Flavonoid Glucuronides and a Chromone from the Aquatic Macrophyte *Stratiotes aloides*

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The first phytochemical analysis of the aquatic macrophyte *Stratiotes aloides* afforded two new flavonoid glucuronides, luteolin 7-*O*-*β*-D-glucopyranosiduronic acid-(1→2)-*β*-D-glucopyranoside (**1**) and chrysoeriol 7-*O*-*β*-D-glucopyranosiduronic acid-(1→2)-*β*-D-glucopyranoside (**2**), as well as the new 2-(2-hydroxypentyl)-5-carboxy-7-methoxychromone (**5**) and chrysoeriol 7-*O*-*β*-(6-*O*-malonyl)glucopyranoside (**3**), which has been assigned via NMR data for the first time. Additionally, free amino acids such as tryptophan, arginine, leucine, isoleucine, phenylalanine, and tyrosine along with choline, *cis*-aconitic acid, the phenolic glycoside α-arbutine, the chlorophyll derivative phaeophorbide *a*, and the flavonoid glycoside luteolin 7-*O*-*β*-(6-*O*-malonyl)glucopyranoside (**4**) were isolated. Despite the low quantities obtained in some cases (between 50−300 *μ*g), the structures of all compounds were unambiguously elucidated by extensive NMR and MS experiments. With a delay of 2 days compound **1** (10 and 50 *μ*M test concentration) strongly inhibited the growth of human SH-SY5Y neuroblastoma cells in a dose-dependent manner, whereas only a moderate growth inhibition of human Patu 8902 carcinoma cells could be observed. Compounds **1** and **2** showed no activities against the bacteria *Escherichia coli* BW25113, *Pseudomonas pudida* KT2440, and *Enterobacter cloacae* subsp. *dissolvens*.

The aquatic macrophyte *Stratiotes aloides* L. (Hydrocharitaceae), also known as "water soldier", is native to Central Europe and Siberia.[1] *S. aloides*, which was selected as the flower of the year in Germany in 1998, has been under strong legal protection. Possible reasons for protection are the fact that *S. aloides* represents the only known habitat of the dragonfly *Aeshna viridis*[2] and that a strong decline in the stands of *S. aloides* has been observed for the last decades.[3] *S. aloides* plays an important role in meso and eutrophic waters, because it can produce large amounts of biomass, thus accumulating nutrients. In former times the plant was used as both famine vegetable and animal food.[4] Because of its sensitivity to water pollution, e.g., sulfates,[5] it serves also as an ecological indicator species for water quality.[6] Investigations on the elemental composition of several water plants showed that *S. aloides* is able to accumulate heavy metals—especially manganese—radioactive nuclides, and iodide.[7a−f] On the basis of the observation that there is often a notable lack of phytoplankton in the stands of *S. aloides*,[1] it was suggested that *S. aloides* extrudes allelopathic compounds. Despite studies showing allelopathic effects, the structures of these compounds are not known.[8a−d] As for the identity of extruded chemicals or plant metabolites in general, only a limited number of publications can be found. However, reports comprising investigations on sodium, calcium, and potassium contents[9] as well as the carbon source of *S. aloides* have been published.[10] Furthermore, comparative studies on the pigment complex[11] and the total phenolic content in the leaves[12] are reported, but in both cases without isolation of any individual compounds. Due to the overall lack of detailed information on constituents of *S. aloides*, we carried out a primal phytochemical analysis of the aqueous acetone as well as aqueous EtOH extracts of 30 individuals of *S. aloides* collected in a pond on the campus of this university. ^{1}H NMR analysis revealed that the predominant polar fractions of both extracts consisted of a mixture of carbohydrates, the major component being free glucose (data not shown). We now report the isolation and structure elucidation of two new flavonoid glucuronides, luteolin and chrysoeriol 7-*O*-*β*-D-glucopyranosiduronic acid-(1→2)-*β*-D-glucopyranoside (**1**, **2**), a new chromone derivative, 2-(2-hydroxypentyl)-5-carboxy-7-methoxychromone (**5**), and chrysoeriol 7-*O*-*β*-(6-*O*-malonyl)glucopyranoside (**3**), which has not been assigned via NMR data before. Additionally, six amino acids (tryptophan, arginine, leucine, isoleucine, phenylalanine, tyrosine) and five known secondary metabolites including choline, *cis*-aconitic acid,[13] α-arbutine,[14] luteolin 7-*O*-*β*-(6-*O*-malonyl)glucopyranoside (**4**),[15] and the chlorophyll derivative phaeophorbide *a*[16] were isolated and identified by NMR and MS data. Despite low quantities of pure compounds (ca. 50−300 *μ*g) obtained in some cases, e.g., **3** and **5**, we were able to unambiguously identify the structures of all isolated constituents by extensive 1D and 2D NMR experiments using 3 mm SHIGEMI tubes. We also report the results of a screening against bacteria *Escherichia coli* BW25113, *Pseudomonas pudida* KT2440, and *Enterobacter cloacae* subsp. *dissolvens* of **1** and **2** and present the cytotoxic activities of **1** toward human Patu 8902 carcinoma cells as well as human SH-SY5Y neuroblastoma cells.

Results and Discussion

Luteolin 7-*O*-*β*-D-glucopyranosiduronic acid-(1→2)-*β*-D-glucopyranoside (**1**) was obtained as yellowish powder, $[\alpha]^{25}_{D}$ −85. Its molecular formula, $C_{27}H_{28}O_{17}$, was established by +HR-FT-ESIMS, which exhibited an $[M + H]^+$ ion at *m/z* 625.14032 (calcd for $C_{27}H_{29}O_{17}$, 625.13993). Analysis of the ^{1}H NMR and the COSY spectra revealed an aromatic ABM splitting system consisting of the protons at *δ* 7.87 (d, *J* = 2.0 Hz, H-2′), 7.22 (d, *J* = 8.3 Hz, H-5′), and 7.48 (dd, *J* = 2.1, 8.5 Hz, H-6′), a set of two *m*-coupled protons at *δ* 7.07 (d, *J* = 1.9 Hz, H-6) and 7.09 (d, *J* = 2.0 Hz, H-8), and a singlet at *δ* 6.84 (H-3). The ^{13}C NMR chemical shifts of their corresponding carbons C-2′, C-5′, C-6′, C-6, C-8, and C-3 were assigned by a GHSQCAD experiment (Table 1; Supporting Information, S1). Combining all ^{1}H−^{13}C long-range correlations (HMBC) of the aromatic protons and the remaining nine quaternary unsaturated carbons, a luteolin structure was deduced for the flavonoid aglycone (Figure 1). Furthermore, the ^{1}H NMR spectrum of **1** displayed two anomeric protons at *δ* 5.78 (d, *J* = 7.6 Hz, H-1″) and 5.50 (d, *J* = 7.8 Hz, H-1‴) along with 10 protons in the *δ* 3.79−4.70 region, suggesting the presence of two sugar moieties.

