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New Natural Products from the Sponge-Derived Fungus *Aspergillus niger*

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Fractionation of the EtOAc extract of a static culture of *Aspergillus niger* isolated from the Mediterranean sponge *Axinella damicornis* yielded eight secondary metabolites, out of which seven compounds (**2**–**8**) proved to be new natural products, whereas one was identified as the known fungal pigment cycloeucomelone (**1**). The new compounds included the 3,3'-bicomarin bicoumanigrin (**2**), the structurally unusual 4-benzyl-1*H*-pyridin-6-one derivatives aspernigrins A and B (**3** and **4**), and pyranonigrins A–D (**5**–**8**), the latter featuring a novel pyrano[3,2-*b*]pyrrole skeleton hitherto unprecedented in nature. All structures were elucidated on the basis of extensive one- and two-dimensional NMR spectroscopic studies (¹H, ¹³C, COSY, HMQC, HMBC, NOE difference spectra) and mass spectral analysis. For the two chiral molecules **4** and **5**, the absolute configurations were established by quantum chemical calculations of their circular dichroism (CD) spectra. In each case, two independent methods, i.e., a molecular dynamics approach taking into consideration the molecular flexibility, and a conformational analysis followed by Boltzmann weighting of the single CD spectra calculated for the conformers thus obtained, led to identical results without the need of any empirical comparison of chiroptical data reported for reference compounds. Bicoumanigrin (**2**) showed moderate cytotoxicity against human cancer cell lines in vitro. In addition, aspernigrin B (**4**) was found to display a strong neuroprotective effect against glutamic acid.

In the search for new bioactive natural products from marine organisms increasing attention is being given to microorganisms such as bacteria and fungi.^{1–4} Filter-feeding marine invertebrates, such as sponges, have been shown to host a variety of microorganisms (especially bacteria) that do not merely reflect the microbial communities present in the surrounding seawater but appear to constitute a more specialized association between sponge hosts and microbial associates.^{5,6} Sponges are, however, not only rich in bacteria but also known to harbor fungi.⁷ The nature of the association between sponges and fungi is not yet fully understood. For example, it is not clear whether fungal cultures that can be readily isolated from marine sponges originate from spores that had previously been accumulated through filter feeding or whether some fungi may also divide and propagate within their hosts. Seemingly, most fungi hitherto isolated from marine sponges are taxonomically related to strains already known from the terrestrial environment,^{8–12} which would lend support to the assumption that most fungi residing in sponges perhaps have their true origin on the land and are more or

less accidentally washed into the sea and accumulated by sponges and other marine invertebrates through filter feeding. However, it should also be taken into account that many, if not most, of the fungal strains cannot be identified with the currently available taxonomic methods, and furthermore the majority of these organisms might not even be cultivated with the techniques developed for terrestrial fungi. Thus, many strains will not be amenable to scientific investigation at this point, and further research is urgently required to test the different hypotheses on the true origin of marine invertebrate-derived fungi.

Irrespective of the nature of sponge–fungi associations, sponge-derived fungal cultures have repeatedly been shown to be interesting sources of new bioactive secondary metabolites previously unknown from terrestrial strains of the same species. Examples of unusual fungal metabolites published by our own group include hortein, a new polyketide from the fungus *Hortaea werneckii* isolated from the sponge *Aplysina aerophoba*,⁹ new anthraquinone and betaenone derivatives as well as new γ -pyrones from the fungus *Microsphaeropsis* sp., also obtained from *A. aerophoba*,^{8,13} new spiciferone derivatives from the fungus *Drechslera hawaiiensis* derived from the sponge *Calyspongia aerizusa*,¹⁰ and new xestodecalactones produced by the fungus *Penicillium* cf. *montanense*, which was isolated from the sponge *Xestospongia exigua*.^{14,15}

Fungi of the genus *Aspergillus* have repeatedly been isolated from marine sources such as sponges or algae. Even though terrestrial strains of *Aspergillus* spp. have been extensively studied in the past with regard to their natural products chemistry, marine-derived strains of the

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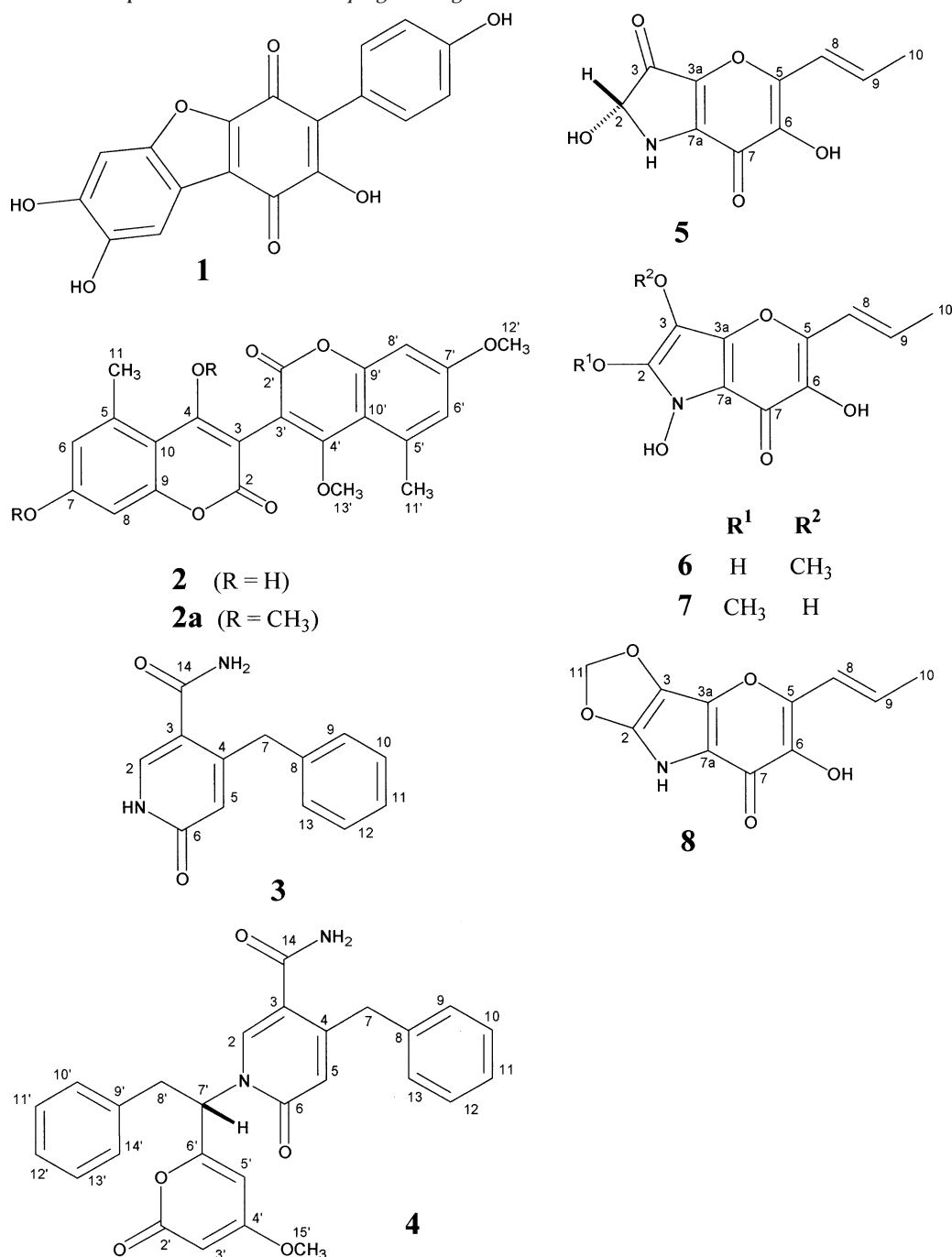
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Chart 1. Structures of Compounds Isolated from *Aspergillus niger*

same genus nevertheless proved to be prolific sources of new secondary metabolites up to now unknown from terrestrial fungi. Examples include a strain of *A. versicolor* isolated from the sponge *Xestospongia exigua*, which yielded new chromone derivatives,¹⁶ or drimane lactones featuring an unusual nitrobenzoyloxy substituent reported from another strain of *A. versicolor* that had been isolated from the marine green alga *Penicillus capitatus*.¹⁷ *A. niger* had also been isolated from marine sponges before.¹⁸ Fungal strains isolated from the sponge *Hyrtios proteus*, for example, yielded a plethora of new natural products including asperic acid, asperazine, hexylitaconic acid, and malformin C.¹⁸

In continuation of our studies on sponge-derived fungi we now report on the secondary metabolites of a strain of *A. niger* that we obtained from the Mediterranean sponge *Axinella damicornis*. Besides the known fungal pigment

cycloleucmelone (**1**), this chemically prolific fungal strain yielded only new compounds including the 3,3'-bicycoumarin bicoumanigrin (**2**), the 6-pyridinone¹⁹ derivatives aspernigrins A and B (**3** and **4**), and the pyrano[3,2-*b*]pyrroles pyranonigrins A–D (**5**–**8**). Structural characterization of the new derivatives by NMR spectroscopy and mass spectrometry, determination of the absolute configuration by quantum chemical calculation of CD spectra, and the results of biological activity studies are reported. Most importantly, aspernigrin B (**4**) was found to display a potent neuroprotective activity, thus efficiently preventing glutamic acid-caused neuronal cell death.

