METABOLIC PRODUCTS OF MICROORGANISMS. 244† COLABOMYCINS, NEW ANTIBIOTICS OF THE MANUMYCIN GROUP FROM *STREPTOMYCES GRISEOFLAVUS*

I. ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

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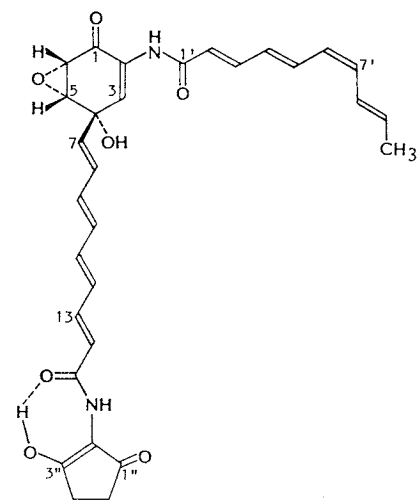
The yellow colabomycins A to C, three new antibiotics of the manumycin group produced by *Streptomyces griseoflavus* (strain Tü 2880), were detected by chemical screening. They were isolated from mycelium extracts by column chromatography on various adsorbents, followed by preparative reversed phase HPLC. The main compound, colabomycin A (**1**), was characterized and shown to be chiefly biologically active against Gram-positive bacteria and stem cells of murine L1210 leukemia.

The colabomycin-complex was detected in the course of our chemical screening program. On silica gel TLC plates (Rf values see Table 1) the bright yellow compounds showed reactions with various staining reagents, *e.g.*, blue tetrazolium reagent (brown), vanillin-sulfuric acid (dark violet) and molybdatophosphoric acid (black), indicating their reducing character. Under examination with UV light (366 nm) the color of colabomycin spots changed rapidly to red-brown. In this paper we describe the fermentation, isolation, physico-chemical and biological properties of the colabomycins, especially of colabomycin A (**1**). A second paper[2] will deal with the structure elucidation of colabomycin A (**1**) by two-dimensional (2D) NMR and CD spectroscopy.

Table 1. Rf values of colabomycins A to C on silica gel TLC plates.

Solvent system	A	B	C
$CHCl_3$ - MeOH (9 : 1)	0.47	0.45	0.46
$CHCl_3$ - MeOH (98 : 2)	0.05	0.04	0.03
EtOAc - MeOH (9 : 1)	0.43	0.43	0.42
EtOAc - MeOH - H_2O (6 : 2 : 1)	0.66	0.68	0.68



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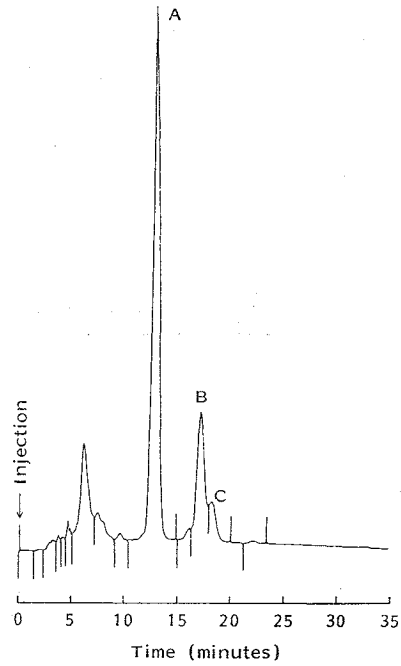
† See ref 1.

Fermentation and Isolation

The producing organism was a new soil isolate from Mexico, classified as *Streptomyces griseoflavus* subsp. *canus* (strain Tü 2880). Production of colabomycin was conducted in a 200-liter fermentor in a medium consisting of mannitol 2% and soybean meal 2%. Colabomycin production was maximal after 96 hours. Due to the extreme instability of colabomycin to daylight all separation steps were carried out under dark room conditions or using aluminum foil coated glassware. Because of the low solubility of purified colabomycin, dioxane solutions were used for column chromatography.

