3-ALKANOYL-5-HYDROXYMETHYL TETRONIC ACID HOMOLOGUES AND RESISTOMYCIN: NEW INHIBITORS OF HIV-1 PROTEASE

I. FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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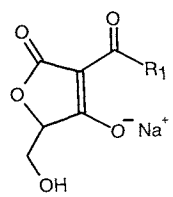
In the course of a screening program for HIV-1 protease inhibiting activity, six new homologues of 3-alkanoyl-5-hydroxymethyl tetronic acids (**1**~**6**) and the known natural product resistomycin (**7**) were isolated from cultures of the *Actinomycete* strain DSM 7357. The substituted tetronic acids belong to a recently described structural class of secondary metabolites. The HIV-1 activity of resistomycin (**7**) has not been reported before.

Human immunodeficiency virus (HIV) is the etiological agent responsible for the development of acquired immunodeficiency syndrome (AIDS) in man. Replication of HIV depends on the cleavage of two viral polyproteins by a virally-encoded protease (HIV-1 protease) to yield structural proteins and essential viral enzymes[1]. Inhibition of this enzyme results in the formation of immature viral particles and hence suppression of infection *in vitro*. Several synthetic transition state mimetics of this aspartyl protease have been designed, which display potent antiviral activity, and which are currently in clinical evaluation[2]. The availability of structural information through X-ray analysis allowed the rational design and optimization of enzyme inhibitors. The identification of natural HIV-1 protease inhibitors could be a further important step leading to a therapeutic agent against AIDS[3].

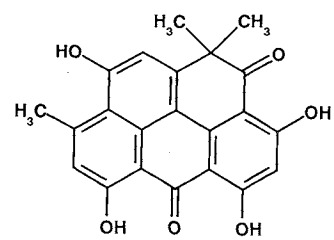
In the course of a screening program for inhibitors of HIV-1 protease an *Actinomycete* strain (DSM 7357) was found which produced six new 3-alkanoyl-5-hydroxymethyl tetronic acid homo-

Scheme 1. Chemical structures of six new homologues of 3-alkanoyl-5-hydroxymethyl tetronic acid (**1**~**6**).



1 $R_1=(CH_2)_{12}CH_3$
2 $R_1=(CH_2)_{13}CH_3$
3 $R_1=(CH_2)_{11}CH(CH_3)_2$
4 $R_1=(CH_2)_{14}CH_3$
5 $R_1=(CH_2)_{12}CH(CH_3)_2$
6 $R_1=(CH_2)_{12}CH(CH_3)(CH_2CH_3)$

Scheme 2. Chemical structure of resistomycin (**7**).



logues (**1**～**6**) (Scheme 1) belonging to a recently described[4] new structural class of secondary metabolites and the known antibiotic resistomycin (**7**)[5] (Scheme 2). In the following the cultivation of the producing microorganism, as well as the isolation and biological activity of these natural products is described.

Materials and Methods

Microorganism: Isolation and Preservation

The *Actinomycete* strain was isolated from a soil sample collected in Budapest, Hungary and has been deposited at DSM (Deutsche Sammlung für Mikroorganismen und Zellkulturen; Braunschweig, Germany) under the accession number DSM 7357. The strain was grown on SC agar (potato starch 1.0%, casein 0.1%, yeast extract 0.1%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, and agar 2%, pH 7.0), incubated for ten days at 28°C until complete sporulation, and stored at −20°C.

Fermentation

A loopful of a mature slant culture of the *Actinomycete* DSM 7357 was inoculated into an Erlenmeyer flask (500 ml) with one baffle containing 100 ml of the following seed-medium: glucose 2%, Pharmamedia (Archer Daniels Midland Corp., U.S.A.) 1.5%, $(NH_4)_2SO_4$ 0.3%, $ZnSO_4 \cdot 7H_2O$ 0.003%, $CaCO_3$ 0.4%. Before sterilization, the pH was adjusted to 7.0 with sulfuric acid (98%) or sodium hydroxide (30%). This first seed culture was shaken under aerobic conditions for 48 hours at 28°C and an agitation rate of 250 rpm. Five ml were then transferred into a second series of Erlenmeyer flasks (2,000 ml) with four baffles containing 500 ml of seed medium each. This second seed culture was incubated under the same conditions as the previous one. Six hundred ml of this culture was then transferred into a fermentor (50 liters) containing 30 liters of the following production medium: glycerol 3%, meat extract (Difco, U.S.A.) 3%, tryptophan 0.1%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.03%, $CaCO_3$ 0.3%. Before sterilization, the pH was adjusted to 7.0 with sulfuric acid (98%) or sodium hydroxide (30%). The fermentation was carried out at 28°C for 93 hours with an aeration rate of 30 liters/minute and an agitation rate of 600 rpm.

