Lipocarbazoles, Secondary Metabolites from *Tsukamurella pseudospumae* Acta 1857 with Antioxidative Activity†

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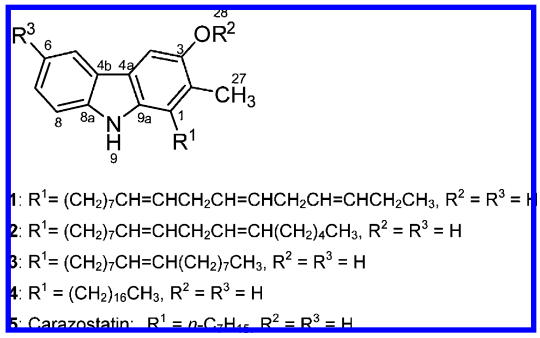
A family of new secondary metabolites with a carbazole moiety and an alkyl side chain was isolated from *Tsukamurella pseudospumae* strain Acta 1857. They were named lipocarbazoles in accordance with their chemical structures, which were determined by mass spectrometry and NMR spectroscopy. Lipocarbazoles are free radical scavengers showing antioxidative activity.

Actinomycetes from various terrestrial, limnetic, and marine ecosystems were investigated in our HPLC-diode array screening program for the production of novel secondary metabolites with the aim of detecting new drugs for pharmaceutical applications. Strain Acta 1857 was isolated from activated sludge foam collected at Stoke Bardolph Water Reclamation Works near Nottingham, UK, and was characterized using a polyphasic taxonomic approach as a new taxon within the genus *Tsukamurella*, namely, *Tsukamurella pseudospumae* sp. nov.[1] Tsukamurellae are members of the suborder *Corynebacterineae*, which contains the mycolic acid-containing aerobic actinomycetes. Some *Tsukamurella* are implicated as agents of disease in humans,[2−5] and others cause foaming in activated sludge sewage treatment plants.[6] Strain Acta 1857 was found to be of special interest, as it gave a cell extract that contained a family of lipophilic metabolites in the HPLC analysis with nearly congruent UV−visible spectra that differed from those of 867 reference compounds stored in our HPLC-UV−vis database.[7] The new metabolites were named lipocarbazoles, and their fermentation, isolation, and structure elucidation are reported here.



Results and Discussion

The strong foaming properties shown by *T. pseudospumae* Acta 1857 during cultivation in an aerated and stirred bioreactor were used to harvest the orange-colored cell mass via the air-outlet tube between 24 and 48 h of fermentation. The cells in the foam contained a family of lipophilic compounds, the lipocarbazoles, which were detected in the cell extract by HPLC-diode array monitoring (Figure 1). The foam mass of a 10 L fermentation was centrifuged, and the cells were extracted by EtOAc/cyclohexane. The compounds were separated and purified by subsequent column chromatography on silica gel, Sephadex LH-20, and Toyopearl HW-40.

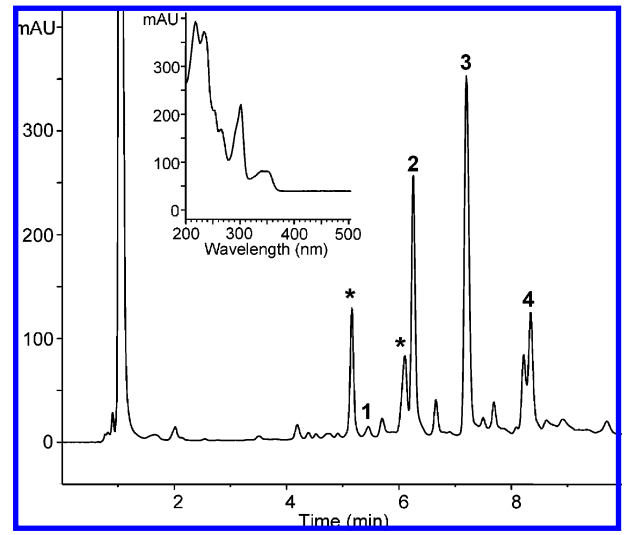


Figure 1. HPLC analysis of a cell extract of *Tsukamurella pseudospumae* Acta 1857 at a fermentation time of 72 h, monitored at 260 nm, and a representative UV−visible spectrum of lipocarbazole A3 (**3**). Compounds marked with asterisks are further noncharacterized lipocarbazoles.

