Ambigol C and 2,4-Dichlorobenzoic Acid, Natural Products Produced by the Terrestrial Cyanobacterium *Fischerella ambigua*

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The new natural products 3,5-bis(2,4-dichlorophenoxy)-2,6-dichlorophenol (ambigol C, **1**), a highly chlorinated aromatic compound, and 2,4-dichlorobenzoic acid (**2**) were isolated from the terrestrial cyanobacterium *Fischerella ambigua* together with the known compounds ambigol A (**3**) and tjipanazole D (**4**). All structures were secured by extensive spectroscopic analysis (1D and 2D NMR, MS, UV, IR). Ambigol C has moderate activity against *Trypanosoma rhodesiense*.

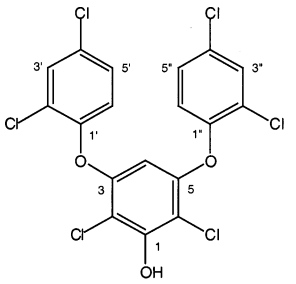
Chemical investigations into the secondary metabolite content of cyanobacteria have led to the isolation of numerous biologically active compounds. Cyanobacteria of the genus *Fischerella* have been shown to contain diverse secondary metabolites, e.g., antifungal fischerindole-type alkaloids,[1] algicidal pyrrolidinone-based fischerellins,[2,3] and the polyhalogenated ambigols,[4] which have antimicrobial activities. The ambigols are especially interesting because of their unusually high level of halogenation and the biological activities exhibited by halogenated phenols in general[5] and by compounds previously isolated from *F. ambigua*.[4]

The current study of *F. ambigua* aimed at identifying the natural products produced by this cyanobacterium under diverse culture conditions and at the assessment of the biological activity of these compounds in pharmacological assays.

In contrast to our previous studies, in which Z-medium was used as the culture medium,[4] BG-11 medium was used in this trial, in an attempt to modify the secondary metabolite spectrum produced by the cyanobacterium. Taking the biosynthesis of the polyhalogenated aromatic ambigols into account, two modified BG-11 media were also tested: one, where all chlorides were replaced by equimolar amounts of potassium bromide, and another where they were replaced with potassium iodide.

After cultivation, isolated biomass was frozen, freeze-dried, and extracted with dichloromethane (CH_2Cl_2), followed by methanol (MeOH). Normal-phase vacuum liquid chromatography (VLC) of the CH_2Cl_2 extracts, employing a step gradient from *n*-hexane to EtOAc, and finally to MeOH, yielded several fractions that were used for chemical (TLC, ^{1}H NMR) and biological (antimicrobial, antiviral, ecotoxicological) screening. The culture medium of the BG-11 trial was concentrated on XAD-16 resin, and retained compounds were eluted with MeOH. Analysis of fractions originating from the cyanobacterial biomass grown in BG-11 medium led to the isolation of the new natural product ambigol C (**1**) together with the known metabolites 2,4-dichlorobenzoic acid (**2**), ambigol A (**3**), and tjipanazole D (**4**).

Chemical and biological screening of the CH_2Cl_2 and MeOH extracts from both the biomass and the culture medium, obtained with media devoid of any chlorine, showed that no ambigol-type compounds nor any related compounds were formed. Bioassays performed with these extracts showed them to be devoid of any activity attributable to the ambigols.



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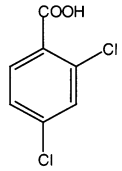

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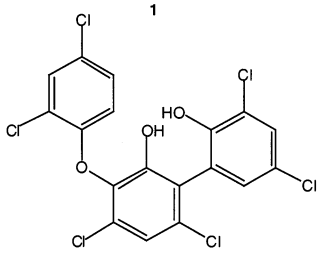

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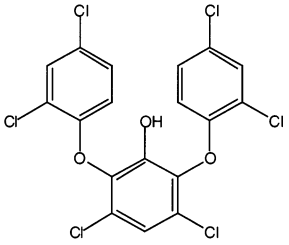

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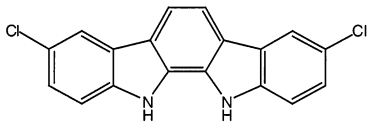

