ANTIBIOTICS FROM BASIDIOMYCETES. XIII[1]

THE ALLIACOLS A AND B FROM *MARASMIUS ALLIACEUS* (JACQ. *ex* FR.) FR.†

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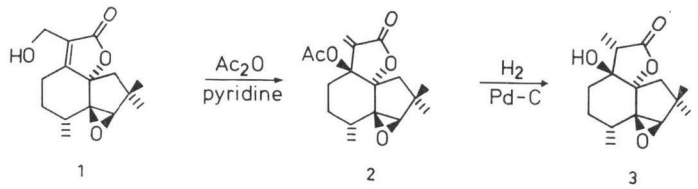
Two antimicrobial and cytotoxic metabolites were isolated from fermentation broth of *Marasmius alliaceus*. The structures of the two crystalline antibiotics, alliacols A (**6**) and B (**1**) were elucidated by spectroscopic methods and chemical correlation with alliacolide (**3**). The alliacols show weak antibacterial and antifungal activity. Both antibiotics strongly inhibit DNA synthesis in cells of the ascitic form of Ehrlich carcinoma at concentrations of 2~5 μg/ml. Both alliacols A and B react with cysteine to form adducts with strongly reduced biological activities.

Marasmius alliaceus (*Tricholomataceae*) is a medium sized mushroom frequently growing on decaying beech wood in late summer and fall. The species is very well characterized by its intense garlic odor. As has been shown by GMELIN *et al.*[2], the odorous components of the fruit bodies are derived by enzymatic cleavage of γ-glutamyl marasmine, a dipeptide containing an unusual cysteine sulfoxide moiety. During a screening for polyacetylenes KING *et al.*[3] isolated alliacolide (**3**), a new sesquiterpene lactone, from submerged cultures of *Marasmius alliaceus*. From cultures of the same fungus PA 789/80, a polyacetylenic antibiotic, has been described by PALMA and KNAUSEDER[4]. In our continuing search for biologically active metabolites from basidiomycetes we found that our strains of *Marasmius alliaceus* produced two antibiotics. In the following we wish to report on the isolation, structure determination, and biological characterization of these metabolites.

Results and Discussion

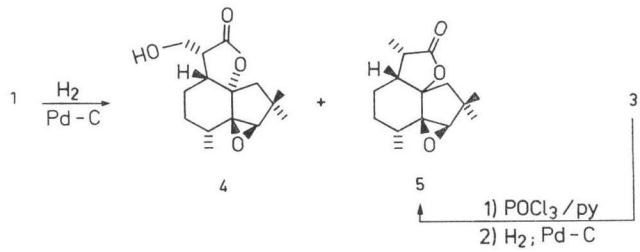
The alliacols A and B were detected in cultures of our strains of *Marasmius alliaceus* because of their antimicrobial and cytotoxic properties. Both antibiotics could be purified easily from the culture fluid by extraction with ethyl acetate and chromatography on silica gel. Alliacolide was isolated from our fermentations only as a minor, antibiotically inactive compound. Direct comparison with the compound isolated by KING[3] proved its identity. Alliacol B, $C_{15}H_{20}O_4$, shows a UV maximum at 238 nm and IR bands (KBr) at 1775 and 1670 cm^{-1} which point to the presence of an α,β-unsaturated lactone system. This is further corroborated by the ^1H NMR spectrum ($CDCl_3$) which lacks the quartet at δ 2.70, typical for 10-H in the spectrum of **3**. The presence of signals for a CH_2OH group (δ 4.40 (2H); 1.75 (OH)), an oxirane proton (δ 3.42) and three methyl groups (δ 0.95 (d), 1.18 (s), 1.26 (s)) suggests structure **1** for alliacol B.

† Parts of the results have been presented at the International Research Congress on Natural Products as Medicinal Agents in Strasbourg, France, July 6~11, 1980.



