Dicranin, an Antimicrobial and 15-Lipoxygenase Inhibitor from the Moss Dicranum scoparium

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DICRANIN, AN ANTIMICROBIAL AND 15-LIPOXYGENASE INHIBITOR FROM THE MOSS *DICRANUM SCOPARIUM*

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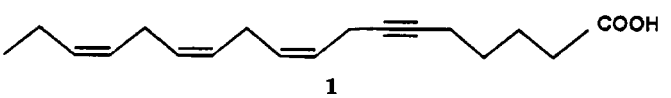
ABSTRACT.—Extracts of nine mosses, collected in Switzerland, were screened for antimicrobial, antioxidative, and 15-lipoxygenase (15-lpo) inhibitory activities. The CH_2Cl_2 extract of *Dicranum scoparium* was found to possess pronounced antimicrobial activity against *Bacillus cereus, Bacillus stearothermophilus, Bacillus subtilis, Staphylococcus aureus,* and *Escherichia coli.* In addition, inhibition of soybean 15-lpo occurred at very low concentration. Phytochemical investigation of this extract afforded *Z,Z,Z*-octadeca-6-yne-9,12,15-trienoic acid [**1**] by a combination of chromatographic techniques. This compound, named dicranin [**1**], was found to be responsible for most of the biological activity. The strongest antimicrobial effect was observed against *Streptococcus faecalis* (disc diffusion assay). In contrast to the CH_2Cl_2 extract of *D. scoparium,* dicranin was inactive against *E. coli.* Dicranin was identified by ir, ms, and nmr spectroscopy. A 2D INADEQUATE experiment confirmed the structure and yielded the assignment of all ^{13}C-nmr signals.

Oxidation and microbial contamination are two major causes of food and cosmetic spoilage, leading to unacceptable products. Synthetic preservatives are often applied to prevent such spoilage, but legislation restricts their use. Thus, there is a need for new preservation strategies making use of natural preservatives. Mosses contain a variety of rare and novel natural compounds, including new structure types. Their lipid content is of special interest, as they could be an alternative source of long chain polyunsaturated fatty acids, such as arachidonic and eicosapentaenoic acids (1,2). Earlier studies reported antimicrobial activities of several bryophytes (3–5). In a search for natural preservatives we therefore examined nine mosses for their antimicrobial, antioxidant, and soybean 15-lipoxygenase inhibition properties.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Si gel 60, 40–63 μm (Merck) was used for normal phase cc. Reversed-phase chromatography was done on a Büchi B-681 mplc system with Lichroprep rp-18, 25–40 μm (Merck), and a gradient of 20% MeCN in H_2O to pure MeCN (total 8 liters, flow rate 5 ml/min) as solvent. The final purification of the active fraction was done by cc with Si gel 60, 40–63 μm, and a gradient of 1–8% iPrOH in petroleum ether (total 400 ml). Tlc was performed on 60 F_{254} Si gel coated Al sheets (Merck) with CH_2Cl_2-EtOAc (95:5). Coated plates rp-18 F_{254} (Merck) were used for hptlc with MeCN-H_2O (95:5). The chromatograms were examined under uv light (254 and 366 nm) and with Godin reagent (6). Hplc was done on a Kontron 200 system equipped with Altex 110A pumps and a photodiode array detector HP-1040A. The separations were performed on a Lichrosorb rp-18 column, 10 μm (Macherey-Nagel), with 65% MeCN in H_2O containing 0.05% H_3PO_4, at 1.5 ml/min. Chromatograms were monitored at 205 nm. Gc was done on a Carlo Erba strumentazione HRGC 5160 Mega series instrument with a carbowax 20 M 25 m capillary column. The carrier gas was H_2 at 65 KPa, and the samples were injected at 80° by the autosampler. The following temperature program was used: 2 min at 80°, followed by a gradient of 15°/min for 5 min, 1 min at 145°, a gradient of 3°/min for 16.7 min, 1 min at 195°, then 5°/min for 1 min, and 15 min at 220°. Mass spectra were recorded on a Finnigan/MAT 8430, SS300 ei/ci instrument; the source temperature was 220°. Dicranin derivatives were prepared according to Traitler *et al.* (7) and Lepage and Roy (8). Nmr spectra were acquired on a Bruker AM 360 spectrometer at 360.13 MHz for ^1H and 90.56 MHz for ^{13}C. The cooling gas temperature was 20.5°. The nmr data were obtained from a sample containing 98.9 mg dicranin, 0.381 g of 99.95% deuterated CD_3OD (Dr. Glaser AG, Basel, Switzerland), and 400 μl of TMS