Table 1. Molecular Ions and Retention Times in RP-HPLC/MS Analysis of Lipocarbazoles **1**−**4**

compound	$[M + H]^+$	$[M - H]^-$	t_R HPLC (min)
1 (A1)	430.3	428.3	5.4
2 (A2)	432.4	430.4	6.3
3 (A3)	434.4	432.4	7.2
4 (A4)	436.4	434.4	8.3

The mass spectra derived from HPLC-ESIMS chromatograms revealed molecular ions for **1** [$(M - H)^- = 428.3$], **2** [$(M - H)^- = 430.3$], **3** [$(M - H)^- = 432.5$], and **4** [$(M - H)^- = 434.4$] (Table 1). This led to the hypothesis that the differences between **1**, **2**, **3**, and **4** lie in the presence or absence of double bonds. The exact molecular ions were measured by high-resolution ESI-FT-ICR-MS, revealing the molecular formulas $C_{30}H_{39}NO$ (**1**), $C_{30}H_{41}NO$ (**2**),

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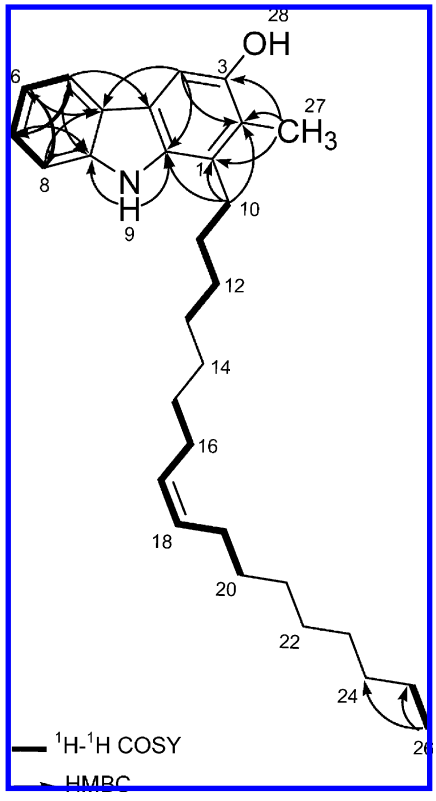
$C_{30}H_{43}NO$ (**3**), and $C_{30}H_{45}NO$ (**4**). The molecular formulas supported the hypothesis that the compounds **1**, **2**, **3**, and **4** vary in the occurrence of double bonds.

The structure elucidation of **1**, **2**, **3**, and **4** was done on the basis of NMR experiments as exemplarily described for derivative **3** as follows. The ^{1}H NMR spectrum of **3** showed five signals in the aromatic region, one signal at 5.3 ppm, one broad signal at 8.8 ppm, and one signal at 10.6 ppm. Seven signals, including a distinct signal between 1.2 and 1.3 ppm, were found in the aliphatic region between 3.0 and 0.8 ppm. The integral of the major signal between 1.2 and 1.3 ppm indicated that this signal corresponds to 18 protons. ^{13}C NMR and DEPT spectra clearly revealed the presence of two methyl, seven aromatic, and seven quaternary carbons. Additional ^{13}C NMR signals belonging to methylene groups were found between 32 and 22 ppm. As some of these signals were overlapping, it was not completely clear from the DEPT spectrum how many methylene groups were present in **3**. Comparing the results of the ^{13}C NMR and DEPT spectra to the molecular formula of **3**, $C_{30}H_{43}ON$, 14 methylene groups should be present in the molecule.