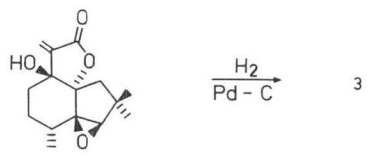
1 2 3

Final proof was obtained by direct correlation of **1** with **3**. Acetylation of **1** with Ac_2O - pyridine proceeds with allylic rearrangement and yields acetate (**2**). Its structure follows from the ^1H NMR spectrum which exhibits two singlets at δ 5.78 and 6.42, typical for exomethylene protons conjugated to a carbonyl group. This unusual allylic rearrangement of a primary alcohol into a tertiary acetate may be explained by the high internal strain caused by the endocyclic double bond. Hydrogenation of **2** in methanol leads to a mixture of products from which alliacolide (**3**), identical with the authentic compound, could be separated. Since the relative[5] and absolute[5] configuration of **3** is known, this transformation establishes the stereochemistry of **1**. Hydrogenation of **1** yields a 2: 1 mixture of alcohol (**4**) and deoxyalliacolide (**5**). The latter is identical with the compound derived from **3** by dehydration with $POCl_3$ - pyridine[6], followed by catalytic hydrogenation. In all cases the approach of hydrogen occurs from the less hindered β-face of the molecule.



4 5

Alliacol A is isomeric with **1**, and because of the presence of a UV maximum at 225 nm, IR bands at 1750 and 1680 cm^{-1}, and two singlets in the ^1H NMR spectrum at δ 5.88 and 6.37, structure **6** can be ascribed. This was confirmed by catalytic hydrogenation of alliacol A, which afforded **3** in high yield.



6

After we had completed our investigation, Dr. THALLER, Oxford, kindly informed us that compound **6** had already been isolated in his laboratory[6]. The structures of **1** and **6** have been elucidated independently by Prof. J. R. HANSON[5].

The alliacols belong to the group of α,β-unsaturated sesquiterpene lactones which are among the typical constituents of plants mainly of the family Compositae[7] and from liver worts[8]. α,β-Unsaturated γ-lactones exhibit a variety of interesting biological activities. Some have been found to possess antibacterial, antifungal, phytotoxic or schistosomicidal properties, others act as feeding deterrents for insects. Sesquiterpene lactones have also been implicated in allergic contact dermatitis in man and livestock poisoning (for review see[9]). A great many sesquiterpenes containing one or two α-methylene butyrolactone moieties show high levels of cytotoxicity against tumor cells *in vitro*. Since some of these

Table 1. Antimicrobial spectra of the alliacols.

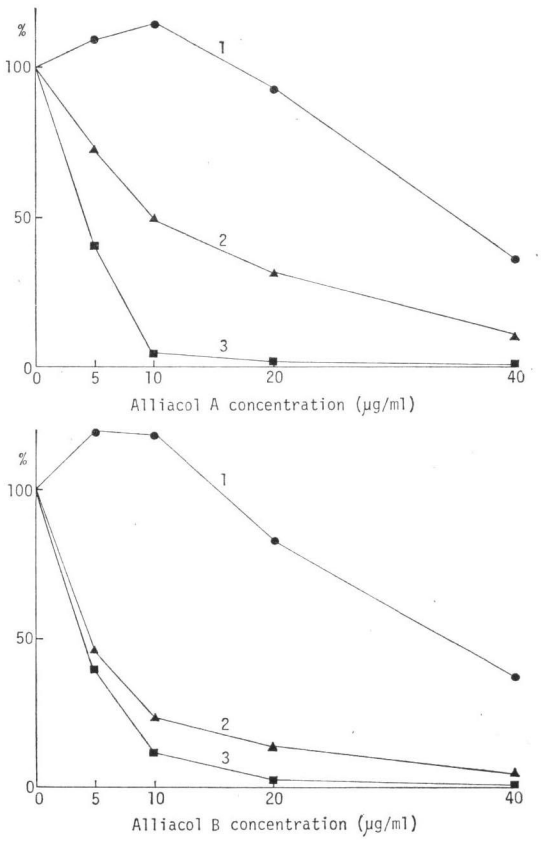
Test organism	Alliacol B	Alliacol A
Serial dilution assay	MIC (μg/ml)	
Aerobacter aerogenes	>50	>50
Arthrobacter citreus	>50	>50
Bacillus brevis	25~50	25~50
Bacillus subtilis	>50	>50
Escherichia coli	>50	>50
Micrococcus roseus	>50	>50
Micrococcus luteus	>50	>50
Mycobacterium phlei	25~50	25~50
Proteus vulgaris	>50	>50
Staphylococcus aureus	>50	>50
Streptomyces sp. ATCC 23836	50	50
Candida albicans	>50	>50
Nadsonia fulvescens	>50	>50
Rhodotorula glutinis	50	50
Saccharomyces cerevisiae is 1	>50	>50
Plate diffusion assay	Diameter inhibition zone (mm) with 100 μg/disc	
Ceratocystis fimbriata	15	8
Phytophthora infestans	35	30
Fusarium cubense	13	8
Phythium debaryanum	—	—
Penicillium notatum	15	18

— No inhibition.