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vapor as an internal standard in a 5 mm o.d. precision tube. The 2D INADEQUATE experiment was performed using a single four-pulse sequence with a last pulse of ca. 125° (9), to suppress unwanted image peaks, and the complete 128-step phase cycling as proposed by Bax (10). The relaxation delay was 6.78 sec, the acquisition time 0.524 sec; 11.2 μsec pulse duration corresponded to a 180° pulse; 256 transients were acquired for each of the 256 t_1 increments. The spectral width was 15625 Hz in both dimensions for a matrix of 16384×512 data points, and the experiment was optimized for a 62 Hz one-bond coupling constant. Total acquisition time was ca. 135 h. Quadratic shifted sinebell filtering was applied before the 2D Fourier transformation. Spin-lattice relaxation times (T_1) were measured by an inversion-recovery experiment and calculated by the DISR88 Bruker software, the DISR90 version containing errors. The ir spectra were obtained with a Perkin-Elmer model 1600 FT-IR.

PLANT MATERIAL.—The mosses [*Plagiomnium undulatum* (Hedw.) T. Kop. (Mniaceae), *Brachythecium rutabulum* (Hedw.) Schimp. (Brachytheciaceae), *Polytrichum formosum* Hedw. (Polytrichaceae), *Plagiomnium affine* (Bland.) T. Kop. (Mniaceae), *Hylocomium splendens* (Hedw.) Schimp. (Hypnaceae), *Pleurozium schreberi* (Hedw.) Mitt. (Hypnaceae), *Rhytidiadelphus triquetrus* (Hedw.) Warnst. (Hypnaceae), *Dicranum scoparium* Hedw. (Dicranaceae), and *Ctenidium molluscum* (Hedw.) Mitt. (Hypnaceae)] were collected in Switzerland near Chalet-à-Gobet and Diemtigen at about 1000 m altitude, and were identified by Dr. N. Schnyder (Botanical Garden of Zürich) and Dr. P. Geissler (Botanical Garden of Geneva), where voucher specimens were deposited. The mosses were washed carefully to remove soil and debris, and the clean gametophytes were lyophilized. The dried moss tissues (200 g) were then extracted twice with CH_2Cl_2 (500 ml, 12 h). The residue was further extracted with MeOH (2×500 ml, 12 h).

ISOLATION OF DICRANIN [**1**].—A part of the CH_2Cl_2 extract of *D. scoparium* (3 g, corresponding to ca. 30 g of dried moss) was separated by Si gel cc with petroleum ether-toluene (1:1) (1.5 liters), toluene (2 liters), $CHCl_3$ (2 liters), EtOAc (1.5 liters), and MeOH (2.5 liters) to afford 5 fractions of 161 mg, 102 mg, 820 mg, 594 mg, and 640 mg, respectively. Biological tests showed that 15-lipoxygenase (15-lpo) inhibitory activity and antimicrobial activity (disc diffusion assay; *Bacillus stearothermophilus*) were mainly present in fraction 4. Direct bioautographic assay of fraction 4 with *Bac. stearothermophilus* demonstrated that the antibacterial activity was mostly due to a spot characterized by an R_f value of 0.48 [Si gel, petroleum ether-iPrOH (95:5)]. Analysis of the control tlc plate showed that the corresponding spot reacted positively when stained with 2′,7′-dichlorofluorescein. The presence of lipids was confirmed by gc analysis of fraction 4 after transesterification with acetyl chloride. Our results indicated the presence of a compound (Rt 25.9 min) accounting for 76% of the fatty acids. The internal standard $C_{22:0}$ was used for quantitative determination. The active compound was further purified by reversed-phase mplc and Si gel cc to give 22 mg (ca. 0.07% of the dry moss wt) of a slightly yellow oil which was labeled as dicranin [**1**]. The isolation procedure was repeated to obtain enough material for biological tests and spectroscopy. Hplc quantitation indicated that dicranin found in the CH_2Cl_2 extract accounted for 3.2% of the dry moss wt.

