222. New Peptide Lactones of the Streptogramin Family (Group B). Structures of Virginiamycin S_5 and Viridogrisein II

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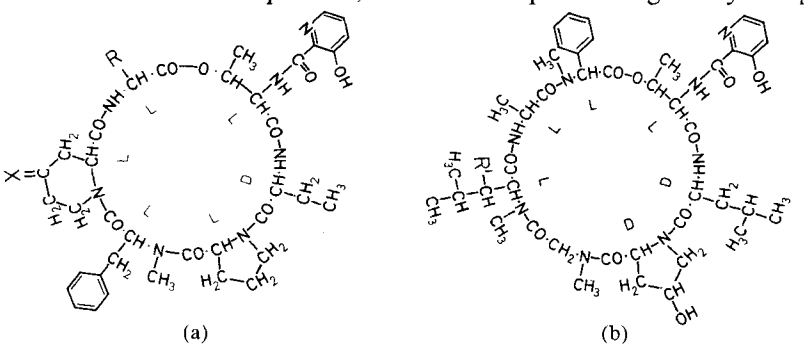
Summary

Virginiamycin S_5 and viridogrisein II were isolated by liquid chromatography from the crude antibiotics virginiamycin and viridogrisein, respectively. Their structures were elucidated by amino-acid analysis and mass spectrometry.

Streptogramin antibiotics are mixtures of several chemical components which are classified in two groups, A and B, according to their structure. They are mainly active against *Gram*-positive bacteria. Mixtures of antibiotics of the two groups show a marked synergism. Evidence has been presented that the streptogramins act by binding to the bacterial ribosome and blocking of protein biosynthesis [1]. In addition, the B components interact with membranes and facilitate the transport of cations across phospholipid bilayer membranes [2].

The structures of the streptogramins of group B (see *Fig. 1*) are characterized by a macrocyclic peptide-lactone ring containing 6 to 7 amino-acid residues. The *N*-terminal threonine is invariably *N*-acylated with 3-hydroxypicolinic acid. The other amino-acid residues exhibit some variability. Virginiamycin is known to contain four different B components, the main component virginiamycin S_1 [3] and



(a) (b)

Fig. 1. a) *Structure of virginiamycin* S_1 ($R = C_6H_5$; $X = O$) *and virginiamycin* S_5 ($R = CH_3$; $X = OH, H$); b) *Structure of viridogrisein I* ($R' = CH_3$) *and viridogrisein II* ($R' = H$)

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three minor components S_2, S_3, S_4 [4] [5]. Viridogrisein (etamycin) [6] was considered to consist of a single B component. Recently, however, congeners of this antibiotic [7] have been reported.

Thin layer chromatography (TLC.) on silica gel plates revealed that our crude virginiamycin sample contained, besides its main B component, virginiamycin S_1, three minor B components. Two of them could be assigned to virginiamycin S_2 and S_4 (MS.), whereas the third was an unknown component which we denoted virginiamycin S_5. Similar studies on our crude viridogrisein samples also led to the discovery of an unknown minor component. The main component is denoted viridogrisein I and the minor one, viridogrisein II. To determine the structures of virginiamycin S_5 and viridogrisein II, both compounds were isolated by chromatography.

In order to characterize the amino-acid constituents of the new components, their total hydrolyses were performed. The amino-acid identification was based on thin layer electrophoresis (TLE.) on cellulose plates. 3-Hydroxypicolinic acid could be detected directly by its fluorescence (excitation at 366 nm), while the other amino acids became visible after spraying the plates with a ninhydrin-copper nitrate reagent. Comparison of the hydrolysates of virginiamycin S_1, S_4 and S_5 showed that the latter lacks phenylglycine and 4-oxopipecolic acid, but contains, like S_1, 2-aminobutyric acid (2-Abu), proline (Pro), *N*-methylphenylalanine (*N*-MePhe) and threonine (Thr). Instead of the two missing amino acids, alanine (Ala) and an unidentified amino acid were present in the hydrolysate of S_5. Compared with viridogrisein I, the hydrolysate of viridogrisein II indicated that only *N*,*β*-dimethylleucine was substituted by another, not readily identifiable amino acid.