Fig. 1. HPLC analysis of the colabomycin-complex.



The raw material, which was obtained by extraction of the mycelium of strain Tü 2880 with acetone followed by evaporation of the solvent, was extracted with dioxane - methanol (2:3). The evaporation residue was degreased by petroleum ether, mixed with sand, applied to the head of a fast running silica gel column and developed with chloroform - methanol (9:1). Repeated chromatography of the resultant substances of the main yellow zone on Sephadex LH-20 in chloroform and chloroform - methanol (9:1) yielded the colabomycin-complex. The pure colabomycins were obtained by reversed phase HPLC (see Fig. 1) with acidic acetonitrile - water (2:1). The ratio of colabomycins A, B and C amounted to 1:0.1:0.1, the yield of compound A was approximately 0.1 mg per liter culture broth.

After lyophylization the colabomycins were obtained as yellow amorphous powders. They are easily soluble in *N*,*N*-dimethylformamide, moderately soluble in dioxane or dimethyl sulfoxide, hardly soluble in alcohols, acetone, chloroform and insoluble in water or alkanes. The chirality is demonstrated by the optical rotation and CD spectra, respectively.

Characterization of Colabomycin A (1)

The field desorption mass spectrum (FD-MS, solvent: dioxane) gave a molecular ion at *m/z* 530, corresponding to the molecular formula $C_{30}H_{30}N_2O_7$, which is in agreement with the elemental analysis of the monohydrate. The UV spectrum (methanol) showed one broad absorption band at 345 nm (ε 62,100), suggesting an unsaturated system, and a smaller band at 262 nm. There were only small changes under basic (339 nm) or acidic (348 nm) conditions. The IR spectrum (Fig. 2) displayed a complex pattern of absorption bands. A broad band at 3410 cm^{-1}, indicating NH and OH groups, and strong but unresolved absorptions at 1670 and 1615 cm^{-1}, which are due to the presence of carbonyls and olefins, were observed.

The ^1H NMR spectrum displayed seventeen olefinic protons in the range between δ 5.9 and 7.4. Besides an olefinic methyl group at δ 1.82 (10′-H_3), a broad signal for four protons at δ 2.49 (4″-H_2 and 5″-H_2) and two one proton resonances at δ 3.68 (6-H) and 3.78 (5-H) were also detected. The

Fig. 2. IR spectrum of colabomycin A (1) in KBr.

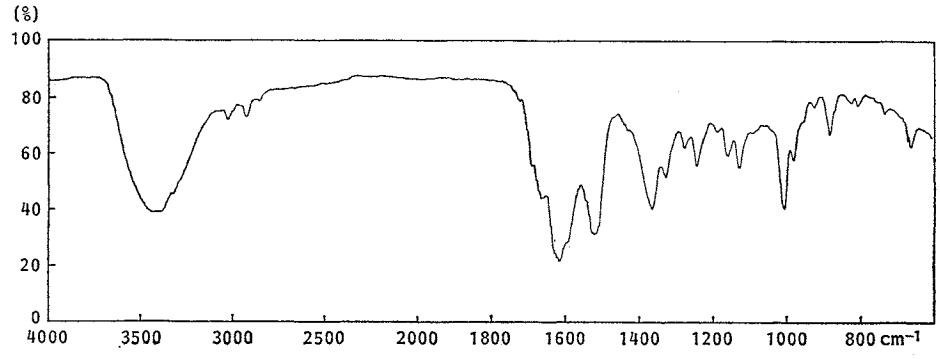
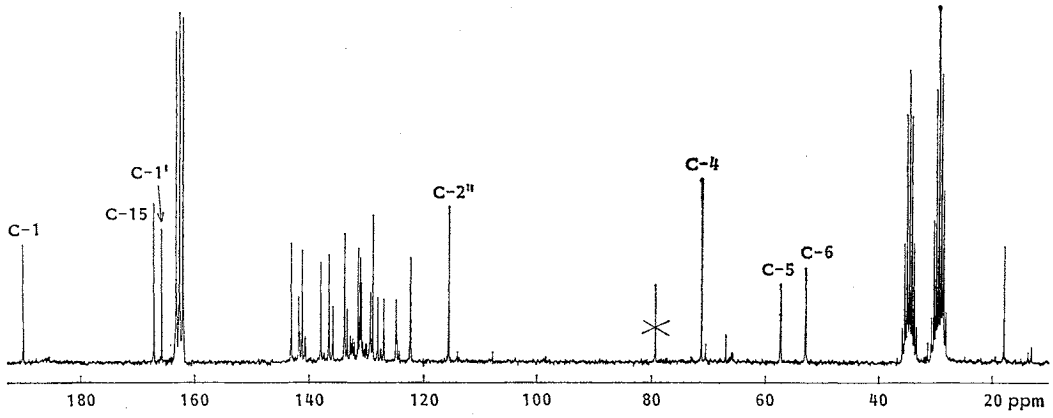