BIOASSAYS.—*Antioxidant assays.*—A solution (60 μl) containing 0.01% β-carotene (Merck) and 1% linoleic acid (Merck) in $CHCl_3$ was mixed with 20 μl of moss extract (20 mg/ml in CH_2Cl_2 or MeOH). The solution was then diluted to 2 ml with CH_2Cl_2 or MeOH. The absorbance was read at 450 nm before and after irradiation with uv light (ca. 5 min; Philips TUV 30 W G 30 TB at 253.7 nm). Blanks were prepared by omitting the extract or β-carotene.

15-Lpo inhibition.—The tests were run according to Sircar *et al.* (11) with arachidonic acid (40 μM) as substrate.

Antimicrobial assays.—The strains used for these experiments were *Bacillus stearothermophilus* (NCA 1518), *Escherichia coli* (B74-1, Nestec culture collection), *Bacillus cereus* (NCIB 3329), *Bacillus subtilis* (NCIB 3610), *Staphylococcus aureus* (T-AQ 49/YF29, Nestec culture collection), *Salmonella typhimurium* (NCIB 10248), *Pseudomonas aeruginosa* (ATCC 10145), *Pseudomonas putida* (T-AQ 18/YF18, Nestec culture collection), and *Streptococcus faecalis* (NCIB 775). The culture media were nutrient broth (nb: Oxoid CM 67), brain heart infusion (bhi: Oxoid CM 225), tyrptone salt (ts: 0.85% NaCl, 0.1% tryptone, in distilled H_2O), soft nutrient agar (sna: nb+0.7% agar), and plate count agar (pca: Difco). For the disc diffusion assays extracts or fractions (1 mg/disc) were applied on small sterile filter paper discs (6 mm) which were then placed in the center of a Petri dish containing 15 ml pca to which 3 ml sna with 1% of the test organism culture (grown for 16–18 h) had been added. Antimicrobial activity was detected by measuring the diameter of the area surrounding the filter paper disc where the organism did not grow. The direct bioautographic tlc assays were performed according to Hamburger and Cordell (12) with bhi as medium. The liquid culture assays were done with sterile Cookes Microtiter U-form plates. Bhi (200 μl) bacterial inoculum (containing 103–105 cfu/ml, 50 μl) and the required concentration of dicranin (dissolved in H_2O as the sodium salt) were added to the wells. Duplicate samples of 100 μl were pipetted off at times 0, 2, 4, 6, 8, and 24 h and diluted

with ts solution to obtain 10–500 bacteria/pca plate. The culture was incubated at its optimal growth temperature, and the colonies were counted after 24 h.

DICRANIN [**1**].—*Z,Z,Z*-Octadeca-6-yne-9,12,15-trienoic acid: eims *m/z* (rel. int.) $[M]^+$ 274 (0), 173 (32), 145 (30), 131 (43), 119 (21), 117 (71), 105 (50), 91 (100), 79 (69), 67 (46), 55 (25); ^{13}C nmr see Table 1.

RESULTS AND DISCUSSION

The extracts of the mosses (see Plant Material) were screened for their antioxidant and antimicrobial properties. Antioxidant assays carried out by measuring the rate of β-carotene bleaching in the linoleic acid/β-carotene system showed that the values were generally low (5–36% protection). The best result was obtained with the MeOH extract of *Po. formosum*.

