Structure–Activity Relationships of Phenyl- and Benzoylpyrroles

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Antitumor, antimicrobial, and phytotoxic activities of the marine antibiotic pentabromopseudilin (1a) and related phenyl-, benzyl- and benzoyl pyrroles were compared. All activities depended strongly on the substituent pattern, with the natural compound 1a being the most active one. As judged from model reactions, a covalent bond of nucleophiles to the pyrrole system may be involved in the inhibition of macromolecular syntheses.

Key words pseudilin; phenylpyrrole antibiotic; structure–activity relationship; antitumor

According to the literature, pentabromopseudilin[1,2] [(2,3,4-tribromo-5-(3,5-dibromo-2-hydroxyphenyl)-1*H*-pyrrole, **1a**], first isolated from the marine bacterium *Alteromonas luteoviolaceus*, is the most active member in a group of more than 20 pyrrole antibiotics: **1a** interferes effectively with the macromolecular syntheses in gram-positive and gram-negative bacteria, has antifungal activity, and inhibits various enzyme systems and the biosynthesis of cholesterol. It has potential value for plant protection.[3]

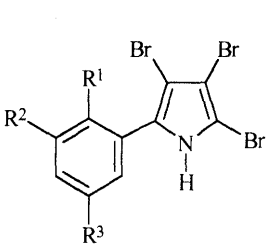
In addition, **1a** shows pronounced *in vitro* activity against experimental leukemia and melanoma cell lines, with an IC_{50} of 0.8 μg/ml, comparable to that of adriamycin. However, the *in vivo* results against L1210 leukemia and B16 melanoma in mice were negative (Table 1) tending to rule out the usefulness of **1a** as an antitumor lead. In NMT I mice it showed an LD_{50} of 50 mg/kg as a single intraperitoneal dose and was ten times more toxic after applications on days 1, 4 and 7 (LD_{50} = 5 mg/kg).

Results and Discussion

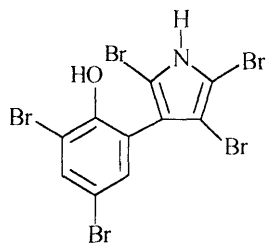
We have synthesized[4–6] numerous 2- and 3-phenyl- as well as 2-benzoylpyrroles and tested these analogs for biological (antimicrobial, herbicidal and insecticidal) and pharmacological (enzyme-inhibitory and cytotoxic) activities. The results can be summarized as follows:

Most halogenated 2-phenylpyrroles **1a—h** are strong inhibitors of nonspecific liver esterase, pentabromopseudilin (**1a**) being the most active one (85% inhibition at 0.5 μg/ml, 65% at 0.05 μg/ml, 15% at 0.5 ng/ml). Acetylcholinesterases, arylesterases, tyrosine kinase and alkaline phosphatase are not inhibited, but various other enzymes are blocked (Table 2); *e.g.*, the aminopeptidases A and M, human leucocyte elastase, dipeptidylpeptidase IV, urokinase, alkaline phosphatase, leucine-aminopeptidase (IC_{50} about 5 μg/ml each).

The cytotoxic activity of 2-phenylpyrroles is due to the inhibition of macromolecular syntheses, as Table 3 shows. Compound **1a** is the most potent even at 0.05 μg/ml, and most of its analogs tested are 5—10 times less active (Tables 3—5). Generally, lowering the halogen content leads to a decrease and finally to a complete loss of activity. This is also true of the introduction of a spacer, as the inactivity of the benzylpyrrole **23** shows. Chlorinated derivatives are less cytotoxic than corresponding bromine derivatives, but the same relation does not always hold for antibiotic



	R^1	R^2	R^3
1a	OH	Br	Br
1b	OH	Br	H
1c	OH	H	Br
1d	OMe	Br	Br
1e	OMe	Br	H
1f	OH	Cl	Cl
1g	OMe	Cl	Cl
1h	H	Br	Br



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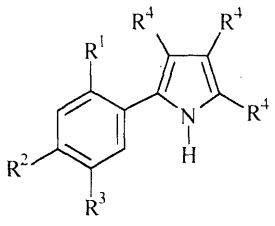

	R^1	R^2	R^3	R^4
3a	Br	Br	OMe	H
3b	Br	Br	OMe	Br
3c	Br	Br	OH	Br
3d	H	OMe	Br	Br

Chart 1

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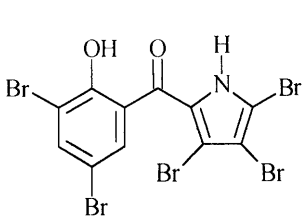
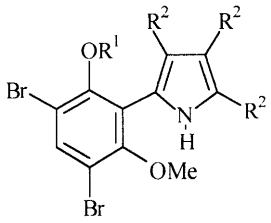
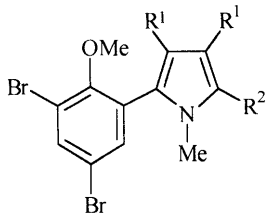


4a
4b: Cl instead of Br
4c: NH_2 instead of OH

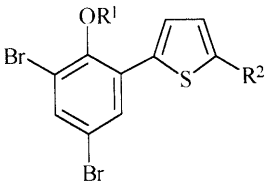


5a: $R^1 = H$, $R^2 = Br$
5b: $R^1 = Me$, $R^2 = H$
5c: $R^1 = Me$, $R^2 = Br$



6a: $R^1 = R^2 = Br$
6b: $R^1 = R^2 = H$
6c: $R^1 = H$, $R^2 = I$
6d: $R^1 = R^2 = H$; OH instead of OMe

Chart 2



7a: $R^1 = H$, $R^2 = Br$
7b: $R^1 = Me$, $R^2 = H$

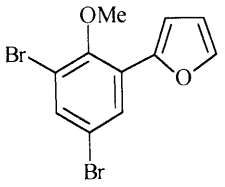


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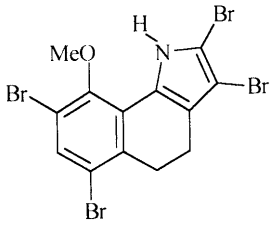
Chart 3



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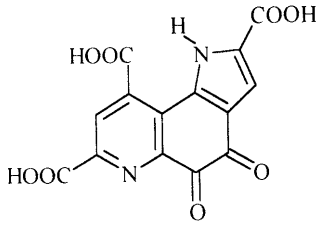


10

Chart 4

activity.

