ANTIBIOTICS FROM BASIDIOMYCETES. XI[1]

THE BIOLOGICAL ACTIVITY OF SICCAYNE, ISOLATED FROM THE MARINE FUNGUS *HALOCYPHINA VILLOSA* J. & E. KOHLMEYER

JUTTA KUPKA, TIMM ANKE, WOLFGANG STEGLICH* and LOTHAR ZECHLIN*

Institut für Biologie I der Universität, Auf der Morgenstelle 1,
D-74 Tübingen, FRG
*Institut für Organische Chemie und Biochemie der Universität,
Gerhard-Domagk-Str. 1, D-53 Bonn, FRG

(Received for publication January 5, 1981)

From submerged cultures of the marine basidiomycete *Halocyphina villosa* we isolated siccayne (4-(2,4-dihydroxyphenyl)-2-methyl-1-buten-3-yne) (**1**), a metabolite first described from fermentations of the deuteromycete *Helminthosporium siccans*. Siccayne is a moderately active antibiotic, which inhibits Gram-positive bacteria and some fungi at concentrations of 10~50 μg/ml. Its cytotoxic effect is much more pronounced on both normal and Rous-sarcoma-virus transformed chicken embryo fibroblasts as compared to cells of the Ehrlich ascites carcinoma. Siccayne apparently interferes with the uptake of nucleoside precursors into eucaryotic cells as well as with the *in vitro* incorporation of nucleotides into DNA and RNA.

During the last years the search for new natural products extending into the realm of marine organisms has uncovered a variety of new secondary metabolites active upon mammalian systems and terrestrial microorganisms. Most of the biologically active compounds have been isolated from algae or sponges, only a minority from marine bacteria and fungi (for reviews see references[2,3,4]). In the course of our screening program we examined *Halocyphina villosa*, one of the four known marine basidiomycetes. The fungus develops its minute, cup-shaped fruiting bodies on mangrove roots and similar substrates close to mussels and other marine organisms[5,6]. The basidiospores of this unusual fungus are to our knowledge not directly discharged into the seawater but first embedded in a slimy drop and then released together-already germinating. Submerged cultures of *Halocyphina villosa* were found to produce an antimicrobial metabolite, which could be isolated and identified as siccayne (**1**). Besides the note that siccayne "shows an antibiotic activity against *Piricularia oryzae* and *Staphylococcus aureus*"[7] no data on its antibiotic properties have been published. In the following we wish to report on the fermentation, isolation and biological characterization of this compound.

Experimental

Isolation of Siccayne

A mycelial culture (strain No. 573) of *Halocyphina villosa* was obtained from a spore print. For the maintenance on agar slants and for seed cultures the fungus was grown in a medium containing 0.4% yeast extract, 1% malt extract, and 0.4% glucose. Well-grown seed-cultures (300 ml) were used to inoculate 10 liters of fermentation medium composed of (per 1 liter): maltose 20 g; glucose 10 g; peptone 2 g; yeast extract 0.2 g; KH_2PO_4 0.5 g; $MgSO_4$ 0.5 g; $FeCl_3$ 10 mg; $ZnSO_4$ 1 mg; $CaCl_2$ 50 mg; thiamine 50 μg; biotin 1 μg; folic acid 100 μg; inositol 50 mg[8]. The pH of the medium was adjusted to 5.5 before sterilization. The culture was incubated at 22°C for 20 days in a New Brunswick FS 314 fermentor, aerated with 3 liters per minute and agitated at 150 rev./minutes. Antibiotic produc-

tion was followed by paper-disc/agar-diffusion assay using *Bacillus brevis* as test organism. After fermentation the mycelia were separated from the culture fluid by filtration. The mycelia, containing one third of the antibiotic, were extracted with methanol - acetone (1: 1) and the culture broth, which contained two thirds of the antibiotic, was extracted with ethyl acetate. The combined extracts were applied to a column of silica gel (Mallinckrodt, 100 mesh) which was eluted with $CHCl_3$ - EtOH (99: 1), The fractions containing the antibiotic were combined to give crude siccayne (260 mg) which was crystallized from MeOH to yield yellowish prisms, m.p. 114°C (Ref.[7] 115~116°C).