The moss extracts were also tested as inhibitors of soybean 15-lpo (Fluka). Lipoxygenases are a group of closely related enzymes responsible for the dioxygenation of various polyenoic fatty acids containing all-*cis* methylene-interrupted double bonds, often linked to the production of undesirable flavors. Low to intermediate activities (<50%) were noted for most of the extracts, except for the CH_2Cl_2 extract of *D. scoparium*, which showed 90% 15-lpo inhibition at 100 μg/ml. For comparison: pure catechol produced 96% inhibition at 33 μg/ml.

In the test of moss extract activities at 1 mg/disc against both Gram-positive and Gram-negative bacteria (*E. coli, Bac. stearothermophilus, Bac. cereus, Bac. subtilis,* and *Sta. aureus*), the CH_2Cl_2 extract of *D. scoparium* was found to be the most active and the only one active against all the above microorganisms. Further tests with *Sal. typhimurium, Ps. aeruginosa,* and *Ps. putida* did not show any activity of the moss extracts.

The combined results obtained from the preliminary screening of the moss extracts indicated that *D. scoparium* (especially its CH_2Cl_2 extract) presented the most interesting biological activities among the tested mosses. Thus, a phytochemical investigation of *D. scoparium* was undertaken in order to isolate the active compounds.

The chromatographic separation of the CH_2Cl_2 extract of *D. scoparium* (see Experi-

TABLE 1. ^{13}C-nmr Data of Dicranin [**1**] in CD_3OD.

Carbon	Shift (ppm)	T_1(sec)	$^1J_{C-C}$ (Hz)[a]
C-1	177.25	20.4	55.1
C-2	34.36	1.6	34.4
C-3	25.14	1.6	34.9
C-4	29.44	1.5	34.2
C-5	19.12	1.4	68.0
C-6	80.17	7.8	n.m.[b]
C-7	79.25	8.1	68.7
C-8	17.75	2.6	42.5
C-9	126.47	5.0	71.1
C-10	129.94	4.1	42.0
C-11	26.28	4.2	42.1
C-12	128.35	5.9	n.m.[b]
C-13	129.62	6.0	42.1
C-14	26.32	6.1	42.1
C-15	128.03	10.3	70.3
C-16	132.71	9.8	42.3
C-17	21.41	13.8	34.3
C-18	14.67	10.0	

[a]Coupling constant with the next carbon in the chain from 2D INADEQUATE spectrum (± ca. 1 Hz).

[b]n.m.=not measured because only the inner lines of strongly coupled AB systems were observed.

mental), monitored by biological assays, afforded one main active compound which was labeled as dicranin [**13**]. The compound was submitted to dcims with NH_3 as reactant gas. The spectra, recorded in the negative ion mode, showed a quasi molecular ion at *m/z* 273 $[M-H]^-$. Dimer formation was observed at *m/z* 547 $[2M-H]^-$. In the positive ion mode, the quasi molecular ion appeared at *m/z* 292 $[M+NH_4]^+$. These spectra indicated that dicranin has a mol wt of 274. This was confirmed by cims of dicranin methyl ester from reaction with acetyl chloride, which gave a quasi molecular ion at *m/z* 306 $[M+NH_4]^+$. No extensive fragmentation was observed in these spectra. Eims of methylated dicranin showed a fragmentation pattern very similar to that of methylated octadeca-6-yne-9,12,15-trienoic acid isolated from *Riccia fluitans* (13). Double bond positions were determined by measuring eims of pyrrolidide and oxazoline derivatives, but the position of the triple bond could not be obtained because the corresponding peaks were lost in the bulk of all other fragments.

Preliminary nmr experiments also indicated an unsaturated C_{18} fatty acid with one triple bond and three double bonds. The positions of the triple bond and the double bonds could be determined by homonuclear proton-proton and heteronuclear carbon-proton correlation. The ^{1}H-nmr spectrum of dicranin showed a close similarity (within 0.04 ppm) with earlier data on the octadeca-6-yne-9,12,15-trienoic acid methyl ester in $CDCl_3$ (13). However, the six olefinic and two of the aliphatic proton signals could not be resolved individually at 360 MHz field strength; thus, the assignment of all the carbon signals was not possible via the above correlation experiments. The ^{13}C-nmr assignment given in Table 1 was therefore determined directly and the molecular structure was confirmed by a 2D INADEQUATE experiment. The contour plots of the relevant parts of this spectrum are shown in Figure 1. The skew line bisecting the horizontal doublet pairs is displayed to help interpretation. In order to improve the digital resolution in the double quantum frequency direction, the vertical spectral width was chosen such that foldback of the purely aliphatic correlation signals occurred. All weakly coupled signal pairs were identified, while for the strongly coupled AB systems between C-13 and C-