The correlation of ^{1}H NMR signals to the corresponding ^{13}C atoms was carried out in a heteronuclear single quantum coherence (HSQC) NMR experiment. Two signals in the ^{1}H NMR spectrum could not be assigned to any C atom, suggesting the presence of one hydroxy and one amino group linked to an aromatic ring system, which was identified as a carbazole. The carbazole core structure was fully elucidated using COSY and HMBC spectra. The ^{1}H−^{1}H-COSY experiment revealed correlations from H-5 to H-8. The correlations from H-4 to C-2, C-4b, and C-9a, from H-5 to C-4a, C-7, and C-8a, from H-6 to C-4b and C-8, from H-7 to C-5 and C-8a, from H-8 to C-4b and C-6, from H-9 to C-8a and C-9a, from H-10 to C-1, C-2, and C-9a, and from H_3-27 to C-1, C-2, and C-3 in combination with the chemical shifts gave proof for the carbazole core structure of **3**, especially for the position of the methyl group, of the hydroxy function, and of the alkyl chain.



The molecular formula of the missing substituent, the alkyl chain, was $C_{17}H_{33}$, consisting of two methine, one methyl, and 14 methylene groups. The COSY spectrum revealed correlations from H_2-10 to H_2-13, from H_2-15 to H_2-20, and from H_3-26 to H_2-25. The whole spin system from H_2-10 to H_3-26 could not be established since the chemical shifts of the protons H_2-14, H_2-21, H_2-22, H_2-23, H_2-24, and H_2-25 are similar (Table 2). Thus the structure of this moiety could not be fully elucidated using the HMBC spectrum since the chemical shifts of the CH_2 groups 13, 14, 15, 20, 21, 22, and 23 were almost identical (Table 2); hence the position of the double bond in the alkyl chain had to be established with the help of MS/MS experiments. We used LC-ESIMS/MS experiments to determine the positions of the double bonds.

Ions generated by electrospray ionization undergo charge remote fragmentations (CRFs) to yield two series of product ions reflecting losses of C_nH_{2n+2} and C_nH_{2n+1} for molecules with a fully saturated alkyl chain.[8] Figure 2 shows the negative MS/MS spectra of **1**, **2**, **3**, and **4**, all having a side chain with 17 carbon atoms. In the negative MS/MS spectrum of **4**, which bears a saturated alkyl chain, masses belonging to two series spaced by 14 amu were observed. Such fragment ion series are characteristic of linear unbranched saturated alkyl chains.[9] In contrast, **3**, with one double bond in the alkyl chain, shows a typical gap of 54 amu formed by the peaks at *m*/*z* 333 and 279 in the MS/MS spectrum.[9] Furthermore, this localizes the double bond between C-17 and C-18. Compound **2**, with two double bonds in the alkyl chain, gives a MS/MS spectrum with a characteristic pair of gaps of 54 amu formed by the peaks at *m*/*z* 373 and 319 and of 40 amu formed by the peaks at *m*/*z* 319 and 279.[9] These fragments indicate that the double bonds are located between C-17 and C-18 and between C-20 and C-21. The observed fragments in the MS/MS spectrum of **1**, with three double bonds in the alkyl chain, locate the double bonds between C-17 and C-18, between C-20 and C-21, and between C-23 and C-24. With the help of the above-mentioned MS/MS experiments and the NMR data (Table 2) the structures of **1**, **2**, **3**, and **4** could be elucidated.

The configurations of the double bonds were determined with the help of the ^{13}C NMR data. The aliphatic α-carbon atoms to the olefinic carbons in the side chain show a chemical shift that gave proof for the *Z*-configuration of the double bonds ($\delta = 26.3$ ppm).[10] In addition, total syntheses of **2**, **3**, and **4** fully confirmed the structures due to the identical NMR chemical shifts and MS/MS fragmentation patterns.[11] The biosynthetic origin of the lipid side chain of the lipocarbazoles is most likely a fatty acid, and bacterial fatty acids mostly have the *Z*-configuration.[12] Therefore, our experimental data agree with the data for the configuration of the double bonds in bacterial fatty acids, pointing to oleic acid, linoleic acid (ω-6 fatty acid), and α-linolenic acid (ω-3 fatty acid) as possible biosynthetic precursors of the lipocarbazoles **3**, **2**, and **1**.