IR (KBr) cm^{-1}: 3200~3450 (st, br), 2200 (w), 1860 (w), 1780 (w), 1610 (st), 1440 (st), 1370 (st), 1230 (st), 1160 (st), 1110 (st), 990 (m), 930 (m), 890 (st), 865 (st), 830 (st), 820 (st), 780 (st).

MS (AEI MS 50, 70 eV): *m/z* 174.0666 (100%, M^+, calcd. for $C_{11}H_{10}O_2$ 174.0681), 172 (10.9), 159 (24.8, $C_{10}H_7O_2$), 147 (14.6, $C_9H_7O_2$), 145 (5.3, $C_{10}H_9O$), 134 (9.2, $C_8H_6O_2$), 131 (14.9, C_9H_7O), 127 (9.0, $C_{10}H_7$), 115 (13.5, C_9H_7), 51 (10.0, C_4H_3).

^{1}H-NMR (acetone-d_6): δ=1.95 dd (J=1.6+1.1 Hz) [3H]; 5.31 dq (J=2.2+1.6 Hz) [1H]; 5.33 dq (J=2.2+1.1 Hz) [1H]; 6.76 m [3H]; 7.76 broad [2OH].

Antimicrobial Activity

The minimum inhibitory concentrations (MICs) were determined by the conventional serial broth dilution method. All test strains were grown in antibiotic medium 3 (Difco) and incubated as described previously[9].

Macromolecular Syntheses in Chicken Embryo Fibroblasts

Chicken embryo fibroblasts (CEF) were prepared and grown in a humidified CO_2 incubator as described by VOGT[10]. The secondary culture medium was SCHERER's medium supplemented with 20 mM bicarbonate buffer, 0.3% tryptose phosphate broth, 5% calf serum and 0.1% beef embryo extract. For normal cells, primary cultures were detached by trypsinization, reseeded at 1×10^6 cells per 60 mm plastic culture dish and grown at 36°C to subconfluence before use. To obtain transformed cells primary cultures were infected with the temperature-sensitive mutant of the Rous-sarcoma-virus tsNY68 immediately after seeding. The secondary cultures were grown at 41°C for 2 days and at 36°C (permissive temperature) afterwards. For the incorporation studies the medium was removed by aspiration and the cell monolayer was washed with HANKS balanced salt solution supplemented with 0.1% glucose. The dishes were then preincubated for 30 minutes at 36°C with 5 ml of the same buffer alone or with siccayne. After further incubation with the labelled precursors (1 μCi ^{3}H-thymidine (6 Ci/mmol), 1 μCi ^{3}H-leucine (39 Ci/mmol) or 2 μCi ^{3}H-uridine (25 Ci/mmol)) for one hour at 36°C the cells were rinsed twice with cold HANKS buffer followed by precipitation with 5 ml 5% trichloroacetic acid (TCA). The dried acid-insoluble material was dissolved in 3 ml of 0.1 N NaOH and aliquots were measured for radioactivity in a liquid scintillation counter.

Transport Studies in Cells of the Ascitic Form of Ehrlich Carcinoma (ECA)

Macromolecular syntheses in ECA cells grown in female mice were performed as described previously[9]. For a comparison of uptake and incorporation of the labelled precursors the following modifications were applied: After preincubation 1 ml of the cell suspension was incubated in Eppendorf cups with 0.1 μCi ^{14}C-leucine (354 mCi/mmol), 0.1 μCi ^{14}C-uridine (25 mCi/mmol) or 0.1 μCi ^{14}C-thymidine (58 mCi/mmol) for 10 minutes at 37°C. The cells were immediately centrifuged and the pellet either suspended in 5% TCA (incorporation) or directly added to the liquid scintillation fluid (uptake).

Incorporation of ^{3}H-UTP by Isolated Nuclei from ECA Cells

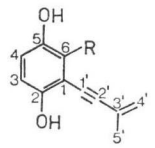
Nuclei from ECA cells were isolated according to MURAMATSU *et al.*[11] The nuclei were resuspended in glycerol-buffer and immediately used for the assay. The reaction mixture (final volume 0.25 ml) contained: Tris-HCl pH 8.0, 30 mM; saccharose 250 mM; EDTA 1 mM; DTE 0.1 mM; $(NH_4)_2SO_4$ 130 mM; $MnCl_2$ 3 mM; $MgCl_2$ 1 mM; ATP, GTP, and CTP 0.33 mM each; 0.5 μCi ^{3}H-UTP (1 Ci/mmol); 50 μl of the nuclear suspension containing 5×10^6 nuclei. Incubation was carried out for 30 minutes at 37°C and 300 rev./minute on a rotary shaker. The reaction was stopped by the addition of 2 ml of cold 5% TCA. The acid-insoluble precipitate was collected on cellulose nitrate filters and the radioactivity measured.