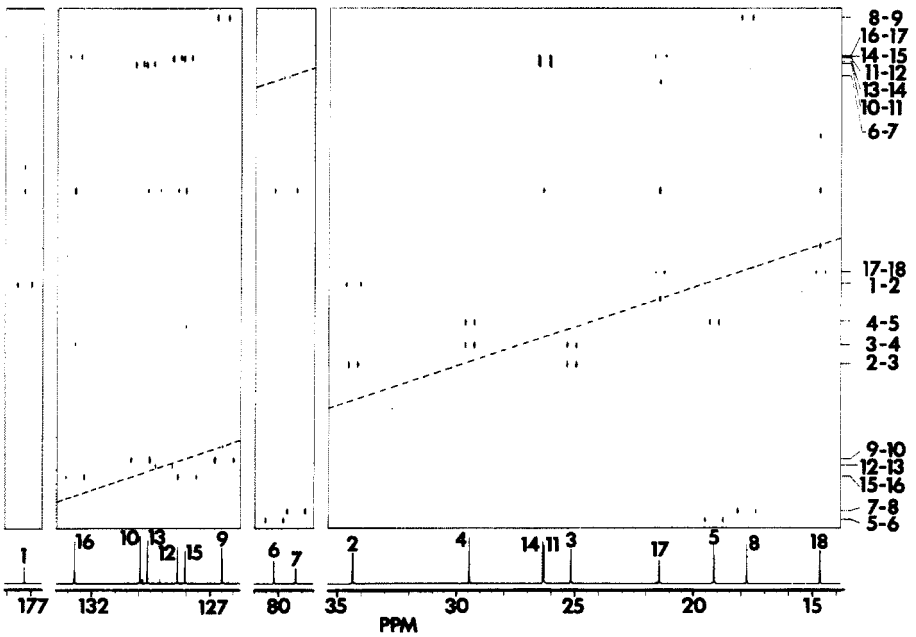

FIGURE 1. Relevant parts of ^{13}C-nmr 2D INADEQUATE spectrum of dicranin, with one-bond correlations indicated on the right.

12 and between C-6 and C-7 only the two inner lines could be clearly distinguished. Thus, the corresponding C-C bonds were verified, but their coupling constants could not be reliably determined. The near degeneracy of C-14 and C-11 presented a special problem, requiring good horizontal digital resolution. Figure 2, an enlargement of the two crowded regions in Figure 1, clearly reveals the olefinic coupling partners of C-14 and C-11. The ^{13}C-induced isotope shifts were verified for all the well determined doublets and were found to be small, although in four cases negative shifts of 0.028 to 0.055 ppm were noted. This is comparable to the shift difference between C-14 and C-11, and thus a shift inversion between the single quantum and the double quantum spectrum due to isotope effects could not be excluded with certainty. However, the T_1 relaxation time permitted a clear distinction. Comparing only methine or methylene carbons among themselves, the T_1 values have a tendency to increase from C-5 towards the end of the chain due to additional motional freedom, if one compares carbons separated by more than one bond. This probably indicates head to head association of the acid molecules in the MeOH solution. The T_1 of 6.1 sec vs. 4.2 sec thus clearly distinguishes between C-14 and C-11. No previous ^{13}C-nmr spectrum of *Z,Z,Z*-octadeca-6-yne-9,12,15-trienoic acid was found in the literature, and in earlier partial ^{13}C-nmr data of its methyl ester in $CDCl_3$ only 9 out of 19 signals were individually listed and assigned (13). The 2D INADEQUATE experiment which yielded both the unequivocal molecular structure and the complete ^{13}C signal assignment is a straightforward alternative to the elegant but labor-intensive structure elucidation by combined chemical degradation and optical spectroscopy reported earlier (14). The ir spectrum of dicranin indicated a cis configuration for the double bonds by the absence of a CH deformation band at 960–970 cm^{-1} and the presence of a CH deformation band at 710 cm^{-1}. Considering all of the above data, dicranin was identified as Z,Z,Z-octadeca-6-yne-9,12,15-trienoic acid.