Results and Discussion

Following chromatographic separation of the combined EtOAc extracts obtained by extraction of the mycelium and the culture broth from the sponge-derived fungus *Aspergil-*

Table 1. ^1H NMR, ^{13}C NMR, ROESY, and HMBC Data of Bicoumanigrin (**2** and **2a**)^a

	2				2a
	δ_{H} (J Hz)	δ_{C} ^b	HMBC (H→C)	ROESY	δ_{H} (J Hz)
2		161.8 s			
3		94.3 s			
4		165.3 s			
5		139.2 s			
6	6.61 d (2.0)	116.1 d	7, 8, 10, 11	OH-7, 11	6.83 d (2.5)
7		160.5 s			
8	6.57 d (2.0)	100.3 d	6, 7, 9, 10	OH-7	6.91 d (2.5)
9		156.2 s			
10		106.1 s			
11	2.62 s (3H)	23.3 q	5, 6, 9, 10	6	2.60 s (3H)
12					3.85 s (3H)
13					3.80 s (3H)
2'		161.4 s			
3'		99.1 s			
4'		168.8 s			
5'		138.3 s			
6'	6.79 d (2.5)	115.4 d	7', 8', 10'	11', 12'	6.83 d (2.5)
7'		161.5 s			
8'	6.87 d (2.5)	98.6 d	6', 7', 9', 10'	12'	6.91 d (2.5)
9'		155.6 s			
10'		109.4 s			
11'	2.58 s (3H)	23.1 q	4', 5', 6', 9', 10'	6', 13'	2.60 s (3H)
12'	3.84 s (3H)	55.8 q	7'	6', 8'	3.85 s (3H)
13'	3.76 s (3H)	59.6 q	4'	11'	3.80 s (3H)
OH-4	11.28 s		10		
OH-7	10.49 s		6, 7, 8	6, 8	

^a Recorded in DMSO-*d*₆. ^b Obtained from HMQC and HMBC spectra.

lus niger, eight secondary metabolites (**1**–**8**) were isolated. Spectroscopic analysis by NMR spectroscopy and by mass spectrometry indicated that seven compounds (**2**–**8**) were new natural products, whereas one compound was identified as the known pigment cycloleucomelone (**1**), a terphenylquinone that had previously been reported from higher basidiomycetes such as *Boletopsis leucomelaena*,²⁰ *Paxillus* spp.,²¹ or *Anthracyphyllum* spp.²²

Compound **2** has the molecular formula C₂₂H₁₈O₈ as determined by HRESIMS, in agreement with the ^1H and ^{13}C NMR spectra. As a prominent feature of the ^1H and ^{13}C NMR spectra of **2**, six sets of paired signals drew our attention immediately on reviewing the spectra (Table 1). Two aromatic methyl groups (δ 2.62 and 2.58, H₃-11 and H₃-11'), two methoxy groups (δ 3.84 and 3.76, H₃-12' and H₃-13'), two pairs of *meta*-coupling protons (δ 6.61/6.57 and 6.79/6.87, H-6/H-8; H-6'/H-8', respectively), and two hydroxy protons (δ 11.28 and 10.49, OH-4 and OH-7) in the ^1H spectrum as well as the respective ^{13}C signals (C-2–C-11 and C-2'–C-11', see Table 1) indicated that **2** was a heterodimer. Beginning with the *meta*-coupling protons, the gross structure of **2** was assembled by inspection of the HMBC spectra. Correlations of OH-7 to the doublets at δ 100.3 (C-8) and 116.1 (C-6) and to a singlet at δ 160.5 (C-7) proved the *meta*-coupling protons to be separated by a quaternary carbon carrying a hydroxy substituent. The strong upfield shift of C-8 further indicated its orientation as *ortho* to two phenolic substituents. A strong cross-peak from C-6 to the methyl group, H₃-11, was observed. These methyl protons showed additional correlations with quaternary carbons at δ 139.2 (C-5) and 106.1 (C-10) and two weak cross-peaks over four bonds with oxygenated carbons at δ 156.2 (C-9) and 165.3 (C-4), respectively. Similar to C-8, C-10 (δ 106.1) also displayed a prominent upfield shift. HMBC correlations of H-8 with C-9 and C-10 allowed for closing of the aromatic ring system. A second phenolic or enolic hydroxy group was found to reside either at C-4 or

at C-9 on the basis of a cross-peak between its OH signal at δ 11.28 and C-10 in the HMBC spectrum. Following the same strategy as described so far, the constitution of the structurally analogous second aromatic ring with *meta*-coupling protons was confirmed, differing only in the fact that H-6' and H-8' were separated by a methoxy substituent (δ 3.84, H₃-12') in this case (Table 1).

A set of two pairs of sp²-carbons for each subunit of the proposed heterodimer resonating at δ 94.3 and 161.8 (C-3/C-2) as well as at δ 99.1 and 161.4 (C-3'/C-2') still remained unassigned since no HMBC correlations could be detected. The two low-field signals were attributed to carbonyl functions, also in agreement with a prominent absorbance at 1686 cm⁻¹ in the IR spectrum of **2**. The remaining two out of the total of 14 degrees of unsaturation required by the molecular formula called for the presence of two additional rings. Thus, each subunit had to contain a lactone ring, leading to the conclusion that **2** was a heterodimeric coumarin. The chemical shifts of C-3/C-3' (δ 94.3 and 99.1) and C-4/C-4' (δ 165.3 and 168.8) could only be explained by two β -oxygenated α,β -unsaturated carbonyl substructures, thus leaving only positions 3 and 3' of the two lactone rings as plausible linkage sites for the two coumarin monomers. This assignment was corroborated by the ROESY spectrum (Table 1). The observed spatial proximity between OCH₃-13' and H₃-11' indicated the attachment of the oxygenated substituents at C-4' and C-4, respectively, and consequently the connection of both subunits through C-3 and C-3'. Additionally, comparison of the respective chemical shifts of **2** with those of the known bicoumarins aflavarin (8,3'-linkage)²³ and 7-*O*-demethyl-3,8'-bisiderin,²⁴ (3,8'-linkage), both likewise isolated from *Aspergillus* cultures, confirmed the proposed structure. Aflavarin was obtained from a terrestrial *Aspergillus flavus* strain from a collection at the USDA National Center for Agricultural Utilization Research, IL,²³ and 7-*O*-demethyl-3,8'-bisiderin was produced by a culture of *Petromyces alliaceus*, the teleomorph of *Aspergillus alliaceus*, which was derived from a soil sample from Shandong Province, China.²⁴ Prior to the isolation of compound **2** from *A. niger*, toddasiatin (3,3'-linkage) from the plant *Toddalia asiatica* (family Rutaceae)²⁵ and 4,4'-biisofraxidin (4,4'-linkage) from root cultures of *Impatiens balsamina* (Balsaminaceae)²⁶ had been the only examples of naturally occurring bicoumarins that are linked via the lactone rings of their monomeric subunits. Since no ^{13}C NMR data for toddasiatin have so far been published, we present here for the first time respective spectroscopic data for a naturally occurring 3,3'-bicoumarin, **2**, for which we propose the name bicoumanigrin A.

The structure of bicoumanigrin A was further confirmed by reaction of **2** with methyl iodide, resulting in the formation of the *O,O*-dimethylated derivative **2a**. The molecular weight was 438, as indicated by low-resolution ESIMS, and the ^1H NMR spectrum was considerably simplified due to the symmetry of the molecule, now displaying only five signals, i.e., two signals for the *meta*-coupling protons, two methoxy signals, and one signal for the aromatic methyl groups (Table 1).

