ANTIBIOTICS FROM BASIDIOMYCETES

XXXVIII. 2-METHOXY-5-METHYL-1,4-BENZOQUINONE, A THROMBOXANE A_2 RECEPTOR ANTAGONIST FROM *LENTINUS ADHAERENS*

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In the course of our screening for antithrombotic compounds using platelet rich plasma from bovine slaughter blood, 2-methoxy-5-methyl-1,4-benzoquinone (**1**) has been isolated from mycelial cultures of *Lentinus adhaerens*. The compound inhibits the U46619-induced aggregation of human blood platelets with an IC_{50} of 2.5 μg/ml (16.45 μM) and is a new thromboxane A_2 receptor antagonist.

This is the first report on an inhibitor of platelet aggregation derived from secondary metabolism of basidiomycetes.

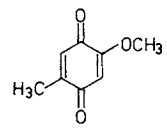
Platelets are multiresponding cells and it is well established that *in vivo* and *in vitro* a series of responses like shape change, aggregation and granule secretion is caused by many diverse agonists and that each of these agonists has its own specific receptor on the platelet surface[1].

Thromboxane A_2 (Tx A_2) is a main product of the arachidonic acid metabolism in platelets and is a very potent inducer of platelet aggregation and vasoconstriction. It is generally accepted that Tx A_2 is involved in the pathogenesis of a variety of vascular disorders such as arterial thrombosis, myocardial infarction or angina pectoris[2].

Aspirin, the classical antiaggregating compound, inhibits cyclooxygenase and therefore redirects the arachidonic acid metabolism to the lipoxygenase products, the leucotrienes, which are responsible for some of the adverse side-effects of this drug[3].

Thus antiaggregating agents acting on the Tx A_2 receptor level may offer therapeutic advantages[4].

In the following we wish to describe the fermentation, isolation, structure elucidation and biological activities of 2-methoxy-5-methyl-1,4-benzoquinone (**1**), a platelet aggregation inhibitor from fermentations of *Lentinus adhaerens*.



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Materials and Methods

L. adhaerens (A. and S. ex Fr.) Fr., Strain 7856

The producing strain was derived from spore prints of fruiting bodies collected in Deißlingen, Germany.

The specimen showed the characteristics of the genus and species[5]. The strain is deposited in the culture collection of the Lehrbereich Biotechnologie of the University of Kaiserslautern.

Fermentation: For maintenance on agar slants the fungus was grown in YMG medium (yeast extract 0.4%, glucose 0.4%, malt extract 1%, pH 5.5). Fermentations were carried out in 5-liter Erlenmeyer flasks or in a 20-liter Biolafitte C-6 fermenter containing the following medium (g/liter): glucose 4 g, yeast extract 4 g, malt extract 10 g, KH_2PO_4 0.3 g, pH 6.0. The fermenter was incubated at 22°C with aeration (1.5 liters air/minute) and agitation (150 rpm).

Isolation: After 16~19 days the culture broth (8 liters) was extracted with an equal volume of ethyl acetate. Evaporation of the organic phase yielded a crude extract (835.5 mg) which was applied to a flash column containing silica gel (Merck 60, 5 × 15 cm). After elution with cyclohexane - ethyl acetate (3 : 1) an enriched product (141 mg) was obtained. This was further purified by a preparative medium pressure liquid chromatography on LiChroprep diol (Merck; elution with cyclohexane - ethyl acetate (1 : 1) 71 mg) and following chromatography on Sephadex LH-20 (elution with MeOH, 40 mg). 2-Methoxy-5-methyl-1,4-benzoquinone (**1**) (29 mg) was obtained by HPLC (Silica gel Merck 60, 7 μm, column 2.5 × 25 cm, elution with cyclohexane - ethyl acetate (7 : 3)). During purification **1** was localized using the platelet aggregation assay as described below, or TLC.

