Collybial, a New Antibiotic Sesquiterpenoid from *Collybia confluens* (Basidiomycetes)

Brigitte Simon[a], Timm Anke[a], U. Anders[b], M. Neuhaus[b] and F. Hansske[b]

[a] LB Biotechnologie der Universität, Paul-Ehrlich-Str. 23, D-67663 Kaiserslautern, Bundesrepublik Deutschland
[b] Boehringer Mannheim GmbH, Sandhofer Str. 116, D-68305 Mannheim, Bundesrepublik Deutschland

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A new antibiotic was isolated from fermentations of an american strain of *Collybia confluens*. Its structure was elucidated by spectroscopic methods as 2,10,10-trimethal-4-oxo-tricyclo[7.2.0.0^{2,5}]undec-6-en-carbaldehyde (with the relative stereo chemistry 1*S*, 2*R*, 5*R*, 9*R*) (**1**). The inhibitor, which was named collybial, is structurally related to koraiol, a sesquiterpenoid isolated from Pinus koraiensis (Khan V. A. (1979), Khim. Prir. Soedin **5,** 652–658).

Collybial inhibited the growth of Gram-positive bacteria at concentrations starting from 21.5 μM. The propagation of vesicular stomatitis virus (VSV) in baby hamster kidney (BHK-21) cells was inhibited by 21.5 μM collybial. Cytotoxic effects on BHK cells were observed at 5 fold higher concentrations.

Introduction

Basidiomycetes provide an interesting source for new secondary metabolites with a variety of different biological activities (Anke, 1988). However, up to now only a few antiviral substances like the purine derivatives of *Collybia maculata* (Leonhardt *et al.,* 1987) have been reported. In the course of a screening for inhibitors of the multiplication of vesicular stomatitis virus (VSV) in baby hamster kidney (BHK) cells we detected that *Collybia confluens* produced a compound with antiviral, cytotoxic and antimicrobial activities. In the following we describe the fermentation, isolation, structural elucidation, and biological characterization of collybial, a natural compound with a very rare tricyclo[7.2.0.0^{2,5}]undecen structure.

Experimental

General

Collybia confluens strain 90293 was derived from the spore print of a fruiting body collected in USA. Mycelial cultures and voucher specimen are deposited in the culture collection of the LB Biotechnologie, University of Kaiserslautern.

Reprint requests to Prof. Dr. T. Anke.
Telefax: (0631) 2052999.

Melting points were determined on a Büchi 510 apparatus and are uncorrected. IR spectra were measured with a Bruker IFS 48 spectrometer. The UV spectra were recorded with a Perkin Elmer Lambda 16 spectrophotometer. Proton and carbon spectra were recorded on a Bruker AMX 500 spectrometer operating at 500.14 MHz for ^{1}H. The concentration of the sample of **1** was 72 mM in $CDCl_3$. All spectra were recorded at 305 K and TMS was used as internal reference. For the DQF-H,H-COSY (Aue *et al.,* 1976; Marion *et al.,* 1983) 512 experiments of 16 scans each (4 dummy scans before the experiment) were recorded with a relaxation delay of 4 s, an acquisition time of 0.44 s and 4K of data size. The hetero-nuclear inverse experiments were recorded with 512 experiments of 2K data size and an acquisition time of 0.22 s each (16 dummy scans before the experiment). HMQC (Bax *et al.,* 1986a) and HMQC-TOCSY (Lerner *et al.,* 1986) were carried out with BIRD-filter. For the later a 40 ms spin-lock of 10 kHz with DIPSI2 (Shaka *et al.,* 1988) and two 2.5 ms trim-pulses were applied. The delay for evolution of the long range couplings in the HMBC (Bax *et al.,* 1986b) was 40 ms. The H,C-COLOC experiment (Kessler *et al.,* 1984) was recorded with a relaxation delay of 3 s, an acquisition time of 0.08 s, 4K of data size and 188 experiments. The delay for evolution of the long range couplings

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was 25 and 30 ms, respectively. For the ROESY spectrum (Bothner-By *et al.*, 1984; Kessler *et al.*, 1987) a mixing time of 700 ms was used (T_1^{min} app. 900 ms). The spin-lock of 2 kHz was produced by 15° pulses with an inter-pulse delay of 18.7 μs.