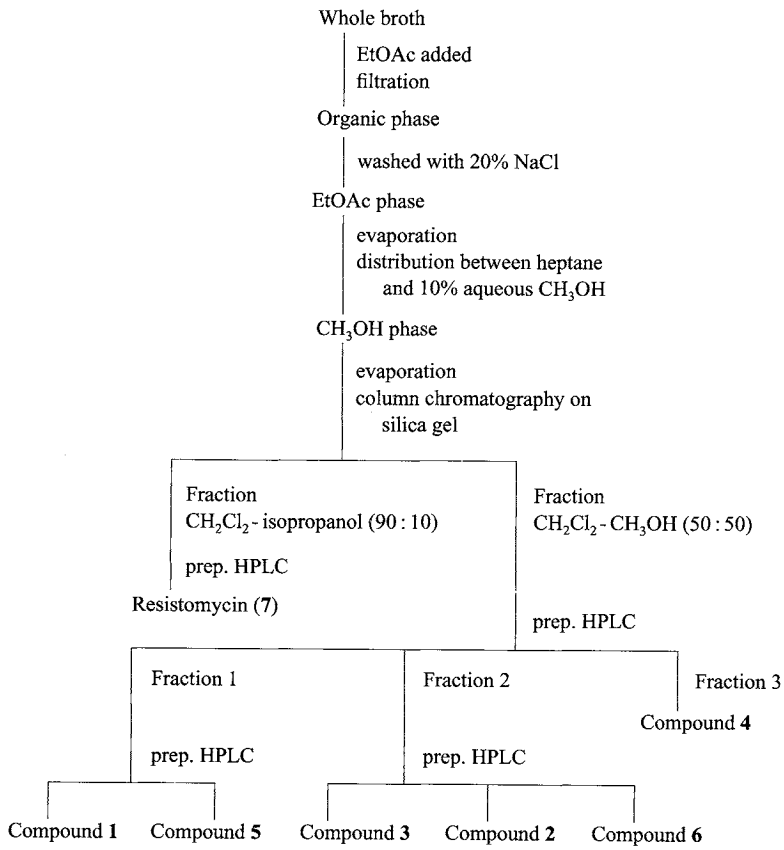
Isolation

Chromatographic systems used: Preparative normal-phase liquid chromatography with silica gel (Lichroprep Si 60, 12～25 μm; Merck, Germany) as stationary phase was carried out using the following system: dual-pump gradient system (Gilson pumps type 303 and 306; Gilson, France), preparative glass column (49×460 mm; 870 ml; Büchi, Switzerland), a UV-detector equipped with a cell of 0.5 mm optical pathlength (Shimadzu type UV-120-02; Shimadzu, Japan) and a fraction collector (Büchi, type 684). Preparative reversed-phase liquid-chromatography was performed with a silica based C-18 modified stationary phase (Lichroprep RP-18, 15～25 μm; Merck, Germany), using the following system: preparative pump (Latek 500; Latek, Germany) in combination with a low-pressure gradient former (Labomat VS200; Labomatic, Switzerland), a conical glass column (26～49×230 mm; 260 ml; Büchi, Switzerland), a UV-detector equipped with a cell of 0.5 mm optical pathlength (Shimadzu type UV-120-02; Shimadzu, Japan) and a fraction collector (Büchi, type 684).

TLC-analysis of the fractions from silica gel chromatography was performed on HPTLC precoated plates (Silica gel 60 F_{254}, which concentrating zone; Merck, Germany) using CH_2Cl_2-isopropanol 90:10 (v/v) as mobile phase and anisaldehyde spray reagent (1% in a solution of 2% sulphuric acid (98%) in acetic acid) or UV-measurements (254 nm, 316 nm) as detection methods.