Isomeric phenylpyrroles (*e.g.*, **2a** and **3c**), all phenol ethers and the aniline **4c** show virtually no antibiotic activity against any of the strains tested. But in most cases they are still cytotoxic; even oxygen-free phenylpyrroles such as **1h** show a remarkable cytotoxic activity [**1h**, IC_{50} (L 1210) = 3.8 μg/ml] if the bromine content is high enough. It is clear that a chelated free 2'-hydroxy function which flattens the rings by hydrogen bonding is essential for antibiotic activity (Tables 5 and 6).

Insertion of a carbonyl group in the bond between the rings of phenylpyrroles of type **1a**, yielding benzoyl pyrroles of type **4a**, further enhances the antibacterial activity but considerably lowers the antifungal and cytotoxic activities.

In tests for phytotoxicity, moderate to high activity and a similar dependence on substituent pattern to that seen for cytotoxicity (see above) were observed in simple model systems (Tables 7 and 8). The results of experiments with whole plants (see the experimental part) did not, however, encourage further development. Furthermore, insecticidal activities were generally low.

The Function of Hydrogen Bonds In solution, the benzene and pyrrole rings of 2-phenylpyrroles (type **1a**) are fixed in a hetero-*cis*-arrangement by hydrogen bonding ($\delta_{OH} \approx 12$, $\delta_{NH} \approx 9$, 10), forcing the ring systems into coplanarity. At a hypothetical receptor the intramolecular hydrogen bond may be replaced by an intermolecular one. Its strength is indicative of antibiotic activity: this is most obvious in **4a** which shows a far downfield OH signal at δ 13.66 and is about ten times more active against *Bacillus subtilis* and *Escherichia coli* than **1a** itself.

This hypothesis explains why all phenol ethers and larger substituents disturbing coplanarity (such as OMe in **5a**) are deactivating. As recently described for pyrrolomycins,[7] *N*-methylation of **1d** also results in a decrease in activity. Examination of the isopentabromopseudilin structure type, hitherto not found in nature, shows a corresponding result: **2** and all of its derivatives[4] are virtually inactive against *Candida albicans*, *Streptomyces viridochromogenes* (Tü 57), *Mucor miehei* (Tü 284), and *E. coli*, and the 3,2-phenylpyrrole **3c**, the dibromophenylpyrrole **1h**, the thiophene **7** and the furan **8** are also antibiotically inactive.

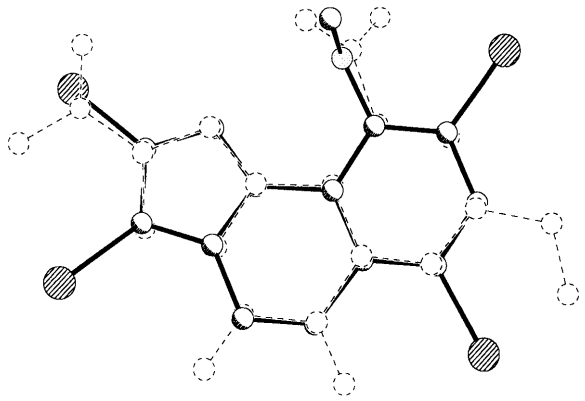
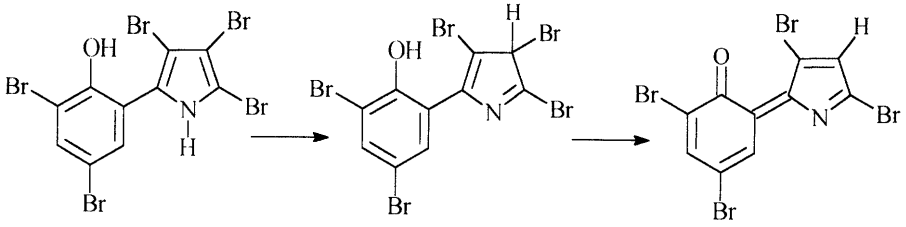


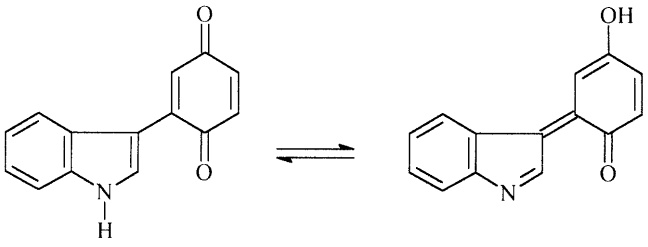
Fig. 1. Molecular Conformations of the Tetrabromopseudilin **9a** (Bold Lines), Methoxatin (**10a**, Dotted Lines)

In biological systems **1a** may act as a competitive inhibitor of a physiological effector. In a hydrogen-bridged hetero-*cis*-conformation, **1a** and its conformationally rigid derivative **9**[4] might act as mimics of the bacterial coenzyme methoxatin (**10**), having similar bond lengths, dipole moments and even nearly superimposable conformations (Fig. 1). However, activity of methoxatin-free glucose oxidase after incubation with **1a** or **9** is completely restored[8] by addition of **10**, so that there have to be other reasons for the biological activity.



1a 11 12

Chart 5



13 14

Chart 6

In contrast to the high antimicrobial activity, the cytotoxicity of **4a** against leukemia cells was disappointingly low. On the other hand, all phenols of type **1a** restained most of their cytotoxicity on methylation, and even the oxygen-free **1h** is cytotoxic, indicating that the antibiotic and cytotoxic activities have different mechanisms.

For antitumor activity, only the binding site (or reactivity) of the brominated pyrrole seems to be essential, but again the phenol and pyrrole systems have to be coplanar. This is in accordance with the observation that 6-methoxypentabromopseudilin (**5a**), whose rings are twisted through 63° (MMX calculation) and in which the intramolecular hydrogen bond is weak, is inactive in both respects.

Model Reactions to Evaluate the Action of Pentabromopseudilin (1a) Phenylpyrroles may bind to a receptor *via* hydrogen bonds, but covalent bond formation under physiological conditions also seems possible: in contact with **1a**, human skin is stained green, and partially brominated hydroxyphenylpyrroles decompose on the TLC sheet on warming, yielding red and blue compounds of unknown structure. These observations may be explained by the formation of **1a** prototropic isomers by hydrogen shift and elimination of hydrogen bromide at suitable pH values, yielding coloured quinonoid systems (*e.g.* **12** from **1a**). These intermediates may add to SH or NH groups of proteins of the cell and in this way inhibit their biocatalytic activity.

However, **1a** is stable in basic solution under a nitrogen atmosphere. On oxidation with potassium dichromate, silver oxide, ammonium-ceric(IV) nitrate or potassium hexacyanoferrate(III)–the latter in alkaline solution–the expected green or blue oxidation products of type **12** were not obtained. Instead, a complex mixture of yellow and orangy-red compounds was isolated in low yield. The two main components showed molecular ions at *m/z* 407 and 408 respectively in the MS, indicating that not more than three bromine atoms are present and that the pyrrole ring had been degraded. The ^{1}H-NMR spectra show the *meta*-coupled protons of the phenol ring; this observation and the chemical shift exclude both *ortho*- and *para*-benzoquinones.