For a complete structure elucidation, both new antibiotics were further subjected to analysis by mass spectrometry. The electron impact (EI.) mass spectrum of virginiamycin S_5 showed, besides structurally informative fragment ions, a pronounced molecular ion M^+ at *m/z* 763 *(Fig. 2a)*. This is in agreement with the results of chemical ionization (CI.) mass spectrometry (CH_4 reagent gas, DCI. technique): in the positive ion mode, $(M+H)^+$ ions are observed as practically a solitary peak at *m/z* 764 and, likewise, in the negative mode M^- ions at *m/z* 763. For viridogrisein II a molecular weight of 864 was similarly found (*e.g.* M^+ at *m/z* 864 in the EI. spectrum, *Fig. 2b*). In addition to molecular-weight information (virginiamycin S_5 is 60 daltons less than S_1, viridogrisein II only 14 less than I), amino-acid identification as well as sequence information were provided by the EI. fragmentation patterns. Thus, for virginiamycin S_5 a prominent loss of 99 daltons from M^+ (*Fig. 2a*, *m/z* 763 → 664), followed by a loss of 43 daltons (*m/z* 664 → 621) was found closely analogous to the characteristic sequential loss of 97 and 43 daltons in virginiamycin S_1. This latter double loss, triggered by preferential rupture of a tertiary amide bond, reflects the ejection of C_5H_7NO (4-oxo-1,2-dehydropiperidine)/HNCO from the 4-oxopipecolic acid moiety [4b]. Hence, the presence of a hydroxy instead of the missing oxopipecolic acid is inferred (*Scheme*, path *b* leading to ion B_2 *via* B_1). Fragments at *m/z* 100 and 82 (the protonated azomethine 4(?)-hydroxy-1,2-dehydropiperidine and its H_2O-elimination

