyl singlet at δ_H = 2.05 ppm. Compared to the other metabolites of this family the main absorption in the UV spectrum of compound **4** (m/z 220.1331, $C_{13}H_{17}NO_2$) was shifted from 278 to 285 nm due to the replacement of the C7 carbonyl group by a *trans*-double bond (J = 14.5 Hz) conjugated to the aromatic system. The minor compounds **5** and **6** were lipophilic isomers with a common molecular formula of $C_{14}H_{19}NO_3$ corresponding to their $[M + H]^+$ ions m/z 250.1447. Their NMR structure elucidation revealed the difference of their side chains, i.e., an isopentyl group in **5** and an *n*-pentyl group in **6**.

The biosynthetic precursors of the main metabolite **1** were determined by feeding experiments with deuterium-, ^{13}C-, and ^{15}N-labeled amino acids in 100 mL cultures supplemented with traces of natural methionine to increase metabolite production.

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Table 2. ^{13}C Incorporation in Labeled 1 from Feeding Experiments with [$^{13}C_9$,^{15}N]-Tyrosine

position	^{13}C incorporation [%] from ^{13}C NMR	^{13}C incorporation [%] from ^{1}H NMR
1	51−53[a]	
2, 6	51−53[a]	51[d]
3, 5	51−53[a]	53[d]
4	51−53[a]	
7	53[b]	
8	51−53[a]	n.a.[e]
9	8[c]	n.a.
10	7−8[a]	n.a.
11	7−8[a]	n.a.
12, 13	7[c]	n.a.

[a] Incorporation transferred from the calculated incorporation of ^{13}C from the data of ^{1}H and ^{13}C NMR spectra [b] Calculation with reference C9. [c] Calculated from labeled to natural ^{13}C signal ratio. [d] Calculated ^{13}C enrichment from the ^{1}H NMR spectrum. [e] n.a. = signals were not analyzed.

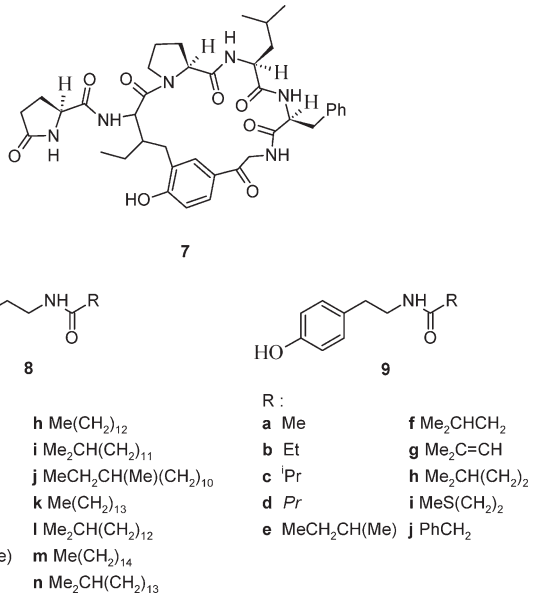

Figure 3. Hibispeptin A (**7**), the family of arylethylamides (**8a**−**n**), and 10 acylated tyramides (**9a**−**j**).

HPLC-HRESIMS analysis of the sample after feeding of [d_{10}]-leucine showed two molecular ion clusters, one at m/z 236.1268 (intensity 53%) for $[M + H]^+$ of the unlabeled natural part of metabolite **1** and another one at m/z 245.1834 (intensity 100%) for $[M + 9 + H]^+$ of the [d_9]-labeled metabolite **1**, thus indicating an incorporation of 65% of the precursor in compound **1**. Consistent with the high incorporation of deuterium, the ^{1}H NMR spectrum of the extract contained decreased ^{1}H-signal intensities for the acyl residue (spectrum 39, Supporting Information).