The homonuclear coupling constants were determined with WIN-DAISY from a 1D spectrum by iterative simulations of the 10 spin-system of coupled protons .

The structure was refined with Sybyl 6.03 (Tripos Associates, Inc.) in vacuo. The distances, determined from the ROESY spectrum by quantitative integration of the cross peaks, were used as constraints for a molecular dynamics calculation (MD). The force constant for the distance pseudo potential was K_{dc} = 840 kJ·mol^{-1}·nm^{-2}. The calculation was carried out over 20 ps, where during the first 5 ps the temperature was 1000 K. Afterwards, the temperature was lowered to 300 K. The final structure was energy minimised (EM).

Mass spectra were measured with a Fisons AutoSpec spectrometer.

Fermentation

For the maintenance on agar slants and for submerged cultivation *C. confluens* was grown in an YMG medium composed of: Yeast extract 0.4%, malt extract 1%, glucose 0.4%. The pH was adjusted to 5.5. Fermentations were carried out in 100 l of YMG medium in a Biostat U fermentor (Braun-Diessel) with aeration (20 l air/min) and agitation (100 rpm) at 22°C. Inoculum: 10 l of well grown fermentation in the same medium. The production of collybial was followed by plate diffusion assay with *Bacillus brevis* as test organism.

Isolation of collybial

After 160 hours of fermentation the mycelia were separated from the culture fluid by filtration. Collybial was adsorbed from the culture fluid to HP21-resin (Mitsubishi) and eluted with acetone. The crude product was purified by chromatography on silica gel (Merck 60, eluant: cyclohexane/ethyl acetate 80:20) and then preparative HPLC (column 25×250 mm; Merck LiChrosorb Diol, 7 μm; eluant: cyclohexane – *tert.* butylmethylether 80:20). Yield: 10 mg.

Collybial (**1**)

White needles, m.p. 121 °C soluble in methanol, acetone, ethylether, R_f = 0.82 [toluene – acetone – HAc 70:30:1, silica gel 60 F_{254}, Merck], UV (MeOH) λ_{max} (ε): 236 (8571), $[\alpha]_D^{20}$ = + 17.1 (*c* = 1, MeOH), IR (KBr) ν_{max}: 3449, 3356, 2959, 2934, 2860, 1779, 1688, 1629, 1465, 1417, 1398, 1379, 1368, 1332, 1317, 1285, 1251, 1221, 1192, 1179, 1164, 1141, 1123, 1113, 1089, 1057, 1024, 968, 832, 820, 756, 709, 668, 604 cm^{-1}. MS- and NMR-Data see below.

Antimicrobial activity

The minimal inhibitory concentrations were determined in the serial dilution assay as described earlier (Kupka *et al.*, 1979) with some modifications (Simon, 1994).

Cell culture, assay for cytotoxicity, and macromolecular syntheses

L1210 (ATCC CCL 219) and HeLa S3 (ATCC CCL 2.2) cells were grown in F-12 medium containing 20 % of horse serum or 10 % of fetal calf serum, respectively. BHK-21 cells (ATCC CCL 10) were grown in G-MEM medium containing 10% tryptose phosphate broth and 10 % of fetal calf serum, HL-60 (ATCC CCL 240) and U937 (ATCC CRL 151) in RPMI 1640, with 10 % of fetal calf serum aded. All media contained 65 μg/ml of penicillin G and 100 μg/ml of streptomycin sulfate, cells were grown in a humidified atmosphere containing 5% of CO_2 at 37°C.

Cytotoxicity was measured by the method described by Mirabelli *et al.* (1985) with modifications (Erkel, 1990).

