TETRONIC ACID DERIVATIVES FROM *ASPERGILLUS PANAMENSIS*

PRODUCTION, ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITY*

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When *Aspergillus panamensis*, CBS 120.45, was grown on a medium with high carbon and low nitrogen content, it was found to produce six antibiotically active metabolites. All compounds were identified as tetronic acid derivatives; three of them were found to be identical with gregatins A, B, and D, previously reported as metabolites of *Cephalosporium gregatum*. The other three compounds are new. All six compounds show antibacterial activity towards Gram-negative and Gram-positive bacteria. Five of the antibiotics exhibit inhibitory effects on the macromolecular syntheses in cells of the ascitic form of EHRLICH carcinoma (ECA) of mice.

During our search for new metabolites from those species of the genus *Aspergillus*, which so far have not been reported to produce biologically active metabolites, *Aspergillus panamensis* was found to produce several antimicrobial compounds.

In this paper we wish to report the production, isolation, characterization, and biological activity of these antibiotics.

Materials and Methods

Cultivation of organisms

Bacteria (except Actinomycetales) were grown on nutrient broth (Difco). Actinomycetales and fungi were grown on a medium composed of: malt extract 10 g; yeast extract 4 g; and glucose 4 g per liter (HA medium).

Fermentation of *Aspergillus panamensis*

In order to select the most suitable production medium and culture conditions fermentations were carried out in 500-ml Erlenmeyer flasks, with one intrusion containing 150 ml of medium. The flasks were incubated on a rotary shaker (120 rpm) at 27°C. The conditions for the fermentors were: (a) 25 liters (type b 20, Braun Melsungen AG, Melsungen, FRG) with "intensor" system: 800 rpm, 6 liters air/min., 27°C; (b) 200 liters (type b 200, AG für biologische Verfahrenstechnik, Giovanola Frères SA, Monthey, Switzerland) with "intensor" system: 800 rpm, 50 liters air/min., 27°C. To prevent foaming, silicone antifoam (Merck) was added. During fermentation the pH was measured with a glass electrode (Ingold, Zürich, Switzerland).

Assays

The disc-diffusion assay with *Bacillus brevis* (ATCC 9999) was used for measuring the antibiotic

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content of the culture filtrate and mycelial extracts.

The effect of the isolated compounds on the macromolecular syntheses in bacteria and EHRLICH carcinoma ascitic cells (ECA cells) was tested as previously described by ANKE[3].

Mice bearing ECA were a gift from H. G. PROBST, Tübingen.

Chemicals

TLC plates were precoated with silica gel 60 F_{254} or Al_2O_3 F_{254} (Merck); for column chromatography silica gel (Mallinckrodt, 100 mesh) was used. For the chemical detection of metabolites molybdatophosphoric acid spray reagent (Merck) was employed. Organic solvents were from Merck and reagent grade. Radioactively labeled compounds were purchased from Amersham Buchler.

Physico-chemical and spectroscopic measurements

$[\alpha]_D$ values were measured in $CHCl_3$ at room temperature; UV spectra were recorded in MeOH: ^{1}H-NMR spectra in $CDCl_3$ at 250 MHz (Bruker WM 250) or 90 MHz (Varian EM 390) with TMS as the internal standard. Mass spectra were run on a Varian-MAT 312 with an EI/EID ion source (at 70 eV) using the direct inlet system.

Chemical reactions

Hydrogenations were carried out in ethyl acetate in the presence of palladium on charcoal (10%) at room temperature (12 hours) under 760 torr hydrogen.

Chemical degradations on 704-I were performed according to known procedures[5,6].

