NEW ANTHRACYCLINE ANTIBIOTICS PRODUCED BY INTERSPECIFIC RECOMBINANTS OF STREPTOMYCETES

III. ISOLATION AND STRUCTURE OF IREMYCIN

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The structure of the anthracycline antibiotic iremycin isolated from *Streptomyces violaceus* subspecies *iremyceticus* has been elucidated as 10-(α-L-rhodosaminyl)-γ-rhodomycinone (**I**) on the basis of spectroscopic analyses and chemical reactions.

Recently, we described the selection and characterization of an interspecific recombinant phenotype obtained by hybridization experiments with mutants of various *Streptomyces* species blocked in antibiotic production[1]. The anthracycline, iremycin, is the main component of the red antibiotic complex produced by cultures of the selected interspecific recombinant designated as *Streptomyces violaceus* subspecies *iremyceticus* strain IMET 43615[2]. The antibiotic possesses antimicrobial and cytostatic activity[3]. This paper describes the isolation of iremycin; furthermore, the spectral and physicochemical properties of the antibiotic and the elucidation of its structure are reported. Iremycin is a red crystalline solid, soluble in chloroform, dioxane and acetone, sparingly soluble in methanol and insoluble in water and hydrocarbon solvents. The weakly basic antibiotic forms a hydrochloride (**Ia**) with methanolic hydrogen chloride, which is readily soluble in methanol and water. On acid hydrolysis (0.1 N HCl) iremycin yields a red aglycone and one sugar component. The aglycone (**II**) was identified by comparision of its UV/VIS, IR, CD and mass spectra with those of γ-rhodomycinone[4~6]. The sugar is levorotatory and its Rf value corresponds to that of rhodosamine. Acetylation of iremycin afforded a tetraacetate (**Ib**) (M^+, *m/z* 695, $C_{36}H_{41}NO_{13}$).

The ^{1}H-NMR, ^{13}C-NMR and the mass spectra demonstrated that iremycin consists of one mole each of rhodosamine and γ-rhodomycinone. Generally, anthracyclines cannot be analysed by conventional mass spectrometric analysis due to their extremely low volatility. In most cases the electron impact mass spectra of these compounds fail to show molecular ions. In contrast, the mass spectrum of iremycin (Fig. 1) shows a relatively intense molecular ion peak and a number of intense and characteristic fragments of the aglycone and sugar moiety, respectively.

The main fragmentation pathways and the elemental composition of the major fragment ions, derived from metastable ion and high resolution measurements are presented in Fig. 2. Primary cleavage of the glycoside linkage with charge retention on the sugar portion leads to the rhodosaminyl ion *m/z* 174 and the relatively abundant pyrilium ion *m/z* 158. On the other hand, cleavage with charge

Fig. 1. Electron impact mass spectrum of iremycin.
× 10% of the peak intensity.

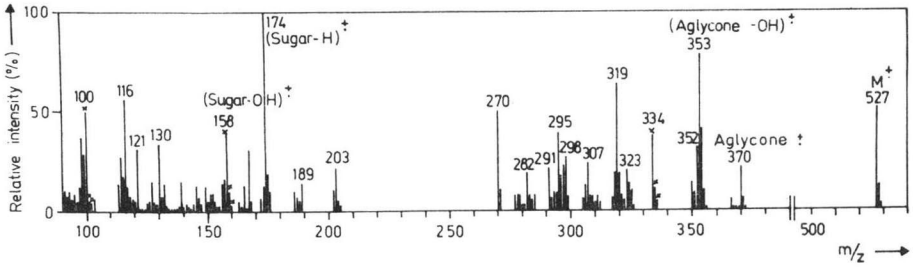


Fig. 2. Fragmentation scheme of iremycin.
× metastable transitions observed.