After feeding [$^{13}C_9$,^{15}N]-tyrosine metabolite **1** was ^{13}C-labeled in both subunits of the molecule. Significant incorporation was found in the *p*-hydroxyacetophenone unit and a lower ^{13}C incorporation in the acyl residue, due to partial scrambling of the label by primary metabolism to doubly labeled 1,2-^{13}C-acetate. A rate of 7−8% ^{13}C incorporation was estimated for the scrambling, which was calculated from the integral of the doublet signals of the ^{13}C-labeled C9 (integral 0.16) and the methyl groups C12,13 (0.81) compared to their corresponding natural singlet signals (C9 0.02; C12,13 0.11) with the natural ^{13}C-abundance of 1.1%.[13] The ^{13}C NMR of the *p*-hydroxyacetophenone unit showed the expected complex ^{13}C signal patterns due to direct and long-range ^{13}C,^{13}C coupling after incorporation of intact $^{13}C_9$-labeled tyrosine. A ^{13}C incorporation of 53% was calculated for carbonyl C7 compared to the natural singlet signal of carbonyl C9. Similar adequate reference ^{13}C signals of the other carbon types were not available for calculations. In addition, the ^{1}H NMR spectrum showed broad doublets for the aromatic protons of the ^{13}C-labeled part of the sample with a direct ^{13}C,^{1}H coupling $^{1}J_{C,H} \approx 150$ Hz.[14] Compared to the unlabeled aromatic ^{1}H signals H-2,6 and H-3,5 (integral 3.74 and 2.88), the doublets for H-2,6 and H-3,5 (integral 3.81 and 3.22) indicated 52% ^{13}C incorporation, fully supporting the ^{13}C NMR result (spectrum 37, Supporting Information). In order to assess the incorporation of ^{15}N, the broad ^{1}H doublet signal of the ^{15}N-labeled NH proton (integral 0.42) with a direct coupling of $^{1}J_{N,H} \approx 95$ Hz was compared to the broad unlabeled singlet NH signal (integral 1.0) in the ^{1}H NMR spectrum to give a ^{15}N incorporation of 30% (spectrum 37, Supporting Information). This result was supported by the HRESIMS spectrum of the sample consisting of isotopomeres of **1**, which showed the natural key fragment $[C_8H_9NO_2 + H]^+$ at m/z 152.0701 with a share of 41% and two differently labeled monoisotopic fragment ions (spectrum 41, Supporting Information). One monoisotopic fragment at m/z 161.0938 for $[^{13}C_8H_9{}^{15}NO_2 + H]^+$ with a portion of 29% and a second monoisotopic fragment with a portion of 30% at m/z 160.0966 for $[^{13}C_8H_9NO_2 + H]^+$ was missing the labeled nitrogen. The total ^{13}C incorporation of 59% for the fragment ion of **1** is in good agreement with the results from the NMR analyses.

The combined data unambiguously indicate that tyrosine is a precursor of the *p*-hydroxyacetophenone amine moiety, including the amide nitrogen, and that leucine is a precursor of the aliphatic side chain of **1**. Thus the *Cystobacter* strain is able to oxidize tyrosine to give an atypical carbonyl function at C7. Degradation of tyrosine to *p*-hydroxybenzoyl-CoA would be feasible to form the ketone at C7 too, but the high ^{15}N incorporation in the amide and the high ^{13}C incorporation of C8 from tyrosine require a different biosynthetic route. The same tyrosine degradation pathway also enables the formation of 4-hydroxy cinnamate as a conceivable precursor for the double bond in **4**.[15] However, the nitrogen of tyrosine would be lost in that pathway too. The feeding experiments and the observation of different aliphatic amide residues suggest that the modified side chains of these metabolites result from incorporation of amino acids or fatty acid precursors.

