ANTIBIOTICS FROM BASIDIOMYCETES. XVII[1]

THE EFFECT OF MARASMIC ACID ON NUCLEIC ACID METABOLISM

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From submerged cultures of *Lachnella villosa*, *Lachnella* sp. 541, and *Peniophora laeta* we isolated marasmic acid (**1**), a metabolite first described from surface cultures of *Marasmius conigenus*. The sesquiterpenoid exhibits potent antimicrobial and cytotoxic properties. In cells of the ascitic form of Ehrlich carcinoma RNA and DNA syntheses are preferentially inhibited. Marasmic acid inhibits RNA synthesis in isolated nuclei, but does not interfere with the transport of nucleoside precursors into the cells. RNA polymerase II and capping enzyme (mRNA guanylyltransferase), two enzymes of nucleic acid metabolism, are markedly affected after preincubation with marasmic acid. We assume that marasmic acid acts on nucleic acid syntheses by direct inhibition of some of the enzymes involved. This mode of action would also explain its mutagenic properties.

The preparation and testing of two derivatives, **2** and **3**, revealed that the α,β-unsaturated aldehyde is essential for the antimicrobial and cytotoxic activity of marasmic acid.

In the course of the first extensive screening of basidiomycetes a highly potent antibacterial compound, marasmic acid, was isolated from cultures of *Marasmius conigenus*[2]. The sesquiterpenoid structure of marasmic acid was elucidated in 1966[3]. GREENLEE and WOODWARD achieved the first total synthesis in 1976[4] and only recently new synthetic approaches have been published[5]. Except for some data on the antimicrobial activity in the first publication, the mode of action of this antibiotic had not been investigated.

During our screening of basidiomycetes for antimicrobial and cytotoxic compounds, marasmic acid was detected in the culture broth of several, taxonomically not related species. In this report we wish to describe our studies on its biological activities both in whole cells and in cell-free preparations.

Results and Discussion

Lachnella villosa, Lachnella sp. 541, and *Peniophora laeta* were grown in submerged culture in complex media as described earlier[1].

The antibiotic was extracted from the culture broth with ethyl acetate and purified by several chromatographic steps.

The identity of the metabolite with marasmic acid was determined by MS and ^{1}H NMR spectra. Authentic marasmic acid, kindly provided by P. DE MAYO, London, Canada, was used for comparison.

Strobilurus stephanocystis (Hora) Sing. has been suggested by SINGER[6] to be synonymous with *M. conigenus* (Pers. ex Fr.) Karst, from which marasmic acid had been first described. A strain of *S. stephanocystis* was therefore tested for the production of marasmic acid. None could be detected, our

strain produced strobilurins A and B as the sole antibiotics like the other species of this genus[7].

Marasmic acid exhibits a broad antimicrobial spectrum. As already determined by KAVANAGH *et al.*[2] and confirmed with our test-organisms, marasmic acid inhibits Gram-negative and Gram-positive bacteria at concentrations of $1 \sim 10$ μg/ml. In addition, we found a pronounced inhibition of the phytopathogenic fungi *Ceratocystis fimbriata, Botrytis cinerea* and *Sclerotinia fructigena* by marasmic acid at $10 \sim 50$ μg per disc in the plate diffusion assay. $NaBH_4$-reduction[3] of the molecule led to the derivative **2** which no longer showed antibiotic activity. In contrast, methylation with diazomethane[8] resulted in methyl marasmate (**3**) and no loss in antimicrobial activity. We therefore assume that the α,β-unsaturated aldehyde or in the case of **3** the dialdehyde group is essential for the antibiotic activity of marasmic acid and methyl marasmate. Interestingly the addition of cysteine has no effect on the antimicrobial and cytotoxic activities of marasmic acid.

The effect of marasmic acid and methyl marasmate on eucaryotic macromolecular syntheses was tested in cells of the ascitic form of Ehrlich carcinoma (ECA). Both compounds strongly interfere with the incorporation of thymidine and uridine into DNA and RNA, respectively. At a concentration of 3 μg/ml of compound **1** or **3** DNA and RNA syntheses were almost completely inhibited, whereas protein synthesis was much less impaired.