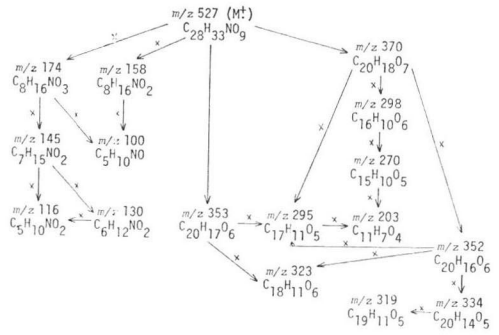
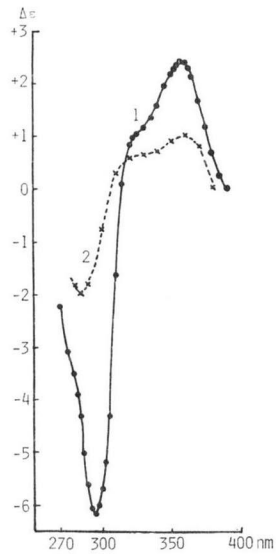


Fig. 3. CD curves of iremycin (**1**) and γ-rhodomycinone (**2**) in dioxane.



retention on the aglycone portion leads to the fragments *m/z* 370 (H-transfer) and *m/z* 353 characteristic of the γ-rhodomycinone moiety.

The molecular formula of iremycin was confirmed by elemental analysis and ^{13}C-NMR spectroscopy. The ^{1}H- and ^{13}C-NMR spectra contain besides the signals assignable to the γ-rhodomycinone moiety the signals which neatly fit for the rhodosamine part of the molecule, both with respect to their chemical shifts and coupling constants. The sugar has α-configuration at C-1′ since (i) the linewidth of H-1′ signal is 6 Hz only; (ii) the direct coupling of H-1′ to carbon δ_C 96.5 is 172.0 Hz[7,8].

As both the iremycin and γ-rhodomycinone show very similar electronic absorption, the sugar moiety is obviously attached to one of the alcoholic hydroxyl groups, and since iremycin forms only a tetra-acetate (**Ib**) and not a penta-acetate, the tertiary alcoholic group at C-9 must be intact.

Comparison of ^{13}C-NMR spectra of iremycin and γ-rhodomycinone (**II**) (Table 1) reveals nearly identical chemical shifts of C-4, C-6, C-11 and C-9 together with 5.4 ppm downfield shift of C-10. These facts indicate that the sugar is attached to C-10.

The CD spectrum of the aglycone (**II**) is identical with that of γ-rhodomycinone[5] demonstrating the same configuration at the chiral centers C-9 (*R*) and C-10 (*R*). That there is no change in stereochemistry at C-10 of iremycin during hydrolysis follows from a comparison of the CD spectra of iremycin and its aglycone (**II**). It follows from all the data presented that iremycin (**I**) is 10-(α-L-rhodosaminyl)-γ-rhodomycinone. Iremycin is probably identical with γ-rhodomycin I described by BROCK-

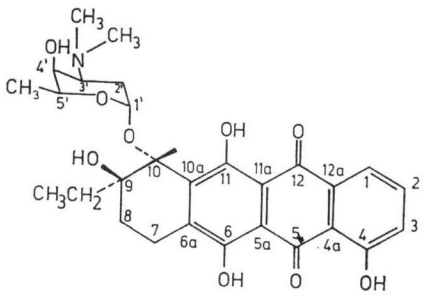
Table 1. ^{13}C-Chemical shift and assignments* of compounds I~IV.**

Carbon No.	I	II	III	Carbon No.	I	II	III	IV
1	119.4	119.3	119.5	10a	140.9	140.2	140.2	—
2	136.8	136.9	137.0	11a	110.2	×	110.3	—
3	124.4	125.3	124.3	12a	133.7	×	133.9	—
4	162.6	160.1	162.7	1′	96.9	—	91.0	98.1
5	190.9	×	191.0	2′	29.2	—	26.5	28.5
6	156.3	157.0	156.5	3′	59.7	—	59.8	59.8
7	27.0	26.6	24.7	4′	66.1	—	77.4	66.1
8	21.1	20.9	21.0	5′	66.5	—	66.5	65.2
9	71.7	71.7	66.0	6′	17.3	—	16.5	17.1
10	71.0	65.6	69.8	NMe_2	42.0	—	42.0	42.1
11	158.3	158.7	158.3	1″	—	—	97.9	—
12	185.8	×	185.6	2″	—	—	29.5	—
13	30.8	30.9	29.8	3″	—	—	59.5	—
14	6.6	6.6	7.0	4″	—	—	66.3	—
4a	116.4	×	116.3	5″	—	—	66.5	—
5a	110.7	×	110.4	6″	—	—	17.2	—
6a	138.2	135.8	136.9	NMe_2	—	—	42.0	—