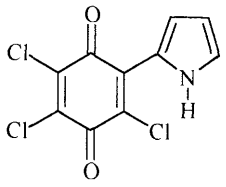
We have therefore synthesized model systems related to **12**. Indole reacts with 1,4-benzoquinone in ethanol–hydrochloric acid to give bluish-violet 3′-indolyl-1,4-benzoquinone,[9,10] which may be represented by formula **13**, or as the tautomer **14**.

The corresponding reaction products of pyrrole with benzoquinone are unknown. Yet on the TLC sheet, pyrrole gives, after spraying with chloranil solution, an intense blue color reaction on warming, indicating that **15** or its tautomer may be present; a similar reaction occurs with the 2-phenylpyrrole **16d**. However, reaction of pyrrole or **16d** with chloranil in dichloromethane under neutral, acidic or basic conditions gave only black inseparable products. This may be interpreted as a result of a catalytic action of the silica gel surface during the color reaction on the TLC sheet. And indeed, the reaction of pyrrole or **16d** with chloranil in dichloromethane at 4 °C gave, in the presence of silica gel, the blue products **15** and **17** respectively, although in low yield.

High-resolution mass spectra of the blue pigments show that addition of the pyrrole systems to chloranil had occurred with loss of hydrogen chloride, as expected. Both ^{1}H-NMR spectra show broad, one-proton singlets at δ 11.75 and 11.45 respectively, which are exchanged only very slowly with D_2O, indicating NH rather than OH protons. Also the coupling constants ($J = 4$ Hz, $J' = 2.8$ Hz in **17**, $J = 3.8$ Hz, $J' = 1.5$ Hz, $J'' = 2.2$ Hz in **15**) of both pyrrole adducts show that pyrrole had reacted at the α-rather than the β-position, and that the *p*-quinonoid tautomers predominate. For the UV absorption of **17**, a maximum at 473 nm is predicted by PPP calculations)[11]; the experimental value (593 nm) fits better with the expected value (594 nm) for the methide tautomer (type **14**).

In the agar diffusion test, both compounds were inactive against *C. albicans*, *M. miehei*, *E. coli*, *B. subtilis* and *S. viridochromogenes* (Tü 57) at concentrations of up to 10 μg/disk. At 100 μg/disk, *E. coli* was weakly inhibited by **15**, but not by **17**; the minimal inhibitory concentrations of **1a**, and **15** or **17** differ by more than three orders of magnitude. Chloranil and **15** had similar inhibitory potency against *E. coli*.

If a covalent bond is formed at all, it may happen by nucleophilic substitution rather than by an elimination–addition sequence as discussed above. In triethylamine



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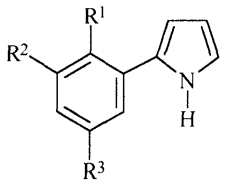

	R1	R2	R3
16a	OH	Br	Br
16b	OMe	Br	H
16c	OMe	H	Br
16d	OMe	Br	Br
16e	OMe	Cl	Cl
16f	H	Br	Br
16g	H	H	OMe
16h	H	OMe	H

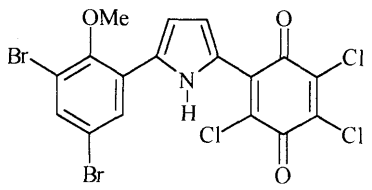

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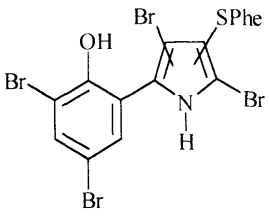
Chart 7



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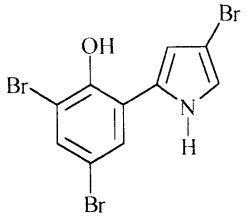

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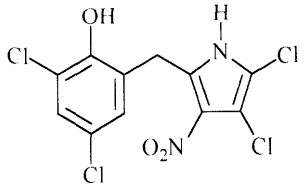

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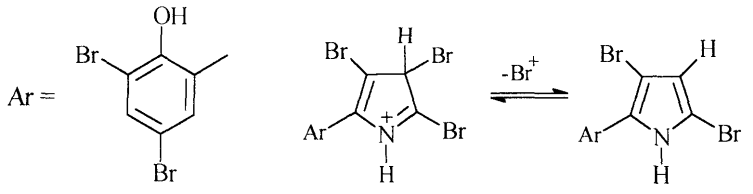
Chart 8



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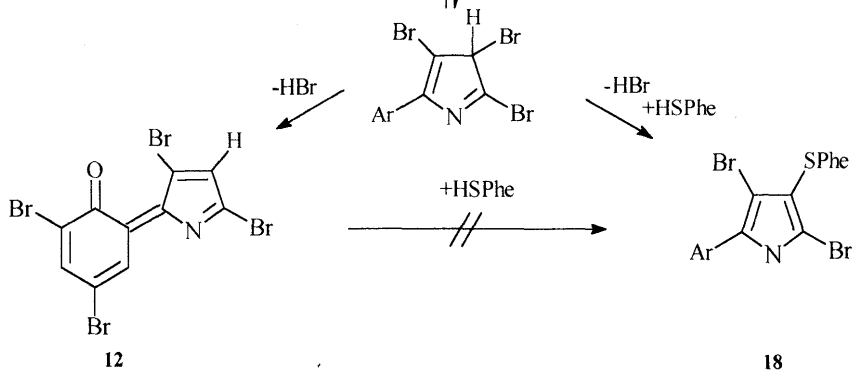


12

18

Chart 9

containing dichloromethane, besides polar decomposition products and unchanged starting material, pentabromopseudilin (**1a**) slowly gave, with thiophenol under nitrogen gas and at room temperature, a substitution product $C_{16}H_9Br_4NSO$ (MS), which showed the spectral data expected for **18**. As the unchanged signal pattern of the benzenoid ring showed, substitution had occurred in the pyrrole ring, but structure determination was not done.

As a chromatographically inseparable by-product, **18** always contained a second compound, $C_{10}H_6Br_3NO$ (MS) showing two one-proton signals in the ^{1}H-NMR spectrum (δ 6.87 and 6.62) as doublets of doublets each with coupling

constants of $J = 1.5$ and $J = 2.8$ Hz; the latter dropped after D_2O exchange and was thereby identified as being due to NH coupling. The value of $J = 1.5$ Hz is characteristic for a $^4J_{3,5}$ coupling. As both *meta*-couplings of the benzene system in **1a** as well as the NH and OH signals still existed, this by-product is the hitherto unknown tribromopseudilin **19**, as was further proved by comparison with its known isomers. Obviously, a part of **1a** was reduced by thiophenol: this agrees well with our finding that under anaerobic conditions, diphenyldisulfide is formed as oxidation product.