Physical and Spectroscopic Data: IR spectra were measured on a Bruker/FS 48 spectrometer. Mass spectra were determined with a Finnigan MAT CH 7A spectrometer. NMR spectra were recorded on a Bruker AMX 500 spectrometer.

Biological Assays

The antimicrobial spectra and the cytotoxicity of **1** were measured as described previously[6,7].

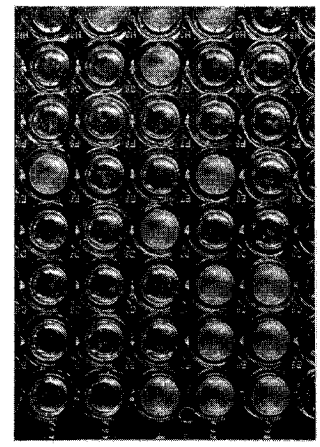
Test for Mutagenicity: Mutagenicity was tested as described by AMES *et al.*[8]. Mutants of *Salmonella typhimurium*, strain TA 98 and strain TA 100 were used for the spot test with and without rat liver microsomes.

Preparation of Platelet Rich Plasma (PRP): 9 volumes of fresh bovine slaughter blood were mixed with 1 volume of the following anticoagulant (AC) buffer: 93 mM sodium citrate; 140 mM glucose; pH adjusted to 7.4 by 1 M citric acid. The anticoagulated blood was centrifugated at 150 × g for 15 minutes at room temperature to obtain PRP. PRP contained $3 \sim 4 \times 10^5$ platelets/μl.

Preparation of Platelet Poor Plasma (PPP): PPP was obtained by centirifugation of anticoagulated blood at 1,000 × g for 10 minutes.

Platelet Aggregation Assay[9]: Bovine platelet aggregation was carried out in a spectrophotometer (Hitachi, model 100-60) with temperated and stirred plastic cuvettes. After a preincubation for 10 minutes aggregation was stimulated with aggregating agent and the change of transmittance was monitored at 600 nm with PPP as reference. For screening purposes the aggregation test with collagen as stimulator was performed in 96-well-microtiter-plates (Renner) and aggregation evaluated at 600 nm in a Bio-Rad-EIA-Reader (Fig. 1).

Fig. 1. Aggregation of bovine platelets in microtiter-plates.



Human platelet aggregation was carried out without preincubation in an aggregometer (Colora).

Phopolipase C: The effect of **1** on phopholipase C (from *Bacillus cereus*, Sigma) was measured as described by KURIOKA and MATSUDA[10].

Effect of **1** on Thromboxane A_2 Synthesis: Bovine-PRP was washed twice with prewarmed AC-buffer (1,000 × g, 5 minutes) and the platelets resuspended in a solution of NaCl 145 mM, KCl 5 mM, $MgCl_2$ 1 mM, HEPES 10 mM, glucose 10 mM, pH 7.4. To 1 ml of this suspension containing 4.25×10^8 platelets/ml collagen (140 μg) or thrombin (1 U) was added to stimulate Tx A_2 synthesis. After incubation for 5 minutes the test tubes were rapidly frozen in liquid nitrogen. After thawing the pH was

adjusted to pH 3 and the samples were applied to C 2 Amprep minicolumns (Amersham), which were equilibrated with H_2O. After washing with water, 10% EtOH, and *n*-hexane thromboxane B_2 was eluted with methyl formate. After evaporation of the solvent thromboxane B_2 was determined using the scintillation proximity assay (Amersham Buchler).

Results and Discussion

Fermentation

During fermentation of *L. adhaerens* (Fig. 2) the production of 2-methoxy-5-methyl-1,4-benzoquinone (**1**) starts 9 days after inoculation. The highest antibiotic content (0.15 mg/liter) is reached after 13 days. At this time the free glucose in the medium has been used up completely. The product yield is higher in 5-liter Erlenmeyer-flasks (3.6 mg/liter).