As shown in Fig. 1 the same concentration of marasmic acid did not inhibit the transport of the precursors thymidine and uridine into the cells in a test system as described earlier[8], thus pointing to a direct interference of marasmic acid with nucleic acid synthesis. The same effect, a preferential inhibition of DNA and RNA syntheses without interference with transport of the precursors was found with *Bacillus brevis* cells. DNA and RNA syntheses were directly tested in toluene-treated *B. brevis* cells and in isolated nuclei from ECA cells. As shown in Table 1 marasmic acid inhibits the incorporation of dTMP into DNA at relatively high concentrations. The cell density in this test, however, was about two orders of magnitude higher as compared to the cell density used for the evaluation of the minimal inhibitory concentration (for *B. brevis* 2 μg/ml in the serial dilution assay).

The effect of marasmic acid on eucaryotic RNA synthesis is more pronounced. As shown in Table

Fig. 1. Comparison of uptake and incorporation of [^{14}C]leucine, [^{14}C]uridine, and [^{14}C]thymidine in ECA cells.

The experiment was carried out according to reference 8. The assays containing 3 μg/ml marasmic acid were compared to controls containing no antibiotic (=100%).

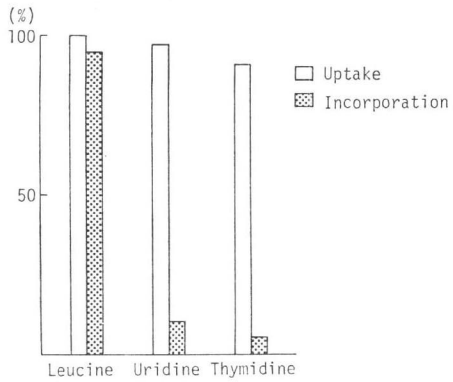
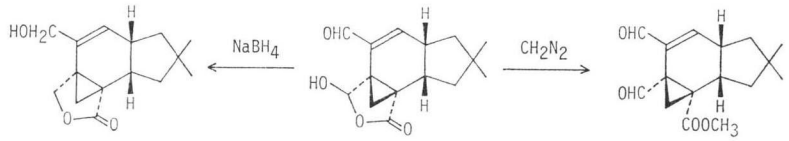


Table 1. Effect of marasmic acid on DNA synthesis in toluene-treated cells of *Bacillus brevis* (1.6×10^8 cells/ml).

Marasmic acid (μg/ml)	Incorporation of [^{3}H]dTMP (pmole)	% of control
0	3.75	100
100	2.06	55

Table 2. Effect of marasmic acid of RNA synthesis in isolated nuclei of ECA cells.

Antibiotic added (μg/ml)	Incorporation of [^{3}H]UMP (pmole)	% of control
None	2.14	100
α-Amanitin 2	0.87	41
Marasmic acid 8	1.22	57
80	0.59	28



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2, already 8 μg led to a 43% inhibition of RNA synthesis in nuclei derived from ECA cells. A 72% inhibition, higher than the one obtainable with α-amanitin, was observed at 80 μg/ml. One of the enzymes which appeared to be a target of marasmic acid is the α-amanitin-sensitive RNA polymerase II, which constitutes about 60% of the UMP-incorporating activity in this test.

Using RNA polymerase II from wheat germ and calf thymus DNA as template the incorporation of [^{3}H]UMP into acid-precipitable material was followed.

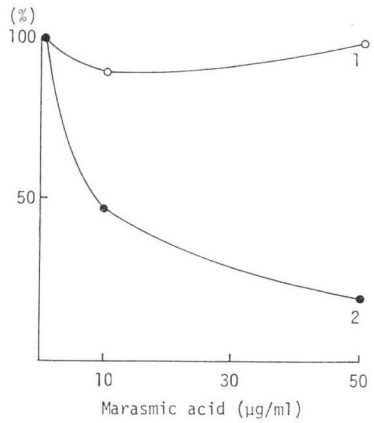
In contrast to α-amanitin, marasmic acid inhibited the enzyme at rather high concentrations (Table 3). Preincubation of marasmic acid with DNA template was less inhibitory than preincubation with the polymerase. The most pronounced inhibitory effect could be obtained after preincubating marasmic acid with enzyme and precursor-nucleotides together. The inhibition of RNA polymerase II is much less pronounced than the effect on UMP-incorporation in ECA nuclei. It therefore seems likely that not RNA polymerase II alone, but other nuclear RNA polymerases as well are targets of marasmic acid. It is also possible that other components or factors necessary for accurate transcription[9] which are not involved in the purified polymerase system are impaired.

Table 3. Effect of marasmic acid on RNA polymerase II (wheat germ).

Antibiotic added (μg/ml)		Preincubation of antibiotic with	Incorporation of [^{3}H]UMP % of control
None		—+	100*
α-Amanitin	2	—	0
Marasmic acid	80	—	80
	80	DNA template	64
	80	Enzyme	38
	80	Enzyme+(GTP, ATP, CTP)	3

+— No preincubation.
* Incorporation of control: 20 pmole [^{3}H]UMP.