Fig. 3. ^{13}C NMR spectrum of colabomycin A (1) in DMF-d_7 at 125.7 MHz.



signals at δ 3.25, 9.15 and 9.90 were exchangeable with D_2O and therefore attributed to NH or OH protons, respectively.

Only five signals of the twenty-five observed in the ^{13}C NMR spectrum (Fig. 3) could be detected upfield δ 120. They were attributed to an olefinic methyl group (δ 18.5, C-10'), two epoxide CH groups (δ 53.4 and 57.8, C-6 and C-5) and two quaternary carbons (δ 71.6 and 115.7, C-4 and C-2''). Three carbonyl resonances were displayed at δ 190.2 (α,β-unsaturated ketone, C-1), and δ 167.1/165.8 (amide carbonyls, C-15/C-1') besides sixteen olefinic methins (one of these with double intensity) and one quaternary olefin (δ 129.1, C-2).

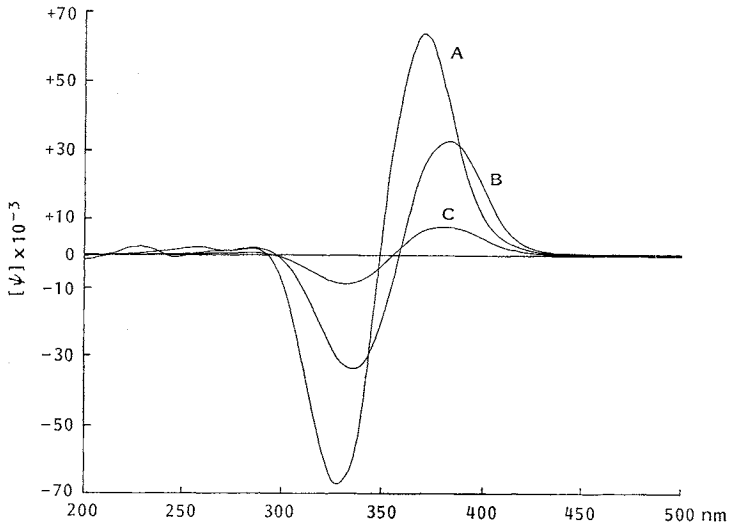
The IR spectrum in connection with the typical resonances of the ^1H and ^{13}C NMR spectra suggested colabomycin A to be a member of the manumycin group of antibiotics[2~10]. The ^1H NMR spectra of this class show characteristic chemical shifts and coupling constants of the epoxide protons 5-H and 6-H (δ 3.78, J=4.1 Hz; 3.68, J=4.1 and 2.8 Hz) and of 4''-H_2 and 5''-H_2. Additionally some ^{13}C NMR signals were found to be in good accordance within the group (see Table 2). The occurrence of the signals of C-1, C-2, C-4, C-5 and C-6 proved the presence of the 5,6-epoxycyclohex-2-enone ring in colabomycin A. The chemical shift of C-2'' (δ 115.7), the only observable signal of the 2-amino-3-hydroxycyclopent-2-enone moiety, is very characteristic. Carbons C-1'', C-3'', C-4'' and C-5'' are usually not detectable. We attribute this effect to tautomeric changes in the range of the NMR time scale, resulting in nearly complete coalescence and hence corresponding linebroadening.