1 has been isolated previously as a weakly antimicrobial metabolite from *Coprinus similis* and *Lentinus degener*[11].

2-Methoxy-5-methyl-1,4-benzoquinone (**1**)

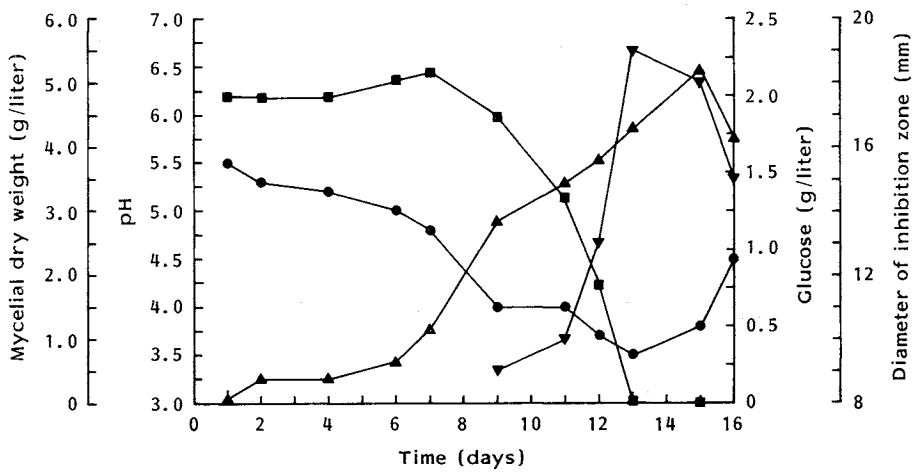
The structure was confirmed by the following spectroscopic data: Slightly yellow crystals; mp 173~175°C (174~175.5[12]); Rf 0.46 cyclohexane - ethyl acetate - acetic acid (120 : 40 : 5); 0.77 toluene - ethyl acetate (9 : 1); UV λ_{max}^{MeOH}: 263; IR (KBr) cm^{-1}: 1672, 1650, 1602; EI-MS *m/z* 152; ^1H NMR (DMSO-d_6): δ (ppm) 1.96 (3H, d, J=1.6 Hz, CH_3), 3.77 (3H, s, OCH_3), 6.09 (1H, s, h-quinoid, α-position to methoxy), 6.65 (1H, qu, J=1.6 Hz, H-quinoid, α-position to methyl); ^{13}C NMR (DMSO-d_6): δ (ppm) 15.05 (CH_3), 56.21 (OCH_3), 107.48 (–*C*H=COCH$_3$), 130.99 (–*C*H=*C*CH$_3$), 146.00 (CH$_3$–*C*=), 158.50 (H$_3$C–C–*C*=), 181.70 (C=O, 14 β-position to OCH_3), 187.45 (C=O, β-position to methyl).

Effect on Platelet Aggregation

The influence of **1** on the collagen-induced bovine platelet aggregation is shown in Fig. 3. **1** inhibits platelet aggregation at concentrations of 5~6 μg/ml. It does not effect the primary aggregaion of human

Fig. 2. Fermentation of *Lentinus adhaerens*.

● pH, ▲ dry weight, ■ glucose, ▼ antibacterial activity.



The antibacterial activity was followed up by agar diffusion assay using *Bacillus subtilis* as test organism.

and bovine platelets induced by ADP (Fig. 4A and 4B) and shows only weak influence on secondary thrombin-stimulated aggregation of human platelets (Fig. 5). Thrombin, the most potent platelet aggregating agent, acts *via* phosphoinositol hydrolysis by phospholipase C[13]. This enzyme (from *B. cereus*) was not inhibited up to 100 μg/ml of **1**.

Collagen-stimulated aggregation of human platelets was inhibited at concentrations of 5~10 μg/ml (Fig. 6).