Fig. 1. Effect of the alliacols A and B on macromolecular syntheses in Ehrlich carcinoma ascitic cells as a percentage of the controls without antibiotic.

Controls without antibiotic (=100%) incorporation per 3 ml cell suspension: [^{14}C]leucine 29340 cpm, [^{14}C]uridine 11645 cpm, [^{14}C]thymidine 4312 cpm.

(1) protein synthesis, (2) RNA synthesis, (3) DNA synthesis.



compounds exhibit antitumor activity *in vivo*, they might be of interest as possible future medicinal agents[10,11].

Table 1 shows the antimicrobial spectrum of the alliacols. The inhibitory concentrations for sensitive bacteria and fungi are rather high. As shown in Fig. 1 the cytotoxic activity of the alliacols against cells of the ascitic form of Ehrlich carcinoma (ECA) is much more pronounced. Incorporation of thymidine into acid-insoluble material (DNA) is inhibited 50% at concentrations of 4~5 μg/ml of alliacol A or B. The incorporation of uridine is somewhat less affected. The incorporation of leucine into protein is stimulated by lower concentrations of the antibiotics, a 50% inhibition is observed only at concentrations of 35~40 μg/ml. α,β-Unsaturated γ-lactones react readily with nucleophiles, especially cysteine and other thiols[12,13]. The cysteine adducts have been found to be devoid of cytotoxic and *in vivo* antitumor activity. It is assumed that the reaction of the unsaturated lactones with biologically important sulfhydryl groups *e.g.* in enzymes is responsible for most of the biological activities of these compounds[14]. Cysteine adducts of the alliacols A and B were prepared as described in the experimental section. When tested for inhibition of macromolecular syntheses in ECA cells the cysteine adducts ex-

Table 2. Effect of alliacolide and the cysteine adducts of the alliacols A and B on macromolecular syntheses in cells of the ascitic form of Ehrlich carcinoma.

Addition	nmole/ml	Incorporation in % of the controls without antibiotics		
		Precursor		
		Leucine	Uridine	Thymidine
Alliacol B	40	109	39	15
Cysteine adduct of alliacol B	40	103	78	72
Alliacol A	40	114	50	22
Cysteine adduct of alliacol A	40	110	89	95
Alliacolide	160	93	70	77

Table 3. Effect of the alliacols A and B on the uptake and incorporation of precursors in cells of the ascitic form of Ehrlich carcinoma.

Antibiotic	μg/ml	Uptake/incorporation (pmole/10^7 cells)		
		Precursor		
		Leucine	Uridine	Thymidine
Alliacol B	0	234/175	363/78	43/15
	20	146/104	179/12	30/0.5
Alliacol A	0	286/171	418/80	50/12
	20	317/198	252/30	35/3

hibited much less activity than the alliacols (Table 2). Alliacolide which contains a saturated lactone ring was found to be almost devoid of cytotoxic activity. Table 3 shows the effect of the alliacols on the total uptake (radioactivity in TCA-soluble and insoluble fraction of cells) and incorporation (radioactivity in TCA-insoluble material) of leucine, uridine, and thymidine in ECA cells. The alliacols interfere much more strongly with the incorporation of uridine and thymidine into RNA and DNA, compared with the total uptake. This indicates that the macromolecular syntheses themselves and not the transport systems for the precursors are the preferential targets of the alliacols.