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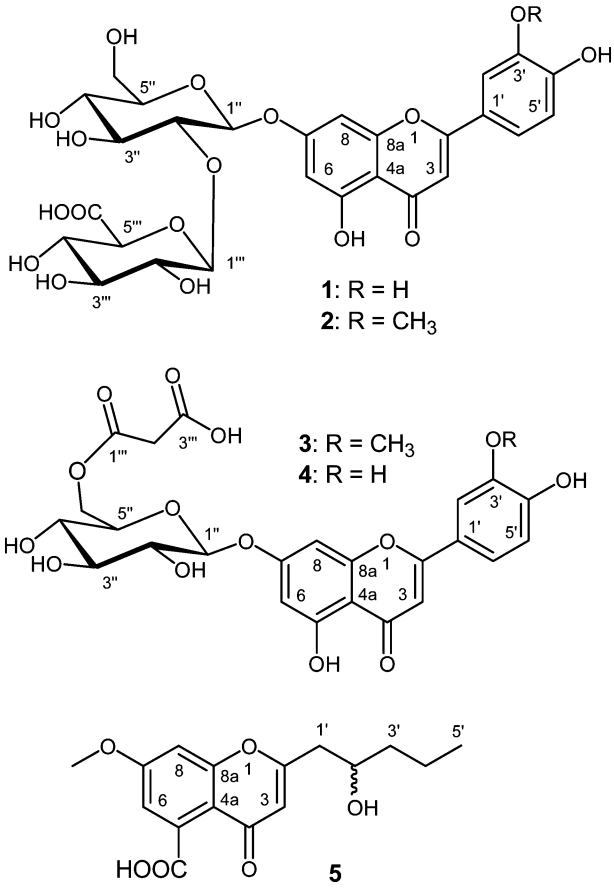

Selective 1D-TOCSYs on both anomeric protons exhibited a seven-proton spin system in the case of H-1″ and a five-proton spin system in the case of H-1‴ (Supporting Information, S1). Evaluation of the DQFCOSY, GHSQCAD, and GHMCAD spectra and analysis of the coupling constants established a β-glucopyranosyl moiety of the seven-proton spin system H-1″−H-6″α/β with a $^4C_1$ conformation in the case of a D-configured pyranose. Similarly, a β-glucopyranosyl-derived configuration of the five-proton spin system H-1‴−H-5‴ could be deduced. The major differences between the glucopyranosyl moiety and the five-proton spin system were as follows: H-5‴ appears as a doublet in the ^{1}H NMR with only one vicinal correlation in the DQFCOSY spectrum in lieu of a ddd pattern of H-5″. Furthermore, an HMBC correlation between H-5‴ and a carboxylic carbon at δ 172.6 indicated an oxidized C-6, thus establishing a glucopyranosiduronic acid moiety in **1** with a $^4C_1$ conformation. An HMBC correlation between H-1″ and C-7 of the aglycone at δ 163.8 along with ROEs between H-1″ and H-6 as well as H-8 located the glucopyranosyl unit at C-7 of the aglycone. HMBC correlations between both H-1‴ (glucopyranosiduronic acid) and C-2″ (glucopyranosyl) and H-2″ and C-1‴, respectively, unambiguously established the 1 → 2 interglycosidic linkage between the carbohydrate residues (Figure 1). Higher energy collision dissociation (HCD) fragmentation during the −HR-FT-ESIMS^2 of **1** yielded four predominant fragments, $[M - H_2O - H]^-$ with *m/z* 605.11137, $[M - H_2O - CO_2 - H]^-$ with *m/z* 561.12084, [M − glucopyranosiduronic acid − H]$^-$ with *m/z* 447.08687, and [M − glucopyranosiduronic acid − glucopyranosyl − H]$^-$ with *m/z* 285.03131 (Supporting Information, S2). The observation of these fragments independently confirmed the structure of **1** on the basis of MS experiments.

Chrysoeriol 7-*O*-β-D-glucopyranosiduronic acid-(1→2)-β-D-glucopyranoside (**2**) was isolated as yellowish powder, $[\alpha]^{25}_D$ −94. +HR-FT-ESIMS revealed a molecular formula of $C_{28}H_{30}O_{17}$ as indicated by its $[M + H]^+$ ion at *m/z* 639.15509 (calcd for $C_{28}H_{31}O_{17}$, 639.15558). Compared to **1**, compound **2** showed a closely related ^{1}H NMR spectrum except for an additional singlet

Table 1. ^{1}H NMR and ^{13}C NMR Data for Compounds **1**−**3** (500 MHz)

	1[a]		**2**[b]		**3**[a]	
position	δ_C[b], mult.	δ_H[c] (*J* in Hz)	δ_C[b], mult.	δ_H[c] (*J* in Hz)	δ_C[b], mult.	δ_H[c] (*J* in Hz)
2	165.1, C		165.4, C		166.6, C	
3	103.6, CH	6.84, s	104.0, CH	6.90, s	104.4, CH	6.69, s
4	181.8, C		183.0, C		184.0, C	
4a	106.5, C		107.1, C		107.2, C	
5	162.4, C		162.0, C		162.2, C	
6	105.3, CH	7.07, d (1.9)	99.8, CH	6.39, d (2.1)	101.1, CH	6.51, d (2.1)
7	163.8, C		164.1, C		165.0, C	
8	95.4, CH	7.09, d (2.0)	96.4, CH	6.78, d (2.0)	96.1, CH	6.82, d (2.1)
8a	157.6, C		158.5, C		159.1, C	
1′	122.5, C		123.2, C		123.1, C	
2′	114.3, CH	7.87, d (2.0)	109.7, CH	7.55, d (1.9)	110.6, CH	7.51, d (2.1)
3′	147.6, C		149.3, C		149.6, C	
3′-OMe			55.8, CH_3	3.84, s	56.6, CH_3	3.98, s
4′	151.7, C		152.2, C		152.7, C	
5′	116.4, CH	7.22, d (8.3)	116.4, CH	6.93, d (8.5)	116.8, CH	6.96, d (8.6)
6′	119.3, CH	7.48, dd (2.1, 8.5)	121.3, CH	7.57, dd (1.9, 8.2)	122.0, CH	7.56, dd (2.2, 8.4)
1″	99.7, CH	5.78, d (7.6)	98.5, CH	5.16, d (7.5)	101.4, CH	5.09, d (7.2)
2″	84.0, CH	4.40, t (8.4)	81.4, CH	3.53[d]	74.7, CH	3.50[d]
3″	77.3, CH	4.47, t (9.3)	76.8, CH	3.46[d]	77.3, CH	3.51[d]
4″	70.4, CH	4.31, t (9.1)	69.2, CH	3.21, t (8.9)	71.0, CH	3.49[d]
5″	78.5, CH	4.09, br ddd (1.9, 4.9, 9.1)	75.2, CH	3.54[d]	75.3, CH	3.79, ddd (2.4, 5.3, 9.3)
6″a/b	61.7, CH_2	4.49, br d (12.2)	60.3, CH_2	3.72, br d (11.0)	64.5, CH_2	4.44, dd (2.3, 12.0)
		4.34, dd (4.6, 12.4)		3.46[d]		4.36, dd (5.2, 12.0)
1‴	106.4, CH	5.50, d (7.8)	102.8, CH	4.53, d (7.9)	170.0[e], C	
2‴	75.5, CH	4.21, t (8.3)	73.8, CH	2.99, dd (7.9, 8.5)	45.1[e], CH_2	3.77, s[e]
3‴	77.4, CH	4.36, t (9.3)	76.3, CH	3.15, t (8.7)	172.8[e], C	
4‴	73.0, CH	4.57, t (9.4)	71.9, CH	3.07, t (9.2)		
5‴	77.7, CH	4.71, t (9.7)	73.4, CH	3.24, d (9.6)		
6‴	172.6, C		172.8, C			