Fig. 7 shows the influence of **1** on the U46619-induced aggregation of human platelets. Five μg/ml (33 μM) of **1** inhibit platelet aggregation completely. The IC_{50}-value was determined to 2.5 μg/ml (16.45 μM). It is generally accepted that the prostaglandin analog and thromboxane A_2 mimic U46619 binds directly to the thromboxane A_2 receptor-site[14] and for that reason **1** was thought to interfere either with thromboxane directly or with its receptor.

The influence of **1** on thromboxane synthesis and thromboxane itself was investigated using serum-free platelet suspensions. After stimulation with collagen and thrombin the extent of thromboxane A_2 was determined *via* the stable thromboxane B_2 by the scintillation proximity technique. In this assay no significant effect of **1** on Tx A_2 synthesis could be observed. As a control acetylsalycylic acid,

Fig. 3. Effect of **1** on collagen-stimulated bovine platelet aggregation.

134 μg/ml collagen, (a) control, (b) 5 μg/ml of **1**, (c) 5.4 μg/ml, (d) 6 μg/ml.

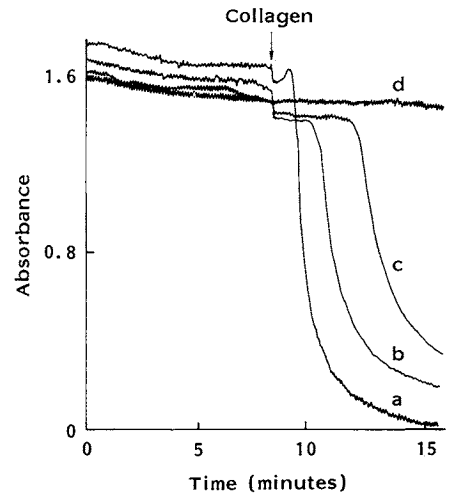


Fig. 4. Effect of **1** on ADP-induced bovine platelet aggregation and effect of **1** on ADP-induced human platelet aggregation.

(A) Bovine platelet, ADP 25 μM; (a) control, (b) 10 μg/ml of **1**. (B) human platelet, ADP 2.5 μM; (a) control, (b) 5 μg/ml of **1**, (c) 10 μg/ml, (d) 15 μg/ml.

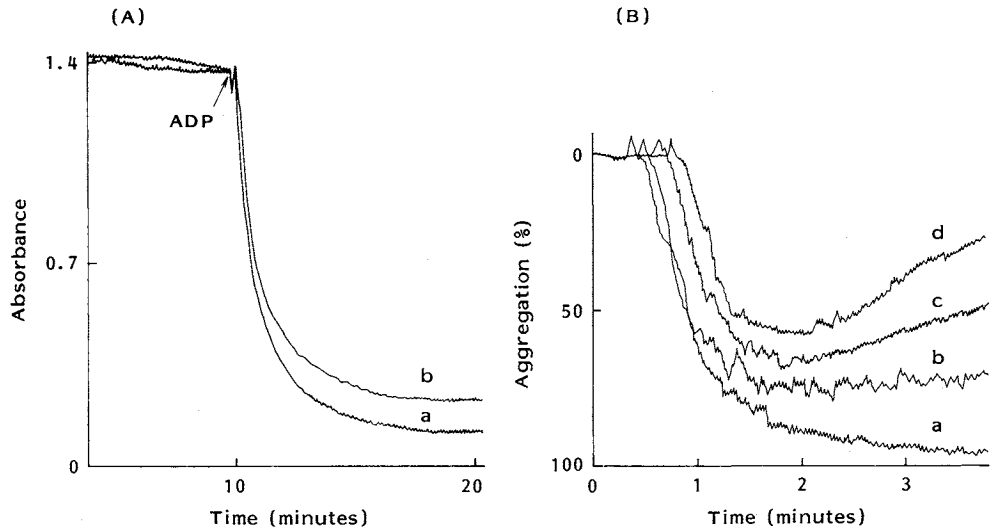


Fig. 5. Effect of **1** on thrombin-stimulated aggregation of human platelets.