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The molecular formula of ambigol C (**1**) was deduced as $C_{18}H_8Cl_6O_3$ by accurate mass and NMR measurements (*m/z* 481.8605). The EIMS data of **1** showed a molecular ion peak cluster characteristic of a molecule containing six chlorine atoms. Other fragment ion peak clusters corresponding to $[M - Cl]^+$, $[M - Cl_2]^+$, $[M - Cl_3]^+$, $[M - C_6H_3OCl_2]^+$, $[M - Cl - C_6H_3Cl_2]^+$, and $[M - Cl - C_6H_3OCl_2]^+$ supported this deduction. Further, from the UV [λ_{max} (EtOH) 280, 284, 308 nm], IR (ν_{max} 3450, 1580, 1470 cm^{-1}), and NMR data (see Table 1), it was possible to deduce the presence of an extended aromatic ring system and a hydroxyl group. The ^{13}C NMR spectrum of **1** (Table 1) contained only 10 resonances, indicating a number of them to be doubled.

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Table 1. ^{1}H (300 MHz, $CDCl_3$) and ^{13}C (75.5 MHz, $CDCl_3$) NMR Assignments for Ambigol C (**1**)[a]

position	δ_H	δ_C	HMBC[b]
1		144.3 qC	
2/6		125.6 qC	
3/5		138.1 qC	
4	7.18 (s)	121.8 CH	1, 2/4, 3/5
1′/1″		150.9 qC	
2′/2″		123.7 qC	
3′/3″	7.46 (d, J = 2.4 Hz)	130.6 CH	1′/1″, 2′/2″, 4′/4″, 5′/5″, 6′/6″
4′/4″		128.7 qC	
5′/5″	7.11 (dd, J = 2.4, 8.7 Hz)	127.8 CH	1′/1″, 2′/2″, 3′/3″, 4′/4″, 6′/6″
6′/6″	6.55 (d, J = 8.7 Hz)	115.6 CH	1′/1″, 2′/2″, 3′/3″, 4′/4″, 5′/5″

[a] All assignments are based on extensive 1D and 2D NMR experiments, including COSY, HMQC, and HMBC. [b] Numbers in this column represent proton to carbon long-range correlations.

Integration of the resonances in the ^{1}H NMR spectrum of **1** (Table 1) showed the presence of seven aromatic methine protons. From the MS data and the results of HMQC and HMBC experiments, it was evident that all of the ^{13}C signals except those at 121.8 (d) and 144.3 (s) ppm accounted for two carbon atoms. Thus, a total of 18 carbon atoms, consistent with the molecular formula, could be assigned to a symmetrical molecule, almost identical to ambigol B (**5**). The apparent difference between the two molecules was the position of the hydroxyl group, as supported by further NMR studies. NOE difference measurements, which included irradiation at the resonance frequency for H-4 with resultant enhancement of the resonances associated with H-6′/H-6″, revealed the OH group to reside at C-1. Compound **1** is a new natural product for which the trivial name ambigol C is proposed.

Compound **2** had the molecular formula $C_7H_4O_2Cl_2$ as determined by accurate mass measurement (*m/z* 189.9588). Its EIMS contained a molecular ion grouping at *m/z* (rel int) 190 (52), 192 (34), and 194 (5) indicative of a molecule containing two chlorine atoms. Other major fragment ions were observed at *m/z* 173 (100), 175 (68), 177 (10) for $[M - OH]^+$ and 145 (24), 147 (16), 149 (3) for $[M - COOH]^+$. The ^{1}H NMR contained resonances for three aromatic protons with a coupling pattern characteristic of a 1,2,4-substituted aromatic system. The ^{13}C NMR spectrum of **2** contained six resonances for aromatic carbons and a quaternary carbon (168 ppm), indicating a ring substituent. The latter was thus substituted with a carboxyl group and two chlorine atoms. With these data in hand, it was evident that **2** was 2,4-dichlorobenzoic acid, a deduction that was confirmed by comparison of all data with those of an authentic sample. This is the first report of this compound to be produced in nature.

Together with compounds **1** and **2**, the compounds ambigol A (**3**) and tjipanazole D (**4**), previously isolated from *F. ambigua*,[4] were also isolated from the current sample.