[a] **3** in methanol-d_4; **1** in pyridine-d_5; **2** in a mixture of DMSO-d_6 and 0.01%TFA (9:1; v/v). [b] ^{13}C chemical shifts were derived from GHSQC and GHMBC. [c] Observed coupling constants were not averaged. Assignments based on GCOSY, 1D- TOCSY, GHSQC, and GHMBC spectra. [d] Overlapping signals. [e] Due to fast H/D exchange the NMR signals of the malonyl side chain could only be observed in pyridine-d_5.

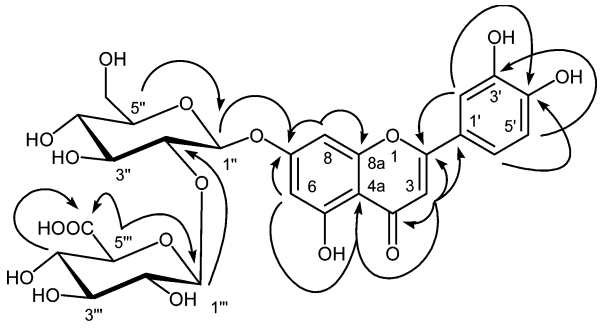

Figure 1. Important HMBC correlations of **1**.

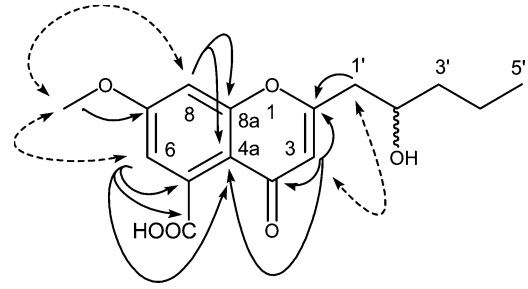

Figure 2. Important ROESY (dashed arrows) and HMBC (plain arrows) correlations of **5**.

at δ 3.84 integrating for three protons. The ^{1}H and ^{13}C NMR chemical shifts δ 3.84 and 55.8 of the latter along with a mass difference of 14 amu indicated the presence of a methoxy group. A ^{1}H−^{13}C long-range correlation between the methoxy and the aromatic carbon C-3′ at δ 149.3 as well as a ROESY cross-peak between the methoxy and H-2″ fixed the 3′-position of the methoxy group. Interpretation of all 1D and 2D NMR data established the identical carbohydrate side chain as in **1** linked via C-7 to the flavonoid aglycone, which itself was assigned as chrysoeriol (Table 1). Acid hydrolysis of compounds **1** and **2** with 2 M TFA (5 h, 100 °C) liberated the sugars into the aqueous layer. The carbohydrate mixture was separated on a Varian MetaCarb 87H column to yield D-glucose, which was identified by ^{1}H NMR and specific rotation data in comparison with an authentic sample. The ^{1}H NMR spectrum of the fraction eluting at similar retention time as the D-glucuronic acid standard showed broad humps at δ 5.4−5.7 and 3.2−3.8 typically for, for example, polymeric material and/or mixtures of many different transformation products derived from sugars. The complete absence of glucuronic acid NMR signals corroborates previous findings on the stability of free glucuronic acid, which tends to undergo decarboxylation and/or dehydration reactions in acidified aqueous solutions.[17a−c] To avoid these reactions, compounds **1** and **2** were alternatively incubated with β-glucuronidase at 37 °C for 2 h. Purification of the reaction mixture by liquid−liquid separation with *n*-BuOH followed by HPLC of the freeze-dried aqueous layer on a DIOL phase yielded D-glucuronic acid. Its ^{1}H NMR and specific rotation data were in good agreement with respective values obtained for the D-glucuronic acid standard.

Complete analysis of the 1D and 2D NMR and MS data of constituents **3** and **4** revealed chrysoeriol and luteolin algycones, respectively. In both cases, a β-glucopyranosyl moiety attached to C-7 of the flavonoid was identified (Table 1). In contrast to **1** and **2**, a malonyl side chain attached to C-6″ instead of a glucuronic acid moiety at C-2″ could be identified: The low-field-shifted diastereotopic glucopyranosyl protons H-6″a/b of **3** showed HMBC correlations to an ester carbonyl C-1‴ at δ 170.0, which showed a further HMBC correlation to a methylene singlet at δ 3.77. Its directly bonded C-2‴ at δ 45.1 and further correlation to the terminal carboxyl carbon C-3‴ at δ 172.8 could only be detected in pyridine-d_5, due to the known H/D exchange in malonyl derivatives in protic deuterated solvents.[18a−c] Thus, the structures of compounds **3** and **4** were unambiguously determined as chrysoeriol 7-*O*-β-(6-*O*-malonyl)glucopyranoside and luteolin 7-*O*-β-(6-*O*-malonyl)glucopyranoside, respectively. Compound **4** is a known compound, whereas no published NMR data of **3** could be found. Only one report exists[19] in which the authors propose the structure of **3** in *Apium graveolens* L. and varieties on the basis of LC MS experiments. The applied MS techniques, however, cannot provide detailed information on the identity and substitution patterns of both sugars and aglycone as well as on relative stereochemical issues, and we therefore carried out extensive NMR studies to unambiguously assign and verify the structure of **3**. Both compounds belong to the class of malonylated flavonoid glycosides known to undergo decarboxylation or to decompose readily by releasing malonic acid.[18b,c,20]