Experimental

Fermentation and Isolation

Mycelial cultures of *Marasmius alliaceus* strains 7629, 7747, and 77228 were obtained from spore prints of fruiting bodies collected near Tübingen. For submerged cultivation and maintenance on agar slants the strains were grown in a yeast extract - malt extract - glucose (YMG) medium composed of (g/liter): yeast extract 4, malt extract 10, glucose 4. Fermentations were carried out in 10 or 20 liters of YMG medium in a New Brunswick FS 314 (1.5 liters air/minute, 150 rpm, 22~24°C) or in a Biolafitte fermentation apparatus (3 liters air/minute, 150 rpm, 22°C). After 7 days 20 liters of culture fluid were extracted twice with 3 liters of ethyl acetate yielding approximately 2 g of crude product. Chromatography of 1 g of crude product yielded alliacolide (30~50 mg) as well as a mixture of alliacols A and B. The alliacols were separated by repeated chromatography on silica gel or by preparative thin-layer chromatography on silica plates (Merck 5554, developed in benzene - acetone - acetic acid, 70: 30: 1) yielding approximately 20~30 mg of alliacol A and 460~500 mg of alliacol B. The yields of alliacolide and the alliacols from fermentations of our strains 7629, 7747, and 77228 were almost identical.

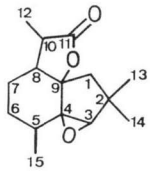
Alliacol B (1)

Rf 0.34 (silica plates Merck 5554; CCl_4 - EtOAc, 1: 1). m.p. 121~123°C. $[\alpha]_D^{20}$ −5.47° (*c* 0.365, $CHCl_3$). UV (MeOH): λ_{max} (ε) 238 nm (1209). IR (KBr): 3530~3490 (m, br), 3000~2980 (m, br), 1750 (st), 1670 (m), 1460 (w), 1310~1290 (w, br), 1250 (w), 1100 (w), 1080 (w), 1030 (m), 1010 (m), 995 (m), 930 (w), 900 cm^{-1} (w). ^1H NMR (Bruker WH 90; $CDCl_3$): See Table 4. MS (A.E.I. MS 50; 150°C, 70 eV): *m/z* 264.1359 (1.5%, M^+, Calcd. for $C_{15}H_{20}O_4$ 264.1362), 190 (30, $C_{12}H_{14}O_2$), 83 (100, C_5H_7O), 55 (14, C_4H_7).

Alliacol A (6)

Rf 0.39 (silica plates Merck 5554; CCl_4 - EtOAc, 1: 1), m.p. 156°C (lit.[6] 155.5~156°C). $[\alpha]_D^{20}$

Table 4. Selected ^1H NMR signals ($CDCl_3$; 90 MHz; TMS as internal standard).



	1	2	3	4	5	6
1-H	1.24; 1.84 (AB-q, J=13)		1.30; 1.98 (AB-q, J=13)	1.51 ("s")	1.50 ("s")	
3-H	3.42 (s)	3.17 (s)	3.25 (s)	3.26 (s)	3.26 (s)	3.20 (s)
5-H	2.02 (m)					
8-H	—			2.52 (m)	2.41 (m)	
10-H	—		2.70(q, J=7.5)	2.94 (ddd, J=7.5+7.5 +6.5)	2.73 (pentet, J=7.5)	—
12-H	4.40 (s)	5.78 (s) 6.42 (s)	1.19 (d, J=7.5)	3.76 (dd, J=11.5+6.5) 3.96 (dd, J=11.5+7.5)	1.22 (d, J=7.5)	5.88 (s) 6.37 (s)
13-H	1.18 (s)	1.11 (s)	1.12 (s)	1.11 (s)	1.11 (s)	1.12 (s)
14-H	1.26 (s)	1.17 (s)	1.12 (s)	1.11 (s)	1.11 (s)	1.12 (s)
15-H	0.95 (d, J=8)		1.14 (d, J=7.5)	1.15 (d, J=7.5)	1.19 (d, J=7.5)	
O ‖ CH_3–C–	—	2.06 (s)	—	—	—	—
OH–	1.75 (s)	—	2.28 (s)	1.50 (s)	—	1.56 (s)

+10.5° (c 1.2, $CHCl_3$). UV (MeOH): λ_{max} (ε) 225 nm (1040). IR (KBr): 3510~3480 (m, br), 1760 (st), 1730 (st), 1460 (w), 1440 (w), 1350 (w), 1280 (w), 1265 (w), 1170 (w), 1145 (m), 1120 (m), 1020 (m), 990 (m), 960 cm^{-1} (w). ^1H NMR (Bruker WH 90; $CDCl_3$): See Table 4. MS (A.E.I. MS 50; 150°C, 70 eV): m/z 264.1355 (29%, M^+, Calcd. for $C_{15}H_{20}O_4$ 264.1361), 249 (17, $C_{14}H_{17}O_4$), 236 (10, $C_{14}H_{20}O_3$), 220 (17, $C_{14}H_{20}O_2$), 193 (100, $C_{12}H_{17}O_2$), 182 (43), 180 (19), 165 (14), 140 (30).