The substitution product **18** was formed – unexpectedly for a reaction with **12** as an intermediate – in the absence of triethylamine under initially neutral, and later under strongly acidic reaction conditions (as a result of the hydrogen bromide evolved) in a shorter time (24 h) and in a higher yield. In addition, two inseparable substitution products were formed, and appeared from the NMR spectrum to be a 1 : 1-mixture of a bis-adduct and a further monoadduct. The corresponding substitution of bromine by thiophenol in the phenol ring of **1a** or its derivatives was not observed.

As the reaction of **1a** with thiophenol is slow under basic, but fast under acidic conditions yielding products of higher purity, it is likely that at first a protonated prototropically isomeric pyrrole (*e.g.* **21**) is formed. This may react with a nucleophile, as occurs in the formation of **15**, through addition to C-2 or C-4 followed by elimination of HBr, or directly through substitution at C-3.

Instead of an elimination–addition reaction, retro substitutions at the pyrrole have also been reported: heavy halogen atoms may be exchanged for fluorine or chlorine by hydrogen halides, whilst the ring-bonded halogen atom is split off as a cation.[12,13] A similar process has been reported for the autocatalytic decomposition of halonaphthols[14] and can easily be demonstrated for **1a**, too: on dissolving **1a** in acetic acid and adding a few drops of hydrobromic acid, the bromine evolved is detectable by its characteristic blue colour reaction with starch–potassium iodide.

Conclusion

A reaction involving quinonoid intermediates would explain why all the tested *N*- and *O*-methylpyrroles show poor antibiotic activities because in these cases methides such as **12** cannot be formed. On the other hand, naturally occurring or synthetic keto- and methylenephenylpyrroles [pyrrolomycin D (**4b**) or pyrrolomycin B (**20a**)] are highly active, although no corresponding conjugated intermediates **12** should exist. Furthermore, it was shown that the ether **1d** reacts with halophiles such as phenol, again through halogen transfer from the pyrrole system.[15] This behavior as well as the lack of antibiotic activity of model compounds definitely excludes a reaction path *via* quinonoid intermediates and favors a direct substitution of bromine in electron-deficient pyrroles. This would also explain why unbrominated pyrroles show only marginal activities.

Experimental

Melting points were determined in open capillaries and are not corrected. IR spectra were taken with a Perkin-Elmer, model 297 (KBr tablets), ^{1}H- and ^{13}C-NMR spectra with Varian FT 80 A, XL 200, and VXR 200 instruments, (tetramethylsilane as internal standard), mass spectra (MS) with a Varian MAT 311 A (70 eV), and high resolution MS with a Varian MAT 731 (peak-matching with perfluorokerosene as a standard, resolution 10000). FAB (fast atom bombardment) MS were obtained with a Finnigan 8200 (glycerol matrix) and UV spectra with a Beckman DB-G (Beckman Instruments, Munich). Thin layer chromatography (TLC) was done on DC sheets Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). For preparative thick layer chromatography (PLC), a slurry of 55 g of Silica gel P/UV_{254} (Macherey-Nagel & Co.) in 120 ml of water was poured on a horizontal glass plate (20 × 40 cm), air-dried overnight and activated for 3 h at 130 °C. For column chromatography (CC), Silica gel 60 (0.05—0.2 mm; Macherey-Nagel & Co.) was used. The columns were filled using the slurry method. All chromatographic zones were numbered in order of decreasing *Rf* values.

Condensation of Pyrrole and Phenylpyrroles with Chloranil General Procedure: Chloranil (1 mmol) was added to 1.6 mmol of the pyrrole and 3 g of silica gel in 20 ml of dichloromethane. After 18 h at 4 °C, the mixture was chromatographed on silica gel using dichloromethane–petroleum ether (1 : 2).

2-(3,5,6-Trichloro-1,4-benzoquinon-2-yl)-1*H*-pyrrole (**15**): Compound **15** (31 mg, 11% yield) was obtained from 0.11 g (1.6 mmol) of pyrrole and 0.25 g (1 mmol) of chloranil as a blue amorphous powder, which decomposed at 132 °C. IR ν_{max}^{KBr} cm^{-1}: 1690, 1680, 1575, 1265, 1240, 1115, 910, 760, 715. UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 593 (3.16), 355 (3.05), 291 (4.30), 282 (4.28). ^{1}H-NMR ($CDCl_3$, 80 MHz) δ: 11.45 (1H, br s, NH), 7.10—7.25 (2H, m, 3-, 5-H), 6.32 (1H, ddd, $^3J_{4,3} = 3.8$ Hz, $^3J_{4,5} = 1.5$ Hz, $^4J_{4,NH} = 2.2$ Hz, 4-H). MS (70 eV) *m/z*: (%) 279 (25), 277 (54), 275 (40) [M$^+$], 249 (30), 247 (29), 240 (14), 212 (10), 186 (17), 184 (30), 151 (18), 149 (54), 125 (54), 118 (100), 114 (40), 110 (14), 87 (18). $C_{10}H_4Cl_3NO_2$ Calcd: 274.9308. Found: 274.9303 (MS).

2-(3′,5′,6′-Trichloro-1′,4′-benzoquinon-2′-yl)-5-(3″,5″-dibromo-2″-methoxyphenyl)-1*H*-pyrrole (**17**): Column chromatography on silica gel using dichloromethane–petroleum ether (1 : 1) afforded 71 mg (13%) of **17** as a blue powder with mp 222 °C from 0.33 g (1 mmol) of **16d** and 0.25 g (1 mmol) of chloranil. IR ν_{max}^{KBr} cm^{-1}: 3390, 3070, 1685, 1660, 1570, 1525, 1460, 1395, 1370, 1305, 1240, 1210, 1175, 1100, 1080, 1000, 980, 820, 795, 755, 705. UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 650 (3.89), 333 (4.42), 293 (4.18). ^{1}H-NMR (DMSO-*d*, 200 MHz) δ: 11.75 (1H, br s, NH), 8.06, 7.86 (AB, $^4J_{4'',6''} = 2.5$ Hz; each 1H, 4″-, 6″-H), 7.30—7.44 (1H, br s, 3-H), 7.12 (dd, $^4J_{4,NH} = 2.8$ Hz, $^3J_{4,3} = 4$ Hz; 1H, 4-H), 3.82 (s; 3H, OCH_3). MS *m/z* (70 eV): (%) 542 (52), 541 (18), 540 (100), 539 (18), 538 (87), 536 (36) [M$^+$], 529 (5), 527 (20), 525 (42), 523 (37), 521 (11) [M$^+ - CH_3$], 462 (10), 461 (9), 460 (11), 459 (9) [M$^+ -$ Br], 348 (9), 291 (16), 289 (28), 287 (12), 248 (20), 210 (15), 186 (14), 151 (11), 91 (23), 87 (24). $C_{17}H_8Br_2Cl_3NO_3$ (540.42) Calcd. C 37.78, H 1.49, Br 29.57, Cl 19.68, N 2.59; Found C 37.89, H 1.45, Br 29.79, Cl 19.93, N 2.60.