Some of the above-mentioned bicoumarins from the literature were reported as optically active due to axial chirality.^{25,26} In addition, for 6,6'-bicoumarins such as isokotanin A, there are reports of both atropo-enantioselective syntheses and the assignment of the absolute configuration by quantum chemical CD calculations.²⁷ However, the CD spectrum of bicoumanigrin A (**2**) in DMSO showed neither an optical rotation nor a Cotton

Table 2. ^1H NMR, ^{13}C NMR, and HMBC Data of Aspernigrins A (**3**) and B (**4**)^a

	3			4		
	δ_{H} (J/Hz)	δ_{C}^b	HMBC (H→C)	δ_{H} (J/Hz)	δ_{C}^b	HMBC (H→C)
1	12.21 br s					
2	8.32 s	142.1 d	4, 6, 14	8.57 s	143.8 d	4, 6, 14, 7'
3		117.4 s			118.9 s	
4		151.0 s			151.7 s	
5	6.30 s	118.2 d	3, 4, 6, 7	5.96 s	121.9 d	3, 4, 6, 7
6		165.5 s			164.5 s	
7	3.90 s (2H)	37.6 t	4, 8, 9/13	A 3.91 d (16.4) B 3.75 d (16.4)	36.9 t	4, 8, 9/13 4, 5, 8, 9/13
8		136.9 s			135.4 s	
9/13	7.34 m (2H)	128.8 (2 × d)	8, 11	7.05 m (2H)	128.9 d	10/12
10/12	7.28 m (2H)	126.9 (2 × d)	9/13, 11	7.32 m (2H)	128.6 d	8, 11
11	7.28 m (1H)	126.9 d	9/13, 10/12	7.27 m (1H)	128.9 d	9/13
14		177.6's			176.1 s	
2'					162.0 s	
3'				5.65 d (2.5)	89.3 d	4', 5'
4'					170.0 s	
5'				6.23 d (2.5)	102.4 d	3', 4', 6'
6'					158.3 s	
7'				5.62 dd (8.2, 6.9)	60.7 d	2, 6'
8'				A 3.55 dd (13.9, 6.9) B 3.27 m ^c	36.7 t	9', 10'/14' 7', 9', 10'/14'
9'					135.0 s	
10'/14'				7.02 m (2H)	129.1 (2 × d)	12'
11'/13'				7.23 m (2H)	127.2 (2 × d)	9', 10'/14'
12'				7.27 m	128.9 d	
15'				3.79 s (3H)	56.7 q	4'
NH ₂ -14	9.47 br s 7.40 br s			9.31 d (4.4) 7.58 d (4.4)		

^a Recorded in DMSO-*d*₆. ^b Obtained from HMQC and HMBC spectra. ^c Obscured by the residual water signal.

effect at two different concentrations, indicating either that the compound was isolated as a racemic mixture of the two possible atropo-enantiomers or that it lacked stable axial chirality due to a low rotational barrier of the 3,3'-linkage.

The molecular formula of aspernigrin A (**3**) was determined as C₁₃H₁₂N₂O₂ by HREIMS. From its ^1H NMR spectrum (Table 2), the presence of a benzyl moiety was immediately conceivable by signals characteristic of a monosubstituted aromatic ring system (δ 7.2–7.4, 5H) in conjunction with a signal at δ 3.90 integrating for two protons (H₂-7). Furthermore, two sharp singlets at δ 6.30 (H-5) and 8.32 (H-2) as well as three broad singlets resonating at low field (δ 7.40, 9.47, and 12.21) were observed (Table 2). Out of the nine degrees of unsaturation implied by the molecular formula, only four accounted for the benzyl substructure. Thus, four of the remaining six carbons had to form two sets of monosubstituted double bonds, while the two most downfield signals were judged to be present as one amide (δ 177.6, C-14) and one lactam carbonyl (δ 165.5, C-6). The latter conclusion was based on the requirement of one additional ring system to account for the remaining degree of unsaturation. This heteroaromatic system was identified as a 6-pyridinone by inspection of the HMBC correlations observed for H-2 and H-5. Position 5 was found to be the α -position of an α,β -unsaturated carbonyl substructure, in agreement with both its chemical shifts (δ_{C} 118.2, δ_{H} 6.30) and cross-peaks between H-5 and signals at δ 165.5 (C-6), 151.0 (C-4), 117.4 (C-3), and 37.6 (C-7). Correspondingly, the benzyl moiety was attached to C-4. The presence of a six-membered ring was corroborated by HMBC correlations from H-2 (δ 8.32) to C-6, C-4, and C-14 (δ 177.6), the latter also confirming the placement of the primary amide function at C-3. Hence, compound **3** was found to be the novel 4-benzyl-6-oxo-1,6-dihydropyridine-3-carboxamide, for which the name aspernigrin A was proposed.

Aspernigrin B (**4**) displayed a molecular ion at m/z 456 in the EI mass spectrum. The ^1H and the ^{13}C NMR spectra

suggested a molecular formula of C₂₇H₂₄N₂O₅, which was confirmed by HREIMS. The ^1H NMR spectrum (Table 2) indicated a close structural relationship to aspernigrin A (**3**) including signals of the pyridine ring, the benzyl unit, and the carboxamide. Since the signals for H₂-7 were split into an AB system (δ 3.91 and 3.75, J = 16.4 Hz), the protons of this methylene group were judged to be diastereotopic, leading to the conclusion that **4** was a chiral molecule. In comparison to **3**, the presence of a second monosubstituted phenyl ring was clearly evident, again being part of a benzyl unit with the CH₂ protons H-8'A and H-8'B resonating at δ 3.55 and 3.27, respectively. The latter group was attached to a methine function carrying electronegative substituents (δ 5.62, dd, J = 8.2, 6.9 Hz), as implied by the chemical shift and the splitting patterns. Further signals in the ^1H NMR spectrum of **4** included two *meta*-coupling protons (δ 6.23, H-5' and δ 5.65, H-3', J = 2.5 Hz) and one methoxy group (δ 3.79, H₃-15'). In combination with the chemical shifts for the remaining carbon atoms (C-2'–C-6', Table 2), all spectral data were in perfect agreement with an additional 4-methoxy- α -pyrone moiety, which is quite frequently found in fungal natural products, for example in the known metabolite pyrophen.^{19,28} This assumption was corroborated by cross-peaks in the HMBC spectrum from H-3' to C-4' (δ 170.0) and C-5' (δ 102.4) and from H-5' to C-3' (δ 89.3) and C-4' as well as C-6' (δ 158.3). The different substructures obtained were connected by key HMBC correlations from H-7' to C-6' in the α -pyrone and C-2 (δ 143.8) in the pyridinone subunits and from H₂-8' to C-10'/14' (δ 129.1), thus establishing the gross structure of aspernigrin B (**4**) as depicted.

As deduced above, **4** is a chiral molecule. For the assignment of its absolute configuration, CD spectroscopy appeared to be the method of choice. Still, the interpretation of the CD spectrum of **4** was difficult, due to its novel-type structural framework, making the empirical comparison with the CD curves of structurally related compounds

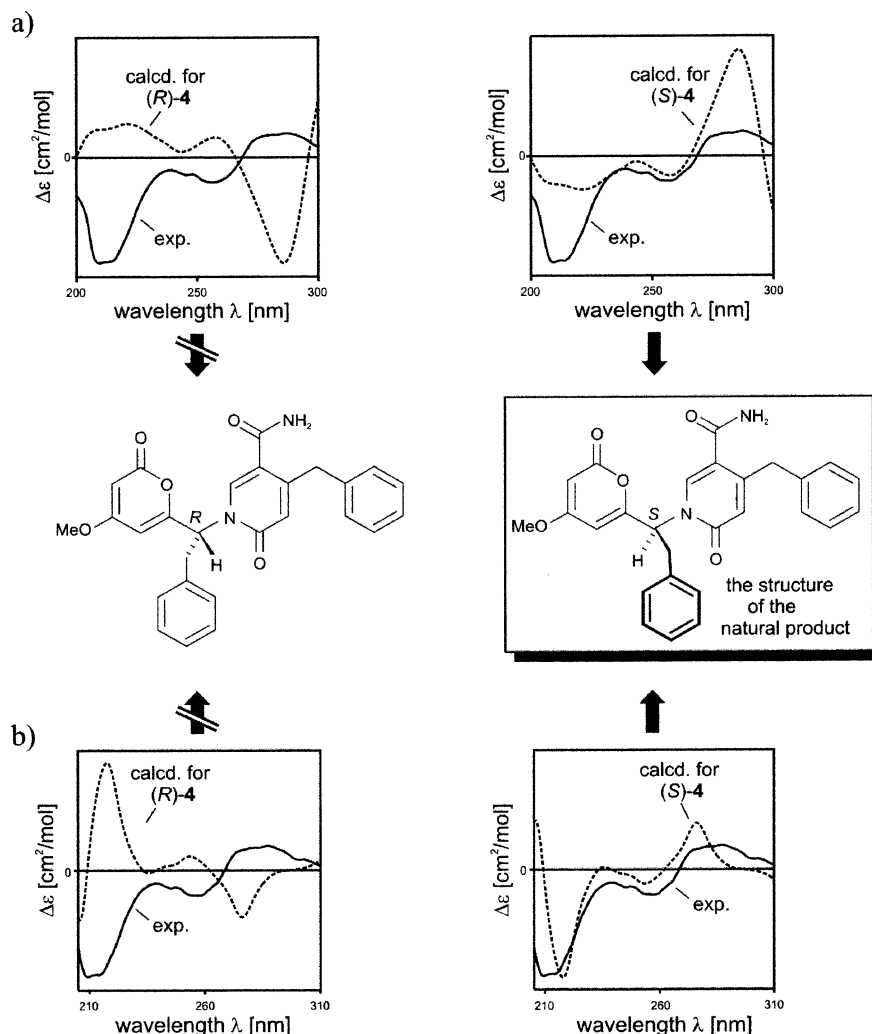


Figure 1. Attribution of the absolute configuration of aspernigrin B (**4**) by comparison of the experimental CD spectrum (in MeOH) with the spectra calculated for (*R*)-**4** and (*S*)-**4**: (a) following the TRIPOS-MD method; (b) according to the AM1-Boltzmann approach.

of known absolute configuration impossible, so that quantum chemical calculations^{29–31} were performed, which predicted the CD spectra expected for the *R*- and the *S*-enantiomers of **4**.