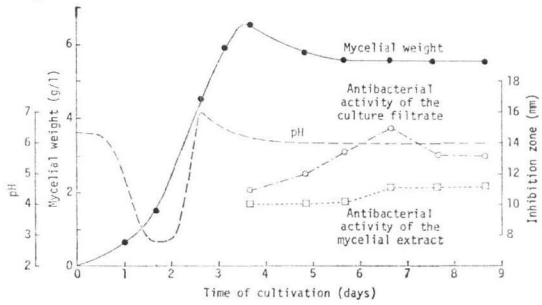
Results

Production of Tetronic Acids by *Aspergillus panamensis*

Production of antimicrobial compounds was only observed under specific culture conditions. No antibiotic production was obtained in media containing high amounts of nitrogen, or even in "balanced" media like RAULIN-THOM, CZAPEK-DOX, asparagine medium or HA medium. Finally the chosen production medium was composed of: glucose 100 g; malt extract 0.4 g; yeast extract 0.4 g; NH_4NO_3 0.4 g; KH_2PO_4 0.4 g; $MgSO_4$ 0.4 g per liter. If the carbon-nitrogen ratio was lowered by raising the NH_4NO_3 content, no antibiotic activity could be detected in the culture broth or in the mycelium. Variation of the starting pH of the culture between 3.5 and 6.5 did not influence the antibiotic production nor did the incubation temperature within the range from 22°C to 32°C. The aeration rate was also not critical for antibiotic production.

Aspergillus panamensis, Tü 704, was maintained on HA agar slants. Spores from one plate (ϕ 12 cm) were used to inoculate 20 liters of the production medium. The production of antibiotically active compounds started after 4~6 days and continued for two or three days. After 6~8 days the culture was harvested. A typical fermentation diagram is shown in Fig. 1. Two thirds of the antibiotic activity was excreted into the culture broth and was extracted from the broth with ethyl acetate. One third could be extracted from the mycelium with acetone. Analysis of the extracts by means of TLC and bioautography revealed, that six antibiotically active compounds were present in the culture filtrate. In the mycelium, however, only one antibiotic metabolite was found. This compound (704-I) was identical with the most lipophilic antibiotic extracted from the culture

Fig. 1. Time-course of fermentation of *Aspergillus panamensis*.



filtrate. During fermentation, even at early stages, the culture filtrate always contained all six components in the same ratio, indicating that all compounds were synthesized *de novo* rather than being derived from each other.

However, during the isolation procedure 704-V and -VI tended to disintegrate. Furthermore the concentration of 704-II increased drastically when methanol was used during the isolation, while the amount of 704-I decreased correspondingly. In order to avoid this "artificial" production of 704-II or other artefacts methanol as well as other alcohols or acids were not used during the isolation procedure.

Isolation of the Antibiotics

The mycelia were separated using a BUCHNER funnel and extracted twice with acetone. The acetone extract was concentrated and the aqueous residue was extracted with chloroform. The antibiotic content of the extract was examined by means of TLC and bioautography. In order to detect impurities not visible under UV light, the chromatogram was sprayed with molybdatophosphoric acid reagent.

The chloroform extract was applied to a silica-gel column and eluted with chloroform. Fractions containing antibiotics were identified by means of the agar diffusion assay with *Bacillus brevis* as test organism and pooled. After evaporation of the solvent the residue was dissolved in carbon tetrachloride - chloroform (1: 1) and rechromatographed over silica-gel using carbon tetrachloride - chloroform (1: 2) as eluent. Last impurities were removed by chromatography over Sephadex LH-20 with chloroform.

Since 704-I proved to be rather unstable in the presence of acid or base, the chloroform was treated with basic Al_2O_3 prior to use and all columns were run in the dark. The isolation scheme for the culture broth is given in Fig. 2. Mixtures of the antibiotics were analyzed by means of TLC (with chloroform and chloroform - methanol (9: 1) as solvents) and bioautography.

Fig. 2. Isolation scheme for the metabolites of *Aspergillus panamensis*.