LC-MS spectra of the purified extract revealed the presence of various other lipocarbazole derivatives, and FT-ICR-MS data suggested an alkyl chain with 15 carbon atoms and various degrees of saturation instead of an alkyl chain with 17 carbon atoms (Figure 1, marked peaks). In comparison to the heptadecyl analogues it was not possible to obtain suitable analytical data to clearly reveal the positions of the double bonds. Nevertheless, on the basis of the results of the LC-DAD, LC-MS, and FT-ICR-MS spectra we assume that the structures of these compounds are closely related to the derivatives described in this work by having a pentadecyl instead of heptadecyl alkyl chain.

Because of the high structural analogy of lipocarbazoles with carazostatin (**5**), compounds **3** and **4** were examined for their antioxidative properties. Carazostatin, a free radical scavenger produced by *Streptomyces chromofuscus*,[13] acts as a potent antioxidant against lipid peroxidation, and in liposomal membranes, it shows a stronger antioxidant activity than α-tocopherol.[14] Two other natural product groups, the hyellazoles and the carbazoquinocins, are closely related to carazostatin.[15,16] Hyellazoles were isolated from the cyanobacterium *Hyella caespitosa*,[15] whereas carbazoquinocins were found in *Streptomyces violaceus* 2448-SVT2 and shown to possess antioxidative properties.[16] These alkaloids are of considerable interest because of their antioxidant activity, and

Table 2. NMR Spectroscopic Data (DMSO-d_6) for Lipocarbazoles A1−4 (**1**−**4**)