Thrombin 0.075 u/ml, (a) control, (b) 10 μg/ml of **1**, (c) 15 μg/ml, (d) 20 μg/ml.

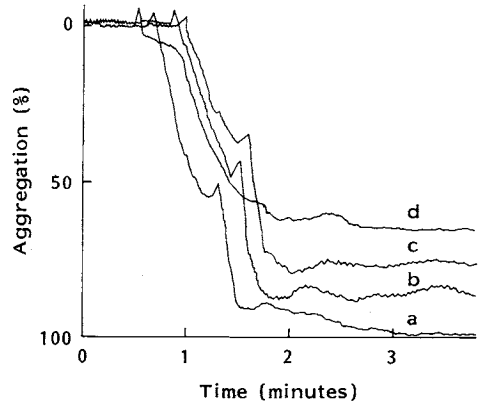


Fig. 6. Effect of **1** on collagen-stimulated aggregation of human platelets.

40 μg/ml collagen, (a) control, (b) 5 μg/ml of **1**, (c) 10 μg/ml.

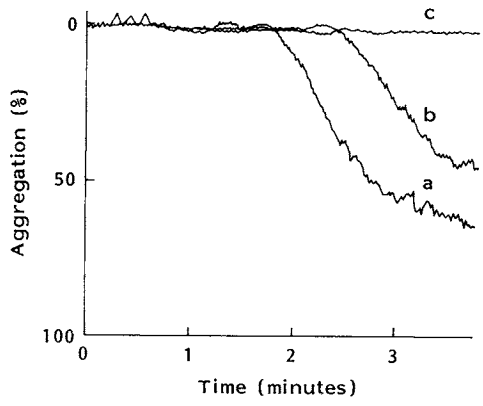


Fig. 7. Effect of **1** on U46619-induced aggregation of human platelets.

U46619 0.45 μM, (a) control, (b) 2.5 μg/ml of **1**, (c) 5 μg/ml, (d) 7.5 μg/ml.

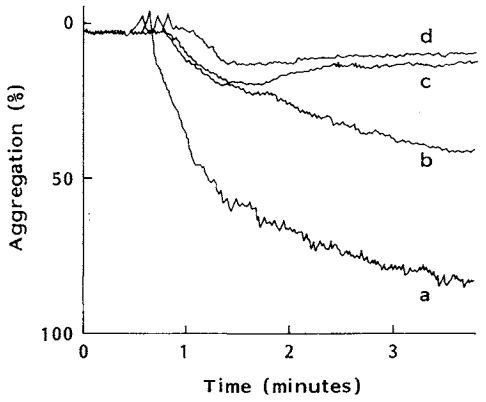
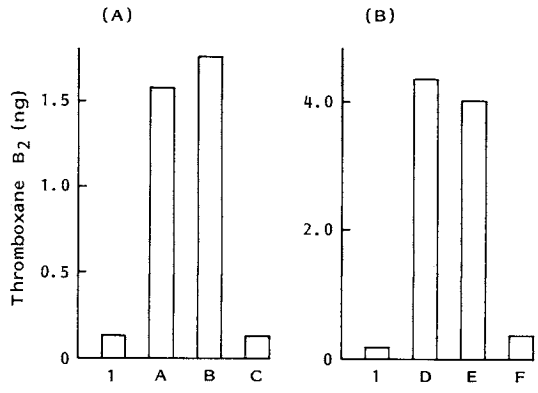


Fig. 8. Influence of **1** on thromboxane synthesis.

1: Platelet suspension, A: control (collagen), B: 15 μg/ml of **1**, C: 300 μg/ml acetylsalicylic acid, D: control (thrombin), E: 15 μg/ml of **1**, F: 300 μg/ml acetylsalicylic acid.



which irreversibly inhibits platelet cyclooxygenase, reduced significantly thromboxane synthesis (Fig. 8). Therefore **1** neither affects thromboxane synthesis nor reacts with thromboxane itself.