The molecular formula $C_{16}H_{18}O_6$ of the new chromone derivative 2-(2-hydroxypentyl)-5-carboxy-7-methoxychromone (**5**) was established by HPLC-HR-FT-ESIMS, which exhibited an $[M + H]^+$ ion at *m*/*z* 307.11700 (calcd for $C_{16}H_{19}O_6$, 307.11761). The ^{1}H NMR spectrum of **5** displayed two aromatic *m*-coupled protons at δ 6.50 (H-6) and 6.78 (H-8), a singlet at δ 5.98 (H-3), a methoxy group at δ 3.82, and six signals for the monohydroxylated aliphatic side chain at δ 3.87 (H-2′), 2.55 and 2.66 (H-1′a/b), 1.41 (H-3′), 1.32 and 1.41 (H-4′), and 0.86 (H-5′). The sequence of the protons in the side chain was established by GCOSY. Together with their directly bonded carbons (GHSQCAD) the structure of the side chain was assigned as a 2-hydroxypentyl moiety as shown in Figure 2. Analysis of the heteronuclear GHMBCAD and GHSQCAD spectra afforded a chromone derivative (Figure 2). A $^3J_{1H-13C}$ long-range correlation between H-6 (δ 6.50) and the carbonyl carbon at δ 172.6 indicated the presence of a carboxyl group at C-5, in accordance with the observed molecular mass. ROESY correlations between the methoxy group and the two aromatic protons H-6 and H-8 established the 7-position of the attached methoxy group. A strong HMBC correlation between the protons of the methoxy group and the aromatic C-7 at δ 163.5 supported the above assignment. Similarly, the C-2/C-1′ connectivity between the chromone and the 2-hydroxypentyl side chain was deduced. Thus, HMBC correlations between both the diastereotopic methylene protons H-1′a/b (δ 2.55 and 2.66) and carbons C-2 (δ 166.4) as well as C-3 (δ 111.5) and the proton H-3 (δ 5.98) and carbons C-2 and C-1′ (δ 42.1) unambiguously determined the C-2/C-1′ bond (Figure 2). However, the very limited amount of this compound present in the extract (ca. 100−200 μg could be isolated) prevented the determination of the absolute configuration at C-2′ by chiroptical methods and chemical derivatization procedures.

Compounds **1** and **2** were tested for their activities against the bacteria *Escherichia coli* BW25113, *Pseudomonas pudida* KT2440, and *Enterobacter cloacae* subsp. *dissolvens* and showed no effect even at the highest concentration applied (128 μg/mL). In order to evaluate potential antiproliferative/cytotoxic effects, human SH-SY5Y neuroblastoma cells and Patu 8902 pancreatic carcinoma cells were cultured in the absence or presence of 10 and 50 μM of the major compound **1** for 1−4 days. Patu cells were only moderately affected by **1**, showing up to 20% decrease of growth at 50 μM of the agent after 4 days (Figure 3, C and D). The growth of SH-SY5Y cells, however, was strongly and dose dependently inhibited up to 75% (Figure 3, A and B; Figure 4, A and B). In the treated cell cultures many apoptotic cells could be detected, showing fragmented DNA (Figure 4, C). Interestingly, both cell lines were unaffected within the first 2 days of treatment, indicating that **1** does not exert a toxic effect immediately. The obvious delay of antiproliferative activity of **1** suggests that the substance is converted into an active form with increasing time. This observation is in line with the fact that flavonoids metabolized as conjugates with glucuronic acid (e.g., during intestinal absorption) are inactive.[21]

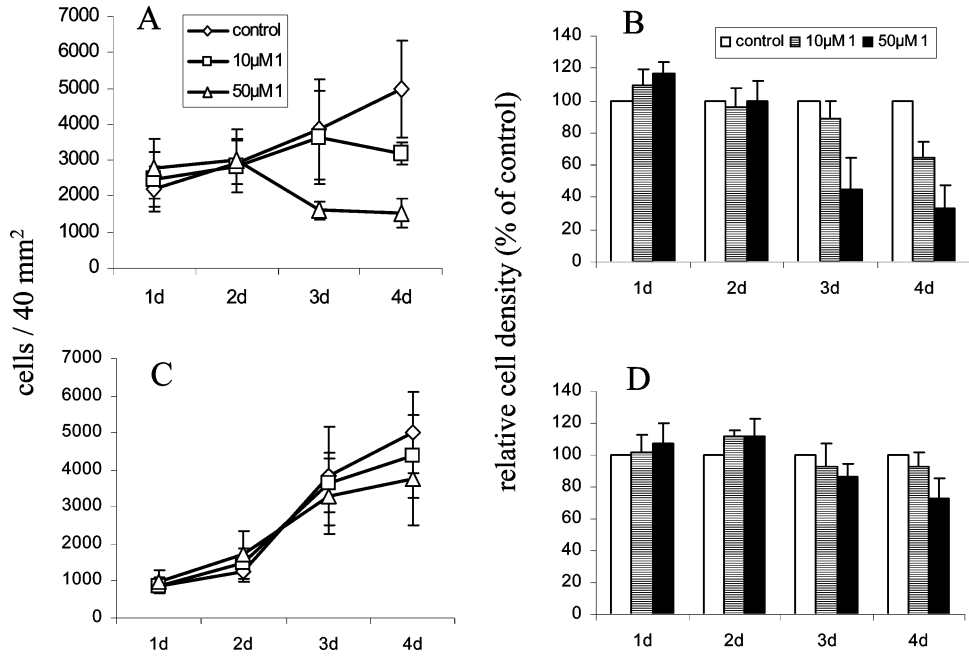

Figure 3. Antiproliferative/cytotoxic activity of **1**. Strong and dose-dependent growth inhibition (up to 75%) of SH-SY5Y neuroblastoma cells (A and B), but only weak inhibition (up to 20%) of Patu 8902 pancreatic carcinoma cells (C and D) occurring after 4 days; no effect of **1** up to 2 days of treatment; data represent means ± SD of 3 experiments with 2 parallel dishes for each point ($n = 6$).