Incorporation of ^{3}H-dTTP in Toluene-treated Cells of *Bacillus brevis*

Exponentially growing cells of *Bacillus brevis* were toluenized as described by MOSES and RICHARDSON[12]. The reaction mixture[13] (final volume 0.125 ml) contained: Tris-HCl pH 7.8, 20 mM; $MgCl_2$ 5 mM; EDTA 0.1 mM; KCl 100 mM; ATP 1 mM; NAD 0.2 mM; dATP, dTTP, dCTP, dGTP 0.02 mM each; 1 μCi ^{3}H-dTTP (66 Ci/mmol) and 25 μl (2×10^{7} cells) of the bacterial cell suspension. The reaction mixture was preincubated with the antibiotic for 5 minutes at 37°C before the addition of the labelled precursor. After further incubation for 15 minutes at 37°C the reaction was stopped by the addition of cold 10% TCA containing 1% $Na_4P_2O_7$. The acid-precipitable material was processed as described above.

Results

The antibiotic from *Halocyphina villosa* was obtained as described in the experimental section. M.p., MS and ^{1}H-NMR spectrum revealed its identity with siccayne (**1**), a metabolite of *Helminthosporium siccans*. This fungus also produces siccanin[7]. The ^{13}C-NMR signals of siccayne were assigned by use of the proton coupled spectrum and chemical shift calculations (Table 1). Comparison with the ^{13}C-NMR data given for frustulosinol (**2**) indicates that the δ-values assigned to C-1 and C-3′ (our numbering) in Ref.[14] have to be interchanged.



(**1**) R=H
(**2**) R=CH_2OH
(**3**) R=CHO

Table 1. ^{13}C NMR spectrum of siccayne (**1**) (δ-values, TMS as internal standard, in $CDCl_3$).

C-1	110.0	d, J=5 Hz	C-1′	82.0	m
C-2	150.8	m	C-2′	97.7	m
C-3	115.6	d, J=162 Hz	C-3′	126.1	m
C-4*	117.5	dd, J=161+4 Hz	C-4′	123.1	tq, J=160+6 Hz
C-5	148.8	m	C-5′	23.4	qtt, J=129+10+6.5 Hz
C-6*	118.1	dd, J=160+5 Hz			

* Assignments may be interchanged.

As seen in Table 2 siccayne has antibacterial activity against *Aerobacter aerogenes* and a variety of Gram-positive bacteria at concentrations of 10~50 μg/ml. Only a few fungi are sensitive to siccayne at 50 μg/ml. The effect of siccayne on macromolecular syntheses was tested with chick embryo fibroblasts and with cells of the ascitic form of Ehrlich carcinoma (ECA). In both normal and Rous-sarcoma-virus-transformed fibroblasts thymidine incorporation into acid-insoluble fraction of cells was more affected than the incorporation of leucine or uridine (Fig. 1). No preferential inhibition of the malignantly transformed fibroblasts could be observed. At 10 μg/2×10^{6} cells in both cell-lines DNA syntheses was reduced to about 10% as compared with the controls. About 3~5 fold higher concentrations of siccayne were needed to inhibit macromolecular syntheses in ECA cells (Fig. 2). At lower concentrations RNA synthesis proved to be the most sensitive, whereas at higher concentrations RNA, DNA and protein syntheses were affected to the same degree.

Since the inhibition of incorporation might be caused by a reduced uptake of the appropriate precursors into the cells, we investigated the uptake of thymidine, uridine, and leucine into ECA cells. In Table 3 the uptake is compared with the incorporation at different concentrations of siccayne.

Table 2. Antimicrobial spectrum of siccayne.