Acetylation of **1**

Five mg of **1** were stirred in 1 ml of pyridine and 0.5 ml of Ac_2O for 30 minutes at room temperature. After evaporation under reduced pressure the residue was purified by PTLC (silica gel, CCl_4 - EtOAc, 1: 1), Rf 0.52. Yield: 3 mg crystalline compound, m. p. 133°C (crude). IR (KBr): 1770, 1765, 1740 cm^{-1}. ^1H NMR see Table 4. MS: m/z 306.1445 (0.5%, M^+, Calcd. for $C_{17}H_{22}O_5$ 306.1423), 264 (24), 246 (17), 236 (63), 218 (17), 203 (14), 193 (24), 191 (18), 178 (11), 96 (25).

Hydrogenation of **2**

Crude **2** (4 mg) in 50 ml MeOH was hydrogenated (10 mg Pd-C, atmospheric pressure) for one hour at room temperature. Purification by PTLC yielded a mixture of several products, from which **3** could be separated (silica gel, CCl_4 - EtOAc, 1: 1), Rf 0.39. **3** was identical with alliacolide by m.p., $[\alpha]_D^{20}$, ^1H NMR, and MS.

Hydrogenation of **1**

Five mg of **1** were reacted as described above. Purification by PTLC yielded 1.2 mg **4**, Rf 0.29 and 2.6 mg **5**, Rf 0.36.

4: crude m.p. 175°C, $[\alpha]_D^{20}$ −30.0° (c 0.06, $CHCl_3$). ^1H NMR see Table 4. MS: m/z 266.1511 (10%, M^+, Calcd. for $C_{15}H_{22}O_4$ 266.1519), 251 (65), 233 (50), 195 (100), 180 (48), 177 (90), 166 (60), 165 (58), 151 (45), 124 (50), 123 (47).

5, Deoxyalliacolide: m.p. 165°C (crude), $[\alpha]_D^{20}$ −2.7° (crude) (c 0.10, $CHCl_3$). IR (KBr): 1770 cm^{-1}. ^1H NMR see Table 4. MS: m/z 250.1583 (35%, M^+, Calcd. for $C_{15}H_{22}O_3$ 250.1572), 235 (34), 222 (15),

195 (58), 194 (25), 193 (22), 182 (32), 179 (28), 177 (75), 166 (40), 165 (88), 125 (55), 97 (56), 55 (100).

Conversion of **3** to **5**

Ten mg of **3** were treated with $POCl_3$-pyridine for 24 hours at 50°C as described[8]. After separation from a yellow oil (Rf 0.64) by PTLC, the dehydration product (Rf 0.36, m.p. 145°C (subl.), Ref.[4] 146~149°C) was hydrogenated as above yielding **5** identical with the compound obtained from **1** (m.p., $[\alpha]_D$, ^1H NMR, MS).

Hydrogenation of **6**

Three mg of **6** were hydrogenated as described for **1**, yielding **3**, identical with the authentic compound.

Preparation of Cysteine Adducts

Five mg of **1** or **6** were dissolved in 0.2 ml EtOH and added to a solution of 2.3 mg L-cysteine in 0.8 ml H_2O. After standing at room temperature for 20 minutes the adduct formation was completed as tested by TLC on silica gel (Merck 5554, BuOH - HOAc - H_2O, 4:1:1, detection with ninhydrin). Cysteine adduct of **1** Rf 0.71, cysteine adduct of **6** Rf=0.73.