Aglycones, liberated by β-glucuronidase, however, can inhibit tumor cell proliferation by induction of cell cycle arrest and apoptosis, as was shown for luteolin in epidemiological and cell culture studies.[22a,b]

In conclusion, the present phytochemical investigation with focus on the qualitative composition of constituents of *S. aloides* provides the first detailed insight into the structural classes and molecules present in this fascinating plant. The presence of detectable amounts of free amino acids could help to explain why the plant was formerly used for nutrition purposes. Furthermore, the isolated phenolic and polar flavonoid metabolites may support previous suggestions on the nature of allelochemicals based on molecules of phenolic origin[23] on one hand and moderately lipophilic compounds on the other.[8c] Further studies will have to evaluate the natural flavonoid−glucuronic acid conjugate **1** as a potential anticarcinogenic agent.

Experimental Section

General Experimental Procedures. Optical rotations were determined with a Perkin-Elmer model 341 polarimeter. UV spectra were recorded using a Varian Cary 4E UV−visible spectrophotometer. FT-IR spectra were obtained with a Perkin-Elmer Spectrum One FT-IR spectrophotometer. NMR spectra were recorded in 3 mm standard NMR or 3 mm SHIGEMI tubes on a Varian Unity Inova 500 MHz spectrometer equipped with a 3 mm ID-PFG probe. The ^{1}H and ^{13}C NMR chemical shifts were referenced to solvent signals at $\delta_{H/C}$ 2.49/39.5 (DMSO-d_6), 3.32/49.0 (methanol-d_4), 4.70 (D_2O), 8.71/149.8 (pyridine-d_5), and 7.27/77.0 ($CDCl_3$) relative to TMS. 1D and 2D homonuclear NMR spectra were measured with standard Varian pulse sequences. Adiabatic broadband and band-selective GHSQCAD and GHMBCAD spectra were recorded using CHEMPACK 4.0 pulse sequences (implemented in Varian Vnmrj 2.1B spectrometer software).

HPLC-APCI-MS spectra of known compounds were measured on a Finnigan MAT TSQ 700 equipped with an APCI ion source in positive and negative ion mode. The HPLC system consisted of a Varian Pro Star 230 pump and an ABI 785 detector. A Phenomenex Aqua 5 μ C18 column (250 × 4.6 mm) eluted with different gradients of (I) 0.01% formic acid in doubly distilled H_2O (v/v) and (II) MeCN were used for HPLC.

HPLC-HR-FT-HRESIMS experiments of new compounds were performed using a Waters Nano-Acquity UPLC system interfaced to a ThermoScientific LTQ Orbitrap XL mass spectrometer with an ESI ion source. The instrument was operated in positive and negative ionization mode and was externally calibrated according to the manufacturer's guidelines. Mass spectra were acquired in the mass range of 50−1200 Da using an Orbitrap mass analyzer operating with a target mass resolution of 60 000 (at m/z 400). MS/MS analysis was performed with the HCD collision cell with direct infusion of 3 μL/min. The spectra were acquired in the negative mode using N_2 as collision gas, the normalized collision energy was set at 21 eV, and fragment ions were detected in the Orbitrap at a resolution of 7500. Chromatographic separations were performed on a Nano-Acquity UPLC employing a ThermoScientific Hypersil Gold column (150 mm × 0.5 mm; 5 μm) at a flow rate of 12 μL/min. The column was eluted with (I) 0.1% formic acid in H_2O and (II) 0.1% formic acid in MeCN.

Analytical and semipreparative HPLC were performed on an HPLC system consisting of two Knauer WellChrom HPLC pumps K-1001 and a Bischoff Lambda 1010 UV/vis detector. For analytical purposes a Phenomenex Aqua 5 μ C18 column (250 × 4.6 mm) was used. Gradient: 0% B for 10 min, 100% B within 30 min, 100% B for another 10 min, 1 mL/min; UV detection $\lambda = 254$ nm. Throughout, the mobile phase consisted of 0.01% TFA in doubly distilled H_2O (v/v, eluent A) and a mixture of MeCN and H_2O, 84/16 (v/v, eluent B).

Plant Material. On May 28 and June 2, 2003, 30 individual plants of *S. aloides* were collected from a freshwater pond in the botanical garden at Universität Hohenheim (48°42′32″ N, 9°12′32″ E), Stuttgart, Germany, and identified by Mr. Norbert Kretschmer, Institut für Zoologie, Universität Hohenheim. Voucher specimens are still cultivated in the pond.

Extraction and Isolation. Immediately after collection, the brownish outer leaves of each of the 30 individual plants of *S. aloides* were discarded, and the whole plants were frozen in liquid N_2. The frozen plants were ground and lyophilized for seven days. The dried material (80 g) was homogenized and successively extracted twice with petroleum ether (bp 30−60 °C, 1.5 L each) for 20 min in an ultrasonic bath, twice with a mixture of Me_2CO and doubly distilled H_2O (ratio 8:2, v/v, 1.5 L each) for 20 min in the ultrasonic bath, and finally twice with a 10:1 mixture of EtOH and doubly distilled H_2O (1.1 L each) for 18 and 70 h, respectively. The organic layers were evaporated to dryness and afforded 1.5 g of petroleum ether extract, 11.5 g of Me_2CO extract, and 6 g of EtOH extract. The Me_2CO extract was dissolved in 100 mL of EtOH and 40 mL of H_2O and extracted three times with petroleum ether (80 mL each). The petroleum ether layer was washed three times with 60 mL of a mixture of EtOH and H_2O (ratio 2:1, v/v).