2,*x*-Dibromo-5-(3′,5′-dibromo-2′-hydroxyphenyl)-*y*-thiophenyl-1*H*-pyrrole (**18**) and 3-Bromo-5-(3′,5′-dibromo-2′-hydroxyphenyl)-1*H*-pyrrole (**19**): A solution of 332 mg (0.6 mmol) of **1a**, 66 mg (0.6 mmol) of thiophenol and 60 mg of triethylamine in 25 ml of dry dichloromethane was kept for 5 d in the dark and then separated by PLC (dichloromethane–petroleum ether 1 : 1), to afford 40 mg of a 1 : 4-mixture (*Rf* = 0.34) of **19** and **18**. ^{1}H-NMR ($CDCl_3$, 200 MHz) of **19** (from mixture) δ: 9.72 (1H, br s, D_2O-exchangeable, NH), 7.62, 7.44 (AB, $^4J_{4',6'} = 2.5$ Hz; each 1H, 4′-, 6′-H), 6.87 (dd, $^3J_{2,NH} = 2.8$ Hz, $^4J_{2,4} = 1.5$ Hz; 1H, 2-H), 6.62 (dd, $^4J_{4,NH} = 2.8$ Hz, $^4J_{4,2} = 1.5$ Hz; 1H, 4-H), 6.05 (1H, br s, D_2O-exchangeable, OH). ^{1}H-NMR ($CDCl_3$, 200 MHz) of **18** δ: 9.59 (1H, br s, D_2O-exchangeable, NH), 8.14, 7.59 (AB, $^4J_{4',6'} = 2.5$ Hz; each 1H, 4′-, 6′-H), 7.32—7.11 (m, 5H, thiophenyl-H), 6.05 (1H, br s, D_2O-exchangeable, OH). ^{13}C-NMR ($CDCl_3$, 50 MHz) δ: 147.5 (C-2′), 135.7 (C-1″), 133.4 (C-4′), 131.3 (C-6′), 129.3 (C-3″, C-5″), 127.1 (C-2″, C-6″), 126.8 (C-1′), 126.4 (C-5), 119.3*), 118.1*), 113.2 (C-5′), 111.9 (C-3′), 100.1*). MS *m/z* (%) (70 eV): 585 (35), 583 (44), 581 (43), 579 (4) [M$^+$], 506 (3), 504 (5), 502 (5) [M$^+ -$ Br], 423 (100), 422 (10), 421 (30) [M$^+ -$ HBr − Ph], 395 (5), 345 (15), 344 (9), 302 (24), 261 (7), 260 (32), 178 (12), 111 (12), 95 (18), 71 (20), 69 (44) [*) assignment uncertain]. $C_{16}H_9Br_4NSO$, Calcd: 578.7138. Found: 578.7134 (MS).

In addition, 110 mg of unchanged **1a** (*Rf* = 0.28) and 60 mg of diphenyldisulfide (*Rf* = 0.63) were isolated. All other pyrroles tested here have been described elsewhere.[4–6]

Biological Methods and Data Acute Toxicity: **1a** was injected intraperitoneally into NMR I mice either as a single dose or 3 × Q3D.

After 2 weeks the total number of dead animals in each group was counted and the LD_{50} (50 mg/kg body weight after 1 × i.p., 5 mg/kg after 3 × i.p. Q3D) was evaluated using the Litchfield–Wilcoxon method.

1. *In Vivo* Drug Effects on L 1210 Leukemia and B16 Melanoma Methods: L 1210 Leukemia: Ascitic fluid was aseptically withdrawn from DBA2 mice (female, 18—20 g) on day 7 after implantation. The ascites was washed 3 times with phosphate-buffered saline (PBS, pH 7.2—7.4), then cells were counted and diluted in PBS to 10^6 cells per 0.2 ml. Aliquots of 10^6 cells in 0.2 ml of PBS were injected i.p. into DBA2 mice (female, 18—20 g) for propagation of the cell line. This transfer was repeated once a week. For testing, aliquots of 10^5 cells in 0.2 ml of PBS were injected i.p. into BDF1 mice (female, 18—20 g). Six animals/group were used at each substance concentration and for the control.

B16 Melanoma: Solid subcutaneously growing tumor was aseptically excised at day 14 to 18 after implantation in C57B1/6 mice (female, 18—20 g). The tumor was minced and treated with collagenase (0.05%) at 37°C for 1 h. The resulting single cell suspension was washed 3 times with PBS, then the cells were counted and diluted to 10^6 cells/0.2 ml. Aliquots of 10^6 cells in 0.2 ml of PBS were injected subcutaneously into C57B1/6 mice (female, 18—20 g) for maintainance of the cell line. For testing, aliquots of 10^6 cells in 0.2 ml of PBS were injected i.p. into BDF1 mice (female, 18—20 g). Six animals/group were used at each substance concentration and for the control.

2. Evaluation of Antitumor Effects a) The animals were weighed on day 1 and 5 after tumor cell implantation. Weight loss of more than 20% at 5 d after the last injection was taken as an indication of toxic effects.

b) At the end of experiment (death of all animals or on day 60) the median survival time of the animals in all groups with more than 65% survivors on day 5 was evaluated according to standard procedures. The median survival time was evaluated for dying animals only. Long-term survivors (LTS) were excluded from this evaluation and are listed separately. From the median survival time of treated (MST_T) and control

Table 1. *In Vivo* Activity of Pentabromopseudilin (**1a**) against L 1210 Leukemia in BDF_1 Mice

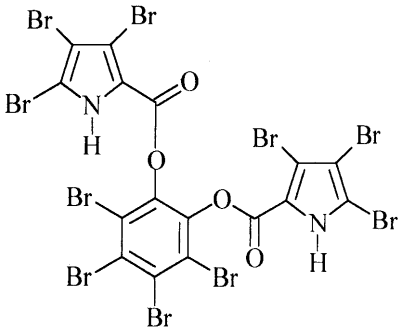
		L 1210			
	Schedule	*T/C*	Dose (mg/kg/inj.)	LTS[a]	Tox. deaths
1a	2 × i.p./i.p. Q3D	83.0	3.00	—	—
		66.0	4.00	—	5
		71.0	5.30	—	3
	3 × i.p./i.p. Q3D	108.0	1.40	—	—
		100.0	2.10	—	1
		104.0	3.00	—	1
			B16		
	3 × i.p./i.p. Q4D	91.0	5.60	—	—
		89.0	7.40	—	—
		91.0	10.00	—	—

a) Long-term survivor.