The incorporation of labelled thymidine, uridine, and leucine into DNA, RNA, and protein of L1210 cells was tested as described previously (Weber *et al.*, 1990). The cells were grown in roller bottles for 72 hours and suspended in phosphate buffered saline (PBS, g/l: NaCl 8, KCL 0.2, $Na_2HPO_4 \times 2H_2O$ 1.44, KH_2PO_4 0.2; pH 7.4, 2×10^7 cells/ml).

Vesicular Stomatitis Virus (VSV)

VSV (ATCC VR 158, Indiana Strain) was propagated in BHK-21 cells grown in microtiter plates as described earlier (Leonhardt *et al.*, 1987).

Virus titers were assayed by withdrawing 10 µl aliquots in 6 h intervals and determining the plaques formed on BHK-21 cells after suitable dilution of the virus-containing samples. RNA- directed RNA- polymerase in lysed VSV was assayed as described previously (Kuschel *et al.*, 1994)

Results and Discussion

Production and isolation of collybial

A typical fermentation of *Collybia confluens* strain TA 90293 is shown in Fig. 1. The production of collybial as followed by the plate diffusion assay with *Bacillus brevis* starts 100 hours after inoculation, the highest content is reached after 160 hours.

Structural elucidation

Collybial was isolated as described in the experimental section. The molecular weight was determined by chemical ionisation (CI) MS (with isobutane). The CI spectrum shows significant peaks at *m/z* 233 (pseudo molecular mass MH^+; rel. int. 100%), *m/z* 215 (loss of H_2O; rel. int. 28%) and *m/z* 191 (loss of CH_2CO from MH^+; rel. int. 74%).

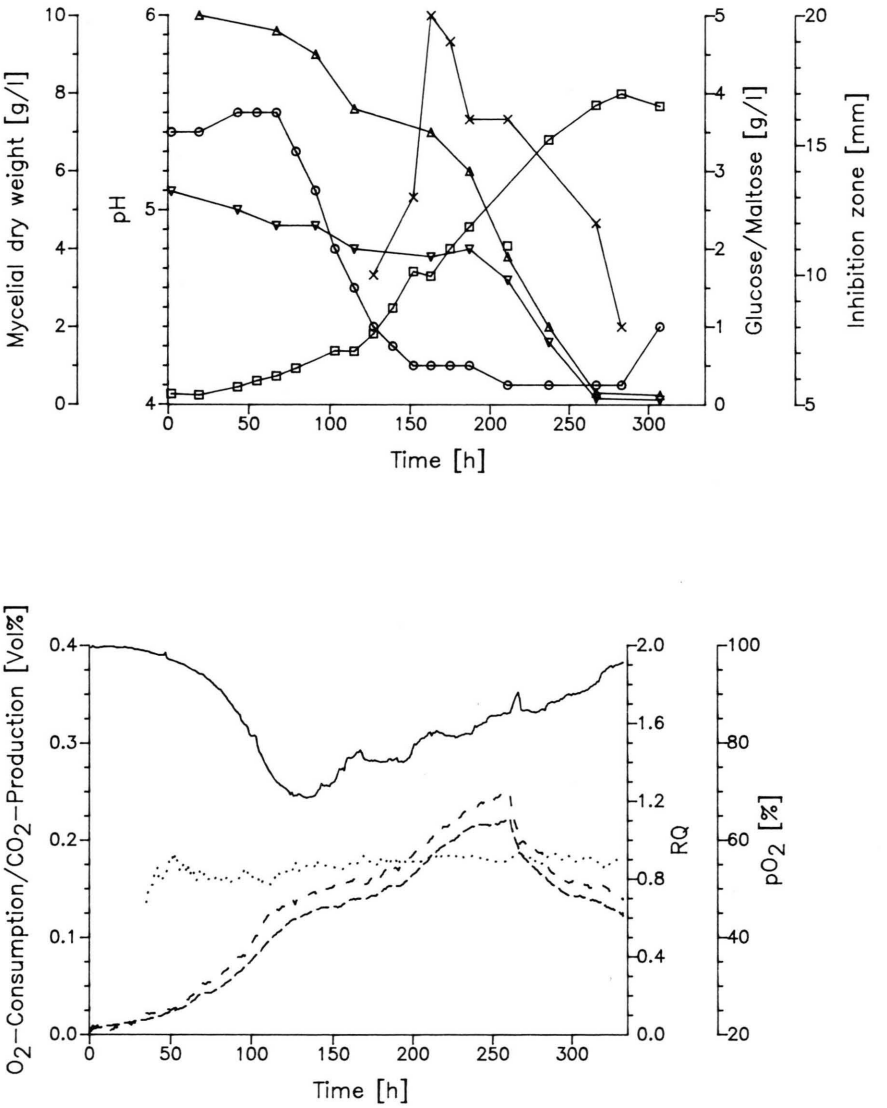
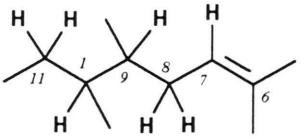
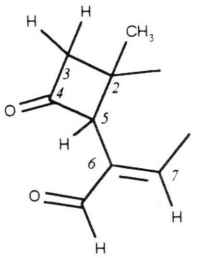