As shown in Fig. 9 the effect of **1** on the U46619-induced aggregation of human platelets can be completely reversed by the addition of 1.8~2.4 μM U46619. It is therefore concluded that 2-methoxy-5-methyl-1,4-benzoquinone is a competitive Tx A_2 receptor antagonist.

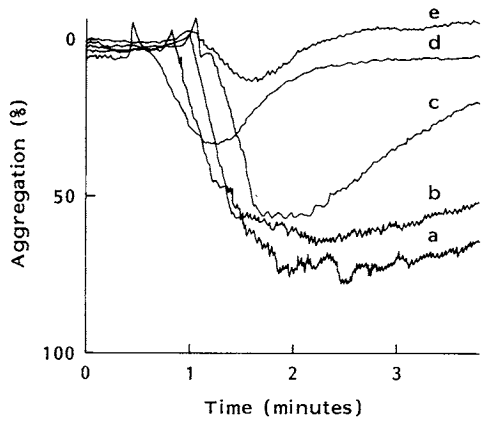
Other Biological Activities

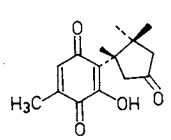
1 exhibits weak antibiotic activities. In the serial dilution assay the MICs for *Bacillus brevis* and *Proteus vulgaris* were determined to 10~50 and 50~100 μg/ml, respectively. The antifungal activities of **1** are very low. *Nematospora coryli*, one of the most sensitive species was inhibited at a MIC of 50~100 μg/ml.

In the test for mutagenicity according to AMES *et al.*[8] no induction of revertants of *S. typhimurium* TA 98 and TA 100 could be observed with 100 μg of **1**/disk (spot test with and without addition of rat

Fig. 9. Influence of variable inductor concentrations on the inhibitory effect of **1** (15 mg/ml).

(a) 2.4 μM U46619, (b) 1.8 μM U46619, (c) 1.2 μM U46619, (d) 0.6 μM U46619, (e) 0.3 μM U46619.





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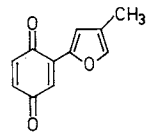

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liver microsomes).

The cytotoxic activity of **1** was tested with Ehrlich ascites carcinoma cells (H. PROBST, University of Tübingen) and HeLa S3 cells (ATCC CCL 2.2). **1** reduces the proliferation of ECA and HeLa cells at concentrations of 50~100 μg/ml and 25~50 μg/ml, respectively. Lysis of HeLa cells started at concentrations of 50~100 μg/ml.

Comparison with Other Benzoquinones

Lagopodin B (**2**)[15] and omphalon (**3**)[16] were isolated as metabolites of *Coprinus cinereus*[17] and *Lentinellus omphalodes*[18]. Using the same assays as described above for **1** both lagopodin and omphalon inhibited the collagen-induced aggregation of bovine platelets at concentrations of 5~7 μg/ml and 25~50 μg/ml, respectively. The primary aggregation of bovine and human platelets was not affected by **2** and **3** whereas the secondary aggregation of human platelets was inhibited at 10~15 μg/ml of **2** and at 25~50 μg/ml of **3**. The U46619-induced aggregation of human platelets was inhibited by 15 μg/ml (72 μM) of lagopodin B and 10 μg/ml (53 μM) of omphalon.