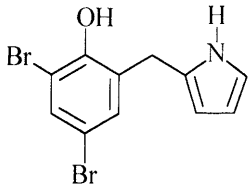
Table 2. Inhibition of Various Enzymes by Phenylpyrroles (Concentrations in μg/ml)

Substance	APA	APM	PMN-ELA	LAP	DPIV	Uro
3a	0	0	56	0	0	0
3c			0		0	0
3d	0	0	0	0	0	0
5a			32		0	0
5b	0	0	0	0	0	0
7a	0	0	23	0	0	31
8	27	33	4.2	32	21	20
16a	0	0	36	0	0	28
16c	0	0	23	0	0	42
16e	0	0	41	0	0	0
16f	0	0	0	0	0	0
16h	0	0	74	0	0	0

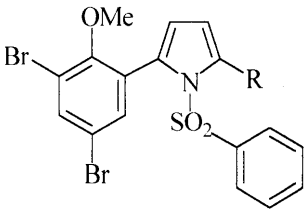
APA = aminopeptidase A (Glu-*p*NA), APM = aminopeptidase M (Leu-*p*NA), LAP = leucine aminopeptidase (Leu-*p*NA), PMN-ELA = human leukocyte elastase (MeSuc-Ala-Ala-Pro-Val-*p*NA), DPIV = peptidyl dipeptidase (Gly-Pro-*p*NA), Uro = urokinase (H-Pyr-Glu-Gly-Arg-*p*NA), no activities were seen with alkaline phosphatase and pp60vv src tyrosin kinase.



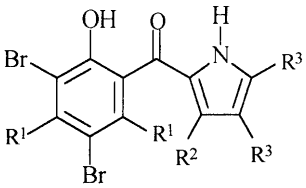
22



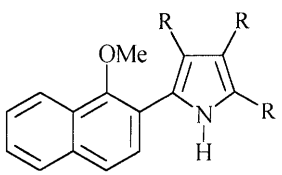
23



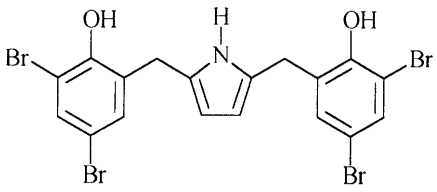
24a: R = H
24b: R = Ac



	R^1	R^2	R^3
25a	H	H	H
25b	H	H	Br
25c	H	I	I
25d	Br	Br	Br



26a : R = H
26b : R = Br



27

Chart 10

groups (MST_C) the antitumor effect (T/C) was evaluated according to the equation

$$T/C\% = \frac{MST_T}{MST_C} \times 100$$

T/C values of more than 125% were regarded as indicating a significant antitumor effect of the test substance. The values of the dose producing the greatest antitumor effect (optimal dose) as well as one dosage level below and above it are summarized in Table 1 (treatment schedule: starting on day 1 after implantation).

The effects on macromolecular syntheses were measured as described before.[16] *B. brevis* was grown in nutrient broth (Difco, Detroit) to an A_{578} of 0.5, and the indicated amounts of the compounds were added, followed 10 min later by the radioactive precursor (0.1 μCi/ml). After 30 min of incubation, the incorporation was stopped by adding an equal amount (v/v) of cold trichloroacetic acid (TCA, 10% in H_2O). The acid-insoluble material was collected on membrane filters (pore size 0.45 μm) and washed twice with TCA (5%). The filters were dryed, 5 ml of scintillator fluid (Quickszint 501, Zinsser, Frankfurt a.M.) was added, and the radioactivity was determined in a Betaszint BF 5001 A.

Nematospora coryli was grown in YMG medium (yeast extract, 1 g; malt extract, 10 g; and glucose, 4 g/l) to an A_{578} of 1.2 and then prozessed as described above. The radioactive precursors [1-^{14}C]leucine (52 mCi/mM), [2-^{14}C]thymidine (52 mCi/mM) and [2-^{14}C]uridine (52.4 mCi/mM) were purchased from New England Nuclear, Dreieich, Germany.

Effect on Stem Cells of L 1210 Cells The assay was performed according to the procedure of Salmon *et al.*[17] with the following modifications. Conditioned medium was replaced by McCoy 5A. Number of cells plated was reduced to 5×10^2 cells/plate (L 1210) due to the high plating efficiency of the tumor cell line. Cells were incubated with various concentrations of the test substance for 1 h at 37 °C, washed twice with McCoy 5A and finally plated in an agar upper layer according to the method of Salmon *et al.*[17] Parallel experiments were performed by continuous incubation with various concentrations of the test substance admixed in the upper layer prior to plating. Plates were stored in an incubator (5% CO_2, 20% O_2 and 95% relative humidity) for 5—7 d at 37 °C. After this time, colonies with a diameter >60 μm were counted using an automated image analysis system (FAS II, Bausch & Lomb).

Results were expressed as the number of colonies formed from treated cells over that in an untreated control (percent). The coefficient of variation of repeated experiments was less than 15%. From the dose–response curves the IC_{50} values for the continuous and 1 h exposures were evaluated.

Proliferation Assay (MTT-Reduction) Exponentially growing L1210, A 549 or WIDR tumor cells at a density of 5×10^3/ml in RPMI were incubated in a 96-well microtiter plate for 72 h (37 °C, 5% CO_2, 95% relative humidity) with various concentrations of each test compound. The control consisted of cells exposed to fresh medium only. Quadruplicate wells were prepared for each drug concentration and for the control. After 65 h 50 μl of MTT (3-[4,5-dimethylthiazolyl-2-yl]-2,5-

Table 3. Effects of **1a**, **6c**, **6d**, **16a**, and **16d** on Macromolecular Syntheses (Incorporation of Thymidine, Uridine and Leucine into TCA-Precipitable Material) in *Bacillus brevis* in Nutrient Broth and Effect of **1a** on Protein and RNA Synthesis in *Nematospora coryli* in YMG Medium

	Conc. (μg/ml)	Leucine (%) cpm (%)	Uridine (%) cpm (%)	Thymidine (%) cpm (%)
Control (*B. brevis*)		5600 (100)	14520 (100)	2100 (100)
1a	0.05	0	3	90
	0.1	0	2	81
	0.2	0	0	0
6c	20	90	65	100
6d	4.0	80	88	85
	20	0	0	0
16a	0.2	76	67	93
	1.0	4	2	2
16d	4	93	92	86
	20	0	2	0
24b	20	85	94	100
Control (*N. coryli*)		6707 (100)	38400 (100)	—[a]
1a	50	5098 (76)	39017 (100)	—[a]
	100	3420 (51)	12315 (32)	—[a]

a) Thymidine was not incorporated due to lack of thymidine kinase in yeast cells.