Fig. 1. Fermentation of *Collybia confluens*. ○—○, pH; △—△, maltose; ▽—▽, glucose; □—□, mycelial dry weight; ×—×, diameter inhibition zone *Bacillus brevis;* – – –, O_2-consumption; – –, CO_2-production; ·····, RQ; ——, pO_2.

Furthermore HR-EIMS (ion energy 70 eV, source temperature 250 °C, acc. volt. 8kV) was used to determine *m/z* 304.183105 (rel. int. 10 %) $M^{+\cdot}$ of mono-TMS derivative (trimethylsilyldienolether: $C_{18}H_{28}O_2Si$ requires 304.185859). The molecular formula results in $C_{15}H_{20}O_2$. Fragment ion *m/z* 190.13443 gives $C_{13}H_{18}O$ ($M^{+\cdot}$ – CH_2CO ; requires 190.135765).

In the DQF-H,H-COSY NMR spectrum, a spin system comprising of 7 protons is found:



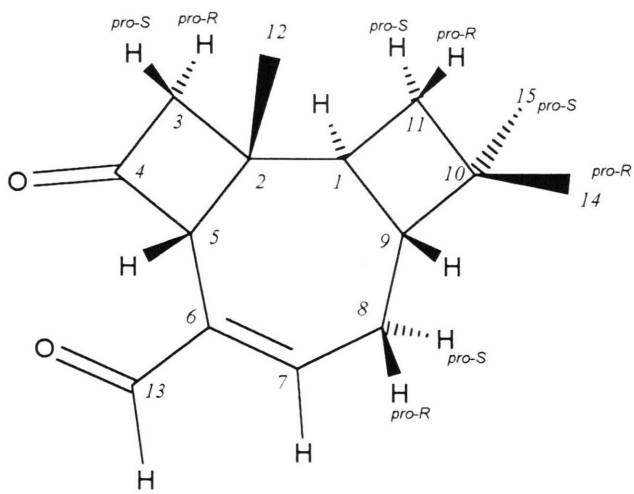


In addition, a CH_2 group with strongly different chemical shifts of the proton resonances shows a small coupling to a CH. There are three methyl groups that are bound to quaternary carbon atoms as they show only singlets in the proton spectrum. Furthermore, an aldehyde functional group is found (cf. Table I).

Heteronuclear long range couplings ($^3J_{H,C}$) between two of the methyl groups (H-14 and H-15) can be assigned in the HMBC spectrum. Furthermore, the protons of both methyl groups couple to C-10 ($^2J_{H,C}$), C-9 and C-11 ($^3J_{H,C}$). Both methyl groups must be bound to the quaternary carbon C-10 while C-9 and C-11 are the two neighbouring carbon atoms of C-10. In the DQF-H,H-COSY spectrum, H-9 and H-11 both show connectivities to H-1 with $^3J_{H,H}$ coupling constants between 8.0 and 10.5 Hz (cf. Table I). Henceforth, C-9, C-1, C-11 and C-10 form a four-membered ring in this part of the molecule.