p-Hydroxyacetophenone acetamide **3** has previously been described as a synthetic product in the partial synthesis of chloramphenicol derivatives[16] and in the synthesis of tyrosine kinase inhibitors.[17] The *p*-hydroxyacetophenone amides **1**−**6** are closely related to the arylethylamides **8a**−**n**. The group **8a**−**f** was first isolated from three limnic strains of a new subspecies of bacillus,[18] while further derivatives were obtained from numerous extracts of *Cytophaga*, *Frigoribacter*, and marine *Streptomyces* strains.[19] In addition, the same authors described 10 acylated tyramides, **9a**−**j**, from *Vibrio* sp. featuring a tyrosine moiety.[19] Arylethylamide **8e** showed weak activity against the microalgae

Chlorella sorokiniana, *Chlorella vulgaris*, and *Desmodesmus subspicatus*.[17] Thus, the *Cystobacter* amides (**1**−**6**) were screened against various microalgae (*Scenedesmus bajacalifornicus*, *Chlamydomonas* sp., *Bracteacoccus* sp., *Pseudococcomyxa simplex*, or *Botryococcus braunii*), with only **4** showing growth inhibition of *Pseudococcomyxa simplex* at the highest tested concentration (100 μg/mL). The crude extract of Cb G35 inhibited the growth of *Mucor hiemalis*, *Escherichia coli*, and *Staphylococcus aureus*. The activity against *Mucor hiemalis* was attributed to the presence of myxalamide C in the crude extract. The *p*-hydroxyacetophenone amides were inactive at 100 μg/mL against *M. hiemalis* or against Gram-positive bacteria (*Staphylococcus aureus*, *Nocardia flava*, *Micrococcus luteus*), Gram-negative bacteria (*Escherichia coli*, *Chromobacterium violaceum*), yeasts (*Candida albicans*, *Rhodotorula glutinis*, *Pichia anomala*, *Schizosaccharomyces pombe*), and human leukemic U-937 cells.

EXPERIMENTAL SECTION

General Experimental Procedures. Analytical RP-HPLC: Agilent 1100 series HPLC system; column 125 × 2 mm, Nucleosil 120 EC (5 μm, C_{18}) (Macherey-Nagel); temperature 40 °C; solvent A: H_2O/ACN (95/5) + 5 mmol/L NH_4Ac + 40 μL/L acetic acid; solvent B: H_2O/ACN (5/95) + 5 mmol/L NH_4Ac + 40 μL/L acetic acid, gradient 10% B rising to 100% B in 30 min; 100% B for 10 min; flow rate 0.3 mL/min; UV detection at 210−500 nm. Preparative RP-HPLC: Preparative Agilent 1100 series HPLC system; RP-column 250 × 21 mm, Nuleodur 100 EC (10 μm, C_{18}) (Macherey-Nagel); flow rate 20 mL/min; UV detection 210−500 nm. Preparative RP-MPLC: Two pumps C-605, control unit C-620, fraction collector C-660, and photometer C-635 (Büchi); column 480 × 30 mm, ODS-AQ, 120 Å, S 16 μm, C-18; flow rate 30 mL/min; UV 280 nm. IR spectrometry: Tensor 27 IR spectrometer (Bruker). UV spectrometry: UV−vis spectrophotometer UV-2450 (Shimadzu), methanol Uvasol (Merck). NMR spectroscopy: AM-300 (Bruker ^{1}H 300 MHz, ^{13}C 75 MHz), ARX-400 (Bruker ^{1}H 400 MHz, ^{13}C 100 MHz), ARX 600 (Bruker ^{1}H 600 MHz, ^{13}C 150 MHz). HPLC-HRMS: Agilent 1200 series RRLC system, ESI-TOF-MS Maxis (Bruker), column 2.1 × 50 mm, Acquity UPLC BEH C-18, 1.7 μm (Waters). Thin-layer chromatography (TLC): aluminum sheets with 0.2 mm silica gel 60 F_{254} (Merck, Darmstadt); solvent ethyl acetate/dichloromethane/*n*-heptane (3/3/4); staining was done with aqueous cer-dye (10 g cer(IV)-sulfate, 25 g phosphor-molybdenum acid, 80 mL sulfuric acid in 1 L) and heating at 120 °C.