Table 2. Typical ^{13}C NMR signals (δ in ppm relative to internal TMS) of colabomycin A (1), manumycin[5], asukamycin[8] and U-56,407[8].

Carbon No.	1[a] (DMF-d_7)	Manumycin ($CDCl_3$)	Asukamycin (DMSO-d_6)	U-56,407 (DMSO-d_6)
C-1	190.2 s	188.9	189.2	189.6
C-2	129.1 s	128.0	127.9	128.2
C-4	71.6 s	71.2	70.5	70.8
C-5	57.8 d	57.4	56.4	56.6
C-6	53.4 d	53.0	52.4	52.6
C-2″	115.7 s	115.4	114.0	115.0
C-1′	165.8 s	168.8	164.7	165.0
C-15/C-13	167.1 s	165.5	165.7	166.1

[a] 125.7 MHz, multiplicity determined by distortionless enhancement by polarization transfer (DEPT).

Fig. 4. CD spectra (CH_3CN, 0.01 mg/ml) of colabomycins A, B and C.



The same effect was observed sometimes in the cases of manumycin and asukamycin. Variation of the solvent was impossible because of the poor solubility of colabomycin A.

The remaining sixteen olefinic CH groups are to be divided into two polyene carboxamide units. The observed bathochromic shift of the absorbance at 345 nm in the UV spectrum of colabomycin A in comparison to manumycin (415 nm) suggested the occurrence of two tetraene chains or one pentaene and one triene chain in colabomycin A.

Characterization of Colabomycins B and C

Colabomycins B and C were obtained by HPLC only in minor amounts. Although full spectroscopic data are not yet available, a close relation to **1** can be stated. The IR spectra of colabomycins B and C are merely identical with that obtained from colabomycin A. The long wavelength UV absorption bands (365 nm, methanol) show bathochromic shifts in comparison with **1**, while the CD spectra (Fig. 4) are very similar. The observed bathochromic shifts (~17 nm) of the zero passes indicate a change in the polyene carboxamide chains, which are responsible for the detected exciton-coupling in colabomycin A[2]. The ^1H NMR spectrum of colabomycin B in *N,N*-dimethylformamide-d_7 suggested a high number of olefinic protons in the range from δ 5.8 to 7.5. A methyl group at

Table 3. Antibacterial and antifungal assays of colabomycin A (1) and manumycin (disc-diffusion assay).

Test microorganism	Inhibition diameter (mm)							
	Colabomycin A (mg/ml)				Manumycin (mg/ml)			
	3.0	1.0	0.3	0.1	3.0	1.0	0.3	0.1
Botrytis cinerea	tr	—	—	—	18	16	13	10
Mucor miehei	—	—	—	—	—/11	—/9	—	—
Paecilomyces	—	—	—	—	12/21	10/19	—/13	—/9
Bacillus subtilis	15	11	8	—	17	15	12	8
B. subtilis α	25	18	12	tr	32	29	25	20
B. brevis	17	13	10	—	23	21	19	16
Clostridium pasteurianum	14	12	10	—	21	20	17	13
Escherichia coli K-12 α	—	—	—	—	—	—	—	—
Achromobacter geminianii	14	10	—/tr	—	20	20	15	10
Arthrobacter aurescens	12	—/8	—/tr	—	20	18	14	10
A. crystallopoides	22	16	12	—	34	30	25	22
Berevibacterium glavum	8/13	—/10	—/tr	—	15	14	10	8
Corynebacterium ruthugi	14	—/9	—	—	19	17	14	10
Saccharomyces cerevisiae	—	—	—	—	—/10	—/8	—	—
S. phaeochromogenes (Tü 4)	20	14	—/10	—	24	21	14	8
Streptomyces griseus (Tü 17)	23	16	—/10	—	31	30	25	17
S. diastatochromogenes (Tü 20)	29	20	15	—	32	30	24	20
S. violaceoruber (Tü 22)	27	21	16	tr	40	35	30	27
S. prasinus (Tü 30)	25	17	10	—	35	33	28	22
S. lavendulae (Tü 35)	14	10	—/tr	—	25	22	16	9
S. glaucescens (Tü 49)	—	—	—	—	28	19	tr	—
S. viridochromogenes (Tü 57)	33	30	28	22	35	30	28	21
S. violaceus-niger (Tü 1418)	24	20	14	10	30	26	22	20