	lipocarbazole A1 (**1**)		lipocarbazole A2 (**2**)		lipocarbazole A3 (**3**)		lipocarbazole A4 (**4**)	
no.	δ_C, mult.	δ_H, (J in Hz)	δ_C, mult.	δ_H, (J in Hz)	δ_C, mult.	δ_H, (J in Hz)	δ_C, mult.	δ_H, (J in Hz)
1	123.9, qC		123.9, qC		123.9, qC		124.0, qC	
2	121.3, qC		121.3, qC		121.3, qC		121.4, qC	
3	148.9, qC		148.9, qC		148.9, qC		149.0, qC	
4	101.9, CH	7.25, m	101.9, CH	7.26, m	101.9, CH	7.26, m	102.0, CH	7.26, m
4a	119.6, qC		119.7, qC		119.7, qC		119.7, qC	
4b	122.7, qC		122.7, qC		122.7, qC		122.8, qC	
5	119.5, CH	7.85, d (7.74)	119.5, CH	7.85, d (7.74)	119.5, CH	7.85, d (7.74)	119.6, CH	7.85, d (7.74)
6	117.5, CH	7.01, dd (7.50, 7.50)	117.5, CH	7.01, dd (7.50, 7.50)	117.5, CH	7.01, dd (7.50, 7.50)	117.6, CH	7.01, dd (7.50, 7.50)
7	124.4, CH	7.25, m	124.4, CH	7.25, m	124.4, CH	7.25, m	124.5, CH	7.25, m
8	110.8, CH	7.39, dd (8.10, 8.10)	110.7, CH	7.39, dd (8.10, 8.10)	110.7, CH	7.39, dd (8.10, 8.10)	110.8, CH	7.39, dd (8.10, 8.10)
8a	140.0, qC		140.0, qC		140.0, qC		140.0, qC	
9		10.6, s		10.6, s		10.6, s		10.7, s
9a	133.2, qC		133.2, qC		133.2, qC		133.3, qC	
10	28.1, CH_2	2.86, t (7.80)	28.1, CH_2	2.86, t (7.80)	28.1, CH_2	2.86, t (7.80)	28.1, CH_2	2.86, t (7.80)
11	29.1, CH_2	1.53, m	∼28, CH_2	1.53, m	29.1, CH_2	1.53, m	29.1, CH_2	1.54, m
12	∼28, CH_2	1.41, m	∼28, CH_2	1.41, m	29.1, CH_2	1.41, m	∼28, CH_2	1.43, m
13	∼28, CH_2	1.30, m	∼28, CH_2	1.31, m	∼28, CH_2	1.31, m	∼28, CH_2	1.31, m
14	∼28, CH_2	1.21, m	∼28, CH_2	1.21, m	∼28, CH_2	1.21, m	∼28, CH_2	1.21, m
15	∼28, CH_2	1.27, m	∼28, CH_2	1.27, m	∼28, CH_2	1.27, m	∼28, CH_2	1.21, m
16	26.6, CH_2	1.96, m	26.6, CH_2	1.96, m	26.6, CH_2	1.96, m	∼28, CH_2	1.21, m
17	129.6, CH	5.30, m	129.7, CH	5.30, m	129.6, CH	5.30, m	∼28, CH_2	1.21, m
18	129.6, CH	5.30, m	129.7, CH	5.30, m	129.6, CH	5.30, m	∼28, CH_2	1.21, m
19	25.2, CH_2	2.73, m	25.2, CH_2	2.73, m	26.6, CH_2	1.96, m	∼28, CH_2	1.21, m
20	129.6, CH	5.30, m	127.7, CH	5.30, m	∼28, CH_2	1.27, m	∼28, CH_2	1.21, m
21	129.6, CH	5.30, m	127.7, CH	5.30, m	∼28, CH_2	1.21, m	∼28, CH_2	1.21, m
22	25.2, CH_2	2.73, m	26.6, CH_2	1.96, m	∼28, CH_2	1.21, m	∼28, CH_2	1.21, m
23	127.8, CH	5.30, m	∼28, CH_2	1.25, m	∼28, CH_2	1.21, m	∼28, CH_2	1.21, m
24	127.8, CH	5.30, m	30.9, CH_2	1.23, m	31.3, CH_2	1.20, m	31.4, CH_2	1.21, m
25	26.6, CH_2	1.96, m	22.0, CH_2	1.22, m	22.1, CH_2	1.22, m	22.2, CH_2	1.22, m
26	13.9, CH_3	0.83, m	13.9, CH_3	0.83, m	13.9, CH_3	0.83, m	14.0, CH_3	0.86, m
27	12.0, CH_3	2.22, s	12.0, CH_3	2.22, s	12.0, CH_3	2.22, s	12.1, CH_3	2.22, s

because of this, total synthesis of these natural products has been achieved.[17,18] In our experiments, lipocarbazoles A3 (**3**) and A4 (**4**) showed a slightly better activity for scavenging the DPPH radical in methanol[19] than the antioxidant ascorbic acid, but were less active than propyl gallate (Figure 3). The IC_{50} values of the tested compounds were calculated as the concentrations of the substrates that caused 50% loss of DPPH activity (Table 3). Interestingly, the double bond in the alkyl chain of **3** has only a small effect on the antioxidative activity in this assay. It can be assumed that the isolated lipocarbazoles act as radical scavengers in biological membranes. Besides the antioxidative activity, lipocarbazoles showed no growth inhibition against Gram-positive or Gram-negative bacteria or against yeasts and filamentous fungi up to a concentration of 1 mg/mL.

Experimental Section

General Experimental Procedures. The UV- spectroscopic data were recorded on an Ultrospec 2100 pro UV/vis spectrophotometer (Amersham Biosciences, UK). Infrared data measurement was carried out on a 881 IR spectrometer (Perkin-Elmer). NMR experiments utilized 400 and 500 MHz Avance NMR spectrometers (Bruker, Germany). LC-MS and LC-MS/MS experiments were performed on an Applied Biosystems QTrap 2000 (Applied Biosystems, Germany) coupled to an Agilent 1100 HPLC system (Agilent, Germany). ESI-FT-ICR mass spectra were recorded on an APEX II FTICR mass spectrometer (4.7 T, Bruker-Daltonics, Germany).