Isolation of the Strain and Fermentation. Strain CbG 35 was identified by partial 16S rRNA analysis showing 99.9% similarity (1031 of 1032 bp) to strain *Cystobacter ferrugineus* (DSM 14716; Acc DQ768112). *Cystobacter ferrugineus*, strain Cb G35 (deposited at Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH; DSM 24415), was transferred to a liquid medium consisting of soybean flour 4 g/L, glucose 2 g/L, starch 8 g/L, $CaCl_2 \times 2\ H_2O$ 1 g/L, $MgSO_4 \times 7\ H_2O$ 1 g/L, HEPES 11.9 g/L, and Fe-EDTA 8 mg/L at pH 7.4. A 70 L sample of the same medium in a 100 L fermentor (Giovanola Frères SA, Monthey, Switzerland; blade impeller 150 rpm, aeration 9 L/min, 30 °C, pO_2 20%, pH regulated between pH 7.15 and 7.25) was inoculated in the presence of 1.4 kg of XAD-16 resin (Rohm & Haas). After 7 days the culture broth was passed through a process filter to collect the absorber resin. Residual cells were removed by decantation with water from the faster settling XAD-16 resin.

Isolation of p-Hydroxyacetophenone Amides ***1***−***3***. The XAD resin was eluted with 4 L of methanol/H_2O (30/70), methanol (4 L), and acetone (4 L) in a column. The organic solvent was evaporated from the methanol fraction to give 550 mL of an aqueous mixture, which was extracted with ethyl acetate (5 portions of 250 mL). The combined ethyl acetate layer was dried *in vacuo* to give 18.6 g of crude extract. The extract was dissolved in methanol and partitioned between methanol and *n*-heptane, to provide 17.5 g of more polar raw material. Then 1.0 g of this extract was separated by silica gel flash chromatography [Flash40 (Biotage), cartridge Si 40 S 2976-1 (Biotage); solvent dichloromethane/methanol/*n*-heptane (18/1/1)]. All fractions from the chromatography were analyzed by thin-layer chromatography and combined to four main fractions. Fraction 3 yielded 124.4 mg, which was further purified in three batches by preparative RP-HPLC [column 250 × 21 mm, Nuleodur 100 EC (10 μm, C_{18}) (Macherey-Nagel); solvent A: methanol/H_2O (10/90 + 0.01% acetic acid), solvent B: methanol/H_2O (90/10 + 0.01% acetic acid); gradient 25% B for 5 min, increasing to 45% B over 35 min, maintained at 45% for 10 min, increasing to 100% B over 5 min; flow rate 20 mL/min, UV detection 210−500 nm]. Fractions were combined peakwise. The organic solvent was evaporated from the fractions after addition of toluene, and the water layer was extracted with ethyl acetate, yielding compound **3** (t_R = 7 min, 3.3 mg), compound **2** (t_R = 19 min, 7.1 mg), and compound **1** (t_R = 29 min, 59.6 mg).

Isolation of Compound ***4***. Fraction 2 (221.2 mg) from the silica gel flash chromatography was separated by preparative RP-HPLC in five batches [column 250 × 21 mm, Nuleodur 100 EC (10 μm, C_{18}) (Macherey-Nagel); solvent A: methanol/H_2O 10/90 + 0.01% acetic acid, solvent B: methanol/H_2O 90/10 + 0.01% acetic acid; gradient 25% B for 5 min, increasing to 35% B in 25 min, increasing to 100% B in 1 min, maintained at 100% B for 20 min; flow rate 20 mL/min, UV detection at 210−500 nm]. Compound **4** (6.9 mg) eluted at t_R = 38 min.