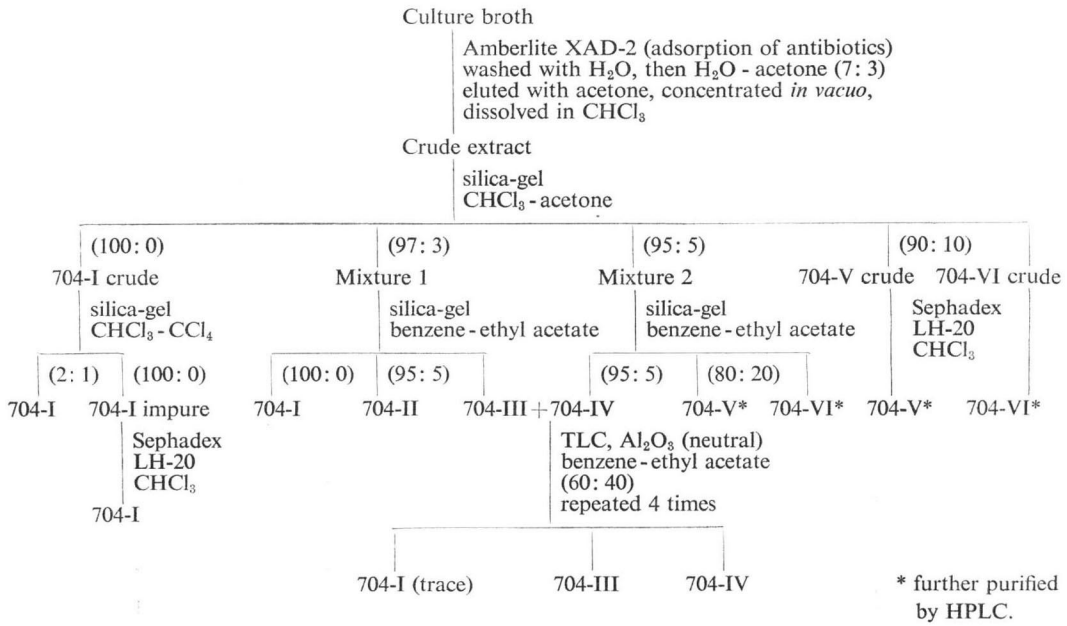


Table 1. Physico-chemical data of metabolites 704-I, -II, -III and -IV.

Metabolite	$[\alpha]_D$	UV: λ_{max} (log ε) [nm]	IR [cm^{-1}]	MS molecular ion	^{1}H-NMR [ppm]	
					Signals, present in all four metabolites:	Signals, characteristic for individual compounds:
704-I m. p. 70~71°C	−144°(*c* 2)	230(4.3) 240sh(4.34) 300(4.03)	1740(sh) 1705	276.1357($C_{16}H_{20}O_4$)	6.24(1H, dd, J_1=15, J_2=10Hz) 6.0~5.7(2H, m) 5.54(1H, d, J=15Hz) 3.83 (3H, s: $OC\underline{H}_3$) 2.06 (2H, m: $C\underline{H}_2$–CH_3) 1.53 (3H, s: >C–$C\underline{H}_3$) 0.96 (3H, t, J=7Hz: CH_2–$C\underline{H}_3$)	7.32(1H, d-broad, J=15Hz: CO–C<u>H</u>=CH–CH_3) 7.18(1H, dq, J_1=15, J_2=6.5Hz: CH=C<u>H</u>–CH_3) 2.05(3H, dd, J_1=6.5, J_2~1Hz: CH=CH–$C\underline{H}_3$)
704-II colourless oil	+28°(*c* 0.5)	235(3.97) 265sh(3.78)	1740(sh) 1705	308.1625($C_{17}H_{24}O_5$)		(90MHz): 3.9~3.8(1H, m: C<u>H</u>OCH_3) 3.4~3.0(2H: CO–$C\underline{H}_2$–$CHOCH_3$) 3.3 (3H, s: $OC\underline{H}_3$) 1.2 (3H, d, J~6Hz: $CHOCH_3$–$C\underline{H}_3$)
704-III colourless oil	+205°(*c* 0.1)	235 (4.03) 265sh (3.85)	1740(sh) 1705	250.1215($C_{14}H_{18}O_4$)		(90MHz): 2.6 (3H, s: CO–$C\underline{H}_3$)
704-IV colourless oil	+72° (*c* 0.1)	235 (3.99) 265sh (3.80)	1740(sh) 1705	294.1456($C_{16}H_{22}O_5$)		4.31 (1H, m: >C<u>H</u>OH) 3.18 (2H, m: CO–$C\underline{H}_2$–CHOH) 1.32 (3H, d, J=7Hz: CHOH–$C\underline{H}_3$)