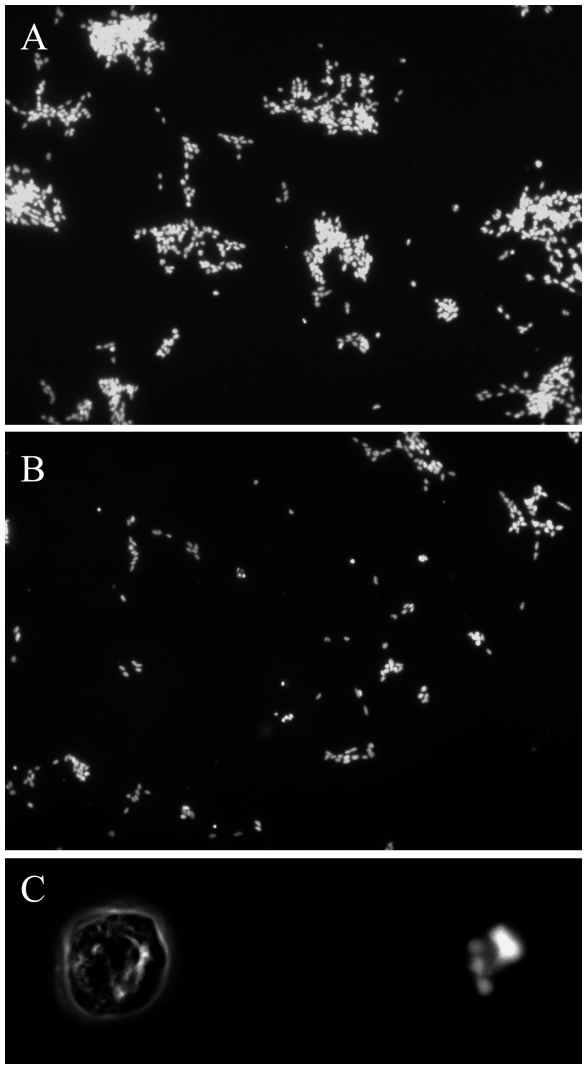

Figure 4. Antiproliferative/cytotoxic activity of **1**. Dapi-stained SH-SY5Y cells cultured for 4 days in the absence (A) or presence (B) of 50 μM of **1**; (C) example of an apoptotic cell. Left: phase contrast. Right: DNA clusters stained with Hoechst 33342.

Both the combined aqueous EtOH layers and the petroleum ether phase were concentrated under reduced pressure. The dried EtOH phase of the Me_2CO extract (10.8 g) was subjected to an MPLC column filled with 70 g of RP18 material (LiChroprep RP18, Merck) and subsequently eluted with 400 mL of doubly distilled H_2O, 500 mL of a mixture of EtOH and H_2O (ratio 1:1, v/v), 400 mL of a mixture of EtOH and H_2O (ratio 9:1, v/v), and 500 mL of a mixture of Me_2CO and EtOH (ratio 3:1, v/v) to yield fractions F1 (9.3 g), F2 (600 mg), F3 (250 mg), and F4 (400 mg). An aliquot of F1 (260 mg) was purified by semipreparative HPLC on RP18 (Bischoff LiChrospher 100 RP18; 250 × 25 mm; 10 μm; gradient: 0−5% B within 10 min, 5−70% B within 35 min; 6 mL/min; UV detection λ = 254 nm) to afford tryptophan and *cis*-aconitic acid. A further liquid−liquid separation step with *n*-BuOH was applied to enrich the flavonoid glycosides, which were present in low amounts only. Therefore, 9 g of fraction F1 was dissolved in H_2O (400 mL) and extracted with *n*-BuOH (4 × 100 mL). The combined organic layers (= Bu) and the aqueous layer (= H) were each lyophilized. The *n*-BuOH fraction (632 mg) was subjected to semipreparative HPLC on RP18 (Merck LiChroCART 250 × 10 mm cartridge; 10 μm; gradient: 0% B for 15 min, 0−50% B within 45 min, 50−100% B within 20 min; 6 mL/min; UV detection λ = 254 nm) to yield 27 subfractions, which were analyzed by ^{1}H NMR. Fraction Bu14 was purified by repeated HPLC on a semipreparative Phenomenex Aqua C18 column (250 × 10 mm; 5 μm; 5 mL/min; UV detection λ = 220 nm) with slightly different gradients of solvents A and B to afford α-arbutine as well as compounds **2** (ca. 1 mg) and **5** (ca. 100 μg). Semipreparative HPLC of fraction Bu16 on a phenyl-bonded Nucleosil 100-7 C6H5 column (Macherey-Nagel; 250 × 10 mm; 7 μm, gradient: 0−20% B within 10 min, 20−40% B within 30 min, 40−50% B within 10 min; 4 mL/min; UV detection λ = 254 nm) to yield compound **4** (10 mg). Fraction Bu18 was subjected to Sephadex LH-20 chromatography with MeOH (1 mL/min; UV detection λ = 254 nm) as eluent to give compound **3** (ca. 500 μg). MPLC of F2 on Polyamide CC 6 (Macherey-Nagel) with gradient elution (gradient: 0.01% TFA for 15 min, 0−100% EtOH within 75 min, 4 mL/min; UV detection λ = 248 nm) yielded eight subfractions, F2.1−2.8. Further purification of F2.2 by repeated semipreparative HPLC on RP18 (Bischoff; 250 × 25 mm; 10 μm; 7 mL/min; and Phenomenex; 250 × 10 mm; 5 μm; 4 mL/min; UV detection λ = 250 nm) with different gradients of solvents A and B resulted in the isolation of tryptophan. Compound **1** (ca. 200 μg) was isolated from the EtOH-soluble part of F2.6 by subsequent semipreparative HPLC on a Phenomenex Aqua C18 column (250 × 10 mm; 5 μm; gradient: 0% B for 10 min, 100% B within 70 min; mL/min; UV detection λ = 250 nm). Semipreparative HPLC of F3 on RP18 (Phenomenex 250 × 10 mm, 5 μm; gradient: 0% B for 10 min, 0−50% B within 20 min, 50−70% B within 10 min, 70−100% B within 30 min, 100% B for 10 min; 4 mL/min; UV detection λ = 220 nm) yielded the chlorophyll derivative phaeophorbide *a*. The EtOH/H_2O-soluble part (ratio 3:7, v/v, 2 mL) of the EtOH extract was subjected to SPE with RP18 (Chromabond C18ec, Macherey-Nagel), and fractions were subsequently eluted with 2 mL of H_2O (fraction EH, 2.2 g), 2 mL of Me_2CO (fraction EA, 231 mg), and 2 mL of $CHCl_3$ (fraction EC, 211 mg). Semipreparative HPLC of fractions EH and EA on RP18 (Merck LiChroCART 250 × 10 mm cartridge; 10 μm; gradient: 0% B for 10 min, 0−100% B within 60 min; 4 mL/min; UV detection λ = 254 nm) afforded subfractions EH1−14 and EA1−15. After NMR analysis similar fractions were combined and finally purified by HPLC on a Phenomenex Aqua C18 column (250 × 10 mm). Again, compound **1** (ca. 1 mg) was obtained from fraction EH8. Purification of fraction EH2 with a semipreparative CN HPLC column (Bischoff Spherisorb CN; 5 μm, 250 × 25 mm; gradient: 0% B for 60 min, 0−100% B within 20 min; 8 mL/min; UV detection λ = 220 nm) yielded arginine, tyrosine, leucine, isoleucine, phenylalanine, and choline. Fraction H was subjected to a Sephadex LH 20 column and eluted with MeOH (1 mL/min; RI detection) to give larger amounts of **1** (9 mg) and **2** (3 mg).