To take into consideration the molecular flexibility of **4**, a molecular dynamics (MD) approach³² was pursued, arbitrarily starting with the *R*-enantiomer of **4**. The MD simulation was carried out at a virtual temperature of 500 K using the TRIPOS³³ force field. For the different geometries of **4**, which were extracted from the trajectory of motion, the single CD spectra were calculated by using the semiempirical CNDO/2S³⁴ method. Subsequent summing up of these CD spectra provided the calculated overall CD curve for the *R*-enantiomer of **4** as shown in Figure 1a (left); it proved to be virtually opposite the experimental one, while the CD spectrum calculated for *S* was found to be in a good agreement with the measured one (Figure 1a, right). From these calculations, aspernigrin B (**4**) was clearly deduced to have the *S*-configuration.

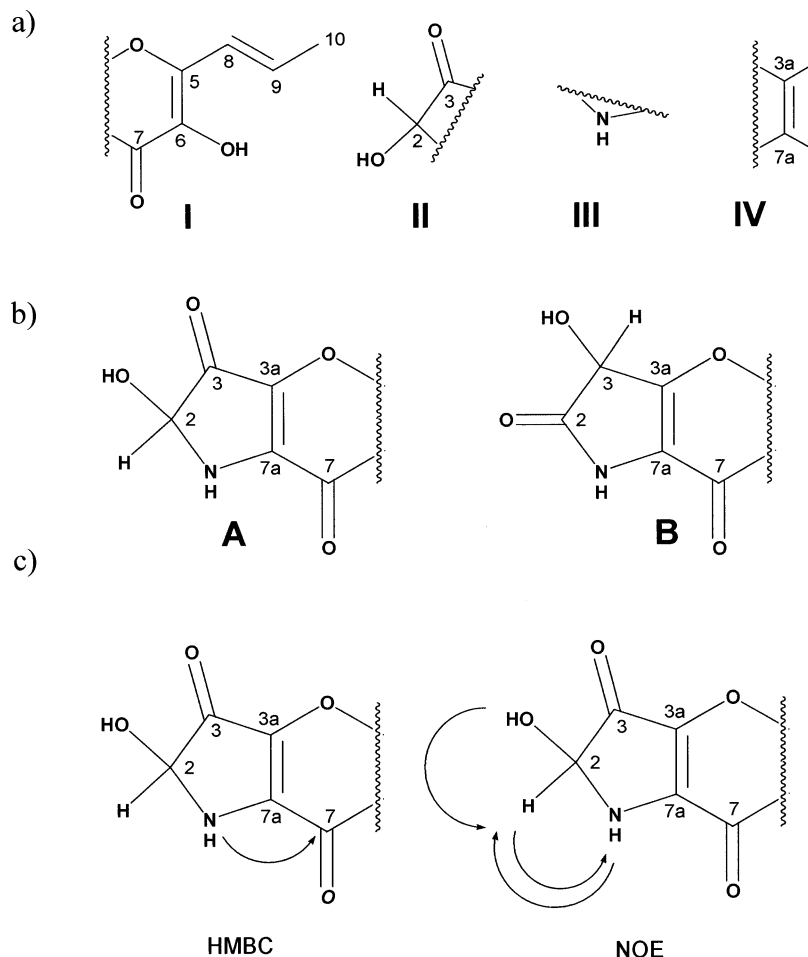
Still, given the fact that **4** represents a new structural type of natural products, a confirmation of this configurational assignment seemed rewarding. Therefore the molecule was also submitted to the second possible approach to CD calculations, based on a conformational analysis, in the course of which no less than 172 conformers were found within a range up to 3 kcal/mol above the global minimum. For each conformer, the respective single CD spectrum was calculated using the same CNDO/2S method as above; all

these single spectra were then added up, now according to their population in the equilibrium mixture, i.e., following the heats of formation of these conformers. The overall calculated CD spectrum resulting from this Boltzmann weighting (Figure 1b) now showed an even better agreement of the measured spectrum with that calculated for *S* and a likewise perfectly opposite curve as compared to the spectrum predicted for *R*, once again clearly confirming the above assignment of the natural product **4** to have the *S*-configuration. Consequently, the structure shown in Figure 1, center right, i.e., with *S*-configuration, represents the full absolute stereostructure of aspernigrin B (**4**).

For pyranonigrin A (**5**), a pseudomolecular ion at *m/z* 224 ($[M + H]^+$) was observed by ESIMS. From the NMR data, the molecular formula of C₁₀H₉NO₅ was deduced, which was corroborated by HRESIMS. From the ¹H NMR spectrum (Table 3), a 1-propenyl moiety with *trans*-configuration was clearly discernible, as indicated by the characteristic splitting patterns observed for the methyl group (δ 1.92, dd, 6.9 and 1.6 Hz, H₃-10) and the olefinic protons (δ 6.45, dq, 15.9 and 6.9 Hz, H-9, and δ 6.57, dq, 15.9 and 1.6 Hz, H-8). In addition, the ¹H NMR spectrum of **5** exhibited four further signals at δ 9.67 (OH-6), 8.60 (H-1), 6.77 (OH-2), and 5.72 (H-2), of which only the last one was due to a proton that was attached to a carbon (δ 75.4, C-2) as judged from the HMQC spectrum. Due to the scarcity of contiguous spin systems, different substructures were identified by close inspection of the HMBC spectra (Figure

Table 3. ^1H NMR, ^{13}C NMR, and HMBC Data of Pyranonigrins A–D (5–8)^a

	5			6			7	8
	δ_{H} (JHz)	δ_{C}	HMBC (H–C)	δ_{H} (JHz)	δ_{C}	HMBC (H–C)	δ_{H} (JHz)	δ_{H} (JHz)
1	8.60 d (1.0)		2, 3, 3a, 7, 7a					8.17 s
2	5.72 dd (8.8, 1.0)	75.4 d	3, 3a, 7a		162.8 s			
3		174.5 s			160.3 s			
3a		165.3 s			150.0 s			
5		146.2 s			145.9 s			
6		142.4 s			141.7 s			
7		169.3 s			171.5 s			
7a		112.0 s			122.1 s			
8	6.57 dq (15.9, 1.6)	119.2 d	5, 6, 9, 10	6.57 br d (15.8)	118.4 d	5, 6, 9, 10	6.54 dd (16.4, 1.3)	6.56 dd (15.8, 1.9)
9	6.45 dq (15.9, 6.9)	131.8 d	5, 8, 10	6.51 dq (15.8, 5.4)	133.5 d	5, 8, 10	6.93 dq (16.4, 6.9)	6.44 dq (15.8, 6.9)
10	1.92 dd (6.9, 1.6, 3H)	18.9 q	8, 9	1.92 br d (5.5, 3H)	18.6 q	8, 9	1.93 dd (6.9, 1.3, 3H)	1.91 dd (6.9, 1.9, 3H)
OH-1				7.65 s		2, 7a	8.15 s	
OH-2	6.77 d (8.8)		2, 3	7.97 s		2		
OH-3							8.26 s	
OH-6	9.67 s		5, 6, 7	10.00 s		5, 6, 7	9.95 s	9.54 s
OCH ₃ -2							3.72 s (3H)	
OCH ₃ -3				3.85 s (3H)	53.3 q	3		
OCH ₂ O								4.31 s (2H)

^a Recorded in DMSO-*d*₆.**Figure 2.** (a) Substructures identified in the course of the structure elucidation of **5**; (b) possible arrangement of substituents in the pyrrole ring of **5**; (c) HMBC correlations and NOE effects important for the elucidation of the constitution of the pyrrole ring of **5**.