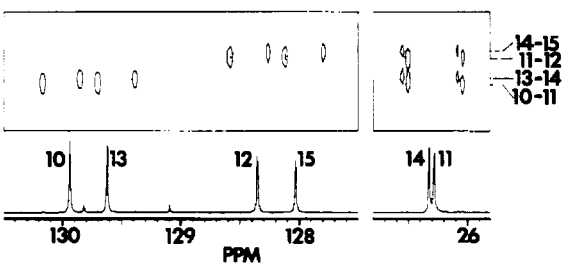

FIGURE 2. Expansion of the two regions of Figure 1 determining the assignments of C-11 and C-14.

The isolated dicranin was tested on 15-lpo and for antimicrobial activity. Down to 3.6 μM of dicranin, 100% inhibition of 15-lpo was observed. At 0.36 μM 37% of inhibition was recorded. For comparison, the potent inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA) gave 95% of inhibition at 4 μM. Although acetylenic compounds are known to bind irreversibly to lipoxygenases, we do not have, at this time, enough information to propose a mechanism of inhibition. Further experiments will be necessary to verify the above hypothesis and to determine the IC_{50} of dicranin.

The known antimicrobial activity of fatty acids (15) led us to choose palmitoleic acid as a standard in our disc diffusion assays. The antimicrobial activity of dicranin was tested against three strains of bacilli and two strains of cocci. The results are shown in Table 2. A positive inhibition of the tested organisms was recorded down to 25 μg of dicranin/disc. The best result was obtained with *Strep. faecalis* which was inhibited down to 10 μg/disc. Further studies on *E. coli, Sal. typhimurium, Ps. aeruginosa,* and *Ps. putida* showed that

TABLE 2. Antibacterial Activity of Dicranin [1]: Diameter of Growth Inhibition in mm.

Test Organism	Control[a]	Dicranin								
	1000[b]	1000	500	250	125	100	75	50	25	10
Bacillus stearothermophilus ...	45	35	32	26	17	16	14	11	9	0
Bacillus cereus	14	25	22	19	16	15	11	10	9	0
Bacillus subtilis	14	20	19	16	14	13	13	11	8	0
Staphyloccus aureus	22	23	22	21	17	16	12	11	10	0
Streptococcus faecalis	20	ND[c]	ND	ND	ND	ND	13	12	10	6

[a]Palmitoleic acid.
[b]Quantity (μg) of antibacterial applied to disc (6 mm diam.).
[c]ND: not determined.

dicranin (1 mg/disc) seems devoid of antibacterial activity against Gram-negative bacteria. For *E. coli,* this is in contrast to the activity recorded with the whole CH_2Cl_2 extract. For comparison *Z,Z,Z,Z*-octadeca-6,9,12,15-tetraenoic acid was tested against the same Gram-positive and Gram-negative microorganisms, and results similar to those for dicranin were obtained. Thus, it seems that antibacterial activity of dicranin is not due to the presence of the triple bond at position C-6.