			MIC (μg/ml)
Bacteria	Pseudomonadales	*Pseudomonas fluorescens*	>50
	Eubacteriales, Gram-negative	*Aerobacter aerogenes*	30~50
		Escherichia coli	>50
		Proteus vulgaris	>50
	Eubacteriales, Gram-positive	*Arthrobacter citreus*	>50
		Bacillus brevis	20
		Bacillus subtilis	>50
		Corynebacterium insidiosum	10
		Micrococcus roseus	50
		Mycobacterium phlei	30~50
		Sarcina lutea	>50
		Staphylococcus aureus	>50
	Actinomycetales	*Streptomyces* PRL 1642	30~50
		Streptomyces ATCC 23836	30~50
Fungi	Ascomycetes	*Candida albicans*	>50
		Nematospora coryli	50
		Nadsonia fulvescens	>50
		Saccharomyces cerevisiae αS 288c	>50
		Saccharomyces cerevisiae FL 200	>50
		Saccharomyces cerevisiae is 1	50
	Basidiomycetes	*Rhodotorula glutinis*	50

Fig. 1. Effect of siccayne on macromolecular syntheses in chicken embryo fibroblasts in % of the controls without antibiotic.

A. Normal fibroblasts: Controls without antibiotic; incorporation per 2×10^6 cells: ^{3}H-leucine, 25,656 cpm; ^{3}H-uridine, 26,708 cpm; ^{3}H-thymidine, 20,058 cpm.

B. Rous-sarcoma-virus transformed fibroblasts: Controls without antibiotic; incorporation per 2×10^6 cells: ^{3}H-leucine, 20,484 cpm; ^{3}H-uridine, 22,152 cpm; ^{3}H-thymidine, 13,440 cpm.

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

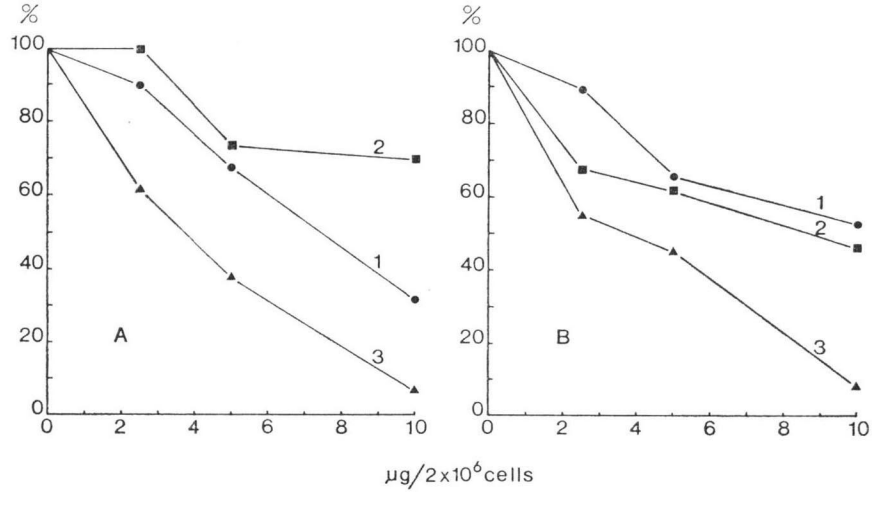


Table 3. Effect of siccayne on uptake (total radioactivity consisting of the acid-soluble and the acid-insoluble fraction of cells) and incorporation (TCA-precipitable material) of ^{14}C-leucine, ^{14}C-uridine, and ^{14}C-thymidine in ECA cells.

Siccayne μg/2×10^6 cells	Leucine		Uridine		Thymidine	
	Uptake (pmol)	Incorporation (pmol)	Uptake (pmol)	Incorporation (pmol)	Uptake (pmol)	Incorporation (pmol)
0	69.2	41.2	880	152	46	12.4
5	50.5	37.1	624	104	40	10.4
10	56.7	37.9	492	77	38	9.5
25	50.5	35.0	272	39	32	5.3

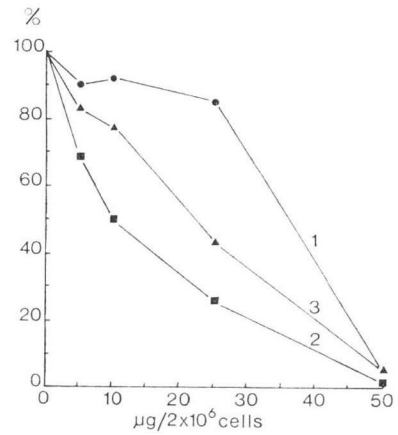
Table 4. Effect of siccayne on RNA synthesis in isolated nuclei of ECA cells.