2a). The site of attachment of the 1-propenyl unit was defined by a correlation of H-9 to a carbon resonating at δ 146.2 (C-5), while the neighboring carbon was identified by cross-peaks from H-8 to C-5 and C-6 (δ 142.4). Chemical shift arguments taken together with correlations observed from OH-6 (δ 9.67) to C-7 (δ 169.3), C-6, and C-5 permitted substructure **I** to be constructed as depicted in Figure 2a. The placement of the secondary alcohol function next to a carbonyl (substructure **II**) was possible through correla-

tions from the corresponding hydroxy proton (δ 6.77, 2-OH) to C-2 and C-3 (δ 174.5). From the chemical shift and the molecular formula, it was evident that the proton resonating at δ 8.60 (H-1) had to reside at a nitrogen atom, but the high number of HMBC correlations displayed by this signal hampered linking of substructure **III** to those identified before. Finally, the double-bond equivalents implied by the molecular formula required the remaining two carbon singlets (δ 169.3, C-7a, and δ 165.3, C-3a) to

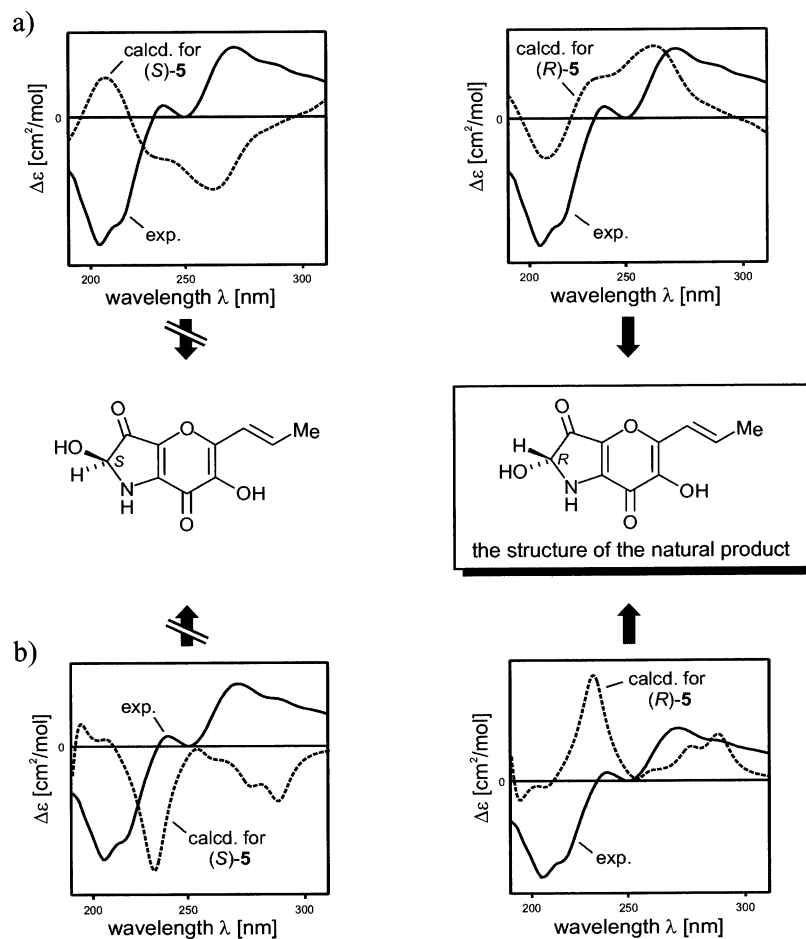


Figure 3. Assignment of the absolute configuration of pyranonigrin A (**5**) by comparison of the experimental CD spectrum (in MeOH) with the curves calculated for (*S*)-**5** and (*R*)-**5**: (a) following the MM3-MD method; (b) according to the AM1-Boltzmann approach.

be connected to a fully substituted double bond (substructure **IV**) as well as the overall presence of two heterocyclic ring systems in **5**. Out of the various possibilities to connect the substructures **I–IV** (Figure 2a), the set of HMBC correlations observed for H-1 (to C-2, C-3, C-3a, C-7, and C-7a) was only in agreement with this NH group being part of a dihydropyrrole ring, thus suggesting that **5** would feature either a pyrano[3,2-*b*]pyrrole or a pyrano[2,3-*b*]pyrrole bicyclic system. This assumption was also corroborated by inspection of the IR spectrum, which showed two prominent carbonyl bands at 1654 and 1636 cm^{-1} , which were characteristic for a γ -pyranone subunit,³⁵ as well as a band at 1715 cm^{-1} , which was indicative of either a lactam substructure or conjugated carbonyl groups.^{35,36} The latter of the two options for connecting the γ -pyranone to the dihydropyrrole ring was clearly ruled out by the strong cross-peak between H-1 and C-7, which was not consistent with a $^4J_{\text{C,H}}$ correlation as required in the case of a pyrano[2,3-*b*]pyrrole, but could easily be explained by a $^3J_{\text{C,H}}$ correlation in a pyrano[3,2-*b*]pyrrole (Figure 2c). Finally, the distinction of the two possible structures arising from opposite connection of substructure **II** into the dihydropyrrole ring (Figure 2b, **A** and **B**) proved nontrivial. Specifically, it remained unclear whether the small magnitude of the coupling constant between H-1 and H-2 ($J = 1.0$ Hz) was indicative of either a $^3J_{\text{H,H}}$ or rather a $^4J_{\text{H,H}}$ coupling. Due to the ring size, both H-1 and H-2 displayed HMBC correlations to all other carbons in the dihydropyrrole ring; thus this NMR experiment could not help to distinguish the two candidate structures. However, in the ROESY spectrum, a cross-peak between H-1 and H-2 was clearly discernible, thus ruling out **B**, leaving **A** as the only

remaining possibility (Figure 2c). Thus, the gross structure of **5** was established as depicted. It should be noted that **5**, for which we propose the name pyranonigrin A, features a unique heterocycle, which to the best of our knowledge is hitherto unprecedented in nature, making it difficult to obtain suitable reference compounds for comparison of the spectral data.

As in the case of aspernigrin B (**4**), the absolute configuration of **5** was elucidated using the combination of experimental and quantum chemical CD investigations, now arbitrarily starting the calculations with the *S*-enantiomer. The first chosen approach was an MD simulation using the MM3³⁷ force field at a virtual temperature of 400 K. The following CNDO/2S-based CD calculations delivered a curve that was virtually opposite compared to the experimental one (Figure 3a, left), whereas the CD spectrum calculated for the *R*-enantiomer showed a good agreement with the measurement (Figure 3a, right). In consequence, the MD approach permitted the assignment of the *R*-configuration to the stereogenic center of **5**.

To further confirm this result, (*S*)-**5** was also submitted to a conformational analysis, resulting in six conformers, optimized with the semiempirical AM1³⁸ method. In this case, the subsequent CD calculations were carried out using the OM2³⁹ approach, and the obtained spectra were energetically weighted according to the Boltzmann statistics. The comparison with the experiment showed again a mirror-image-like behavior for *S* (Figure 3b, left) and matching CD curves for *R* (Figure 3b, right), clearly identifying the stereocenter of pyranonigrin A (**5**) to be *R*-configured.

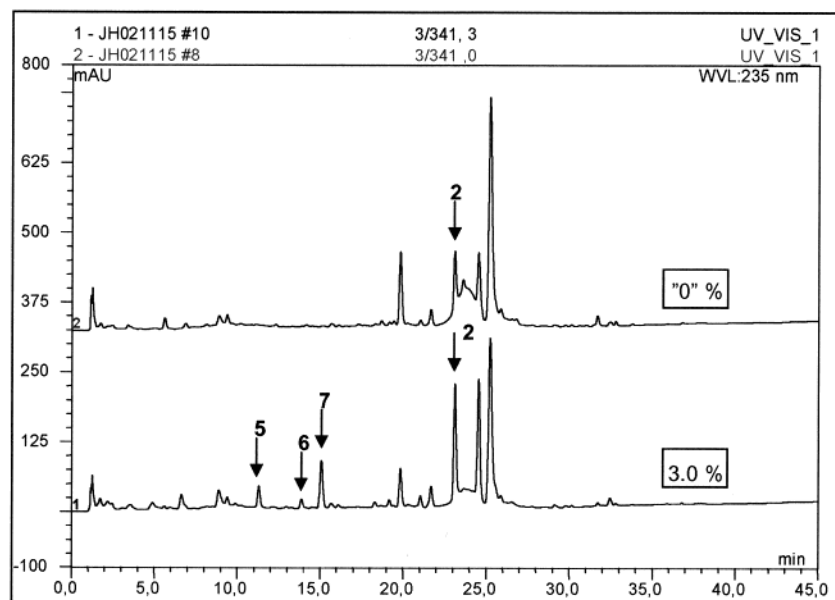


Figure 4. HPLC-DAD chromatogram of extracts of *Aspergillus niger* cultures at different levels of salinity ("0" and 3.0%). The peak of bicoumanigrin (2) is labeled with an arrow as well as those of pyranonigrins A–C (5–7), which appear only at high salt concentrations in the medium.