* In ppm (δ), obtained from $CDCl_3$ solutions containing TMS as internal reference.
×) Signal lost in the noise.
All signals had the multiplicity in the off-resonance spectrum consistent with their assignments.
** Compounds: **I**, iremycin; **II**, γ-rhodomycinone; **III**, roseorubicin B[10]; **IV**, methyl-α-L-rhodosaminide.[13]

MANN *et al.*[9] but no physicochemical properties of the latter have been reported yet. The comparison of ^{13}C-NMR spectra (Table 1) of iremycin and another γ-rhodomycinone glycoside, roseorubicin B[10] shows different chemical shifts of C-1′, C-2′ and C-9, but our values are consistent with the data of several known anthracyclines[11,12]. The upfield shift of the rhodosamine C-1′ attached to C-10 with respect to that bonded to C-7 (101.3~102.2)[11,12] can be used as a diagnostic tool in the study of rhodomycins having hydroxyl groups both at C-7 and C-10.

Fig. 4. Structure of iremycin.



Experimental

General

Melting points are uncorrected. The mass spectra were recorded on a JEOL JMS-D 100 spectrometer (75 eV, temperature of ion source 200°C, temperature of direct inlet system 150~200°C, high resolution measurements were performed using the peak matching technique with PFK as standard). Metastable spectra were recorded on a Varian MAT-311 spectrometer using the DADI technique. The ^{1}H- and ^{13}C-NMR spectra were measured on a JEOL FX-60 NMR spectrometer (FT mode, 59.797 and 15.036 MHz) in $CDCl_3$ at 25°C. Chemical shifts are given in the δ-scale (internal TMS standard), accuracy $\pm$0.005 and $\pm$0.008 ppm. The CD spectra were recorded on a Rousell-Jouan Dichrograph CD 185.

Thin-Layer Chromatography (TLC)

The isolation of the anthracycline was followed by TLC. Samples were applied to layers of silica gel (Silufol, Kavalier, Czechoslovakia) and developed with S_1, chloroform - methanol - acetic acid - water (80 : 20 : 14 : 6 by volume). For TLC of the sugar S_2, *n*-butanol - acetic acid - water (4 : 1 : 1 by volume) was used. After development the plates were air dried and sprayed with 0.93% *o*-phthalic acid and 1.66% aniline in water saturated *n*-butanol and then heated at 110°C to examine color development of sugar.

Iremycin (**I**)

Broth (6 liters) at harvest (pH 7.8) was filtered. The red pigments in the mycelium were extracted sequentially with methanol (0.7 liters) and with methanol - chloroform (1 : 1 by volume, 0.7 liters). The combined methanol-chloroform extracts were concentrated under reduced pressure up to the water phase and reextracted with chloroform. The red pigments in the culture filtrate were extracted with chloroform (0.6 liters). The chloroform extracts were combined, dried over Na_2SO_4 and evaporated to give an oily mixture of red pigments. The oil was dissolved in a small volume of methanol - chloroform (1 : 1 by volume) and the solution was applied to a column of Sephadex LH-20 (80×3 cm) packed in methanol. The column was developed with methanol (300 ml) followed by methanol - chloroform (1 : 1 by volume) and fractions of 20 ml were collected. The fractions 8~15 containing iremycin were combined and evaporated to give a residue (220 mg). The crude iremycin was purified by column chromatography (1.5×40 cm) using chloroform - acetone - methanol (80 : 20 : 6 by volume) on $NaHCO_3$-buffered silica gel and fractions of 10 ml were collected. The fractions 8~14 were combined, evaporated and the residue was dissolved in chloroform. After washing with water the chloroform solution was dried over Na_2SO_4. Evaporation of the solution and recrystallization of the residue from methanol gave 120 mg of pure iremycin.

Iremycin is a red crystalline compound, m.p. 212~214°C, Rf 0.5

Anal. Calcd. for $C_{28}H_{33}NO_9$ (527): C, 63.75; H, 6.30; N, 2.66
Found: C, 63.98; H, 6.66; H, 2.35