The *Fischerella* metabolites, ambigols A (**3**) and C (**1**), were assayed toward various target organisms (Table 2). Both compounds showed significant antibacterial activity toward *B. megaterium*, but were inactive toward Gram-negative *E. coli*. Antifungal and antialgal activity was most evident for ambigol A (**3**). Ambigol C (**1**) only inhibited the growth of the fungus *U. violacea*. Ambigol A also showed activity against *Trypanosoma cruzi*, *T. rhodesiense*, *Plasmodium falciparum*, and *Mycobacterium tuberculosis*, possibly due to its general cytotoxicity. In contrast, ambigol C (**1**), which is not cytotoxic, was also found to have weak antiplasmodial (IC_{50} 1.5 and 2.4 μg/mL) and trypanocidal effects (MIC 11 μg/mL toward *T. rhodesiense*) and may thus be a suitable candidate for chemical modifications in order to optimize its observed activity profile. Compounds **2** and **4** showed no positive activities in any of the applied test systems.

Table 2. Biological Activities for Compounds **1** and **3**

	ambigol C (**1**)	ambigol A (**3**)
E. coli[a]	n.a.[i]	n.a.
B. megaterium[a]	7	8
M. violaceum[a]	5	7
E. repens[a]	n.a.	7
F. oxysporum[a]	n.a.	8
M. microspora[a]	n.a.	4
C. fusca[a]	n.a.	3
T. cruzi[b]	100	33
T. rhodesiense[b]	11	33
P. falciparum,[c] clone K1[d]	1537	846
P. falciparum,[c] clone NF54[e]	2498	1623
M. tuberculosis[f]	>128	64
B. glabrata[g]	n.a.	active
cytotoxicity[h]	100	33

[a] Antimicrobial assays assessed at a concentration of 50 μg/filter disk against *Escherichia coli, Bacillus megaterium, Microbotryum violaceum, Eurotium repens, Fusarium oxysporum, Mycotypha microspora*, and *Chlorella fusca*; numbers indicate the inhibition zone in mm around the filter disk; benzyl penicillin (7 mm), streptomycin sulfate (4 mm), and miconazol (20 mm) were used as positive controls. [b] All values are MIC in μg/mL; trypanosomes were tested in trypomastigote stage; positive control: melarsoprol (MIC = 0.011 μg/mL). [c] All values are IC_{50} in ng/mL. [d] *Plasmodium falciparum* strain is chloroquine resistant; positive control: chloroquine (91 ng/mL) and artemisinin (1 ng/mL). [e] *Plasmodium falciparum* strain is chloroquine sensitive; positive control: chloroquine (5 ng/mL) and artemisinin (3 ng/mL). [f] MIC in μg/mL toward *Mycobacterium tuberculosis*, positive control: rifampicin (MIC = 0.125 μg/mL). [g] Molluscicidal activity toward *Biomphalaria glabrata* at 100 μg/mL; activity causes death of the test organism. [h] MIC in μg/mL toward mammalian cells L-6. [i] n.a. = not active.

The experimental results clearly demonstrate that the secondary metabolism of *F. ambigua* is culture medium dependent. Cultivation using BG-11 medium led to the production of the new compound ambigol C (**1**), at the apparent expense of ambigol B (**5**), which was produced when Z-medium was used.

Experiments aimed at replacing the chlorine substituents in the ambigols with bromine or iodine by altering the composition of the cultivation medium were unsuccessful. This result suggests a high specificity of the halogenating enzymes for chlorine. Additionally, the ambigol pathway seems to be chlorine dependent, as no precursors of these metabolites could be detected in the biomass or culture medium when chlorine was not present in the original culture medium. Another possibility could be a pH-dependent specificity of the enzyme, as observed for the chloroperoxidase of the polychaete *Notomastus lobatus*. This enzyme has unique pH optima for the tested halogen atoms at a defined hydrogen peroxide concentration.[6]

The fact that the extracts and pure compounds produced by *F. ambigua* have antifungal, antibacterial, and antialgal properties may indicate a possible protective role for these substances against other microorganisms in its environment. In relation to our positive controls, ambigols A (**3**) and C (**1**) show antibacterial activity in the range of streptomycin. The observed antifungal activity was either weak (ambigol A) or observed only toward *M. violaceum* (ambigol C).