Once the structure of pyranonigrin A (5) was known, the structure elucidation of the remaining congeners, pyranonigrins B–D (6–8), was straightforward. The molecular formula of 6 was $C_{11}H_{11}NO_6$ as determined by HRESIMS, thus differing from 5 by an additional oxygen and a CH_2 group. All NMR signals due to the γ -pyranone part of 6 were virtually identical to the respective part in 5 (Table 3). The main differences in the 1H NMR spectrum of 6 as compared to 5 consisted of the appearance of a methoxy singlet at δ 3.85, the loss of the signal for H-2, and considerable differences in the chemical shifts for two of the low-field protons attached to heteroatoms (δ 7.65 and 7.97, respectively). These changes could be explained by the presence of a methoxy substituent at C-3, as evident from an HMBC correlation of OCH_3 -3 to C-3 (δ 160.3) on one hand, while the additional oxygen atom was found to reside at N-1 on the other hand. This hydroxylamine function was also evident by a loss of 17 amu in the EI mass spectrum of 6 as a prominent fragment ion at m/z 236 was observed.⁴⁰ The nature and the position of the N-OH group (δ 7.65) were further confirmed by HMBC correlations to both C-2 (δ 162.8) and C-7a (δ 122.1). Thus, the structure of pyranonigrin B (6) formally differed from pyranonigrin A (5) by a 3-*O*-methylation of the corresponding enolic tautomer, followed by an oxygenation at N-1.

The inspection of the low- and high-resolution mass spectra obtained for 7 and comparison to those of 6 immediately revealed the two compounds to be isomers. Since only rather minute differences in the 1H NMR spectrum were observed for the signals corresponding to the N-OH proton (δ 8.15), the methoxy group (δ 3.72), and the OH proton resonating at higher field (δ 8.26), pyranonigrin C (7) was identified as an analogue of pyranonigrin B (6), but with the opposite arrangement of the respective substituents at C-2 and C-3.

Compound 8 displayed a pseudomolecular ion at m/z 236 ($[M + H]^+$), suggesting a molecular formula of $C_{11}H_9NO_5$, which was also in agreement with the 1H NMR spectrum showing again significant similarities to the pyrano[3,2-*b*]pyrrole derivatives described so far (Table 3). The observed singlet at δ 4.31, integrating for two protons, could only be explained with the presence of a methylenedioxy function attached to C-2 and C-3. Thus, the structure of

pyranonigrin D (8) was established as depicted, formally being the dehydrogenation product of either pyranonigrin B (6) or pyranonigrin C (7), which would give rise to the methylenedioxy substructure, while the lack of the hydroxylamine function corresponded to pyranonigrin A (5).

When culture broth and the mycelium of *A. niger* were extracted separately, the pigment cycloleucomelone (1) appeared only in the mycelium, giving the extract its characteristic deep green color. The major compounds, bicoumanigrin (2) and pyranonigrin A (5), as well as the minor compounds, aspernigrins A and B (3, 4), of the crude extract were present in both the media and the mycelium extracts. The minor compounds pyranonigrins B–D (6–8) were identified only in the mycelial extract on the basis of HPLC-DAD or LC-MS detection.

When *A. niger* was raised for 6 days in media differing from each other only with regard to salinity (0, 1.5, and 3.0%), clear differences of the metabolite patterns were observed.

Bicoumanigrin (2) showed the highest intensity in the culture raised at a salinity of 1.5%, whereas in the control culture that lacked sodium chloride only trace amounts of this new natural product were observed (Figure 4). The pyranonigrins were detected only in the extracts of salt-containing cultures (Figure 4). An increase of the sodium chloride concentration from 1.5 to 3.0% resulted in a 2- to 3-fold increase of the concentrations of the respective metabolites, thereby proving an induction of natural product accumulation caused by increasing salinity of the culture medium.

All compounds isolated (apart from 8, of which not enough material was available) were subjected to a panel of four bioassays including a test for insecticidal properties, the brine shrimp assay, an agar plate diffusion assay, and a cytotoxicity testing assay employing human cancer cells. None of the compounds investigated were toxic to brine shrimp or inhibited the growth of the assayed microorganisms in the agar plate diffusion assay. When incorporated into an artificial diet, pyranonigrin A (5) caused a significant growth-inhibiting effect toward neonate larvae of the plant pest insect *Spodoptera littoralis*. At the highest concentration tested (100 ppm), the average weight increase of the larvae over 7 days was reduced to 29%

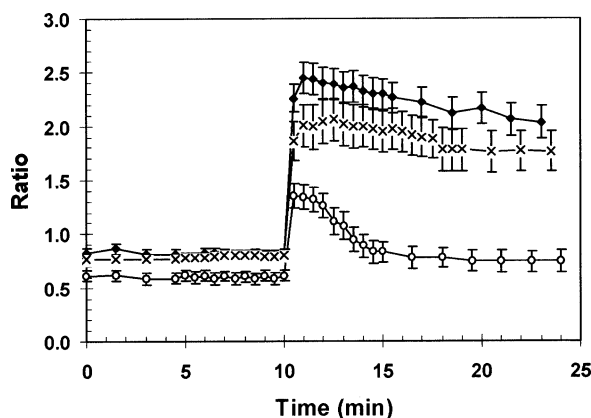


Figure 5. Changes of the $[Ca^{2+}]_i$ level after stimulation of neuronal cells with 200 μ M L-glutamic acid (L-Glu) and 2.5 mM Ca^{2+} (◆); control; 10 min) and after preincubation (5 min) of neurons with 1 (×) and 5 (○) μ g/mL of aspernigrin B (4) following incubation with 200 μ M L-Glu and 2.5 mM $CaCl_2$. The ratios of the 340/380 nm images are shown. The results are expressed as mean values \pm SE; $n = 37$.

compared to untreated controls, while at the same concentration, the survival rate was 85%. With regard to antiproliferative activity toward various leukemia and carcinoma cell lines, bicoumanigrin A (2) showed a moderate inhibitory effect on cell growth when measured with the MTT assay or using incorporation of 3H -thymidine as a marker. Counting of the cells 48 h after addition of 1–20 μ g/mL of bicoumanigrin A (2) resulted in a mean growth inhibition of up to 50% relative to controls depending on the cell line chosen.⁴¹ While pyranonigrin A (5) was inactive at all concentrations tested, both aspernigrins A (3) and B (4) displayed a very moderate cytotoxicity at a concentration of 50 μ g/mL.⁴¹

To assess the potential neuroprotective activity of aspernigrin B (4), its effect on the intracellular calcium ($[Ca^{2+}]_i$) level in neuronal cells was determined. In the first series of experiments the effect of the compound alone on neuronal cells was measured. The results indicated that addition of 1 or 5 μ g/mL of aspernigrin B (4) had no significant impact on the $[Ca^{2+}]_i$ in the neuronal cells (data not shown).

Subsequently, the neuroprotective effect of the compound was quantified. As an agonist L-glutamic acid (L-Glu) was used, and the $[Ca^{2+}]_i$ level in neuronal cells in the presence or absence of aspernigrin B (4) was determined as described in the Experimental Section. Incubation of neurons with 200 μ M L-Glu and 2.5 mM $CaCl_2$ resulted in a strong increase in the $[Ca^{2+}]_i$ after 10 min (Figure 5). At the end of the measurement the 340/380 nm ratio value increased by 1.652 ± 0.128 (284%). This value was set to 100%. Incubation of neurons with 200 μ M L-Glu and 2.5 mM $CaCl_2$ after preincubation of cells with 1 and 5 μ g/mL of aspernigrin B (4) resulted in a strong, statistically significant decrease in the $[Ca^{2+}]_i$ level. A value of 25.5% relative to the control after 1 μ g/mL and 55.0% after 5 μ g/mL of 4 was calculated (Figure 5).