The fractions collected from the preparative chromatography were analyzed using a standardized analytical reversed-phase HPLC method. The instrumentation used consisted of an HPLC pump operated in the low-pressure gradient forming mode (Merck-Hitachi L-6200; Merck, Germany), a UV-detector (Merck-Hitachi L-4250) and an integrator (Merck-Hitachi D-2500). A spherical C-18 modified silica gel based stationary phase with a pore-size of 100 Å and a particle diameter of 5 μm was used (Lichrospher 100 RP-18, 5 μm, column size: 4×125 mm; Merck, Germany). Mobile phase A consisted of 1.85 mM KH_2PO_4 and 0.81 mM Na_2HPO_4 in H_2O resulting in a pH-value of 6.5. Mobile phase B was prepared by mixing 80% CH_3CN with 20% of mobile phase A (v/v). The wavelength for detection was set to 220 nm

Scheme 3. Isolation procedure for tetronic acids (**1**～**6**) and resistomycin (**7**).



and 25 μl of each sample were injected for a chromatogram. A linear gradient was run from 5% to 100% mobile phase B in 25 minutes with a constant flow rate of 1.5 ml/minute.

For the work-up of *Actinomycete* DSM 7357 cultures (Scheme 3) the whole broth (26 liters) was mixed with EtOAc (58 liters). Subsequent filtration was performed using a filter aid (Celite; Celite Corp., U.S.A.). The aqueous and organic phases were separated, the aqueous phase was discarded, while the organic layer was washed with sodium chloride solution (20%, 3 liters) and concentrated *in vacuo*. The EtOAc-extract was solvent partitioned between heptane (500 ml) and aqueous CH_3OH (10%, 500 ml). Evaporation of the CH_3OH phase yielded 25 g of crude extract.

The crude extract (25 g) was suspended in CH_2Cl_2 (100 ml), the suspension was filtered and the filtrate was chromatographed on silica gel with stepwise elution of the following solvents (v/v): CH_2Cl_2-heptane 80:20; CH_2Cl_2; CH_2Cl_2-isopropanol 99:1, 98:2, 95:5, 90:10, 80:20, 50:50; CH_2Cl_2-CH_3OH 50:50 and CH_3OH. The flow rate of the mobile phase was 30 ml/minute, the detection wavelength was set to 254 nm. The fractions were combined based on TLC-analysis (see above), the combined fractions were further analyzed by TLC, HPLC (see above) and in the bioassay. Crude resistomycin (**7**) eluted from the column with the solvent CH_2Cl_2-isopropanol 90:10 (v/v). A mixture of the homologues of 3-alkanoyl-5-hydroxymethyl tetronic acid (**1**～**6**) eluted with the solvent CH_2Cl_2-CH_3OH 50:50 (v/v).

Crude resistomycin (**7**) (0.5 g) was purified by reversed phase preparative HPLC with H_2O as mobile phase A and CH_3CN-H_2O 80:20 (v/v) as mobile phase B. The flow rate of the mobile phase was 25 ml/minute. A gradient was run from 5% to 100% phase B in 80 minutes with the detection wavelength set to 235 nm. The starting material (0.5 g) was suspended in CH_3OH-H_2O 50:50 (5 ml) and applied to the above described reversed phase column. Resistomycin (**7**) (4 mg) eluted with 100% of mobile phase B.

The mixture of the tetronic acid derivatives (**1**～**6**) (3.4 g), which eluted from the silica gel column