Acknowledgments

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References

1) HOLMSON, H.: Platelet secretion, Ch. 24. *In* Hemostasis and Thrombosis. *Ed.*, R. W. COLMAN, pp. 390~403, J. B. Lippincott Company, 1982
2) YANAGISAWA, A.; J. A. SMITH, M. A. BREZINSKI & A. M. LEFER: Mechanism of antagonism of thromboxane receptors in vascular smooth muscle. Eur. J. Pharmacol. 133: 89~96, 1987
3) PATSCHEKE, H.; M. D. C. STAIGER, G. NEUGEBAUER, K. STREIN, R. ENDELE & P. H. STEGMEIER: The pharmacokinetic and pharmacodynamic profiles of the thromboxane A_2 receptor blocker BM 13177. Clin. Pharmacol. Ther. 39: 145~150, 1986
4) DARIUS, H.; J. B. SMITH, A. M. LEFER: Beneficial effects of a new potent and specific thromboxane receptor antagonist SQ-29548 in vitro and in vivo. J. Pharmacol. Exp. Ther. 235: 247~281, 1985
5) MOSER, M. (*Ed.*): Die Röhrlinge und Blätterpilze. Kleine Kryptogamenflora, Bd. II b/2, Gustav Fischer Verlag, 1983
6) KUPKA, J.; T. ANKE, F. OBERWINKLER, G. SCHRAMM & W. STEGLICH: Antibiotics from basidiomycetes. VII. Crinipellin, a new antibiotic from the basidiomyceteous fungus *Crinipellis stipitaria* (FR.) PAT. J. Antibiotics 32: 130~135, 1979
7) LEONHARDT, K.; T. ANKE, E. HILLEN-MASKE & W. STEGLICH: 6-Methylpurine, 6-methyl-9-β-D-ribofuranosylpurine and 6-hydroxymethyl-9-β-D-ribofuranosylpurine as antiviral metabolites of *Collybia maculata* (basidiomycetes). Z. Naturforsch. C42C: 420~424, 1987
8) AMES, B. N.; J. MCCANN & E. YAMASAKI: Methods for detecting carcinogenes and mutagenes with the *Salmonella*/Mammalian mutagenicity test. Mutat. Res. 31: 347~364, 1975
9) LAUER, U.: Isolierung und Charakterisierung eines Thromboxan-A_2-Rezeptor-Antagonisten aus dem

Basidiomyceten *Lentinus adhaerens*. Ph.D. Thesis, Univ. Kaiserslautern, 1990

10) KURIOKA, S. & M. MATSUDA: Phospholipase C assay using pNPPC together with sorbitol and its application to study the metal and detergent requirement of the enzyme. Anal. Biochem. 75: 281~289, 1976
11) ANCHEL, M.; A. HERVEY, F. KAVANAGH, J. POLATNICK & W. ROBINS: Antibiotic substances from Basidiomycetes *Coprinus similis* and *Lentinus degener*. Proc. Natl. Acad. Sci. U.S.A. 34: 498~502, 1948
12) WOODWARD, R. B.; F. SONDHEIMER, D. TAUB, K. HEUSLER & W. M. MCLAMORE: The total synthesis of steroids. J. Am. Chem. Soc. 74: 4223~4251, 1952
13) MICHELL, R. H.: Inositolphospholipids and cell surface receptorfunction. Biochem. Biophys. Acta 415: 81~147, 1975
14) PARISE, L. V.; D. L. VENTON & G. C. LE BRETON: Arachidonic acid-induced platelet aggregation is mediated by a Tx A_2/PGH_2-receptor interaction. J. Pharmacol. Exp. Ther. 1980: 228~240, 1980
15) BOLLINGER, P.: Über die Konfiguration der Lagopodine A, B, C, Ph.D. Thesis, ETH Zürich, 1965
16) MOCEK, U.: Neue Wirkstoffe aus Basidiomyceten. Ph.D. Thesis, Univ. Bonn, 1985
17) BASTIAN, W.: Vergleichende Untersuchung zum Sekundärstoffwechsel coprophiler und erd-oder holzbewohnender Basidiomyceten. Ph.D. Thesis, Univ. Kaiserslautern, 1985
18) STÄRK, A.: Antibiotika aus der Gattung *Lentinellus*. Ph.D. Thesis, Univ. Kaiserslautern, 1985