tr: Trace.
Double numbers: First complete inhibition, second partial inhibition.
—: No activity.

δ 1.82, the signals of the 2-amino-3-hydroxycyclopent-2-enone methylene groups at 2.44 and the epoxide protons 5-H (3.80) and 6-H (3.60) were clearly detected.

Biological Properties

Table 3 shows the results of disc-diffusion assays of colabomycin A (1) in comparison to manumycin against some microorganisms. Less or no inhibition was observed against fungi, yeasts and Gram-negative bacteria. The activity of 1 against Gram-positive bacteria and *Streptomyces* sp. is generally less than that of manumycin. Colabomycin clearly exhibits activity in the proliferation assay (IC_{50} 4.6 μg/ml, manumycin 3.1) and the stem cell assay (IC_{50} 3.4 μg/ml, manumycin 0.9) against murine L1210 leukemia cells.

Discussion

With the colabomycins produced by *S. griseoflavus* (strain Tü 2880) the manumycin group is expanded by three new compounds. The structure of the first compound to be discovered of this group, manumycin[3,4], was published in detail recently[5,6]. The three other substances, asukamycin[7,8], U-56,407[9] and U-62162[10] differ with respect to the stereochemistry at C-6, the configuration and the number of carbons of the olefinic chain and the amide bound side chain. In addition U-62162 does not contain the 2-amino-3-hydroxycyclopent-2-enone moiety. The expanded polyene carboxamide chains of colabomycin A revealed the capability of different *Streptomyces* strains to vary the acetogenic

parts of these molecules. Although the antibacterial activity of these metabolites is fairly poor, it is shown that chemical screening is a useful tool for detecting new secondary metabolites from microorganisms.

Experimental

General

MP's were determined using a Reichert hot stage microscope. UV spectra were recorded using a Kontron Uvikon 860 spectrometer. IR spectra were obtained in pressed KBr discs using a Perkin-Elmer Model 298 spectrometer. The NMR spectra were determined with a Varian VXR-200, Bruker WM 300 or Bruker AM 500, respectively. Chemical shifts (δ in ppm) are reported relative to internal TMS. The electron impact mass spectra (EI-MS) (70 eV) were obtained on a Varian MAT 731 or a Finnigan MAT 311 A, respectively, using direct probe insert, high resolution with perfluorkerosine as a standard, and the FD-MS on a Finnigan MAT 8230. CD spectra were recorded using a Jasco J 500 A spectrometer in combination with a BMC if 800 personal computer. Optical rotations were taken with a Perkin-Elmer Model 241 polarimeter.

Analyticals

TLC was performed on silica gel plates (Macherey-Nagel Sil G/UV 254+366, 0.25 mm silica gel on glass), low pressure column chromatography on Silica gel 60 (0.063~0.04 mm Macherey-Nagel) with N_2 as carrier gas. Sephadex LH-20 (Pharmacia) was used for gel filtration/absorption. Analytical (see Fig. 1) and micropreparative HPLC were carried out with a Waters 6000-A Solvent Delivery System and a Zeiss PM 2 DLC Variable Wavelength Detector using a Macherey-Nagel 7 μm Nucleosil 100-C18 (250×10 mm, 2.0 ml/minute, 70 bar) or a Knauer 7 μm Nucleosil 7C-18 (250×16 mm, 4.5 ml/minute, 105 bar) column, respectively, developed with a mobile phase of acetonitrile - water (2:1). Thecolabomycin-complex was applied as a dioxane solution and detected by UV absorption at 410 nm.