Luteolin 7-*O*-*β*-D-glucopyranosiduronic acid-(1→2)-*β*-D-glucopyranoside (1): yellowish powder; $[\alpha]^{25}_D$ −85 (*c* 0.33, H_2O/DMSO, 5.5:1); UV (H_2O) λ_{max} (log ϵ) 253 (4.11), 262 (4.12), 349 (4.16) nm; IR (ATR) ν_{max} 3360, 2900, 1727, 1652, 1602, 1497, 1448, 1335, 1303, 1260, 1079, 1044 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); +HR-FT-ESIMS *m/z* 625.14032 (calcd for $C_{27}H_{29}O_{17}$, 625.13993) $[M + H]^+$.

Chrysoeriol 7-*O*-*β*-D-glucopyranosiduronic acid-(1→2)-*β*-D-glucopyranoside (2): yellowish powder; $[\alpha]^{25}_D$ −94 (*c* 0.275, H_2O/DMSO, 5.5:1); UV (H_2O) λ_{max} (log ϵ) 252 (4.11), 267 (4.12), 349 (4.17) nm; IR (ATR) ν_{max} 3330, 2911, 1717, 1655, 1600, 1497, 1435, 1339, 1291, 1259, 1078, 1029 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); +HR-FT-ESIMS *m/z* 639.15509 (calcd for $C_{28}H_{31}O_{17}$, 639.15558) $[M + H]^+$.

2-(2-Hydroxypentyl)-5-carboxy-7-methoxychromone (5): white powder; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 6.78 (1H, d, J = 2.4 Hz, H-8), 6.50 (1H, d, J = 2.4 Hz, H-6), 5.98 (1H, s, H-3), 3.87 (1H, m, H-2′), 3.82 (3H, s, OCH_3-7), 2.66 (1H, dd, J = 4.6, 14.3 Hz, H-1′a), 2.55 (1H, dd, J = 8.1, 14.5 Hz, H-1′b), 1.41 (2H, ov, H-3′), 1.41 (1H, ov, H-4′a), 1.32 (1H, m, H-4′b), 0.86 (3H, t, J = 6.9 Hz, H-5′), ov = partially overlapped by other signals; ^{13}C NMR (DMSO-d_6, 500 MHz, indirectly determined by GHSQCAD and GHMBCAD) δ 177.2 (C, C-4), 172.6 (C, *C*OOH-5), 166.4 (C, C-2), 163.5 (C, C-7), 158.8 (C, C-8a), 147.1 (C, C-5), 113.4 (C, C-4a), 111.5 (CH, C-3), 110.3 (CH, C-6), 98.2 (CH, C-8), 68.0 (CH, C-2′), 56.2 (CH_3, O*C*H_3-7), 42.1 (CH_2, C-1′), 39.8 (CH_2, C-3′), 18.9 (CH_2, C-4′), 14.7 (CH_3, C-5′); +HR-FT-ESIMS *m/z* 307.11700 (calcd for $C_{16}H_{19}O_6$, 307.11761) $[M + H]^+$.

Acid Hydrolysis. Compounds **1** and **2** (1.0 mg each) were hydrolyzed in an argon atmosphere (100 °C, 5 h) using 2 mL of 2 M TFA. After cooling to ambient temperature, the solution was extracted four times with EtOAc (2 mL each). The aqueous layer was lyophilized and separated on a Varian MetaCarb 87H column (300 × 6.5 mm; 40 °C; 0.002 M H_2SO_4; 0.5 mL/min; RI detection). The fractions were neutralized with $Ba(OH)_2$, filtered through a PTFE membrane (0.45 μm), freeze-dried, and analyzed by ^{1}H NMR. D-Glucose was identified by ^{1}H NMR and specific rotation ($[\alpha]^{25}_D$ +41.4, *c* 0.14, H_2O) in comparison with an authentic sample purchased from Sigma.

Enzymatic Hydrolysis. Compounds **1** and **2** (0.6 mg each) were dissolved in 1.5 mL of 50 mM KH_2PO_4 (pH = 5.95) and incubated with 0.35 ηkat β-glucuronidase (Type IX-A; from *E. coli*; Sigma) at 37 °C for 2 h. The reaction mixture was extracted with *n*-BuOH (4 × 0.5 mL). The aqueous layer was evaporated to dryness and purified by HPLC on a DIOL phase (Knauer LiChrosorb DIOL 10 μm; 300 × 7 mm; 0.8 mL/min; UV detection λ = 210 nm). MeCN as well as H_2O containing 0.05% TFA (v/v) were used as eluents I and II, respectively. Gradient: 3% II for 10 min, 11−20% II within 25 min. D-Glucuronic acid was unambiguously identified by its ^{1}H NMR and its specific rotation ($[\alpha]^{25}_D$ +25.1, *c* 0.09 H_2O) compared to an authentic sample obtained from Sigma.

Antibacterial Bioassays. Compounds **1** and **2** in serial dilutions [starting test concentration 128 μg/mL, dilution factor $1/2^n$, final test concentration 1 μg/mL; three replicates for each concentration] were tested in microdilution assays in Mueller-Hinton medium according to established protocols.[24] The bacterial strains *Escherichia coli* BW25113,[25] *Pseudomonas pudida* KT2440 (ATTC 47054), and *Enterobacter cloacae* subsp. *dissolvens* (ATTC 23373) served as test organisms. Each strain was grown on a separate 96-well plate along with negative and sterile controls as well as the antibiotics gentamycin and ampicillin as positive controls.

Antiproliferation/Cytotoxic Assay. The human pancreatic carcinoma cell line was a gift of the Bosch Institut für Klinische Pharmakologie (IKP Stuttgart, Germany) and the human neuroblastoma cell line from our own stock. Ham's F12 medium, fetal calf serum (FCS), streptomycine, and penicillin were from Gibco.

The cells were seeded in low density (SH-SY5Y: 50.000 and Patu: 20.000 cells/3.5 cm plastic wells) and cultured in Ham's F12, completed with 10% FCS and 1% penicillin/streptomycine in the absence or presence of 10 or 50 μM of **1** for 1−4 days. For determination of cell densities, cell cultures dishes were washed two times with fresh medium in order to remove detached dead cells, fixed in 4% paraformaldehyde (PFA) in phosphate buffer (PB) for 10 min, permeabilized in 0.5% TRITON X-100/PB for 5 min, and stained with DAPI/PB (1:10 000) for 15 min. From each cell culture dish, digital images of 26 different areas of 1.54 mm^2 were made, and the densities of stained nuclei determined by means of the software Image J, revealing for each dish a total number of cells/40 mm^2. Data are given as means ± SD from six cell culture dishes (three experiments). In order to detect apoptotic cells, viable cell cultures were stained with Hoechst 33342 (1:5000) for 10 min. Apoptotic cells were identified by their strong stain of fragmented DNA clusters.