Physico-chemical and Chemical Properties

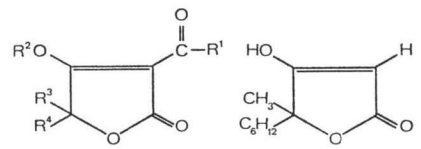
Compounds 704-V and 704-VI are comparatively unstable and, if they are pure, even decompose upon concentration of their solutions; therefore they were subjected to special investigations and their structural elucidation will be reported elsewhere. However, when treated with acid, 704-V, as well as 704-VI, are split into a hydrophilic (alcoholic) and a lipophilic component, the latter being identical with 704-I. The physico-chemical characteristics of compounds 704-I to 704-IV are summarized in Table 1.

Upon catalytical hydrogenation 704-I yields a hexahydro-derivative, whereas metabolites 704-II to 704-IV take up only two equivalents H_2. After hydrogenation the UV absorptions of all four metabolites are identical [λ_{max} (MeOH): 262 nm, $\varepsilon \sim 10^4$] and indicate, that the substances belong to the 3-acyltetronic acid alkyl ether series with cross structure **1**[4] (Fig. 3). This structural assignment was confirmed by alkali-catalyzed hydrolysis of hexahydro-704-I with NaOH[5] (which caused the loss of a CH_3-group), followed by degradation with Br_2 and dehalogenation with H_2/Pd.[6] This sequence of reactions yielded a product ($[\alpha]_D^{20}$ +10° (*c* 0.3)), whose data were identical in every respect(except $[\alpha]_D$) with those published for (−)-5-hexyl-4-hydroxy-5-methylbut-3-enolide (**2**)[7] (Fig. 3).

Table 2. Structures of side chains R^1 in **1**. (R^2=R^3=CH_3; R^4=-(CH=CH)$_2$-C_2H_5)

Metabolite	R^1
704-I	-CH=CH-CH_3
704-II	-CH_2-CHOH-CH_3
704-III	-CH_3
704-IV	-CH_2-CHOH-CH_3

Fig. 3. Cross structure of metabolites and degradation product of 704-I.



1 **2**

From the ^{1}H-NMR data it can be concluded that the ring system **1** in all metabolites is identically substituted with: R^2=R^3=CH_3; R^4=-(CH=CH)$_2$-C_2H_5. The individual metabolites differ only in substituent R^1. The structure of this side chain can also be deduced from the ^{1}H-NMR spectra; the results are listed in Table 2.

704-II, as well as 704-IV, is converted in high yield into 704-I by treatment with acid (*e.g.* acetone/one drop H_2SO_4, 12 hours, room temperature) or base (*e.g.* pyridine, 12 hours, room temperature).

On the other hand, in the presence of catalytic amounts of acid or base, alcohols and water can be added to the double bond in the side chain R^1 of 704-I. This readily occurring reaction raises the question, whether or not 704-II and/or 704-IV are artefacts produced during the isolation procedure. However, since the $[\alpha]_D$-values for 704-II as well as 704-IV are significantly different from those of the corresponding synthetic products formed by addition of methanol or water to 704-I, all isolated compounds are considered to be genuine metabolites.

Metabolites 704-I and 704-IV are identical with gregatin A and gregatin D, respectively, whereas 704-III is identical with gregatin B[8,9]. The gregatins were recently reported as metabolites from *Cephalosporium gregatum*[8,9]. Gregatin A and D are enantiomers of aspertetronins A and B, respectively, from *Aspergillus rugulosus*[7]. However, metabolites 704-II, 704-V and 704-VI from *Aspergillus panamensis* are new tetronic acid derivatives and the subjects of further chemical investigations.