Fig. 2. Effect of marasmic acid on 5′-RNA capping system isolated from rat liver nuclei as a percentage of the controls without antibiotic.
(1) Guanine-7-methyltransferase
(2) Guanylyltransferase



Another step of eucaryotic mRNA biosynthesis which occurs within the nucleus is the 5′-terminal capping reaction. This post-transcriptional process includes mainly the blocking of the 5′-terminal bases of the mRNA by guanylylation and *N*-7 methylation of the attached guanosine residue, afterwards[10]. The effect of marasmic acid was tested on guanylyltransferase and RNA guanine-7-methyltransferase isolated from rat liver nuclei.

Fig. 2 shows that of the two reactions tested only the guanylyl transfer was strongly inhibited. Keeping the amount of marasmic acid constant and varying enzyme, RNA-substrate and GTP-precursor concentration, we did not find a remarkable change in the degree of the inhibition (Fig. 3).

Fig. 3. Effect of marasmic acid on the guanylyltransferase reaction.
Effect of varying the amount of enzyme (A), of RNA substrate (B), and of GTP (C).
(1) Controls without antibiotic
(2) 60 μg/ml marasmic acid added

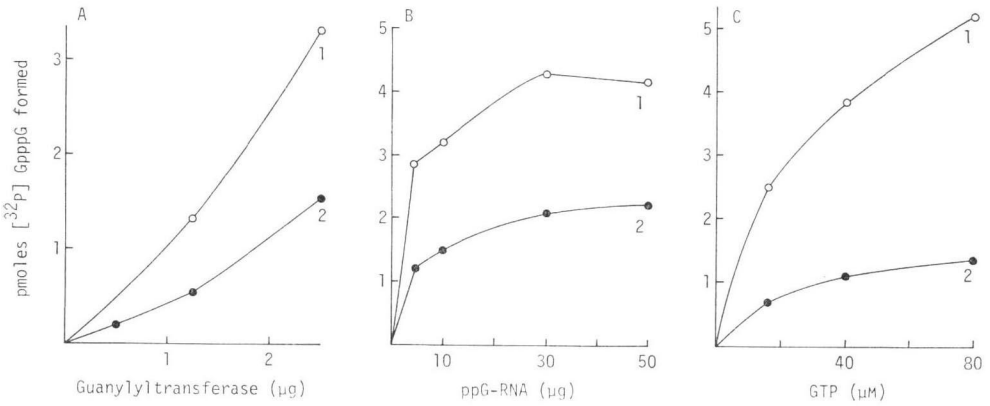


Table 4. Effect of preincubation of guanylyltransferase and RNA with marasmic acid.

Preincubation	Incorporation of [^{32}P]GMP		
	Controls (pmole)	Plus 60 μg/ml marasmic acid	
		(pmole)	(% of control)
None	3.12	1.47	47
Enzyme	2.59	0.67	26
Acceptor RNA	3.06	1.74	57

Table 5. Mutagenicity of marasmic acid and methyl marasmate in the assay of AMES *et al.*[11].

Antibiotic (μg/disc)		Number of revertants$^+$, *Salmonella typhimurium* strain			
		TA 1535	TA 1538	TA 100	TA 98
MNNG*	5	2944	—	20750	—
Marasmic acid	50	—	—	—	344
Methyl marasmate	50	—	27	50	160

$^+$ Background subtracted.
* MNNG = *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine

Again, the inhibitory effect of marasmic acid was most pronounced when the antibiotic was preincubated with guanylyltransferase for 5 minutes before adding GTP and RNA (Table 4).

Preincubation with RNA-substrate did not enhance inhibition as compared to the controls. The effect of marasmic acid on the transguanylation reaction of cap-formation was not affected by the addition of high amounts of bovine serum albumin (up to 2 mg/ml) suggesting that the binding to the target enzyme might be rather selective.

The pronounced inhibitory action on nucleic acid synthesis in whole cells and on some enzymes of nucleoside metabolism prompted us to examine a possible mutagenic activity of marasmic acid in the *Salmonella typhimurium* assay according to AMES *et al.*[11]. In the spot test (Table 5) 50 μg of marasmic acid or methyl marasmate distinctly increased the number of revertants from *S. typhimurium* TA 98 and in the case of methyl marasmate from TA 1638 and TA 100 as well.