Antibiotic added (μg/ml)		Incorporation of	
		^{3}H-UMP (pmol)	% of control
Control	—	2.14	100
α-Amanitin	2	0.87	41
Siccayne	20	1.22	57
Siccayne	40	0.77	36

Table 5. Effect of siccayne on DNA synthesis in toluene-treated cells of *Bacillus brevis*.

Siccayne (μg/ml)	Incorporation of	
	^{3}H-dTMP (pmol)	% of control
0	3.75	100
100	2.32	62

Fig. 2. Effect of siccayne on macromolecular syntheses in Ehrlich carcinoma ascites cells in % of the controls without antibiotic.
(1) protein synthesis, (2) RNA synthesis, (3) DNA synthesis
Controls without antibiotic; incorporation per 2×10^6 cells: ^{14}C-leucine, 29,208 cpm; ^{14}C-uridine, 7,658 cpm; ^{14}C-thymidine, 1,444 cpm.



The data clearly indicate that siccayne interferes with the uptake of the precursors into the cells thus leading to a decreased incorporation into macromolecules.

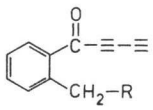
In order to elucidate the mode of action of this compound further, we also tested the influence of siccayne on cell-free RNA and DNA syntheses. *In vitro* RNA synthesis was performed with isolated nuclei of ECA cells. As shown in Table 4 siccayne affects the incorporation of UTP into RNA at concentrations comparable to those needed for the inhibition of RNA synthesis in whole cells. *In vitro* DNA synthesis was tested using toluene-treated cells of *Bacillus brevis*. The incorporation of ^{3}H-dTTP into DNA at 100 μg/ml of siccayne was only slightly affected (Table 5). This might be due to the higher cell density (1.6×10^8 cells/ml) used in this assay as compared to the serial dilution test (inoculum 1×10^6 cells/ml).

These results indicate that siccayne interferes with a variety of enzymatic reactions involved in transport of precursors (*e.g.* nucleosides) or synthesis of essential macromolecules (*e.g.* RNA and DNA syntheses). Siccayne does not cause hemolysis of bovine erythrocytes up to a concentration of 100 μg/ml when tested as described previously[9]. In this respect siccayne differs from other phenolic

compounds exhibiting a high degree of hemolytic and detergent-like mode of action[15].

Discussion

Antibiotically active compounds structurally related to siccayne namely frustulosinol (**2**) and frustulosin (**3**) have been reported from cultures of the basidiomycete *Xylobolus* (*Stereum*) *frustulatus*[14,16]. They differ from siccayne only by the presence of a one-carbon unit at the hydroquinone moiety. Both compounds were found active against *Staphylococcus aureus, Bacillus mycoides* and *Bacillus subtilis* at a concentration of 16 ppm and against several fungal species at somewhat higher concentrations. They were also moderately active against *Vibrio cholera* and *V. cholera* phage[14]. Other acetylenic substances containing an aromatic ring are the antifungal and antitumor antibiotics from *Peniophora affinis*[17] and the antibacterial and antifungal peniophorins B (**4**) and A (**5**)[18].



(**4**) R = CO_2H

(**5**) R = $C{=}CH{-}C{-}C_2H_4OH$ (with OCH_3 and O)

Simple prenylated hydroquinone derivatives have been isolated recently from the marine urochordate *Aplidium*. These substances act as anticancer and antimutagenic agents[19]. Radioprotective and cancer-protective activities have also been found for geranylhydroquinone[20] which is a constituent of higher plants (genera *Phagnalon*[21] and *Phacelia*[22]) as well as of *Aplidium*. The evaluation of the presumed *in vivo* anticancer properties of siccayne is still under investigation.

Acknowledgement

Halocyphina villosa was collected by F. OBERWINKLER in Colombia. We are very grateful to F. LIPMANN, in whose laboratory J. K. had the opportunity to work on chicken embryo fibroblasts. We thank the Deutsche Forschungsgemeinschaft for generous financial support.