Isolation of Compounds ***5*** *and* ***6***. A 4.4 g amount of the methanol extract was subjected to silica gel flash chromatography (300 g silica gel 60, particle size 0.063−0.200 mm, Merck) and eluted stepwise with ethyl acetate/dichloromethane/*n*-heptane (3/3/4) (3 L) and ethyl acetate (6 L). Fifteen fractions were collected and dried *in vacuo* according to TLC analyses. Fraction 5 (48.6 mg) was further separated in three batches by preparative RP-HPLC [column 250 × 21 mm, Nuleodur 100 EC (10 μm, C_{18}) (Macherey-Nagel); gradient 37% B for 30 min, increasing to 100% B over 20 min; solvent A: methanol/H_2O (10/90), solvent B: methanol; flow rate 20 mL/min, UV detection 210−500 nm]. Compound **5** eluted at 33.5 min (0.8 mg).

Fraction 9 (314 mg) of the silica gel chromatography was separated by preparative RP-MPLC [column 480 × 30 mm, ODS-AQ, 120 Å, S 16 μm, C-18 (Macherey-Nagel); solvent A: H_2O/methanol (90/10), solvent B: methanol, gradient: 35% B to 38% B in 3 h, to 100% B in 30 min, isocratic 30 min; flow rate 30 mL/min, UV detection 280 nm). Eleven fractions were collected and evaporated. The remaining aqueous layers were extracted with ethyl acetate. Compound **6** (1.1 mg) eluted at t_R = 185 min.

*N-[2-(4-Hydroxyphenyl)-2-oxoethyl]-3-methylbutanamide (**1**)*: $C_{13}H_{17}NO_3$ (235.28); white needles; mp 188 °C; analytical TLC, R_f = 0.07; UV (methanol): λ_{max} (log ε) = 202 (4.864), 218 (4.724), 278 (4.893) nm; IR (KBr) ν = 3356, 3195, 2960, 1649, 1577, 1546, 1245, 1170 cm^{-1}; NMR (methanol-d_4), Table 1; (+)-HRESIMS calcd for $[M + H]^+$ 236.1281, found 236.1287.

*N-[2-(4-Hydroxyphenyl)-2-oxoethyl]butanamide (**2**)*: $C_{12}H_{15}NO_3$ (221.25); white needles; mp 184 °C; analytical TLC, R_f = 0.05; UV (methanol) λ_{max} (log ε) = 201 (4.736), 219 (4.553), 278 (4.721) nm; IR (KBr) ν = 3358, 3101, 2962, 1633, 1581, 1519, 1243, 1174 cm^{-1}; NMR (methanol-d_4), Table 1; (+)-HRESIMS calcd $[M + H]^+$ 222.1125, found 222.1125.

*N-[2-(4-Hydroxyphenyl)-2-oxoethyl]acetamide (**3**)*: $C_{10}H_{11}NO_3$ (193.20); white needles; mp 187 °C; analytical TLC, R_f = 0.02; UV (methanol) λ_{max} (log ε) = 201 (4.363), 219 (4.211), 278 (4.396) nm; IR (KBr) ν = 3344, 3200, 2931, 1652, 1577, 1548, 1243, 1170 cm^{-1}; NMR (methanol-d_4) Table 1; (+)-HRESIMS calcd $[M + H]^+$ 194.0812, found 194.0809.

*N-[(E)-2-(4-Hydroxyphenyl)ethenyl]-3-methylbutanamide (**4**):* $C_{13}H_{17}NO_2$ (219.28); oil; analytical TLC, R_f = 0.24; UV (methanol) λ_{max} (log ε) = 200 (4.330), 219 (4.454), 285 (4.747) nm; IR (KBr) ν = 3289, 3074, 2969, 1670, 1539, 1228, 1170 cm^{-1}; NMR (methanol-d_4), Table 1; (+)-HRESIMS calcd $[M + H]^+$ 220.1332, found 220.1331.

*N-[2-(4-Hydroxyphenyl)-2-oxoethyl]-4-methylpentanamide (**5**):* $C_{14}H_{19}NO_3$ (249.31); analytical TLC, R_f = 0.07; UV (ethanol) λ_{max} (log ε) = 219 nm (4.228), 279 nm (4.350), 331 nm (3.388); NMR (methanol-d_4), Table 1; (+)-HRESIMS calcd $[M + H]^+$ 250.1438, found 250.1447.