In a further series of experiments the influence of quisqualic acid (QUIS) on the $[Ca^{2+}]_i$ level in neuronal cells in the presence or absence of aspernigrin B (4) was analyzed. Incubation of neurons with 317 μ M QUIS and 2.5 mM $CaCl_2$ resulted in a strong increase in the $[Ca^{2+}]_i$ after 10 min (Figure 6). At the end of the measurement the 340/380 nm ratio increased by 1.207 ± 0.245 (258%). This control value was set again to 100%. A decrease in the $[Ca^{2+}]_i$ was observed after preincubation of cells with 1 (33.6%) and 5 μ g/mL (23.5%) of aspernigrin B (4)

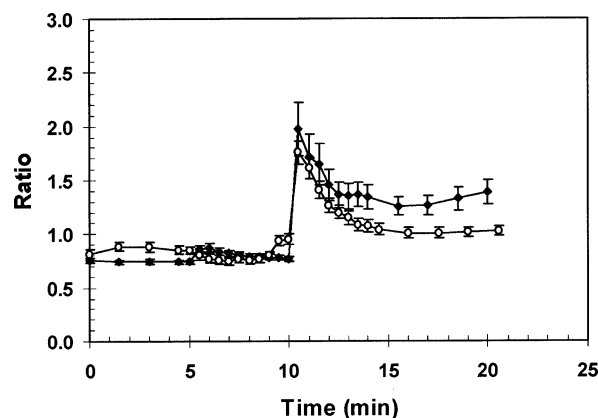


Figure 6. Changes of the $[Ca^{2+}]_i$ level after stimulation of neuronal cells with 317 μ M quisqualic acid (QUIS) and 2.5 mM Ca^{2+} medium (◆); control; 10 min) and after preincubation (5 min) of neurons with 1 μ g/mL (×) and 5 μ g/mL (○) of aspernigrin B (4) following incubation with 317 μ M QUIS and 2.5 mM $CaCl_2$. The ratios of the 340/380 nm images are shown. The results are expressed as mean values \pm SE; $n = 32$.

following stimulation of neurons with 317 μ M QUIS and 2.5 mM $CaCl_2$ (Figure 6).

These data showed that aspernigrin B (4) displays a pronounced neuroprotective effect against stimulation caused by L-Glu or QUIS, a finding that may qualify this compound as a potential drug candidate to ameliorate neurodegenerative diseases.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 LC polarimeter. IR spectra were acquired on a Perkin-Elmer 1600 FTIR spectrometer in KBr. 1H NMR and ^{13}C NMR were recorded on Bruker DRX 500 or DMX 600 NMR spectrometers. EIMS spectra (70 eV, direct inlet) were measured on a Finnigan MAT 8430 mass spectrometer. HREIMS data were determined by peak matching at a resolution of approximately 10 000 (10% valley). ESIMS spectra were recorded on a Finnigan LCQ Deca, and HRESIMS were determined with a Micromass QtoF 2 mass spectrometer.

Biological Material. *Axinella damicornis* Esper was collected at Scoglio della Triglia (40°43'57" N/10°16'13" E) in the south of Elba/Italy by scuba diving at a depth of 20 m in late April 1999. A voucher specimen of the sponge is on file at the Zoological Museum, University of Amsterdam (The Netherlands), encoded ZMA POR 14393.

The sponge material was transferred into a sterilized bag immediately after harvesting and was transported cooled to the nearby laboratory. The isolation of fungi was carried out subsequently. Among the fungi from a 0.1 mL subsample of squeeze water obtained from the sponge a fungal strain was isolated that was identified as *Aspergillus niger* Van Tieghem (CBX, ref: det 146 2002) by the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. The strain was deposited in the Kulturensammlung Mariner Pilze Bremerhaven (KMPB) at the Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven, under accession number E99-3/341.

Mass culture of the fungus for subsequent chemical extraction and analysis was carried out in 1 L Erlenmeyer flasks, each filled with 300 mL of Wickerham seawater medium.⁴² After a growth period of 14 days in static culture at 20 °C, 10 vol % EtOAc was added and the mixture was kept at –80 °C until extraction.

Extraction and Isolation. Culture broth and mycelia of *A. niger* (10 L) were homogenized and exhaustively extracted by stirring with EtOAc. The EtOAc extract was washed with demineralized H_2O (H_2O phase). Following evaporation of solvent, the EtOAc extract was partitioned between cyclohexane and $MeOH-H_2O$ (90:10). Fractionation of the H_2O phase

and the MeOH–H₂O phases was achieved using VLC (silica gel (Merck, Darmstadt, Germany), CH₂Cl₂–*i*-PrOH gradient mixtures), repeated column chromatography employing silica gel (mobile phase: CH₂Cl₂–MeOH, 95:5), and passage over Sephadex LH-20 (Sigma, Deisenhofen, Germany; mobile phase: MeOH). Final purification was obtained using RP-18 Lobar columns (Merck, Darmstadt, Germany; mobile phase: mixtures of MeOH and H₂O), by recrystallization, and by preparative HPLC (MeOH–H₂O gradient, TFA (0.1%) added to H₂O to optimize separation). The separation column (7 or 10 μ m, 300 \times 8 mm, i.d.) was prefilled with Eurospher RP-18 (Knauer, Berlin, Germany). Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. Fractions were monitored by TLC on precoated TLC plates with Si gel 60 F₂₅₄ (Merck, Darmstadt, Germany; mobile phase: CH₂Cl₂–*i*-PrOH, 95:5, or CH₂Cl₂–MeOH, 95:5). Compounds were detected by absorbance under UV at 254 nm, by fluorescence at 366 nm, or after spraying the TLC plates with anisaldehyde reagent as well as by HPLC-DAD and LC-MS. Yields of compounds were (1) 6.8 mg; (2) 35.0 mg; (3) 6.0 mg; (4) 4.7 mg; (5) 33.8 mg; (6) 4.3 mg; (7) 1.5 mg; and (8) 0.9 mg.

Bicoumanigrin (2): white amorphous residue; $[\alpha]_D^{20}$ 0° (c 1.4 and 0.7, DMSO); IR (KBr) ν_{\max} 3079, 2258, 1686, 1611, 1590, 1353, 1335, 1281, 1225 cm⁻¹; ¹H and ¹³C NMR, see Table 1; EIMS (70 eV) m/z 392 [M – H₂O]⁺ (6), 378 [M – CH₃OH]⁺ (100), 363 (21), 335 (16), 306 (9), 279 (5), 185 (11), 129 (7), 101 (7); HREIMS m/z 378.0736 (calcd for C₂₁H₁₄O₇, 378.0740); ESIMS m/z (positive mode) 411 [M + H]⁺, MS/MS 411 @ 35% normalized collision energy m/z 261, 233; HRESIMS m/z 411.1044 (calcd for C₂₂H₁₉O₈, 411.1080).

O-Methylation of Bicoumanigrin (2). A 10 mg sample of **2** was dissolved in 2.5 mL of DMSO and 7.5 mL of acetone. To this solution, 800 mg of K₂CO₃ and 200 μ L of methyl iodide were added. The mixture was allowed to react for 4 h at 40 °C on a water bath. Upon centrifugation, the supernatant was dried using a nitrogen stream followed by freeze-drying.

O-Methylation Product of Bicoumanigrin (2a): white amorphous residue; ¹H NMR, see Table 1; ESIMS m/z (positive mode) 439 [M + H]⁺.

Aspernigrin A (3): white amorphous residue; ¹H and ¹³C NMR, see Table 2; EIMS (70 eV) m/z 228 [M]⁺ (60), 227 (95), 211 (40), 210 (100), 185 (20), 155 (20), 154 (22); HREIMS m/z 228.0880 (calcd for C₁₃H₁₂N₂O₂, 228.0899).

Aspernigrin B (4): colorless oil; $[\alpha]_D^{20}$ +37.8 (c 0.5, DMSO); ¹H and ¹³C NMR, see Table 2; EIMS (70 eV) m/z 456 [M]⁺ (71), 368 (66), 339 (36), 313 (42), 257 (53), 236 (60), 124 (61), 97 (91), 83 (100); HREIMS m/z 456.1681 (calcd for C₂₇H₂₄N₂O₅, 456.1685).

Pyranonigrin A (5): white amorphous residue; $[\alpha]_D^{20}$ +59.9° (c 1.0, DMSO); IR (KBr) ν_{\max} 3286, 1716, 1654, 1636, 1615, 1576, 1488, 1394, 1219, 1035, 960, 792 cm⁻¹; ¹H and ¹³C NMR, see Table 3; ESIMS m/z (positive mode) 224 [M + H]⁺; HRESIMS m/z 224.0576 (calcd for C₁₀H₉NO₅, 224.0559).

Pyranonigrin B (6): white amorphous residue; ¹H and ¹³C NMR, see Table 3; EIMS (70 eV) m/z 253 [M]⁺ (65), 236 (47), 221 (34), 150 (100), 97 (26), 69 (72), 41 (25); ESIMS m/z (positive mode) 254 [M + H]⁺, MS/MS m/z 254 @ 35% normalized collision energy m/z 222, 209, 177; HREIMS m/z 253.0579 (calcd for C₁₁H₁₁NO₆, 253.0586).

Pyranonigrin C (7): white amorphous residue; ¹H and ¹³C NMR, see Table 3; ESIMS m/z (positive mode) 254 [M + H]⁺, MS/MS m/z 254 @ 35% normalized collision energy m/z 222; HREIMS m/z 253.0599 (calcd for C₁₁H₁₁NO₆, 253.0586).

Pyranonigrin D (8): white amorphous residue; ¹H and ¹³C NMR, see Table 3; ESIMS m/z (positive mode) 236 [M + H]⁺.