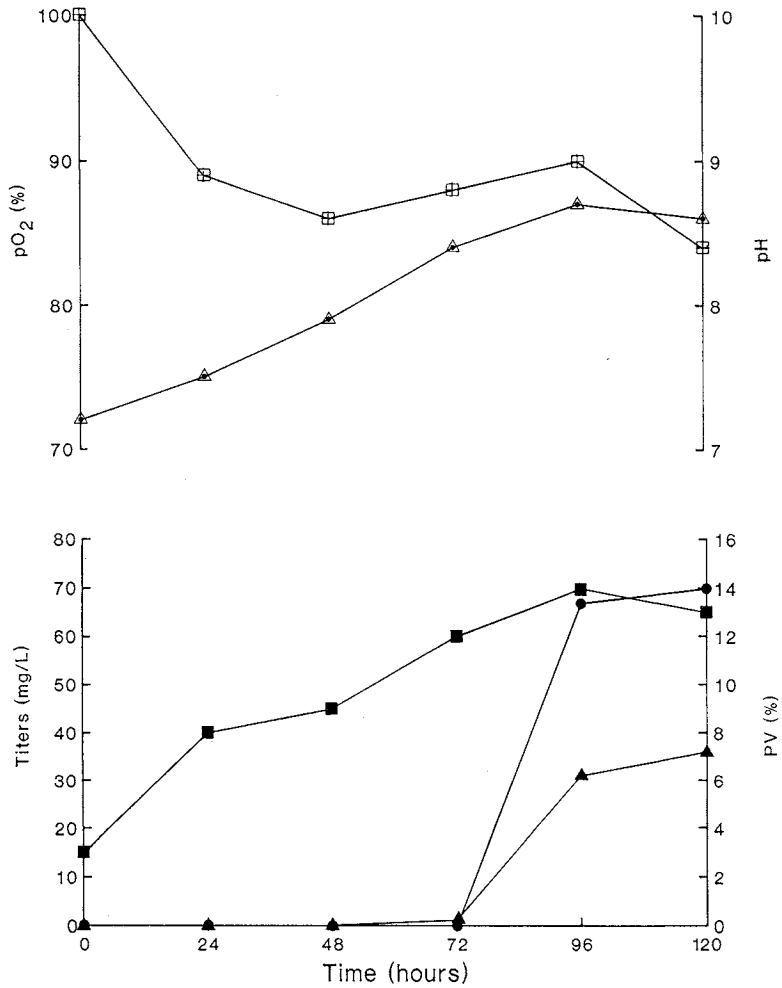
with the solvent CH_2Cl_2-CH_3OH 50:50 (v/v), was further purified by reversed phase preparative HPLC with H_2O as mobile phase A and CH_3CN-H_2O 80:20 (v/v) as mobile phase B with a flow rate of 25 ml/minute. A gradient was run from 10% to 100% phase B in 80 minutes with the detection wavelength set to 235 nm. Portions of 1.1 g or 2.3 g starting material were suspended in CH_3OH-H_2O 10:90 (10 ml or 20 ml) and applied to the column for each chromatogram. Three main fractions were collected according to analytical HPLC-analysis. Fraction 1 eluted with 60% to 70% mobile phase B, fraction 2 eluted with 70% to 78% mobile phase B, fraction 3 was pure compound **4** (300 mg) and eluted with 78% to 80% mobile phase B.

Fraction 1 (700 mg) was rechromatographed on reversed phase running a gradient from 40% to 85% phase B in 60 minutes and from 85% to 100% mobile phase B in 10 minutes. Seven hundred mg of fraction 1 was suspended in CH_3OH-H_2O 50:50 (10 ml) and applied to the reversed phase column. Compound **1** (42 mg) eluted with 45% to 50% mobile phase B and compound **5** (16 mg) eluted with 62% to 64% mobile phase B.

Fraction 2 (540 mg) was separated into compounds **3**, **2** and **6** by a second preparative chromatographic run applying the same column parameters, mobile phases, flow rate, and detection wavelength as before. A gradient was run from 50% to 100% mobile phase B in 60 minutes. Compound **3** (100 mg) eluted with 58% to 60% phase B, compound **2** (41 mg) with 60% to 62% phase B and compound **6** (80 mg)

Fig. 1. Time course of tetronic acid **2** and resistomycin (**7**) production.

■ PV, △ pH, ⊞ pO_2, ▲ tetronic acid **2**, ● resistomycin (**7**).



with 75% to 90% mobile phase B.