Bacterial Strains

The standard strains for the determination of the activity spectrum of Tü 2880 (Table 3) were obtained from the stock culture collection in our laboratories or from ATCC. The antibiotic producing organism was a new soil isolate from Mexico, classified according HÜTTER[11] and BERGEY[12] as *S. griseoflavus* subsp. *canus*.

Fermentation Studies

S. griseoflavus (Tü 2880) was cultured for 96 hours at 27°C in a medium (100 ml in a 500-ml Erlenmeyer flask with one intrusion, the pH of the medium was adjusted to 7.2 before autoclaving) consisting of mannitol 2% and soybean meal 2%. These cultures, 48 hours old, were used as inoculum for a 20-liter fermentor (Laborfermentor b 20, Giovanola Fréres SA), which in turn was used as inoculum for the 200-liter fermentor as well as for test fermentations. Tü 2880 was produced in a 20-liter fermentor under following conditions: 5% Inoculum was transferred to the fermentor containing 19.0 liters of the given medium and run at 27°C for 96 hours under 800 rpm agitation and 6.5 liters/minute aeration. Tü 2880 was produced in 200-liter scale (Laborfermentor b 200, Giovanola Fréres SA) under following conditions: 10% Inoculum, 48 hours old, was transferred to the fermentor containing 180 liters of the given medium and run at 27°C for 96 hours (agitation 800 rpm, aeration 7.5 liters/minute).

Biological Assays

The disc-diffusion assay was used for measuring the antibiotic content of the cultures and to determine the antibacterial and antifungal spectrum of colabomycin A. Complete medium for the activity spectrum: Yeast-extract 0.4%, malt-extract 1%, glucose 0.4%, agar 1.5%, pH 7.3 before autoclaving; minimal medium for the activity spectrum: KH_2PO_4 0.3%, K_2HPO_4 0.7%, NaCl 0.01%, $MgSO_4 \cdot 7H_2O$ 0.01%, $(NH_4)_2SO_4$ 0.1%, glucose 0.4%. Glucose was autoclaved separately.

Isolation of the Colabomycin-complex

The mycelium was extracted with acetone three times. After evaporation and lyophylization of the aqueous residue, half of the resultant raw material (66 g) was extracted three times with 250 ml dioxane - MeOH (2:3), and the pooled extracts were evaporated to dryness under reduced pressure. 200 ml petroleum ether (bp <70°C) were added to the residue and the mixture was kept in an ultrasonic bath for 10 minutes. This procedure was repeated thrice. The grainy brown material (14 g) which resulted after filtration was divided in two, and each portion, mixed with the 1~2-fold of sand, applied on a low pressure silica gel column (20×5 cm, $CHCl_3$ - MeOH, 9:1), the eluates of the yellow main zones were combined yielding 990 mg colabomycin-complex. A second fraction contained another secondary metabolite of the strain (2880-II, 235 mg)[13]. In two portions of 500 mg (dissolved in 1~2 ml dioxane) the colabomycin-complex was further purified by successive chromatography on a Sephadex LH-20 column (100×2.5 cm) in $CHCl_3$ (230 mg) and in $CHCl_3$ - MeOH (9:1) yielding 114.7 mg of the complex. Approx 30 mg were dissolved in 1.5 ml dioxane, filtered and injected in 200 μl portions onto the HPLC column. The fractions (retention times: Colabomycin A: 26.4 minutes, B: 34.4 minutes, C: 37.0 minutes) of several separations were pooled and lyophylized yielding 7.6 mg colabomycin A (**1**) (~0.08 mg/liter culture broth), 1.1 mg B, 0.4 mg C and 0.8 mg mixture of B and C. Due to intermediary partial decomposition, a second run under the same conditions was carried out, yielding the fourth part of the first isolated **1**.