Biological Activity

Antibacterial Activity

All tetronic acid derivatives isolated from the culture broth of *Aspergillus panamensis* show anti-

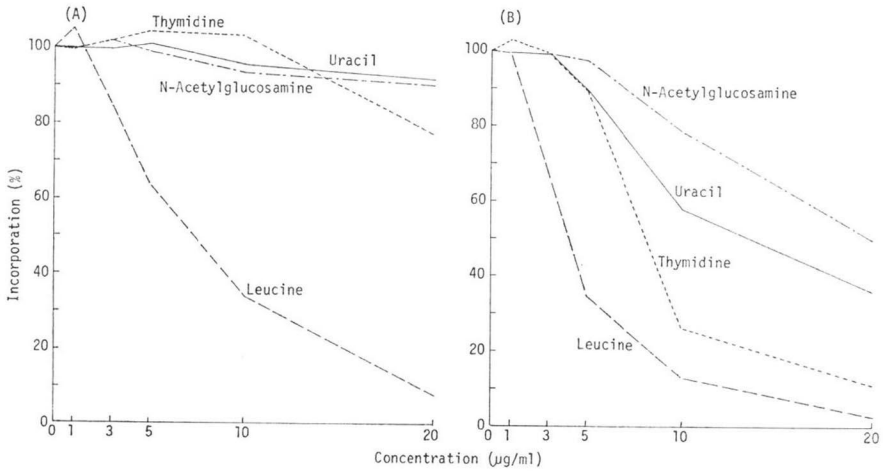
Table 3. Minimum inhibitory concentrations of tetronic acids from *Aspergillus panamensis*.
Serial dilution test in nutrient broth (I), HA medium (II) or minimal medium (III); size of inoculum: 10^6 cells or spores per ml.

Organism	Medium	MIC (μg/ml)					
		704-I	704-II	704-III	704-IV	704-V	704-VI
Acinetobacter calcoaceticus	I	<1	20	50	20	5	5
Bacillus brevis	I	<1	20	50	20	20	15
B. subtilis ATCC 6633	I	5	>50	>50	>50	>50	50
B. subtilis ATCC 6051	I	3	>50	>50	>50	>50	>50
B. subtilis ATCC 6051	III	1	50	>50	50	20	10
Escherichia coli D 22	I	1	50	50	50	15	10
E. coli K 12	I	>50	>50	>50	>50	>50	>50
Proteus vulgaris	I	5	>50	>50	>50	>50	50
Pseudomonas fluorescens	I	>50	>50	>50	>50	>50	>50
Staphylococcus aureus	I	1	50	>50	50	50	50
Streptomyces viridochromogenes	II	1	>50	>50	>50	20	20

bacterial activity. The minimum inhibitory concentrations (MIC) against *Acinetobacter calcoaceticus* and *Bacillus brevis*, the most sensitive organisms, vary from less than 1 μg/ml for 704-I to 50 μg/ml for 704-III, as shown in Table 3. 704-I is the most active among the isolated antibiotics, inhibiting both Gram-negative and Gram-positive bacteria. Strains resistant towards 704-I were found among the Gram-negative bacteria. The MICs are three times higher in complex media than in minimal media.

With cells of *B. subtilis* the effect of 704-I on the macromolecular syntheses was tested. Fig. 4A shows that, in the presence of 7 μg/ml 704-I, the incorporation of leucine is reduced to 50%, whereas the incorporation of thymidine, uracil, and N-acetylglucosamine is not affected. At higher concentrations *e.g.* 20 μg/ml the incorporation of thymidine is also somewhat decreased. In minimal medium, where the MICs are lower, the effect on the macromolecular syntheses is stronger as depicted in Fig. 4B. For

Fig. 4. Effect of 704-I on the incorporation of thymidine, uracil, leucine, and N-acetylglucosamine into TCA-precipitable material of cells of *Bacillus subtilis* in complex medium (A) and in minimal medium (B). The incorporation was calculated as a percentage of the control containing no antibiotic.