As compared to the control mutagen MNNG, however, the activities of marasmic acid and methyl marasmate are minute. The activities of marasmic acid resemble those of many other sesquiterpenoids, especially α-methylene ketones[12] and lactones[13]. Like the other antibacterial and cytotoxic sesquiterpenoids, marasmic acid does not interact with only one specific enzyme of nucleic acid synthesis like α-amanitin, but – dependent on the concentration – with several reactions of DNA and RNA metabo-

lisms both of procaryotes and eucaryotes.

A common feature of the reactions inhibited by marasmic acid is that the enzymes involved transfer nucleotides to nucleic acids. Due to the reactivity of the α,β-unsaturated aldehyde function marasmic acid could covalently bind to nucleophilic (*e.g.* amino) groups of the enzymes or the nucleic acid substrates.

Biological Assays

Macromolecular syntheses in cells of the ascitic form of Ehrlich carcinoma (ECA cells) and incorporation of [^{3}H]UMP in isolated nuclei of ECA cells and incorporation of [^{3}H]dTMP in toluene-treated cells of *B. brevis* were tested as described previously[12,15].

Histidine-auxotroph *S. typhimurium* strains TA 98, 100, 1535, and 1538 were kindly provided by B.N. AMES, Berkeley, USA. The mutagenicity tests as spot tests (without rat liver homogenate) were carried out as described by AMES *et al.*[11]. Sterile 6 mm filter paper discs containing the antibiotics were placed on the surface of the top agar. After 2 days the revertants appearing as a ring of colonies around the discs were counted. Wheat germ RNA polymerase II was purchased from Miles Chemicals. [^{3}H] UMP incorporation into RNA was carried out according to JENDRISAK and BURGESS[16]. Calf thymus DNA was denatured prior to the assay by boiling at 95°C for 10 minutes and subsequent rapid cooling on ice.

The reaction mixture (final volume 0.25 ml) contained: Tris-HCl, pH 7.9 10 mM, $MgCl_2$ 20 mM, $MnCl_2$ 1 mM, $(NH_4)_2SO_4$ 50 mM, ATP, GTP, and CTP 0.4 mM each, 1 μCi [^{3}H]UTP (1 Ci/mmole), denatured DNA 200 μg/ml, bovine serum albumin 500 μg/ml; RNA polymerase 5 μl/assay.

Incubation was carried out for 15 minutes at 25°C. The reaction was stopped by the addition of 1 ml 5% trichloroacetic acid containing 1% $Na_4P_2O_7$. The acid-insoluble precipitate was collected on cellulose nitrate filters and the radioactivity counted.

In some assays marasmic acid was preincubated with template or enzyme in the buffer-salt solution for 10 minutes at 25°C and the reaction started by the addition of the missing components and [^{3}H]UTP.

mRNA guanylyltransferase and mRNA guanine-7-methyltransferase were purified from rat liver nuclei up to the steps of CM-Sephadex column and Sephadex G-150 column respectively as described[17]. The assay for guanylyltransferase was carried out essentially as described[17]. The standard reaction mixture (50 μl) contained: 50 mM tris-HCl (pH 7.9), 1 mM $MnCl_2$, 0.5 mM $Mg(CH_3COO)_2$, 10 mM dithiothreitol, 5~10 μM [α-^{32}P]GTP (2,000~5,000 cpm/pmole). 100 pmole of ppGpCpC-poly (A_2,U_2,G) as a cap acceptor, 25 μg of bovine serum albumin and purified guanylyltransferase. Incubation was for 60 minutes at 30°C. RNA was digested with nuclease P1 and alkaline phosphatase. The digests were electrophoresed on Whatman DE81 paper at pH 3.4 and the radioactivity in [^{32}P]-GpppG spots was determined[17]. In some experiments enzyme or acceptor RNA was preincubated with marasmic acid in a solution (25 μl) containing 50 mM tris-HCl (pH 7.9), 1 mM $MnCl_2$, 0.5 mM $Mg(CH_3COO)_2$, and 10 mM dithiothreitol for 5 minutes at 30°C, then missing components and [α-^{32}P]GTP were added to make 50 μl.

Guanine-7-methyltransferase was assayed by measuring m^7GpppG^m formation from *S*-adenosyl-[methyl-^{3}H]methionine and $GpppG^m$ as described[17]. m^7GpppG^m was isolated by thin-layer chromatography using polyethyleneimine cellulose plates (Macherey-Nagel, Germany), and the radioactivity in [^{3}H]m^7GpppG^m spots was measured.

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