In order to know if dicranin was a bactericide or bacteriostatic against *Bacillus* strains, liquid cultures were initiated. Dicranin was tested from 200 μg/ml to 10 μg/ml against *Bac. cereus, Bac. subtilis,* and *Bac. stearothermophilus.* Figure 3 shows characteristic curves recorded for *Bac. subtilis*. For the mesophilic bacillus *Bac. cereus*, dicranin was bactericidal down to 150 μg/ml. At 100 μg/ml, a bactericidal effect was observed during the first 4 h of incubation. After this time, a slow bacterial growth occurred. Growth inhibition was observed at 50 μg/ml, whereas at 10 μg/ml no antibacterial activity was recorded. For the thermophilic bacillus *Bac. stearothermophilus*, dicranin was bactericidal at 200 μg/ml. From 150 to 50 μg/ml a bactericidal effect was observed during the first 4 h. After this time the compound was bacteriostatic for the surviving bacteria. At 10 μg/ml only a weak growth inhibition was observed. Further experiments are necessary to access exact MIC/MBC values of dicranin.

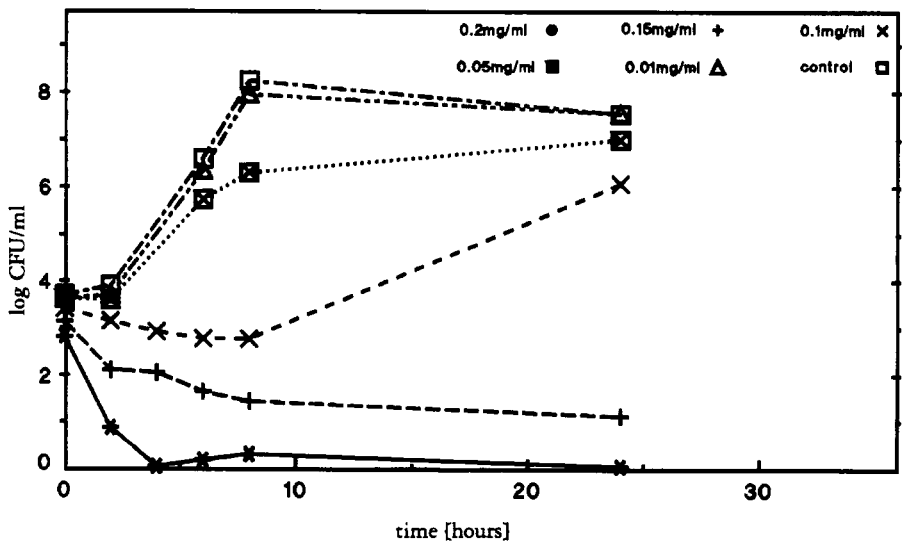

FIGURE 3. Antibacterial activity against *Bacillus subtilis* NC1B 3610. Control: microbial growth without inhibitory agent.

Z,Z,Z-Octadeca-6-yne-9,12,15-trienoic acid was first identified in mosses by Åndersson *et al.* (14). Its biosynthesis in *Ceratodon purpureus* was elucidated (16). Further studies revealed its presence in many Ricciaceae, Ditrichaceae, and Dicranaceae (13,17–20). The dicranin-related compounds *Z,Z*-octadeca-6-yne-9,12-dienoic acid, 1 mono and 1,2 diglyceride of *Z,Z,Z*-octadeca-6-yne-9,12,15-trienoic acid, 13-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrien-6-ynoic acid, and two cyclopentenones were also isolated from *D. scoparium* (18,21,22). Four of these compounds were patented for their antimicrobial activity against *Saccharomyces* species, *E. coli, Bac. subtilis, Ps. aeruginosa,* and *Piricularia oryzae* (21,22).

Our disc diffusion assays performed with dicranin have shown that this compound inhibits the growth of *Bac. stearothermophilus, Bac. cereus, Bac. subtilis, Sta. aureus,* and *Strep. faecalis.* In addition, liquid culture assays demonstrated that dicranin was bactericidal against the mesophilic bacillus *Bac. cereus* and the thermophilic bacillus *Bac. stearothermophilus.* Today natural preservatives are in increasing demand by the consumer. The use of molecules such as antimicrobial fatty acids could be an alternative way of preserving food or cosmetics and could be included in preservation concepts (15). Our study also showed that dicranin is a potent inhibitor of soybean 15-lpo. Human lipoxygenases are involved in various inflammation processes. Therefore the activity of dicranin on these enzymes was also investigated. Important variations of the arachidonic acid metabolism in human platelets were observed (23).

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