Biological Properties

During the purification procedure the anti-HIV-1 protease activity was monitored using the scintillation proximity assay[6] (HIV Proteinase ^{125}I SPA Enzyme Assay System (IMK 8939); Amersham International Plc., Cardiff, UK). All fractions and pure compounds were solubilized in DMSO (final concentration < 5 mM). The IC_{50} values for compound **1** to **12** were determined by a peptide based enzyme assay. The cleavage of the icosapeptide RRSNQVSQNY*PIVQNIQGRR (164 μM) in 20 mM MES (morpholino-ethane-sulfonic acid; pH 6.0) was monitored. The reaction was started by the addition of *Escherichia coli* expressed and purified HIV-1 protease[7]. After 60 minutes incubation at 37 °C the reaction was stopped by adding $HClO_4$ to a final concentration of 0.03 M and product formation was analyzed by RP-HPLC[8].

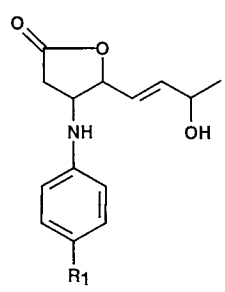
Results and Discussion

The time course for the production of the tetronic acid **2** and resistomycin (**7**) was determined by experiments in a 50-liter fermentor and monitored by HPLC-analysis (Fig. 1).

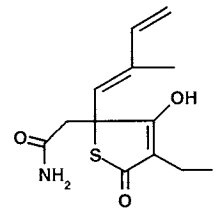
Cultures were worked up after 93 hours of fermentation time. The extraction of the whole broth of the *Actinomycete* strain DSM 7357 by EtOAc and the solvent partitioning of the organic phase between heptane and 10% aqueous CH_3OH yielded crude extract containing the tetronic acid homologues (**1**~**6**) and resistomycin (**7**). The tetronic acid (**1**~**6**) could be separated from resistomycin (**7**) by chromatography on silica gel (Scheme 3). Crude resistomycin was further purified by reversed phase (C_{18}) chromatography. The different tetronic acid homologues (**1**~**6**) were separated by multiple chromatographic steps on reversed phase (C_{18}) using different gradient systems.

The structure elucidation of the tetronic acid homologues (**1**~**6**) and the physico-chemical data of these compounds will be described in the subsequent paper[9]. The UV-, IR-, FAB-MS and ^1H NMR spectra of resistomycin (**7**) are well in accordance with the data from literature[5].

Scheme 4. Chemical structures of compounds **8** and **9**, of thiotetronic acid (**10**), of desacetyl-octanoyl-acetomycin (**11**) and of dibromo-obscurolide (**12**).



8 $R_1 = CH_2OH$
9 $R_1 = CHO$



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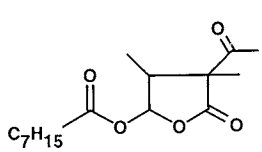

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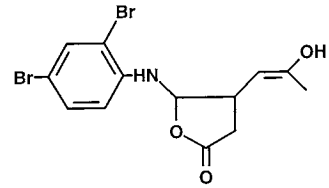

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Table 1. Inhibition of HIV-1 protease by tetronic acids (**1**~**6**), resistomycin (**7**) and compounds (**8**~**12**).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
HIV-1 protease assay IC_{50} (μM)	135*	123*	110*	84*	112*	109*	21*	508	468	191	523	221

* Mean value from three independent determinations.

The 3-alkanoyl-5-hydroxymethyl tetronic acid homologues (**1**~**6**) have been found to be inhibitors of the HIV-1 protease. In addition secondary metabolites (**8**, **9**[10], **10**[11], **11** (unpublished data), **12**[12]) (Scheme 4) containing a five membered lactone ring with a structural analogy to the tetronic acids were tested in the HIV-1 protease assay. The IC_{50} values for all the compounds are summarized in Table 1. The tetronic acid homologues (**1**~**6**) inhibit HIV-1 protease with IC_{50} values varying from 84 μM to 135 μM depending on the length and substitution pattern of the alkanoyl-side chain. The five natural products (**8**~**12**) show weaker inhibition of the HIV-1 protease. The thiotetronic acid (**10**) and the dibromo-obscurolide (**12**) exhibit about two fold higher IC_{50} values than the tetronic acids homolgoues (**1**~**6**), while the compounds **8**, **9** and **11** are five times less active in the HIV-1 protease assay than the tetronic acids.

Resistomycin (**7**) inhibits HIV-1 protease with an IC_{50} value of 21 μM (Table 1). The HIV-1 activity of resistomycin has not been described before. However resistomycin is well known as an antibiotic[13] and as an inhibitor of RNA and DNA polymerase[14].