Computational Methods. The molecular dynamics simulations were performed at a virtual temperature of 500 and 400 K using the TRIPOS³³ and the MM3³⁷ force field, respectively, as implemented in the molecular modeling package SYBYL.³³ The overall simulation time was 500 ps. The single geometries of **4** and **5** were extracted every 0.5 ps.

The conformational analysis was done on Silicon Graphics OCTANE R10 000 workstations by means of the semiempirical

AM1³⁸ method as implemented in the program package VAMP6.5,⁴³ starting from preoptimized geometries generated by the TRIPOS force field.

The wave functions required for the calculation of the rotational strengths for the electronic transitions from the ground state to excited states were obtained by CNDO/2S-CI³⁴ and OM2³⁹ calculations. These calculations were carried out with Linux Pentium III workstations by the use of the BDZDO/MCDSPO⁴⁴ and the MNDO99⁴⁵ program package. In the case of the MD approach, the single CD spectra calculated were added up arithmetically, while in the case of the conformational analysis, they were weighted following the Boltzmann statistics, according to the respective heats of formation. For a better visualization, the rotational strengths were transformed into $\Delta\epsilon$ values and superimposed with a Gaussian band shape function.

Insect Bioassay. The chronic feeding assays were carried out with larvae of the polyphagous pest insect *Spodoptera littoralis* (Noctuidae, Lepidoptera). The larvae were from a laboratory colony reared on an artificial diet under controlled conditions at 26 °C as described previously.⁴⁶ Feeding studies were conducted with neonate larvae ($n = 20$ for each treatment) that were kept on a diet containing extracts or compounds under study. After exposure for 7 days, survival and weights of surviving larvae were recorded and compared with controls that had been exposed to a diet treated with solvent (MeOH) only.

Brine Shrimp Assay. Eggs of *Artemia salina* were hatched in artificial seawater. After 48 h, 20 phototropic nauplii were transferred into each test vial and seawater was added to make 5 mL. Pure compounds were dissolved in 40 μ L of DMSO to give final concentrations of 10 and 100 ppm. Control vials containing 40 μ L of DMSO were also prepared. The percent mortality at each dose level, including control, was determined.⁴⁷

Agar Diffusion Assay. Compounds were tested at concentrations of 50 and 100 μ g per disk, against the following standard strains: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. The agar diffusion assay was performed according to the Bauer-Kirby test (DIN 58940).⁴⁸ Bacteria on Müller-Hinton agar (Difco) plates treated with sample packed sterile filter disks (Oxoid Ltd.) were incubated for 24 h at 37 °C, then antimicrobial activity was recorded as the clear zone of inhibition surrounding the disk (measured in mm). For *C. albicans* yeast autolysate agar (Difco) plates were employed and the incubation temperature was at 27 °C.

Cytotoxicity Assay. The antiproliferative activities of bicoumanigrin A (**2**), aspernigrin A (**3**), aspernigrin B (**4**), and pyranonigrin A (**5**) were examined by the MTT assay, ³H-thymidine incorporation, and direct cell counting,⁴⁹ using a panel of 10 different human leukemia and carcinoma cell lines (DSMZ, Germany). The assays were conducted with different concentrations of the compounds ranging between 1 and 50 μ g/mL (dissolved in DMSO with a final concentration of 0.2%). Experiments were carried out in triplicate, and each cell line was analyzed repeatedly.

Calcium Detection: Materials. Insulin, holo-transferrin (bovine), putrescine, progesterone, poly-L-lysine ($M_r > 300\,000$), quisqualic acid (QUIS), and Dulbecco's modified Eagle's medium/glucose (DMEM) were obtained from Sigma (Deisenhofen, Germany); Hank's balanced salt solution without Ca²⁺ and Mg²⁺ (HBSS), L-glutamine, and trypsin from Biochrom (Berlin, Germany); fetal calf serum (FCS) from Gibco (Berlin, Germany); bovine serum albumin (BSA) from Roth (Karlsruhe, Germany); sodium selenite (Na₂SeO₃) from Fluka (Neu-Ulm, Germany). Fura-2-acetoxymethyl ester was from Molecular Probes (Leiden, The Netherlands), and mouse antineurofilament 68 kDa antibody as well as mouse antiglial fibrillary acidic protein (GFAP) antibody were from Boehringer Mannheim (Mannheim, Germany).

Calcium Detection: Neurons. Rat cortical cell cultures were prepared from the brains of 17–18 day old Wistar rat embryos following a modified procedure.^{50,51} Briefly, after isolation cerebral hemispheres were placed into HBSS without

Ca^{2+} and Mg^{2+} . Brain tissue was dissociated in HBSS using 0.025% of trypsin (10 min; 37 °C); the proteolytic reaction was stopped by addition of 10% FCS. The single cell suspension was centrifuged, and the pellet containing dissociated neuronal cells was resuspended in DMEM, containing 2 mM L-glutamine, 100 mU/L of insulin, and 10% FCS. The cells were seeded into poly-L-lysine ($5 \mu\text{g/mL}$, $300 \mu\text{L}/\text{cm}^2$)-coated plastic dishes at a concentration of 2.0×10^5 cells/ cm^2 . Two days after isolation DMEM/10% FCS was removed and the cells were further cultivated in DMEM serum-free medium supplemented with 0.1% BSA, 2 mM L-glutamine, $100 \mu\text{g/mL}$ of transferrin, 100 mU/L of insulin, $16 \mu\text{g/mL}$ of putrescine, 6.3 ng/mL of progesterone, and 5.2 ng/mL of Na_2SeO_3 . The cells were kept in an atmosphere of 95% air and 5% CO_2 at 37 °C. Seven days after isolation immunostaining was performed with antineurofilament 68 kDa as marker for neurons and anti-GFAP as marker for glial cells. The cultures contained >80% of neurons (on average ~95%); the other cells were GFAP-positive (mainly astrocytes⁵²).

Calcium Detection: Loading of Cells with Fura-2-AM. The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was determined by measuring the fluorescence ratio of the Ca^{2+} -indicator dye fura-2-AM at 340 and 380 nm.⁵³ Neurons were loaded with 6 μM fura-2-acetoxymethyl (AM) ester in DMEM serum-free medium, supplemented with 1% BSA at 37 °C for 60 min. Subsequently, the cells were washed twice with medium and incubated further at 37 °C for 45 min. These time periods were found to be sufficient to load the cells with fura-2-AM and for hydrolysis of the acetoxymethyl ester (fura-2).

Calcium Detection: Preparation of the Ca^{2+} Calibration Curve. A calcium calibration curve was prepared according to the method of Grynkiewicz.⁵³ Fluorescence images at 340 and 380 nm were obtained for each buffer, and the fluorescence ratios of the images (340/380 nm) were calculated and plotted as a calibration curve. One ratio value 340/380 nm equals 228 nM $[\text{Ca}^{2+}]_i$.

Calcium Detection: Monitoring of Intracellular Free Calcium Level. In all sets of experiments the cells were first loaded with fura-2-AM and then stimulated with different compounds in Locke's solution without Ca^{2+} and Mg^{2+} (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO_3 , 5.6 mM glucose, and 10 mM Hepes; pH 7.4). For the experiments the compound (aspernigrin B, **4**) was dissolved in DMSO (conc 10 mg/mL) and kept at -20 °C. In the first set of experiments neurons were stimulated with 0.1% DMSO after 5 min (control) from the beginning of the measurements and then stimulated with 200 μM L-glutamic acid (L-Glu) or quisqualic acid (QUIS) and 2.5 mM CaCl_2 after 10 min. In these cases the $[\text{Ca}^{2+}]_i$ level was measured for at least 20–25 min. In the second set of experiments the primary neurons were incubated with 1 and 5 $\mu\text{g/mL}$ of aspernigrin B (**4**) and 2.5 mM CaCl_2 starting after 5 min. The $[\text{Ca}^{2+}]_i$ level was measured during the whole incubation period of 20–25 min.

For determination of $[\text{Ca}^{2+}]_i$, cells were cultivated on poly-L-lysine-coated borosilicate coverglass in a four-chamber Lab-Tek Chamber Slid System (Nunc, Wiesbaden, Germany). An inverted-stage Olympus IX70 microscope with apochromatic reflected light fluorescence objective UApo40X/340 was used for the fluorescence measurements. The cells were alternately illuminated with light of wavelengths 340 and 380 nm by computer-controlled switching of narrow-band interference filters in front of a 100 W xenon lamp. An additional 0.25 ND filter was used at 380 nm. The fluorescence emissions at 510 nm were monitored by an intensified CCD camera, model C2400-87 (Hamamatsu, Herrsching, Germany). Images were then digitized with 256×256 pixels by 8-bit arrays with a computerized imaging system (Argus-50, Hamamatsu). The fluorescence ratio 340/380 nm was determined by dividing the image pairs. Over 30 determinations were performed; the results were statistically evaluated using the paired Student's *t*-test.⁵⁴

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