Producing Organism. Strain Acta 1857 was deposited as the type species of *Tsukamurella pseudospumae* in two culture collections, the DSMZ (Braunschweig, Germany) and the NCIMB (Aberdeen, Scotland, UK) under the accession numbers DSM 44118^T and NCIMB 13963^T. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain is AY238513.

Screening, Fermentation, and Isolation. The chromatographic system consisted of a HP 1090 M liquid chromatograph equipped with a diode-array detector and a HP Kayak XM 600 ChemStation (Agilent, Germany). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360, 435, and 500 nm, and UV−visible spectra were measured from 200 to 600 nm. A 10 mL aliquot of the fermentation broth was centrifuged, and the cell pellet extracted with 10 mL of EtOAc. After concentration to dryness *in vacuo*, the residue was resuspended in 1 mL of EtOAc. Aliquots (10 μL) of the samples were injected onto an HPLC column (125 × 4.6 mm) fitted with a guard column (20 × 4.6 mm) filled with 5 μm Nucleosil-100 C-18 (Maisch, Germany). The samples were analyzed by linear gradient elution using 0.1% *ortho*-phosphoric acid as solvent A and CH_3CN as solvent B at a flow rate of 2 mL/min. The gradient was from 70% to 100% for solvent B in 7 min with a 3-min hold at 100% for solvent B.

Batch fermentations of strain Acta 1857 were carried out in a 10 L stirred-tank fermentor (Biostat S, B. Braun, Germany) in a medium that consisted of glucose (1%), glycerol (1%), oatmeal (0.5%), soybean meal (1%), yeast extract (0.5%; Ohly Kat), Bacto casamino acids (0.5%), and $CaCO_3$ (0.1%) in tap water; the pH was adjusted to 7.0 (5 N NaOH) prior to sterilization. The fermentor was inoculated with 5% by volume of a shake flask culture grown in the same medium at 27 °C in 500 mL Erlenmeyer flasks with one baffle for 48 h on a rotary shaker at 120 rpm. The cells were harvested between 24 and 48 h of fermentation during the heavy foaming phase via the air-outlet tube by centrifugation. The cells were extracted with EtOAc/cyclohexane (1 L × 3) and contained 2 mg of lipocarbazole A1 (**1**), 40 mg of lipocarbazole A2 (**2**), 65 mg of lipocarbazole A3 (**3**), and 20 mg of lipocarbazole A4 (**4**), as estimated by analytical HPLC-DAD. After concentration to an oily residue (22.3 g) the crude product was dissolved in cyclohexane/CH_2Cl_2 (1:1) and added to a silica gel column (30 × 4 cm, silica gel SI 60, Merck), and the separation was accomplished by a step gradient from 50%, 60%, and 80% CH_2Cl_2 at a flow rate of 6 mL/min. Lipocarbazole-containing fractions were pooled, concentrated and purified subsequently by Sephadex LH-20 and Toyopearl HW-40 chromatography (90 × 2.5 cm) using MeOH/CH_2Cl_2 (2:1) as the eluent at a flow rate of 30 mL/h.