References

1) ANKE, T.; J. KUPKA, G. SCHRAMM & W. STEGLICH: Antibiotics from basidiomycetes. X. Scorodonin, a new antibacterial and antifungal metabolite from *Marasmius scorodonius* (FR.) FR. J. Antibiotics 33: 463~467, 1980
2) SCHEUER, P. J.: Chemistry of Marine Natural Products. Academic Press, New York, 1973
3) BAKER, T. J. & V. MURPHY: Compounds from Marine Organisms. *In*: Handbook of Marine Science, vol. 1. CRC Press, Cleveland, Ohio, 1976
4) FAULKNER, D. J.: Interesting aspects of marine natural products chemistry. Tetrahedron 33: 1421~1443, 1977
5) GINNS, J. & D. MALLOCH: *Halocyphina*, a marine basidiomycete (*Aphyllophorales*). Mycologia 69: 53~58, 1977
6) KOHLMEYER, J. & E. KOHLMEYER: Marine Mycology. The Higher Fungi. Academic Press, New York, 1979
7) ISHIBASHI, K.; K. NOSE, T. SHINDO, M. ARAI & H. MISHIMA: Siccayne: A novel acetylenic metabolite of *Helminthosporium siccans*. Ann. Sankyo Res. Lab. 20: 76~79, 1968
8) MOSER, M.: Die Gattung *Phlegmacium* (Schleimköpfe). *In*: Die Pilze Mitteleuropas 4. Julius Klinkhardt, Bad Heilbrunn, 1960 (published 1961)
9) 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
10) VOGT, P. K.: Focus assay of Rous sarcoma virus. *In*: Fundamental Techniques of Virology. Academic Press, New York, 1969
11) MURAMATSU, M.; Y. HAYASHI, T. ONISHI, M. SAKAI, K. TAKAI & T. KASHIYAMA: Rapid isolation of nucleoli from detergent purified nuclei of various tumor and tissue culture cells. Experimental Cell Res.

88: 345~351, 1974

12) MOSES, R. E. & C. C. RICHARDSON: Replication and repair of DNA in cells of *Escherichia coli* treated with toluene. Proc. Nat. Acad. Sci. U.S.A. 67: 674~681, 1970

13) NÜSSLEIN, V. & A. KLEIN: DNA replication in the cellophane membrane system. *In*: Methods in Molecular Biology, vol. 7, Marcel Dekker Inc., New York, 1975

14) NAIR, M. S. R. & M. ANCHEL: Frustulosinol, an antibiotic metabolite of *Stereum frustulosum*: Revised structure of frustulosin. Phytochemistry 16: 390~392, 1977

15) GIANNETTI, B. M.; W. STEGLICH, W. QUACK, T. ANKE & F. OBERWINKLER: Antibiotics from basidiomycetes. VI. Merulinsäuren A, B und C, neue Antibiotika aus *Merulius tremellosus* FR. und *Phlebia radiata* FR. Z. Naturforsch. 33c: 807~816, 1978

16) NAIR, M. S. R. & M. ANCHEL: Frustulosin, an antibiotic metabolite of *Stereum frustulosum*. Tetrahed. Lett. 1975: 2641~2642, 1975

17) CARRAZ, G.; L. ODDOUX & H. BERRIEL: Antibiotic with fungicidal and antitumor activity. Fr. Demande 2,310,753, 10 Dec. 1976 (Chem. Abstr. 87: 83188n)

18) GERBER, N. N.; S. A. SHAW & H. LECHEVALIER: Structures and antimicrobial activities of peniophorin A and B, two polyacetylenic antibiotics from *Peniophora affinis* Burt. Antimicr. Agents & Chemoth. 17: 636~641, 1980

19) HOWARD, B. M.; K. CLARKSON & R. L. BERNSTEIN: Simple prenylated hydroquinone derivatives from the marine urochordate *Aplidium californicum*. Natural anticancer and antimutagenic agents. Tetrahed. Lett. 1979: 4449~4452, 1979

20) BARANGER, P.: Substituted polyphenols. Fr. Demande M2694. 31, Aug. 1964 (Chem. Abstr. 61: 15940e)

21) BOHLMANN, F. & K.-M. KLEINE: Über ein neues Chinon aus höheren Pflanzen. Chem. Ber. 99: 885~888, 1966

22) REYNOLDS, G. & E. RODRIGUEZ: Geranylhydroquinone: A contact allergen from trichomes of *Phacelia crenulata*. Phytochemistry 18: 1567~1568, 1979