UV/VIS, $\lambda_{max}^{Cyclohexane\text{-}CHCl_3}$ nm ($E_{1cm}^{1\%}$): 238 (595), 254 (522), 295 (142), 469 (222), 489 (266), 498 (327), 521 (230), 535 (273); IR, ν_{max}^{KBr} cm^{-1}: 3450 (OH), 2820, 2770, (NMe_2), 1598 (hydrogen bonded quinone). ^{1}H-NMR ($CDCl_3$ δ): 1.09 t (7.3 Hz, 3H, H-14), 1.36 d (6.8 Hz, 3H, H-6), 1.90~2.15 mt (8H), 2.21 s (6H, NMe_2), 2.92 ddd (6.4, 2.9 and 1.0 Hz, H-3), 3.32 s (1H, OH), 3.67 mt ($J_{3',4'}$=2.9 Hz, H-4'), 3.94 qd ($J_{4',5'}$=1.5 Hz, $J_{5',6'}$=6.8 Hz, H-5'), 4.95 d (1.0 Hz, H-10), 5.40 mt (W-6 Hz, H-1'), 7.26 dd (7.3 and 2.0 Hz, H-3), 7.66 t (7.3 Hz, H-2), 7.87 dd (7.3 and 2.0 Hz, H-1).

Hydrochloride of Iremycin (**Ia**)

Iremycin (264 mg) was dissolved in dry methylene chloride (20 ml), 0.2 N methanolic hydrogen chloride (2.5 ml) was added to the solution followed by ether (80 ml). The red precipitate was collected by filtration and reprecipitated from MeOH with ether to afford 230 mg of **Ia** as an orange-red crystalline powder. m.p. 176~178°C.

Anal. Calcd. for $C_{28}H_{33}NO_9 \cdot HCl$ (564): C, 59.63; H, 6.30; N, 2.48; Cl, 6.29
Found: C, 59.31; H, 6.62; N, 2.33; Cl, 6.14

Tetraacetate of Iremycin (**Ib**)

Acetic anhydride (5 μl) was added to a solution of iremycin (1 mg) in pyridine (5 μl). The reaction mixture was kept at room temperature for 12 hours, then the pyridine and acetic anhydride were evaporated in high vacuum. The dry yellow residue was used directly in the mass spectral studies.

Mass spectrum: (m/z): M^+ 695.2610, calcd for $C_{36}H_{41}NO_{13}$: 695.2577

Hydrolysis of Iremycin

A solution of iremycin (100 mg) in 0.1 N hydrochloric acid (10 ml) was heated at 95°C for 70 minutes. The red precipitate was collected by filtration, and recrystallized from benzene to yield γ-rhodomycinone (**II**) (45 mg) as red needles, m.p. 235~242°C (lit.[4] 230~240°C).

Anal. Calcd. for $C_{20}H_{18}O_7$ (370): C, 64.86; H, 4.90
Found: C, 64.59; H, 5.10

UV/VIS, $\lambda_{max}^{Cyclohexane\text{-}Chloroform}$ nm ($E_{1cm}^{1\%}$): 294 (175), 468 (240), 484 (290), 494 (340), 516 (247), 531 (265); IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1590 (hydrogen bonded quinone); Mass spectrum: (*m/z*): M^+, 370.1067; $(M-H_2O)^+$, 352.0931; $(M-2H_2O)^+$, 334.0838; $(M-C_2H_7O)^+$, 323.0536; $(M-C_4H_8O)^+$, 298.0500; $(M-C_3H_7O_2)^+$, 295.0597; $(M-C_5H_8O_2)^+$, 270.0496. In the filtrate from the hydrolysate a sugar corresponding to rhodosamine was detected at Rf 0.15 on TLC. The filtrate was decolorized with active charcoal, adjusted to pH 5.5 with Amberlite IR-4B (CO_3-form) resin and evaporated under reduced pressure to yield an oily residue (6 mg). $[\alpha]_D^{20}-36°$ (*c* 0.5, water).

Acknowledgements

The authors are indebted to Dr. H. FRITZSCHE for additional evidence of the structure by NMR investigations of iremycin hydrochloride. The NMR spectra have been run at 67.9 MHz (^{13}C, proton-decoupled and off-resonance, respectively) on a Bruker HX-270 at Yale University, New Haven, Connecticut, and at 360 MHz (^1H) on a Bruker WH-360 at the University of Pennsylvania, Philadelphia, Pennsylvania. The spectra of iremycin hydrochloride are in excellent agreement with the NMR spectra of iremycin base and will be reported elsewhere. The authors also thank Dr. M. BECKER of Akademie der Wissenschaften der DDR, Zentralinstitut für Molekularbiologie, Berlin-Buch, for the CD spectra and Dr. J. VOKOUN of Czechoslovak Academy of Science, Institute of Microbiology, Prague, for the MS-DADI spectra.

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