Antioxidant Assay. The assay was carried out according to Sharma and Bath.[19] 1,1-Diphenyl-2-picrylhydrazyl (DPPH), propyl gallate, ascorbic acid, and MeOH (spectrophotometric grade) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Stock solutions were prepared for DPPH (200 μM) and the test compounds (100 μM) in MeOH. All solutions were kept cool and in the dark until use. For each compound different aliquots of the stock solutions were taken

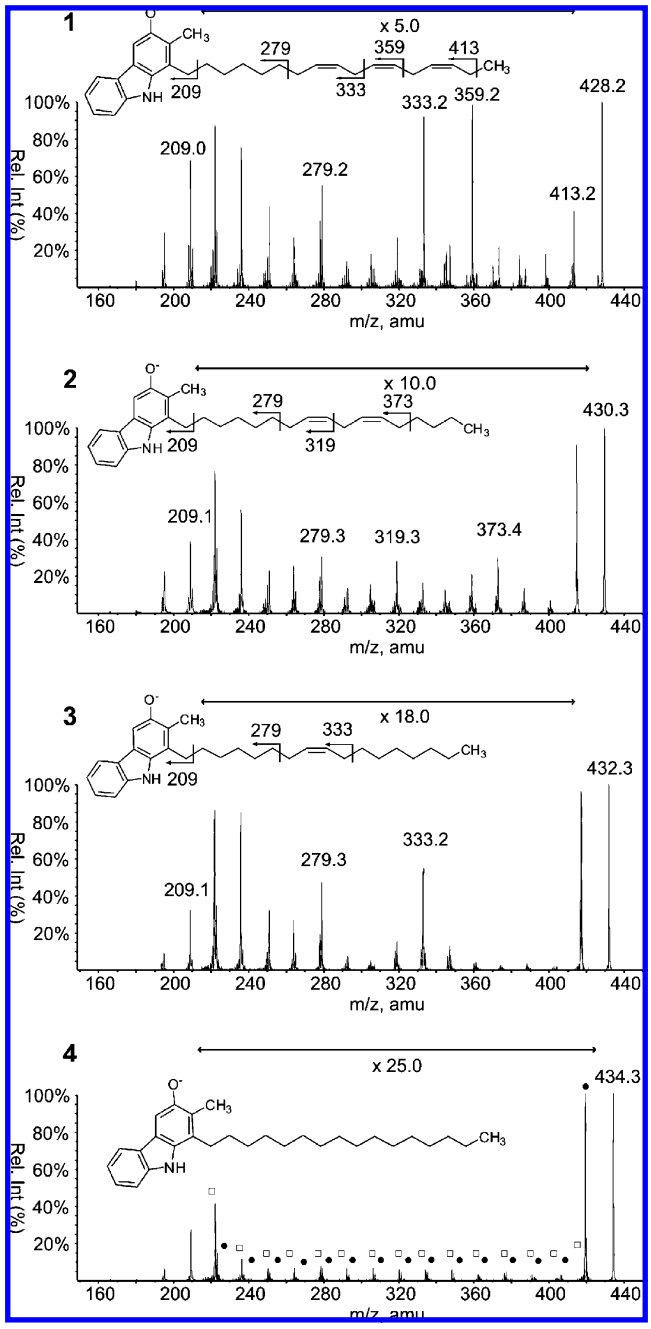


Figure 2. MS/MS spectra in the negative ion mode of **1**, **2**, **3**, and **4**. Fragments belonging to the mass series C_nH_{2n+2} are labeled with ●, and fragments belonging to the mass series C_nH_{2n+1} are labeled with □.

and the volume was adjusted to 3 mL with MeOH. The reaction was started by adding 1 mL of 200 μM DPPH stock solution (final DPPH concentration 50 μM). After shaking for 30 min at 30 °C in the dark the absorbance was measured at 517 nm in microplasic cuvettes (1 cm path length, 70 μL) against MeOH using a Perkin-Elmer Lambda 20 spectrophotometer.

Lipocarbazole A1 (1): amorphous, colorless powder; UV (MeOH) λ_{max} (log ε) 291 (1.94), 302 (2.56), 350 (2.06) nm; IR (ATR) ν_{max} 3438, 3007, 2925, 2854, 1585, 1494, 1452, 1427, 1306, 1255, 1207, 1160, 1147, 1112, 1096, 1015, 830, 768, 737 cm^{-1}; ^{1}H NMR, ^{13}C NMR data, see Table 2; ESI-FTICR-MS *m/z* (M + H)$^+$ 430.31041 [(M + H)$^+_{theor}$ = 430.31044; Δm = 0.07 ppm] corresponding to $C_{30}H_{39}NO$.