Acknowledgment

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References

1) KOHL, N. E.; E. A. EMINI, W. A. SCHLIEF, L. J. DAVIS, J. C. HEIMBACH, R. A. F. DIXON, E. M. SCOLNICK & I. S. SIGAL: Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. U.S.A. 85: 4686~4690, 1988
2) SANDSTRÖM, E. & B. ÖBERG: Antiviral therapy in human immunodeficiency virus infections. Current status (Part II). Drugs 45: 637~653, 1993
3) TANG, J.; Y. LIN, E. CO, J. A. HARTSUCK & X. LIN: Understanding HIV protease: Can it be translated into effective therapy against AIDS? Scand. J. Lab. Invest. 52 (Suppl. 210): 127~135, 1992
4) DOLAK, L. A.; E. P. SEEST, J. I. CIADELLA & M. J. BOHANON (The Upjohn Company): Compounds used for the inhibition of HIV-protease. PCT Int. Appl. 93/04055, Mar. 4, 1993
5) BROCKMANN, H.; E. MEYER, K. SCHREMP, F. REINERS & T. RESCHKE: Resistomycin. IV. Constitution of resistomycin. Chem. Ber. 102: 1224~1246, 1969
6) FEHRENTZ, J. A.; B. CHOMIER, E. BIGNON, S. VENAUD, J. C. CHERMAN & D. NISATO: Statine based tripeptides as potent inhibitors of HIV-1 replication. Biochem. Biophys. Res. Comm. 188: 873~878, 1992
7) BILLICH, S.; M-Th. KNOOP, J. HANSEN, P. STROP, J. SEDLACEK, R. Mertz & K. MOELLING: Synthetic peptides as substrates and inhibitors of Human Immunodeficiency Virus-1 Protease. J. Biol. Chem. 263: 17905~17908, 1988
8) ALTERI, E.; BOLD, W. BECK, E. BOSS, R. COZENS, F. CUMIN, A. FAESSLER, M. GRUETTER, E. HUNGERBUEHLER, T. KLIMKAIT, M. LANG, J. LAZDINS, B. PONCIONI, J. L. ROESEL, H. RUEEGER, P. SCHNEIDER, M. TINTELNOT-BLOOMLEY, M. WALKER & K. WOODS-COOK: From renin inhibitors to CGP 53437, an orally bioavailable HIV-1 protease inhibitor. Bioorg. Med. Chem. Lett., submitted
9) ROGGO, B. E.; P. HUG, S. MOSS, F. RASCHDORF & H. H. PETER: 3-Alkanoyl-5-hydroxymethyl tetronic acid homologues: New inhibitors of HIV-1 protease. II. Structure determination. J. Antibiotics 47: 143~147, 1994
10) HOFF, H.; H. DRAUTZ, H.-P. FIEDLER, H. ZAEHNER, J. E. SCHULTZ, W. KELLER-SCHIERLEIN, S. PHILIPPS, M. RITZAU & A. ZEECK: Metabolic products of microorganisms. 261. Obscurolides, a novel class of phosphodiesterase inhibitors from streptomyces. I. Production, isolation, structural elucidation and biological activity of obscurolides A_1 to A_4. J. Antibiotics 45: 1096~1107, 1992

11) RAPP, C.; G. JUNG, C. ISSELHORST-SCHARR & H. ZAEHNER: A new member of the class of antibiotics with thiotetronic acid structure isolated from *Streptomyces olivaceus* Tü 3010. Liebigs Ann. Chem. 11: 1043～1047, 1988
12) HOFF, H.: Obskurolide: Neue Butyrolactone aus Streptomyceten. Ph. D. Thesis, Univ. Tübingen, 1990
13) BRADLER, G. & K. ECKHARDT: Characterization of a Streptomyces strain producing resistomycin. Isolation and properties of the new antibiotic. Z. Allg. Mikrobiol. 12: 535～545, 1972
14) HAUPT, I.; U. WAEHNERT, C. PITRA, G. LOEBER, G. LUCK & K. ECKARDT: Effects of the antibiotic resistomycin on the synthesis of macromolecules. Z. Allg. Mikrobiol. 15: 411～421, 1975