Table 4. Inhibition of Cell Growth of L 1210, HT 29 and A 549 Cells by Various Phenylpyrroles during Continuous Exposure and after a 1 h Exposure (100% Growth Inhibition)

Substance	MTT-assay IC_{50} (μg/ml) Continuous exposure		Long-term test after 1 h exposure		
	L 1210	HT 29	A 549	L 1210	HT 29
1a	0.12, rep. 0.8	—	—	[a]	
1b	6.1	1.5	8.2		48.0
1c	1.35	1.1	1.6		43.0
1d	8.0, 7.0		7.5		
1f	0.76	0.49	1.32	73	73.0
1h	3.8	3.5	3.56		27.0
4a	3.35	1.36	3.4	67	67.0
6a	3.3		7.0		
6b	4.9, 10		[a]		
15	3.9		[a]		
16c	[a]	6.3	[a]		
16d	4.73		[a]		
16h	[a]	5.8	[a]		
24a	3.8, 4.2		4.2		
24b	4.3, 6.3		6.3		

a) $IC_{50} > 10$ μg/ml, higher concentrations were not tested to avoid unphysiological exposure. Compounds **3a**, **3c**, **3d**, **5a**, **5b**, **7a**, **8**, **16a**, **16e**, **16f**, **22**, and **23** were inactive in all tests.

Table 5. Antimicrobial Spectra: Minimal Inhibitory Concentrations (MIC, μg/ml) in the Serial Dilution Test and Cytotoxic Activity against Cells of the Ehrlich Ascites Carcinoma (ECA Cells) and HeLa S3 Cells

	1a	**1d**	**6a**	**6b**	**6c**	**6d**	**7a**	**8**	**16a**	**16d**	**24a**	**24b**
Acinetobacter calcoaceticus	[a]	[a]	[a]	[a]	[a]	10	[a]	[a]	10	20	[a]	[a]
Bacillus brevis	1.0	[a]	[a]	[a]	20	10	[a]	[a]	2	[a]	[a]	50
Bacillus subtilis	1.0	50	[a]	50	[a]	5	[a]	[a]	2	50	50	[a]
Paecilomyces varioti	50	[a]	[a]	[a]	[a]	[a]	[a]	[a]	20	[a]	[a]	[a]
Penicillium notatum	>10	[a]	[a]	[a]	[a]	20	[a]	[a]	20	[a]	[a]	[a]
Mucor miehei	50	[a]	[a]	[a]	[a]	2	[a]	[a]	2	50	[a]	[a]
Nematospora coryli	[a]	[a]	[a]	[a]	50	[a]	[a]	[a]	50	[a]	[a]	[a]
ECA-cells	12	40	150	100	100	24	nt	nt	100	12	>240	>240
HeLa S-3-cells	<12	<12	40	24	24	<12	nt	nt	<12	<12	24	40

Bacteria were grown in nutrient broth, yeasts and fungi in YMG medium, cell lines were tested as described before[18] (concentrations in μg/ml). nt=not tested. a) MIC>50 μg/ml.

Table 6. Activities of Various Phenylpyrroles against *Escherichia coli*, *Bacillus subtilis*, *Streptomyces viridochromogenes*, *Candida albicans*, and *Mucor miehei*, Using the Agar Diffusion Technique

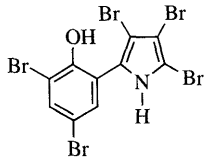
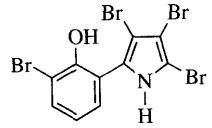
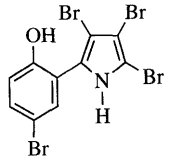
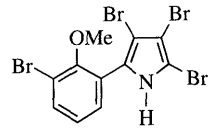
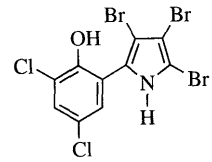
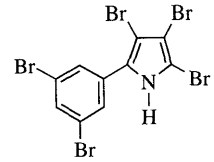
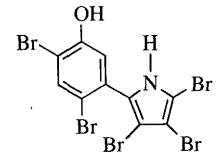
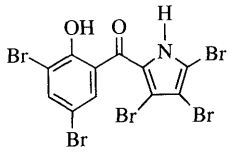
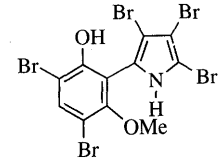
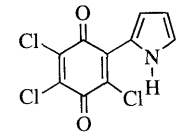
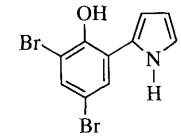
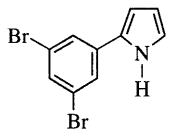
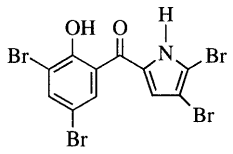
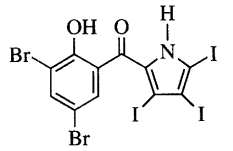
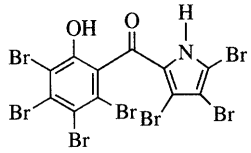
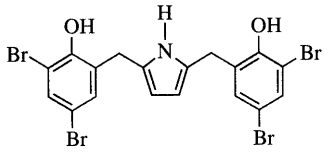
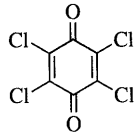
Structure	Compd. No.	Conc. (μg/ml)	Diameter of inhibition zone (mm)				
			E. coli	*B. subt.*	TU 57	*Candida*	*Mucor*
	**1a**	100	14	18	14	0	15
		10	12	16	Weak	0	11
		1	Weak	11.5	0	0	Weak
	**1b**	100	18	26	16	11	27
		10	13	17	Weak	0	~16
		1	Weak	Weak	0	0	~10
	**1c**	100	19	20	14	Weak	19
		10	10	0	0	0	Weak
	**1e**	100	0	0	Weak	0	Weak
	**1f**	100	18	22	19	11	19
		10	14	19	11	0	12
		1	11	13	0	0	0
	**1h**	100	10	*ca.* 13	0	0	0
		10	0	0	0	0	0
	**3c**	100	12	13.5	13	0	14
		10	0	0	0	0	0
	**4a**	100	16	23	14	0	Weak
		10	15	21	10	0	Weak
		1	12	13	0	0	0
	**5a**	100	15	17	Weak	0	Weak
		10	Weak	0	0	0	0
		1	0	0	0	0	0
	**15**	100	13	Weak	0	0	0
		10	10	0	0	0	0
	**16a**	100	11	Weak	0	0	0
		10	0	0	0	0	0