Experimental Section

General Experimental Procedures. Analytical and preparative HPLC was carried out on a Merck-Hitachi-HPLC system, consisting of a L6200A gradient pump, a L4500A diode array detector, and a D6000 interface module; all components were controlled by D-7000 HSM software. All solvents were distilled prior to use and degassed under reduced pressure. Samples were manually injected using a Rheodyne 7725i injector system. For analytical HPLC, pure compounds were used as external standards for calibration purposes. The system was equipped with a Merck LiChrospher RP-18 (250 × 4 mm; 5 μm) column. Sample or standards (10 μL) were injected and analyzed using a low eluent gradient system from 90% MeOH to 100% MeOH in 35 min at a flow rate of 0.8 mL/min. Ambigols were detected at 254 nm (retention times: ambigol A (**3**), 21.37 min; ambigol C (**1**), 23.50 min). All other measurements were performed as described previously.[7]

A sample of *F. ambigua* (Näg.) Gomont, designated strain number 108b, was obtained from the Swiss Federal Institute for Water Resources and Water Pollution Control (EAWAG), Culture Collection of Algae, 8600 Dübendorf, Switzerland.

Precultures were grown in 200 mL Erlenmeyer flasks with 100 mL of the designated medium (BG-11,[8] BG-11/Br, BG-11/I). The cultures were stirred at 60 rpm at 25 °C and illuminated with white fluorescent light. The cyanobacterium was mass cultivated in a 30 L photobioreactor (Model Pluto, Planctotec, Regenstauf) at a constant temperature of 25 °C for 28 days. The pH of the cultures was adjusted to 7.5. Cultures were continuously mixed with sterile air. During cultivation, pH-adjustment was achieved via a pH-electrode coupled magnetic vent, dosing additional CO_2 into the sterile air. Illumination was performed with one white fluorescent lamp (OSRAM L 58W/11-860).

Resultant biomass was freeze-dried (91 g dry weight) and extracted with DCM (4 L) and subsequently with MeOH (5 L). The DCM extract (5.53 g, 6.08%) was separated by normal-phase silica VLC employing a step gradient from 100% hexane to 100% EtOAc, to 100% MeOH, to yield nine fractions each of 80 mL. Fraction 3 (1.84 g, 2.02%) was selected for further separation on the basis of TLC and ^{1}H NMR results that showed the presence of ambigol-type compounds. Normal-phase silica VLC of this fraction using a gradient from 100% hexane to 100% EtOAc yielded a further six fractions each of 70 mL. Of these six fractions, fraction 3 (107 mg, 0.12%) was found to be a single compound, ambigol C (**1**). HPLC separation of fraction 2 (Si 60, hexane/EtOAc, 98/2, 2 mL/min) led to the isolation of 2,4-dichlorobenzoic acid (**2**), ambigol A (**3**), and tjipanazole D (**4**).

Pure compounds and extracts were tested for their antibacterial (*Escherichia coli*, *Bacillus megaterium*), antifungal (*Microbotryum violaceum*, *Eurotium repens*, *Fusarium oxysporum*, *Mycotypha microspora*), and antialgal (*Chlorella fusca*) properties (50 μg/filter disk). Tests were carried out as previously described.[9] Cytotoxicity was assessed toward L6 myoblast cells derived from rat skeletal muscle tissue.[10] For antitrypanosomal tests, strains of *T. rhodesiense* and *T. cruzi* were used.[11] Antiplasmodial activity was tested toward *P. falciparum*.[12] Antimycobacterial assays were performed using *M. tuberculosis*.[13] Molluscicidal tests were undertaken with *Biomphalaria glabrata*.[14] HIV-1 reverse transcriptase inhibition[15] and tyrosine kinase inhibition activities were assessed in ELISA-based test systems.[16]