*N-[2-(4-Hydroxyphenyl)-2-oxoethyl]hexanamide (**6**):* $C_{14}H_{19}NO_3$ (249.31); analytical TLC, R_f = 0.09; UV (ethanol) λ_{max} (log ε) = 218 (4.021), 279 (4.111), 331 (3.150) nm; NMR (methanol-d_4), Table 1; (+)-HRESIMS calcd $[M + H]^+$ 250.1438, found 250.1447.

Feeding Experiments. Three cultures (100 mL of medium described above) were provided with XAD 16 resin (2 mL). The cultures were supplemented with [d_{10}]-DL-leucine (50 mg, 98%, Campro Scientific) or [$^{13}C_9$,^{15}N]-tyrosine (50 mg, 98% ^{13}C, 98% ^{15}N, Campro Scientific). The cultures were incubated at 30 °C with shaking at 180 rpm for 6 days. After sieving off the XAD resin, the resin was eluted with acetone (60 mL) for 16 h. The extracts were evaporated and redissolved in methanol (1 mL) for HPLC-HRESIMS analysis or methanol-d_4 for NMR spectroscopy. The extract from the [$^{13}C_9$,^{15}N]-tyrosine yielded 3.0 mg of **1**, while the extract from the [d_{10}]-leucine yielded 2.3 mg.

*p-Hydroxyacetophenone isobutanamide (**1**) from feeding [$^{13}C_9$,^{15}N]-tyrosine:* ^{13}C NMR (methanol-d_4, 75.5 MHz) δ 194.6 (m, 1 C, C-7, labeled, calculated 50% incorporation rate (ICR)), 176.0 (m, 0.16 C, C-9, labeled, 8% ICR), 176.0 (s, 0.02 C, C-9, unlabeled), 164.2 (m, 0.93 C, C-1, labeled), 131.6 (m, 5.33 C, C-3,5, labeled), 128.0 (m, 3.02 C, C-4, labeled), 116.4 (m, 5.63 C, C-2,6, labeled), 46.5 (m, 3.13 C, C-8,10, labeled), 27.4 (m, 0.86 C, C-11, labeled), 22.8 (d, J = 34.9, 0.81 C, C-12,13, labeled, 7% ICR), 22.8 (s, 0.11 C, C-12,13, unlabeled), % ^{13}C ICR = 1.1 × (integral$_{labeled}$/integral$_{unlabeled}$) − 1.1; ^1H NMR (trifluoroethanol-d_3/H_2O, 1/1, 600 MHz) reference spectrum, δ 0.99 (d, J = 6.6 Hz, 6 H, H-12,13) 2.08 (dq, J = 7.7, 6.6 Hz, 1 H, H-11) 2.23 (d, J = 7.7 Hz, 2 H, H-10) 4.68 (d, J = 5.0 Hz, 2 H, H-8) 7.00 (d, J = 8.8 Hz, 2 H, H-2,6) 7.56 (t, J = 5.0 Hz, 1 H, N-H) 7.94 (d, J = 8.8 Hz, 2 H, H-3,5); labeled spectrum, δ 7.00 (d, 8.8 Hz, 3.74 H, H-2,6, unlabeled), 7.00 (dm, J = 150 Hz, 3.81 H, H-2,6, labeled, 51% ICR), 7.56 (m, 1.0 H, N-H, unlabeled), 7.56 (dm, J = 95 Hz, 0.42 H, N-H, labeled, 30% ICR), 7.94 (d, J = 8.8 Hz, 2.88 H, H-3,5, unlabeled), 7.94 (dm, J = 150 Hz, 3.22 H, H-3,5, labeled, 53% ICR); (% ICR = (integral$_{labeled}$/(integral$_{labeled}$ + integral$_{unlabeled}$)) × 100); (+)-HRESIMS m/z (%) = 250.1677 (2), 249.1647 (14), 248.1663 (11), 247.1584 (10), 246.1584 (9), 245.1515 (42), 244.1537 (51), 237.1272 (27), 236.127 (94), 161.0938 (69), 160.0966 (70), 153.0688 (24), 152.0701 (100%) (% ICR = intensity$_{labeled}$/(intensity$_{labeled}$ + intensity$_{unlabeled}$) × 100).