Table 6. (continued)

Structure	Compd. No.	Conc. (μg/ml)	Diameter of inhibition zone (mm)				
			E. coli	*B. subt.*	TU 57	*Candida*	*Mucor*
	**16f**	100	0	0	12	0	Weak
		10	0	0	0	0	0
	**25b**	100	12	17	0	0	12
		10	Weak	15	0	0	12
		1	0	Weak	0	0	0
	**25c**	100	11.5	13	0	0	0
		10	11	11	0	0	0
		1	0	0	0	0	0
	**25d**	100	13	*ca.* 32	13	0	Weak
		10	Weak	*ca.* 19	0	0	0
		1	0	0	0	0	0
	**27**	100	Weak	0	0	0	0
	Chloroanil	100	11	10	0	0	0
		10	0	0	0	0	0

Compounds **4c**, **5c**, **6b**, **17**, **18**, **22**, **23**, **25a**, **26a**, **26b**, and **27** were not active at 100 μg/ml.

diphenyltetrazolium bromide, Thiazolyl blue; Sigma, 2.5 mg/ml in PBS) was added. The MTT is reduced by viable cells to a red insoluble formazan dye. After an additional 7 to 24 h of incubation (depending on the cells used) the supernatant medium was carefully removed. The formazan dye was solubilized by adding 100 μl of dimethyl sulfoxide (DMSO) to each well followed by gentle shaking. The extinction was measured for each well using a multiscan photometer 340 CC, Fa. Flow, at 492 nm.

Results were expressed as the ratio of the extinction after incubation with test substances to that of the control. The coefficient of variation for replicate experiments was less than 15%. From the dose-response curves, the IC_{50} values were evaluated.

For the cytotoxicity study the following cell lines were used: HeLa S3 ATCC CCL 2.2; ECA cells were obtained from mice bearing Ehrlich ascitic carcinomas.

Agar Diffusion Test: Filter disks (Antibiotica-disks, Schleicher & Schüll, Dassel, Germany, 9 mm diameter) were dipped into a solution of a test substance in dichloromethane (1, 10 and 100 μg/ml), dried for 2 h *in vacuo* and placed on agar plates[18] inoculated with *S. viridochromogenes*, *C. albicans*, *M. miehei*, *E. coli* or *B. subtilis* (0.5 ml spore suspension, spore density ≈ 10^8 cells/100 ml). The cultures were incubated for 2 d at 28 °C (*M. miehei*, at 37 °C).

Cell Suspension Test[19]: Aliquots (2 ml) cell suspension cultures were placed in sterile test tubes. Defined quantities of the compounds were dissolved in acetone and added to the suspensions. After an incubation period of 8 d the conductivity of the culture medium was determined as a growth parameter. The effect of a compound is expressed as growth inhibition (%) with reference to the control, with 0 = no growth inhibition, 100 = total growth inhibition.

Algae Test: The algae test was carried out with *Scenedesmus acutus* (collection 276-3a) in an autotrophic shaken culture at 22 °C under continuous illumination. Algal growth was determined quantitatively by means of a Coulter counter 24 h after compound application. The initial compound concentration was 10^{-4} M. When the growth inhibition was greater than 50%, further tests with lower concentrations were performed.

Azolla Test: *Azolla filiculoides* was cultivated in an autotrophic

Table 7. Inhibition of Cell Growth of Corn and Soybean Cell Suspensions (Growth Inhibition in % with Reference to the Control)

	Corn cell suspension[a]	
	10^{-4} M	10^{-5} M
1a	100[a]	100[a]
1b	100	59
1f	100	100
1g	0	
3a	75	6
3b	0	
6c	21[a]	
7a	2	
16b	23	
16d	16[a]	
16e	4	
16f	82	0
16h	0	

a) Soybean cell suspension.

Table 8. Inhibition of Growth of *Scenedesmus acutus*, *Azolla filiculoides* and *Lemna paucicostata* by Phenylpyrroles (Growth Inhibition in % with Reference to the Control)

	Algae test		*Azolla* test			*Lemma* test		
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-4} M	10^{-5} M	10^{-4} M	10^{-5} M	10^{-6} M
1b	100	100	38	80	20	99 (100B)	99 (99B)	31 (11)
1f	100	88	44	30	0	40 (95B)	(58)	(33)
1g	100	43	7	40	0	90	28	6
1h	36			98	0	28	22	3
3a	100	2	0	80	0	40 (42B)	0	0
3b	12					0		
7a	44					0		
16b	100	50	15	90	0	85	18	2
16e	100	59	11	98	0	40		
16f	100	8	8	20	0	0 (45B)	(0)	(0)
16h	46			0		30	15	0

Values in brackets correspond to mixotrophic conditions. B = bleaching of leaves.

inorganic nutrient without nitrogen. Compounds were added to the culture medium at concentrations of 10^{-4}—10^{-6} M. After 14 d, inhibition of growth was determined visually.

Lemna Test: The *Lemna* test was carried out with *Lemna paucicostata*. The plants were cultured under sterile, autotrophic conditions and continuous illumination. The test compounds were added to the inorganic nutrients at an initial concentration of 10^{-4} M. Eight days after application, *Lemna* growth was determined with an image-analyzing apparatus. When growth inhibition was greater than 50% further tests with lower concentrations were performed.

All compounds in Table 8 were ineffective at 0.025% or barely effective against powdery mildew in wheat and cucumbers, net blotch, gray mold (green pepper) and *Phytophthora* (tomatoes); **1g**, **3b**, **16e**, and **16h** showed slight activity against brown rust in wheat. Only **1h** (good, slight attack in places) and **3b** (good, only slight attack) were effective at 0.05% against downy mildew in grapes. Compound **16d** was ineffective on pigweed, common oat, corn flower, nutsedge, barnyard grass, bedstraw, morning glory, ryegrass, mint, mustard and violet; **6c** and **16d** showed little or no activity against powdery mildew in wheat and cucumbers, brown rust in wheat, downy mildew in grapes, gray mold (green pepper) and *Phytophthora* (tomatoes).

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