50% inhibition of incorporation the following concentrations were needed: leucine: 4 μg/ml; thymidine: 8 μg/ml; uracil: 14 μg/ml; N-acetylglucosamine: 20 μg/ml.

Antifungal Activity

The inhibitory effects of 704-I, -V, and -VI on the growth of yeasts and fungi are summarized in Table 4. Gregatin B (704-III) and gregatin D (704-IV) have been tested against a variety of fungi by KOBAYASHI and UI[9], therefore we omitted these compounds from extensive testing. 704-II (20 μg/ml) showed no inhibitory effect on the tested fungi. The highest inhibitory activity again was found for 704-I. Almost all of the tested strains were sensitive to this compound. The antifungal activity of 704-V and -VI are almost identical. Both compounds were only active at high concentrations (50 μg/disc), whereas 704-I showed inhibitory activity at 10 μg/disc.

Table 4. Inhibition of yeasts and filamentous fungi by 704-I, -V, and -VI.

Agar plate diffusion test; size of inoculum: 10^6 cells (yeasts) or spores per ml HA medium. Discs (φ 6 mm) bearing 10 μg and 50 μg were assayed.

Organism	Inhibition zone (mm)					
	704-I		704-V		704-VI	
	10 μg	50 μg	10 μg	50 μg	10 μg	50 μg
Ascoidea rubescens	10	14	−	−	−	−
Aspergillus deflectus	+	11	−	−	−	−
Aspergillus panamensis	10	15	−	9	−	9
Botrytis cinerea	+	18	−	−	−	−
Candida albicans	+	10	−	+	−	−
Coprinus cinereus	−	+	−	+	−	+
Curvularia spec.	17	22	−	10	−	9
Dipodascus albidus	−	14	−	−	−	−
Endomyces magnusi	+	11	−	−	−	−
Endomycopsis fibuliger	+	12	−	10	−	+
Eremascus fertilis	12	18	−	+	−	+
Eurotium cristatum	14	28	−	−	−	−
Fusarium oxysporum	−	11	−	9	−	9
Hansenula anomala	−	+	−	+	−	+
Mucor miehei	14	20	−	10	−	10
Nadsonia fulvescens	+	10	−	10	−	10
Nematospora corylii	13	20	+	14	−	13
Neurospora crassa	10	18	−	13	−	13
Rhodotorula rubra	−	+	−	+	−	+
Paecilomyces varioti	+	19	+	17	+	16
Penicillium tardum	−	+	−	+	−	+
Pichia farinosa	+	8	−	−	−	−
Saccharomyces cerevisiae	+	10	−	+	−	+
Schizosaccharomyces pombe	12	24	−	14	−	14

−: no inhibition zone; +: very small inhibition zone

Inhibitory Effect on ECA Cells

The incorporation of thymidine, uridine, and leucine into trichloroacetic acid-precipitable material was strongly inhibited in the presence of 704-I as shown in Fig. 5. The addition of 10 μg/ml of 704-I reduced the incorporation of uridine and thymidine to one tenth; protein synthesis was less affected: the incorporation of leucine was only reduced to one half. The other tetronic acid derivatives were less in-

Table 5. Effect of tetronic acids from *Aspergillus panamensis* on the incorporation of thymidine, uridine, and leucine into TCA-precipitable material in cells of the ascitic form of EHRLICH carcinoma.

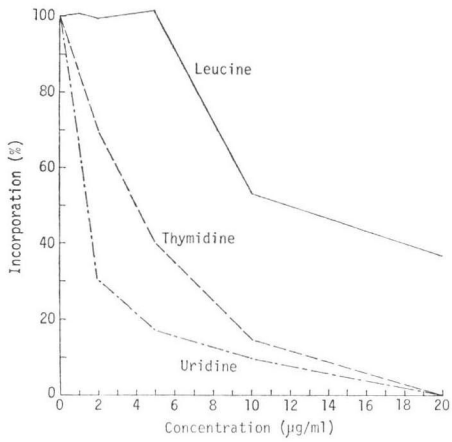
The incorporation was calculated as a percentage of the control containing the appropriate solvent without antibiotic.