*p-Hydroxyacetophenone Isobutanamide (**1**) from feeding [d_{10}]-DL-leucine:* ^1H NMR (methanol-d_4, 300 MHz), identified signals from the crude extract, δ 1.03 (d, J = 6.4 Hz, 2.2 H, H-12,13), 4.66 (s, 2.0 H, H-8), 6.91 (d, J = 8.8 Hz, 2.0 H, H-2,6), 7.94 (d, J = 8.8 Hz, 1.9 H, H-3,5); (+)-HRESIMS m/z (%) = 245.1834 (100), 236.1268 (53) (% ICR = %$_{labeled}$/(%$_{labeled}$ + %$_{unlabeled}$) × 100).

Biological Assays. The minimum inhibitory concentrations (MIC) of the six *p*-hydroxyacetophenone amides were determined against the Gram-positive bacteria *Staphylococcus aureus*, *Nocardia flava*, and *Micrococcus luteus*, as well as the Gram-negative bacteria *Escherichia coli* and *Chromobacterium violaceum*. The yeasts *Candida albicans*, *Rhodotorula glutinis*, and *Pichia anomala* and the fission yeast *Schizosaccharomyces pombe*, as well as the fungus *Mucor hiemalis*, were also tested. MIC tests were conducted in 96 microtiter well plates by serial dilution (1/1) in EBS medium (0.5% casein peptone, 0.5% protease peptone, 0.1% meat extract, 0.1% yeast extract, pH 7.0) for bacteria and MYC medium (1.0% phytone peptone, 1.0% glucose, 50 mM HEPES [11.9 g/L], pH 7.0) for yeasts and fungi, respectively. On each plate a negative control line with only the tested organism was inoculated as well as a control of methanol, and positive controls with oxytetracyclin hydrochloride and nystatin showing the expected growth inhibition in the μg/mL range. Plates were incubated on a microplate shaker at 600 rpm at 30 °C for 24 h. Concentrations tested were 100−0.8 μg/mL for the six *p*-hydroxyacetophenone amides, and no growth inhibition was observed.

The MIC of the six *p*-hydroxyacetophenone amides was also tested against different microalgae of the Chlorophyceae, *Scenedesmus bajacalifornicus*, *Chlamydomonas* sp., and *Bracteacoccus* sp., and the Treboux-iophyceae, *Pseudococcomyxa simplex* and *Botryococcus braunii*. MIC tests were conducted in 96 microtiter well plates in a serial dilution in modified Bold's basal medium with triple nitrate (3 N BBM). The plates were cultivated at room temperature under a light/dark regime of 14 h/10 h and a photon fluency rate of about 25 μmol photons m^{-2} s^{-1} from white fluorescent bulbs until the reference line was overgrown. Concentrations tested were 100−0.8 μg/mL for the six *p*-hydroxyacetophenone amides. The substances were air-dried before the algae suspensions were added. **4** gave growth inhibition at a concentration of 100 μg/mL against *Pseudococcomyxa simplex*; all other samples were inactive.

The cell culture and growth inhibition assays were carried out with the U-937 human leukemic cell line and a WST-1 assay (Roche). The *p*-hydroxyacetophenone amides were tested in 96-well plates by serial dilution (1/1), and no cytotoxicity was observed up to a concentration of 100 μg/mL.

■ ASSOCIATED CONTENT

Supporting Information. Complete ^1H, ^{13}C, and HMBC correlation data of **1**−**6** in methanol-d_4 are provided, together with the 1D and 2D ^1H (COSY) and ^{13}C (HMQC, HMBC) NMR spectra of **1**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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