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Supporting Information Available: ^{1}H NMR and selective TOCSYs and MS/MS spectrum of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Cook, C. D. K.; Urmi-König, K. *Aquat. Bot.* **1983**, *16*, 213–249.
(2) Suutari, E.; Rantala, M. J.; Salmela, J.; Suhonen, J. *Oecologia* **2004**, *140*, 135–139.
(3) Smolders, A. J. P.; Lamers, L. P. M.; den Hartog, C.; Roelofs, J. G. M. *Hydrobiologia* **2003**, *506−509*, 603–610.
(4) Łuczaj, Ł J. *Ethnobiol. Ethnomed.* **2008**, *4*, 4.
(5) Lamers, L. P. M.; Falla, S.-J.; Samborska, E. M.; van Dulken, I. A. R.; van Hengstum, G.; Roelofs, J. G. M. *Limnol. Oceanogr.* **2002**, *47*, 585–593.
(6) Stelzer, D.; Schneider, S.; Melzer, A. *Int. Rev. Hydrobiol.* **2005**, *90*, 223–237.
(7) (a) Gudkov, D. I.; Derevets, V. V.; Kuzmenko, M. I.; Nazarov, A. B. *Radiats. Biol. Radioekol.* **2001**, *41*, 232–238. (b) Fedotov, V. L.; Bogdanov, M. P.; Brysov, N. S. *Vestsi Akad. Navuk BSSR, Ser. Biyal. Navuk 4* **1975**, 16–22. (c) Kaglyan, A. E.; Klenus, V. G.; Fomovsky, M. A.; Nabivanets, Y. B.; Nasvit, O. I.; Belyaev, V. V. In Kranjc, A., Ed.; *Tracer Hydrology 97: Proceedings of the 7th International Symposium on Water Tracing*; A.A. Balkema: Rotterdam, 1997; pp 135−142. (d) Kovacs, M. *Acta Bot. Acad. Sci. Hungary* **1978**, *24*, 273–283. (e) Lyubimova, S. A. *Tr. Inst. Ekol. Rast. Zhivotnykh Ural'skii Nauchnyi Tsentr. Akad. Nauk SSSR* **1971**, *78*, 76−79. (f) Prosvirova, L. V.; Levanidov, L. Y. *Khim. Biokhim. Okislenie Sist., Soderzh. d-Elem.* **1979**, 7–10.
(8) (a) Gross, E. M.; Hilt, S.; Lombardo, P.; Mulderij, G. *Hydrobiologia* **2007**, *584*, 77–88. (b) Kufel, L.; Pasztaleniec, A.; Czapla, G.; Strzalek, M. *Pol. J. Ecol.* **2007**, *55*, 387–393. (c) Mulderij, G.; Mau, B.; van Donk, E.; Gross, E. M. *Hydrobiologia* **2007**, *584*, 89–100. (d) Mulderij, G.; Smolders, A. J. P.; van Donk, E. *Freshwater Biol.* **2006**, *17*, 554–561.
(9) Brammer, E. S.; Wetzel, R. G. *Aquat. Bot.* **1984**, *19*, 119–130.
(10) Prins, H. B. A.; De Guia, M. B. *Aquat. Bot.* **1986**, *26*, 225–234.
(11) Ronzhina, D. A.; Nekrasova, G. F.; P'yankov, V. I. *Russ. J. Plant Physiol.* **2004**, *51*, 21–27.
(12) Smolders, A. J. P.; Vergeer, L. H. T.; Van der Velde, G.; Roelofs, J. G. M. *Oikos* **2000**, *91*, 307–310.
(13) Dinand, E.; Zloh, M.; Brocchini, S. *Aust. J. Chem.* **2002**, *55*, 467–474.
(14) Pawlowska, A. M.; de Leo, M.; Braca, A. *J. Agric. Food Chem.* **2006**, *54*, 10234–10238.
(15) Stein, W.; Anhut, S.; Zinsmeister, H. D.; Mues, R.; Barz, W.; Koester, J. *Z. Naturforsch. B* **1985**, *40*, 469–473.
(16) Cheng, H.-H.; Wang, H.-K.; Ito, J.; Bastow, K. F.; Tachibana, Y.; Nakanishi, Y.; Xu, Z.; Luo, T.-Y.; Lee, K.-H. *J. Nat. Prod.* **2001**, *64*, 915–919.
(17) (a) Anderson, D. M. W.; Garbutt, S. *Talanta* **1961**, *8*, 605–611. (b) Feather, M. S. *Tetrahedron Lett.* **1970**, *48*, 4143–4145. (c) Yamana, T.; Mizukami, Y.; Asahi, S. *Yakugaku Zasshi* **1964**, *84*, 696–699.
(18) (a) Bridle, P.; Loeffler, R. S.; Timberlake, C. F.; Self, R. *Phytochemistry* **1984**, *23*, 2968–2969. (b) Horowitz, R. M.; Asen, S. *Phytochemistry* **1989**, *28*, 2531–2532. (c) Stintzing, F. C.; Conrad, J.; Klaiber, I.; Beifuss, U.; Carle, R. *Phytochemistry* **2004**, *65*, 415–422.
(19) Lin, L.; Lu, S.; Harnly, J. M. *J. Agric. Food Chem.* **2007**, *55*, 1321–1326.
(20) Koester, J.; Bussmann, R.; Barz, W. *Arch. Biochem. Biophys.* **1984**, *234*, 513–521.
(21) Oi, N.; Hashimoto, T.; Kanazawa, K. *ACS Symp. Ser. 993* **2008**, 102–107.
(22) (a) Seelinger, G.; Merfort, I.; Wölfle, U.; Schrempp, C. M. *Molecules* **2008**, *13*, 2628–2651. (b) Cheng, L.; Tan, H.; Wu, X.; Hu, R.; Aw, C.; Zhao, M.; Shen, H.; Lu, Y. *Org. Biomol. Chem.* **2008**, *6*, 4102–4104.
(23) Usenko, O. M.; Sakevich, A. I.; Palamarchuk, V. D. *Int. J. Algae* **2002**, *4*, 20–28.
(24) (a) Vanden Berghe, D. A.; Vlietinck, A. J. In *Methods in Plant Biochemistry*; Dey, P. M., Harborne, J. , Eds.; Academic Press: London, 1991; Vol. 6, pp 52−57. (b) DIN 58940, part 8 In *Medizinische Mikrobiologie und Immunologie*, DIN pocket book 222; Beuth Verlag: Berlin, 1991.
(25) Datsenko, K. A.; Wanner, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 6640–6645.

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