Colabomycin A (**1**)

MP >280°C (dec); $[\alpha]_D^{20}$ +788° (*c* 0.1, dioxane); Rf values see Table 1; IR (KBr, Fig. 2) cm^{-1} 3410 (br), 3030, 2930, 1690, 1670, 1615, 1595, 1520; UV λ_{max}^{MeOH} nm (ε) 345 (62,100), 262 (22,600); $\lambda_{max}^{MeOH-HCl}$ 348 (52,100), 270 (23,800); $\lambda_{max}^{MeOH-NaOH}$ 339 (66,000), 261 (25,200); $\lambda_{max}^{CH_3CN}$ 348 (87,100), 265 (19,000); ^1H NMR (500 MHz, DMF-d_7) δ 1.82 (3H, dd, J=5.8 and 1.2 Hz, 10′-H_3), 2.49 (4H, s, 4″-H_2 and 5″-H_2), 3.25 (1H, b, OH), 3.68 (1H, d, J=4.1 Hz, 6-H), 3.78 (1H, dd, J=4.1 and 2.8 Hz, 5-H), 5.87 (1H, m, 9′-H), 6.00, 6.03 (2H, complex, 7-H and 6′-H), 6.15 (1H, dd, 7′-H), 6.41 (1H, dd, 4′-H), 6.52, 6.54, 6.58, 6.58, 6.63 (5H, complex, 10-H, 12-H, 2′-H, 9-H and 8-H), 6.71 (1H, d, 14-H), 6.78 and 6.78 (2H, complex, 8′-H and 11-H), 7.23 (1H, dd, 5′-H), 7.31, 7.33 and 7.35 (3H, complex, 3′-H, 3-H and 13-H), 9.15 (1H, b, 1′-HN), 9.90 (1H, b, 15-NH), (3′-OH not observed); ^{13}C NMR (125.7 MHz, DMF-d_7) δ 18.5 (q, C-10′), 53.4 (d, C-6), 57.8 (d, C-5), 71.6 (s, C-4), 115.7 (s, C-2″), 122.5 (d, C-14), 125.0 (d, C-2′), 127.1 (d, C-6′), 128.2 (d, C-8′), 129.1 (s, C-2), 129.5 (d, C-3), 131.1 (d, C-4′), 131.4 (d, C-8), 131.7 (d, C-12), 133.6 (d, C-9′), 134.1 and 134.1 (d, C-10 and C-7′), 136.1 (d, C-5′), 136.7 (d, C-9), 138.2 (d, C-7), 141.4 (d, C-11), 142.1 (d, C-3′), 143.3 (d, C-13), 165.8 (s, C-1′), 167.1 (s, C-15), 190.2 (s, C-1); FD-MS *m/z* 530 (M^+); CD $\lambda_{extreme}$ (CH_3CN, see Fig. 4) nm ($[\theta]^{22}$) 370 (+313,400), 328 (−354,200).

Anal Calcd for $C_{30}H_{30}N_2O_7 \cdot H_2O$: C 65.68, H 5.51, N 5.10.
Found: C 65.39, H 5.88, N 5.05.

Colabomycin B

MP >300°C; Rf values see Table 1; IR (KBr) cm^{-1} 3440, 2930, 2860, 1690, 1615, 1580, 1520; UV $\lambda_{max}^{CH_3CN}$ nm ($E_{1cm}^{1\%}$) 365 (948), 261 (198); CD (CH_3CN, see Fig. 4) $\lambda_{extreme}$ nm ($[\psi]^{22}$) 381 (+32,400), 334 (−34,180).

Colabomycin C

MP >300°C; Rf values see Table 1; IR (KBr) cm^{-1} 3410, 2930, 2860, 1730, 1695, 1670, 1620, 1580, 1520; UV $\lambda_{max}^{CH_3CN}$ nm ($E_{1cm}^{1\%}$) 365 (270), 262 (66); CD (CH_3CN, see Fig. 4) $\lambda_{extreme}$ nm ($[\psi]^{22}$) 383 (+8,210), 332 (−8,770).

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