The third methyl group is bound to a quaternary carbon as well. In the H,C-COLOC experiment, strong cross peaks are found to the C-3(CH_2) and C-5(CH) carbons mentioned above. A weak signal to C-1 is seen as well.

Cross peaks due to heteronuclear long range couplings from the proton at the double bond (H-7) can be found to C-5 and the carbonyl carbon



Collybial (**1**)

Table I. Chemical shifts and coupling constants of collybial.

Atom	$^{13}C^{a}$	$^{1}H^{a}$	$^{1}H^{b}$ Multiplicity	Assignment	H,H coupling constants[c]
1	42.95	2.07	m		H1,H9 = 10.5, H1,H11$^{pro\text{-}R}$ = 10.0, H1,H11$^{pro\text{-}S}$ = 8.0, H1,H3$^{pro\text{-}S}$ = 0.3
2	33.57	–			
3	56.77	2.94	dd	*pro-S*	H3,H5 = 1.7, H3,H3 = 15.9
		2.499	dd	*pro-R*	H3,H5 = 1.2
4	202.98	–			
5	67.40	4.35	m		H5,H7 = 1.0, H5,H8$^{pro\text{-}S}$ = 2.0, H5,H8$^{pro\text{-}R}$ = 1.2
6	136.26	–			
7	154.32	6.72	m		H7,H8$^{pro\text{-}S}$ = 2.5, H7,H8$^{pro\text{-}R}$ = 6.5
8	32.06	2.41	m	*pro-S*	H8$^{pro\text{-}S}$,H9 = 11.8, H8,H8 = 19.5
		2.503	m	*pro-R*	H8$^{pro\text{-}R}$,H9 = 4.3
9	42.77	1.97	m		
10	33.88	–			
11	34.73	1.67	dd	*pro-R*	H3,H3 = 19.5
		1.65	dd	*pro-S*	
12	21.85	1.49	s		
13	194.99	9.33	s		
14	30.03	1.08	s	*pro-R*	
15	22.70	1.07	s	*pro-S*	

[a] Chemical shifts rel. TMS = 0.0 ppm.
[b] s = singlet, dd = doublet of doublets, m = multiplet.
[c] Coupling constants in Hz ± 0.2 Hz (from WIN-DAISY simulation).

of the aldehyde functional group. Unambiguous signals are found between H-5 proton the keto carbonyl carbon C-4, the two double bond carbons, and the methyl group carbon C-12. Additionally, the protons H-3 both couple to C-4 and one of the H-3 protons (2.499 ppm) couples to C-5.

Finally, a fairly strong cross peak between H-5 and C-1 is found resulting in a central seven membered ring.

To confirm the structure, the assignment of the pro-chiral protons and to determine the **relative** stereo-chemistry of the four chiral centres, the distances extracted from the quantitative evaluation of the ROESY spectrum were used. For this purpose, the cross-peaks in the ROESY spectrum were integrated and offset-corrected. The corrected volume integrals of the cross-peaks were treated in a simplified manner as being proportional to d^{-6} (e.g. H. Kessler *et al.*, 1990). For the calibration, the distance between the protons H-3$^{pro\text{-}S}$ and H-5 was used as a reference. Methyl group 12 was represented by a pseudo-atom; a pseudo-atom correction of 60 pm was added to the measured distances. The distance between H-5 and the pseudo-atom representing methyl group 12 was used to check the distance calibration.

From the methyl group 12, short distances are found to H-5 and to one of the H-3 protons. A longer distance is found to the other H-3. Therefore, the seven-membered ring and this four-membered ring are *cis*-anelated. With these three distances as reference, it results that H-9 is close to the methyl group 12 and H-1 is on the opposite face. Thus, the other four-membered ring and the seven-membered are *trans*-anelated.