Ambigol C (1): amorphous white powder; mp 197 °C (107 mg, 0.12%); UV λ_{max} nm (EtOH, ϵ) 285 (8193), 294 (9096), 308 (7410); IR (film) ν_{max} 3314, 2925, 1702, 1580, 1471, 1448, 1389 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m/z* (rel int) [M$^+$] 490 (8), 488 (38), 486 (76), 484 (100), 482 (52), [M − Cl]$^+$ 453 (<1), 451 (<1), 449 (6), 447 (7), 445 (5), [M − Cl_2]$^+$ 418 (<1), 416 (7), 414 (16), 412 (11), [M − Cl_3]$^+$ 381 (1), 379 (3), 377 (4), [M − $C_6H_3OCl_2$]$^+$ 326 (<1), 324 (1), 322 (3), 320 (2), [M − Cl − $C_6H_3OCl_2$]$^+$ 292 (2), 290 (7), 288 (19), 286 (20); HR DCIMS (isobutane) *m/z* found 481.8605 [M$^+$] (calcd for $C_{18}H_8Cl_6O_3$ 481.8605).

2,4-Dichlorobenzoic acid (2): (12.3 mg, 0.014%) all physical and spectroscopic data in agreement with those of an authentic sample.

Ambigol A (3): (54.5 mg, 0.06%) all physical and spectroscopic data in agreement with those of an authentic sample.

Tjipanazole D (4): (16.8 mg, 0.02%) all physical and spectroscopic data in agreement with those of an authentic sample.

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References and Notes

(1) Park, A.; Moore, R. E.; Patterson, G. M. L. *Tetrahedron Lett*. **1992**, *23*, 3257−3260.
(2) Hagmann, L.; Juettner, F. *Tetrahedron Lett*. **1996**, *36*, 6539−6542.
(3) Papke, U.; Gross, E. M.; Francke, W. *Tetrahedron Lett*. **1997**, *38*, 379−382.
(4) Falch, B. S.; König G. M.; Wright, A. D.; Sticher, O.; Rüegger, H.; Bernardinelli, G. *J. Org. Chem*. **1993**, *58*, 6570−6575.
(5) Naumann, K. *Chem. Unserer Zeit*. **1993**, *27*, 33−41.
(6) Chen, Y. P.; Lincoln, D. E.; Woodin, S. A.; Lovell, C. R. *J. Biol. Chem*. **1991**, *35*, 23909−23915.
(7) Wright, A. D.; König, G. M.; Angerhofer, C. K.; Greenidge, P.; Linden, A.; Desqueyroux-Faundéz, R. *J. Nat. Prod*. **1996**, *59*, 710−716.
(8) Rippka, R.; Herdman, M. *Pasteur Culture Collection, Catalogue of strains 61;* 1992−1993.
(9) Schulz, B.; Sucker, J.; Aust, H.; Krohn, K.; Ludewig, K.; Jones, P. G.; Döring, D. *Mycol. Res.* **1995**, *99*, 1007−10115.
(10) Likhitwitayawuid, K.; Angerhofer, C. K.; Ruangrungsi, N.; Cordell, G. A.; Pezzuto, J. M. *J. Nat. Prod*. **1993**, *56*, 30−38.
(11) Räz, B.; Iten, M.; Grether-Bühler, I.; Kaminsky, R.; Brun, R. *Acta Trop*. **1997**, *68*, 139−147.
(12) Angerhofer, C. K.; König, G. M.; Wright, A. D.; Sticher O, Milhous WK, Cordell GA, Farnsworth, N. R.; Pezzuto, J. M. In *Advances in Natural Product Chemistry*; Atta-ur-Rahman, Ed.; Harwood Academic Publishers: New York, 1992; pp 311−329.
(13) Collins, L. S.; Franzbla, S. G. *Antimicrob. Agents Chemother*. **1997**, *41*, 1004−1009.
(14) Marston, A.; Hostettmann, K. In *Methods in Plant Biochemistry*, Vol. 6; Harborne, J. B., Dey, P. M., Eds.; Academic Press: London, 1991a; pp 166−174.
(15) Eberle, J.; Seibl, R. *J. Virol. Methods* **1992**, *40*, 347−356.
(16) Wessels, M.; König, G. M.; Wright, A. D. *J. Nat. Prod*. **1999**, *62*, 927−930.

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