Antibiotic	μg/ml	Incorporation (%)			Antibiotic	μg/ml	Incorporation (%)		
		Thymidine	Uridine	Leucine			Thymidine	Uridine	Leucine
704-I	10	15	10	55	704-IV	10	101	108	109
	20	0	0	37		50	102	110	107
704-II	10	98	82	104	704-V	10	100	76	105
	50	55	28	54		50	9	8	7
704-III	10	104	100	108	704-VI	10	102	78	100
	50	91	64	100		50	8	4	7

hibitory than 704-I, as indicated in Table 5. At concentrations up to 50 μg/ml, 704-IV had no effect at all. 704-V and -VI were again equally effective. At 50 μg/ml both compounds completely inhibited the incorporation of all three precursors. 704-II showed weak inhibitory activity at 50 μg/ml, and 704-III (50 μg/ml) reduced only the incorporation of uridine to 60%.

Fig. 5. Effect of 704-I on the macromolecular syntheses in cells of the ascitic form of EHRLICH carcinoma.

The incorporation of precursors was calculated as a percentage of the control containing no antibiotic.



Discussion

Tetronic acids are common secondary metabolites from fungi, especially from strains of the genus *Penicillium*[10,11]. They are also produced by yeasts[10] and species of the genera *Cephalosporium*[8,12] and *Aspergillus*[7]. It is interesting, that three tetronic acids from *Aspergillus panamensis* are identical with compounds produced by *Cephalosporium gregatum*. Furthermore, two of them—namely 704-I (gregatin A) and 704-IV (gregatin D)—are the enantiomers of aspertetronins A and B, which are known metabolites from cultures of *A. rugulosus*[7].

The production of tetronic acids by *Penicillium* and *Cephalosporium* species occurs in media commonly employed for the cultivation of fungi, but both *A. panamensis* and *A. rugulosus* were found to produce these compounds only when grown in media of low nitrogen content.

In all test systems 704-I exhibited the highest inhibitory activities. 704-III showed only weak antibacterial activity towards the most sensitive organisms. 704-II and -IV were slightly more active than 704-III.

704-III is the only tetronic acid from *A. panamensis* with a two carbon side chain in position 3. All others have a longer chain in this position, *e.g.* crotonyl group in 704-I. 704-II, -IV, -V, and -VI are theoretically derived from 704-I through addition of various alcohols or water to the double bond of the crotonyl side chain. The removal of the double bond of the crotonyl group is accompanied by the expected loss of the chemical reactivity of 704-I and a drastic decrease in the biological activity. Thus in 704-IV (gregatin D) reduced phytotoxicity is observed[9] with 100-fold increased MICs for bacteria, and with no inhibitory effects on ECA cells. Therefore the double bond of the crotonyl side chain can be considered to be the structural feature most important for the biological activities of 704-I. A similar

effect has been reported for pyrenocin A, a phytotoxic metabolite of *Pyrenochaeta terrestris*. Pyrenocin A possesses a crotonyl side chain on a furan ring. Addition of water leads to pyrenocin B, a metabolic product of the same fungus. The inhibitory effect upon germination of seeds and root elongation of lettuce and other plants was 50~100-fold higher for pyrenocin A than for B[13]. The formal addition of methanol as in 704-II also leads to a tetronic acid derivative with reduced biological activity as compared to the "parent" compound 704-I. The alcoholic components added to the crotonyl side chain in 704-V and -VI are very readily split off with regeneration of 704-I; this hinders the isolation of these compounds, unless precautions are taken. Therefore it cannot be ruled out, that, particularly in the case of 704-V and -VI, the active species derived in the biological assays during incubation is 704-I possibly regenerated within the cell. This is supported by the fact, that the more readily the double bond is reformed, the greater is the biological activity of the compound.

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