The prochiral methyl groups 14 and 15 can be assigned unambiguously by the specific cross peaks between H-14 and H-9, H-15 and H-1, and H-15 and H-8$^{pro\text{-}S}$ found in the ROESY spectrum.

The measured distances were used as constraints in a molecular dynamics run (for details see experimental section). Constraints for H-8s to H-9 were derived form the corresponding *J*-coupling constants. In order to account for the clear difference between the two coupling constants between H-7, H-8$^{pro\text{-}R}$ and H-8$^{pro\text{-}S}$, the torsion angle was held at 50° and 70°, respectively (cf. Table I). Otherwise torsion angles of about 60° are found in the molecular dynamics run and energy mini-

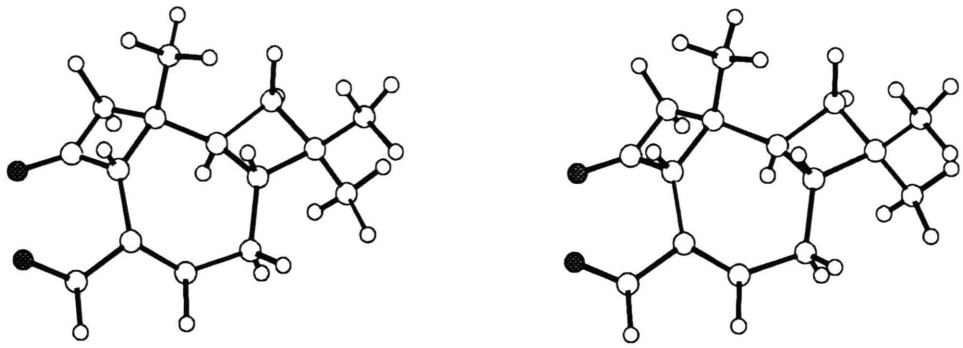


Fig. 2. Stereo view of collybial after restraint MD (relative stereo-chemistry).

misation. The final structure is shown in Fig. 2 (relative stereo-chemistry). The distance constraints are fulfilled within the experimental error.

The pro-chiral protons H-11 can be assigned with aid of the final structure. The torsional angle between H-1 and H-11$^{pro\text{-}R}$ is about 148° and between H-1 and H-9 about 150°. The coupling constants are 10.0 and 10.5 Hz, respectively. Hence, both H-11$^{pro\text{-}R}$ and H-9 are trans with respect to H-1. The torsional angle between H-1 and H-11$^{pro\text{-}S}$ is about 19° and the corresponding coupling constant is 8.0 Hz. Therefore, the assumed assignment is reasonable.

Antibiotic, antiviral, and cytotoxic properties

The antimicrobial activity of collybial in the serial dilution assay is shown in Table II. The antibiotic exhibits a weak antifungal activity against *Mucor miehei*, *Saccharomyces cerevisiae* is 1, and *Ustilago nuda*. The inhibition of bacterial growth is most pronounced for *Bacillus subtilis* and *B. brevis*. However most of the organisms tested were not inhibited by concentrations of up to 100 μg/ml.

Collybial exhibits a remarkable effect on the propagation of VSV in BHK-21 cells starting from concentrations of 5 μg/ml (21.5 μM). 25 Hours after infection the virus titer was reduced by a factor of 10^3 as compared to the control without antibiotic (10^7 PFU/ml).

The cytotoxic activities of collybial after 24–36 h of incubation are listed in Table III. A complete lysis of BHK 21 cells was observed at 25 μg/ml (108 μM). The IC_{50} was determined to 81 μM. In this assay no cytopathic effect could be detected at concentrations causing a marked reduction of VSV multiplication. While the cytotoxic effects on BHK 21, HeLa S3 and L1210 cells are quite modest, HL 60 cells are very sensitive

The effect of the antibiotic on the short time incorporation (30 min) of thymidine, uridine, and

Table II. Antimicrobial spectra of collybial in the serial dilution assay.