Biological Assays

The antimicrobial spectra were determined and the macromolecular syntheses in ECA cells were measured as described previously[15]. For determining uptake and incorporation into acid-insoluble material, 5×10^7 cells in phosphate buffered saline (PBS) containing glucose and heparin[15] were preincubated with or without alliacols at 37°C and gentle shaking. After 10 minutes samples of 1 ml were withdrawn and incubated with 0.1 μCi [1-^{14}C]leucine (59 mCi/mmole), 0.1 μCi [2-^{14}C]uridine (53 mCi/mmole) or 0.1 μCi [2-^{14}C]thymidine (61 mCi/mmole) at the same temperature. After 5 minutes the samples were centrifuged and the cells washed with 1 ml of PBS. For determining the total uptake the cells were dissolved in scintillation fluid (Zinsser Quickscint 504). For determining the incorporation 1 ml of cold 5% TCA was added to the pellet, the cells were resuspended, collected on membrane filters and washed with 10 ml of cold 5% TCA. After drying scintillation fluid was added to the filters and the radioactivity measured by liquid scintillation counting.

Acknowledgement

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References

1) ZECHLIN, L.; M. WOLF, W. STEGLICH & T. ANKE: Antibiotika aus Basidiomyceten. XII. Cristatsäure, ein modifiziertes Farnesylphenol aus Fruchtkörpern von *Albatrellus cristatus*. Chem. Ber. in print
2) GMELIN, R.; H.-H. LUXA, K. ROTH & G. HÖFLE: Dipeptide precursor of garlic odour in *Marasmius* species. Phytochemistry 15: 1717~1721, 1976
3) KING, T. J.; I. W. FARRELL, T. G. HALSALL & V. THALLER: Alliacolide, a new bicyclic sesquiterpene epoxylactone with a novel carbon skeleton from cultures of the fungus *Marasmius alliaceus* (Jacques *ex* FR.)FR.; X-ray structure. J. Chem. Soc., Chem. Comm. 1977: 727~728, 1977
4) PALMA, N. & F. KNAUSEDER: PA 789/80, a polyacetylenic antibiotic from *Marasmius alliaceus* (Jacq. *ex* FR.) FR. J. Antibiotics 33: 1556~1559, 1980
5) HANSON, J.: Plenary lecture, 12th International IUPAC Symposium on the Chemistry of Natural Products, Tenerife, 1980; J. Pure & Appl. Chem., in print
6) FARRELL, I. W.: Ph. D. Thesis, Oxford, 1977
7) FISCHER, N. H.; E. J. OLIVIER & H. D. FISCHER: The biogenesis and chemistry of sesquiterpene lactones. Fortschr. Chem. Org. Naturst. 38: 47~390, 1979
8) ASAKAWA, Y. & T. ARATANI: Physiological active substances found in bryophytes. Misc. Bryol. *et* Lichenol. 7: 144~152, 1977
9) RODRIGUEZ, E.; G. H. N. TOWERS & J. C. MITCHELL: Biological activities of sesquiterpene lactones. Phytochemistry 15: 1573~1580, 1976

10) CORDELL, G. A. & N. R. FARNSWORTH: Experimental antitumor agents from plants, 1974~76. Lloydia 40: 1~44, 1977
11) KUPCHAN, S. M.; M. A. EAKIN & A. M. THOMAS: Tumor inhibitors. 69. Structure-cytotoxicity relationship among the sesquiterpene lactones. J. Med. Chem. 14: 1147~1152, 1971
12) KUPCHAN, S. M.; D. C. FESSLER, M. A. EAKIN & T. J. GIACOBBE: Reactions of alpha methylene lactone tumor inhibitors with model biological nucleophiles. Science 168: 376~377, 1970
13) KUPCHAN, S. M.; T. J. GIACOBBE, I. S. KRULL, A. M. THOMAS, M. A. EAKIN & D. C. FESSLER: Reaction of endocyclic α,β-unsaturated γ-lactones with thiols. J. Org. Chem. 35: 3539~3543, 1970
14) FUJITA, E. & Y. NAGAO: Tumor inhibitors having potential for interaction with mercapto enzymes and/or coenzymes. Bioorg. Chem. 6: 287~309, 1977
15) KUPKA, J.; T. ANKE, F. OBERWINKLER, G. SCHRAMM & W. STEGLICH: Antibiotics from basidiomycetes. VII. Crinipellin, a new antibiotic from the basidiomycetous fungus *Crinipellis stipitaria* (FR.) PAT. J. Antibiotics 32: 130~135, 1979