Lipocarbazole A2 (2): amorphous, colorless powder; UV (MeOH) λ_{max} (log ε) 295 (1.96), 300 (2.64), 353 (2.10) nm; IR (ATR) ν_{max} 3471, 3381, 3009, 2924, 2853, 1593, 1499, 1461, 1436, 1311, 1230, 1146, 1062, 831, 772, 739 cm^{-1}; ^{1}H NMR, ^{13}C NMR data, see Table 2; ESI-FTICR-MS *m/z* (M − H)$^-$ 430.31144 [(M − H)$^-_{theor}$ = 430.31154; Δm = 0.23 ppm] corresponding to $C_{30}H_{41}NO$.

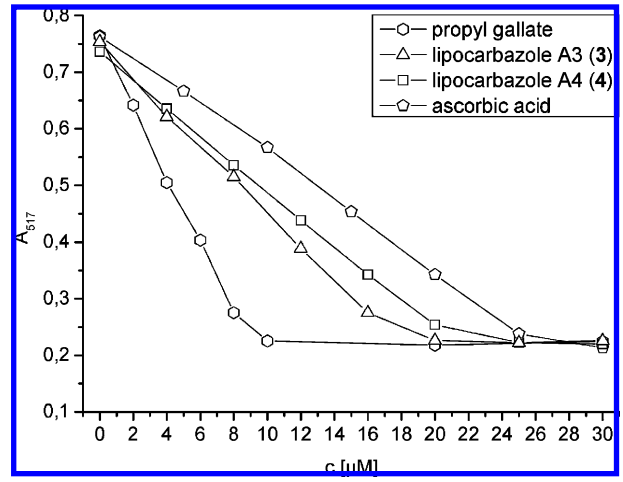


Figure 3. Scavenging of DPPH radical by lipocarbazoles A3 (**3**) and A4 (**4**), propyl gallate, and ascorbic acid.

Table 3. IC_{50} Values of Lipocarbazoles A3 (**3**) and A4 (**4**) Compared with Antioxidants Propyl Gallate and Ascorbic Acid in Scavenging DPPH

	IC_{50} [μM]
3	8.7
4	10.5
propyl gallate	4.4
ascorbic acid	13.4

Lipocarbazole A3 (3): amorphous, colorless powder; UV (MeOH) λ_{max} (log ε) 289 (1.90), 303 (2.62), 350 (2.02) nm; IR (ATR) ν_{max} 3471, 3382, 2923, 2853, 1593, 1500, 1461, 1436, 1311, 1231, 1146, 1062, 831, 772, 739 cm^{-1}; ^{1}H NMR, ^{13}C NMR data, see Table 2; ESI-FTICR-MS *m/z* (M − H)$^-$ 432.32702 [(M − H)$^-_{theor}$ = 432.32719; Δm = 0.39 ppm] corresponding to $C_{30}H_{43}NO$.

Lipocarbazole A4 (4): amorphous, colorless powder; UV (MeOH) λ_{max} (log ε) 292 (1.92), 302 (2.61), 350 (2.11) nm; IR (ATR) ν_{max} 3466, 3388, 2920, 2849, 1500, 1466, 1436, 1311, 1233, 1147, 1064, 830, 772, 738 cm^{-1}; ^{1}H NMR, ^{13}C NMR data, see Table 2; ESI-FTICR-MS *m/z* (M − H)$^-$ 434.34276 [(M − H)$^-_{theor}$ = 434.34284; Δm = 0.18 ppm] corresponding to $C_{30}H_{45}NO$.

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Supporting Information Available: Conditions to determine the antibacterial and antifungal activity spectrum and ^{1}H and ^{13}C NMR spectra of **1**−**4** are available free of charge via the Internet at http://pubs.acs.org.

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