Test organism	MIC [μg/ml]
Bacteria	
Acinetobacter calcoaceticus	>100
Arthrobacter citreus	>100
Bacillus brevis	10–25
Bacillus subtilis	5–10
Corynebacterium insidiosum	>100
Escherichia coli K 12	>100
Micrococcus luteus	50
Mycobacterium phlei	10–50
Salmonella typhimurium TA 98	>100
Streptomyces spec.	50–100
Fungi	
Fusarium oxysporum	>100
Mucor miehei	75
Nadsonia fulvescens	>100
Nematospora coryli	50–100
Paecilomyces varioti	>100
Penicillium notatum	>100
Rhodotorula glutinis	>100
Saccharomyces cerevisiae is 1	50
Saccharomyces cerevisiae S 288	>100
Ustilago nuda	75

Table III. Cytotoxic activities of collybial.

Test organism	IC_{100}* [μg/ml (μM)]
BHK-21	25 (108)
Hela S3	25 (108)
L1210	25 (108)
U937	10 (43)
HL-60	1 (4.3)

* IC_{100}: complete lysis of cells.

leucine into TCA-precipitable material was investigated with resting L1210 cells suspended in PBS buffer (Fig. 3). Collybial inhibited DNA, RNA, and protein syntheses at concentrations beginning from 21.5 μM. Protein synthesis was inhibited 50% at concentrations of 7 μg/ml (30 μM) while the syntheses of RNA and DNA are affected to a lesser extent.

RNA-directed RNA-polymerase of VSV is one of the possible targets for compounds interfering with virus propagation. This enzyme, however, is hardly affected by high concentrations of collybial (IC_{30} = 100 μg/ml).

Other biological activities

In the test for mutagenicity according to Venitt *et al.* (1984) no induction of revertants of *S. typhimurium* TA 98 and TA 100 was observed with 100 μg of collybial/plate (plate pour assay with and without addition of rat liver microsomes).

No hemolytic activity was observed up to a concentration of 100 μg/ml in an assay described previously (Kuschel *et al.*, 1994).

In summary it is assumed that collybial exerts ist antiviral, cytotoxic, and antibiotic activities by interfering with cellular enzymes in a rather unselective manner. As has been found in other antibiotic terpenoids e. g. the striatals (Anke *et al.*, 1977), the a,β-unsaturated aldehyde function is thought to be responsible for most of the biological activities.

Acknowledgement

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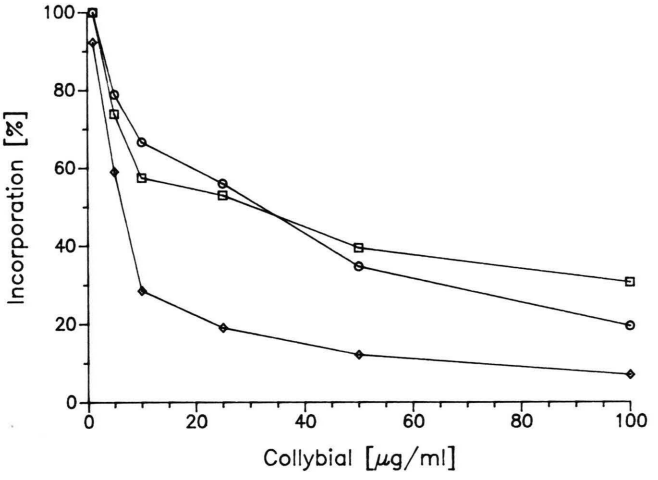

Fig. 3. Inhibition of protein, RNA, and DNA syntheses by collybial. ◇—◇, [^{14}C]leucine (control 34,085 cpm); ○—○, [^{14}C]uridine (control 25,018 cpm); □—□, [^{14}C]thymidine (control 7067 cpm).

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