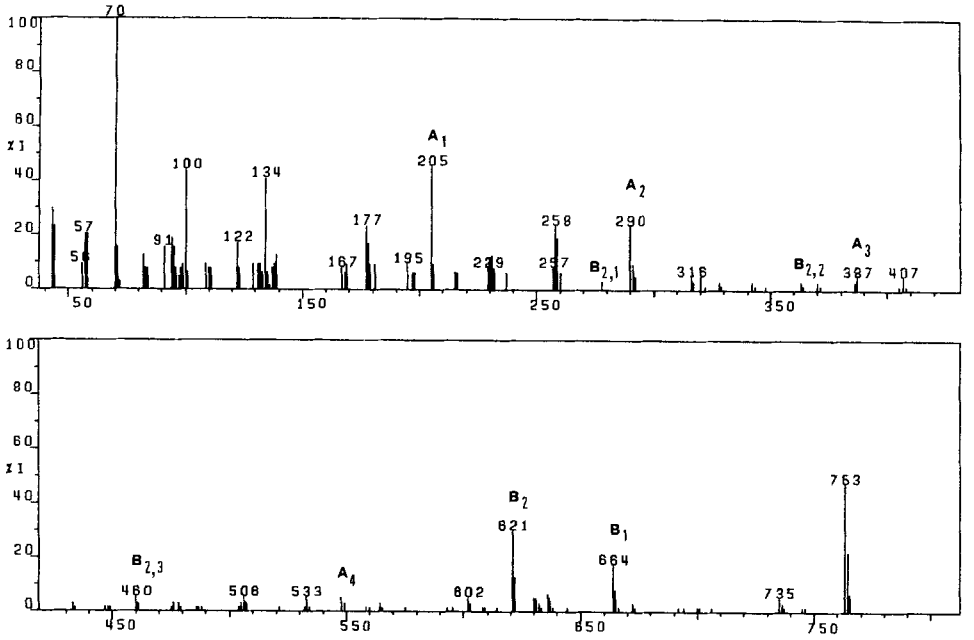


Fig. 2a. *EI.-MS. of virginiamycin S_5*

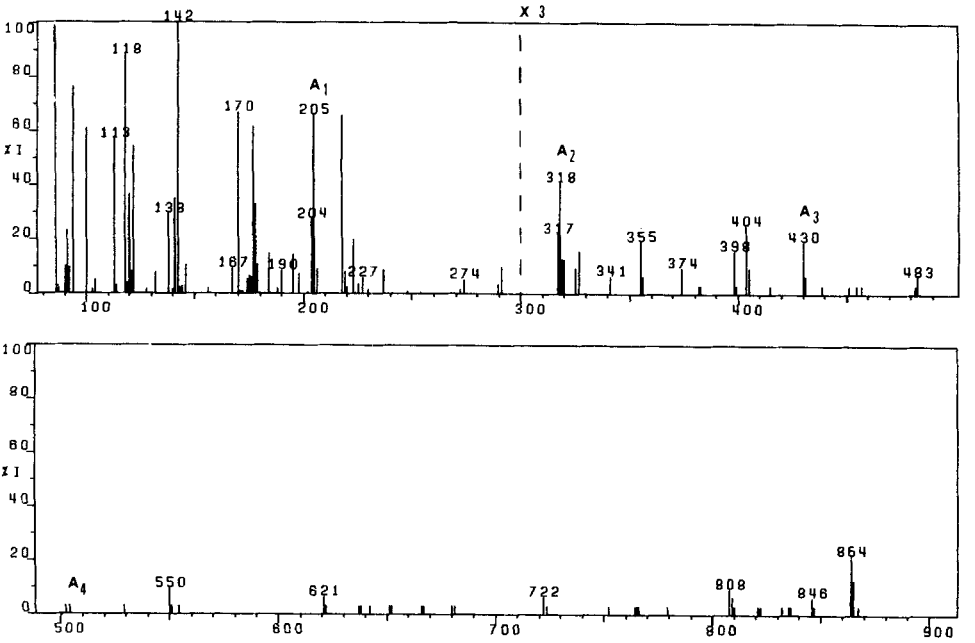
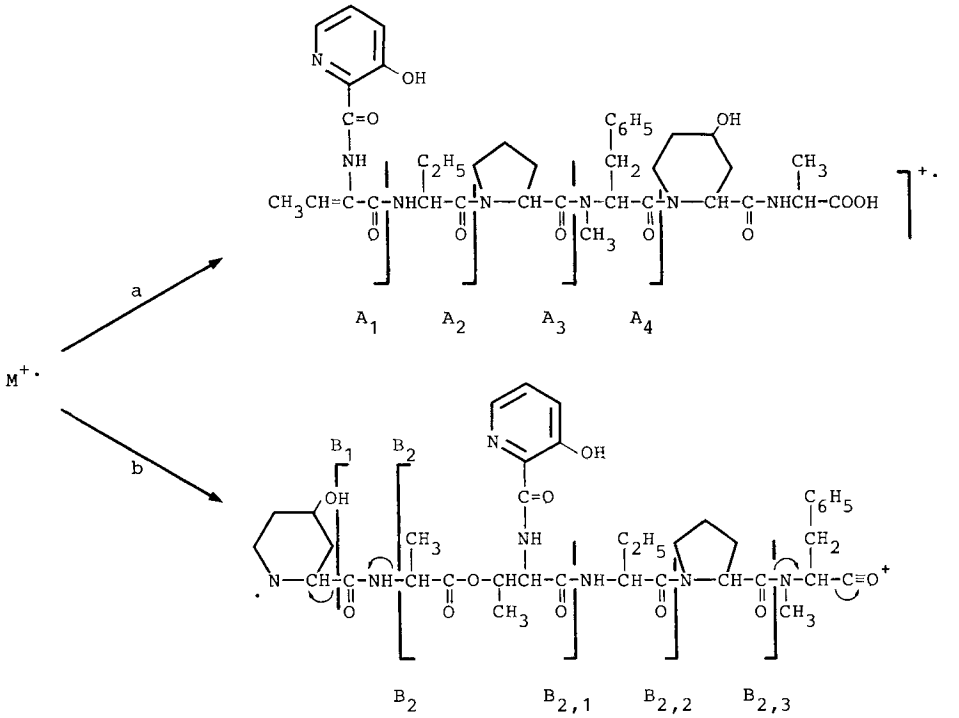


Fig. 2b. *EI.-MS. of viridogrisein II*

Scheme. *Sequence ion formation in virginiamycin S_5*



product, respectively), and the absence of the corresponding ion of S_1, *m/z* 98, support the identity of this amino acid. Most other amino acids, already identified in the total hydrolysate by TLE., are similarly represented in the lower mass range as the protonated azomethines $R\overset{+}{N}H{=}CHR'$ ($R = H, CH_3$).

The site and chirality of the hydroxy group in the hydroxypipecolic acid (expected at the 4-position) was readily determined by a GC./MS. analysis of the total hydrolysate *(Fig. 3a)*. The free amino-acid mixture was converted into *N*-trifluoroacetyl methyl esters ($CF_3CON(R)CH(R')COOCH_3$) in a two-step procedure (MeOH/HCl, followed by $(CF_3CO)_2O$). The bis(trifluoroacetyl) derivative (M = 351, acylated at the hydroxyl function) of the hydroxypipecolic acid present in the hydrolysate was identical with that of *allo*-4-hydroxypipecolic acid (MS. and GC. retention time), but distinctly different from that of its *trans*-diastereoisomer (same MS. but much shorter retention time). Comparison with the two required diastereoisomeric reference amino acids was based on separate GC./MS. analysis of analogously prepared total hydrolysate of *allo*- and *trans*-dihydrovirginiamycin S_1 (together obtained from S_1 by reducing selectively the 4-oxopipecolic acid residue with $NaBH_4$ [8]).

In the case of viridogrisein II, the mass deficiency of 14 daltons relative to viridogrisein I is due to the replacement of *N*,*β*-dimethylleucine by *N*-methylleucine. This is reflected in the elimination of only 56 daltons from M^+ of virido-

grisein II (*Fig. 2b*, *m/z* 864→808, loss of C_4H_8 from the identical side chains of leucine and *N*-methylleucine by *McLafferty* rearrangement) in contrast to the double loss of 56 and 70 daltons from M^+ in viridogrisein I (*m/z* 878→822 and 878→808, losses of C_4H_8 and C_5H_{10} from the leucine and *N*,β-dimethylleucine sites, respectively) [9]. Again, the single amino-acid building blocks are displayed more directly as a series of $R\overset{+}{N}H{-}CHR'$ ions in the low mass range; of the three leucine homologues, both *m/z* 86 (R=H, R'=C_4H_9) and *m/z* 114 (R=CH_3,

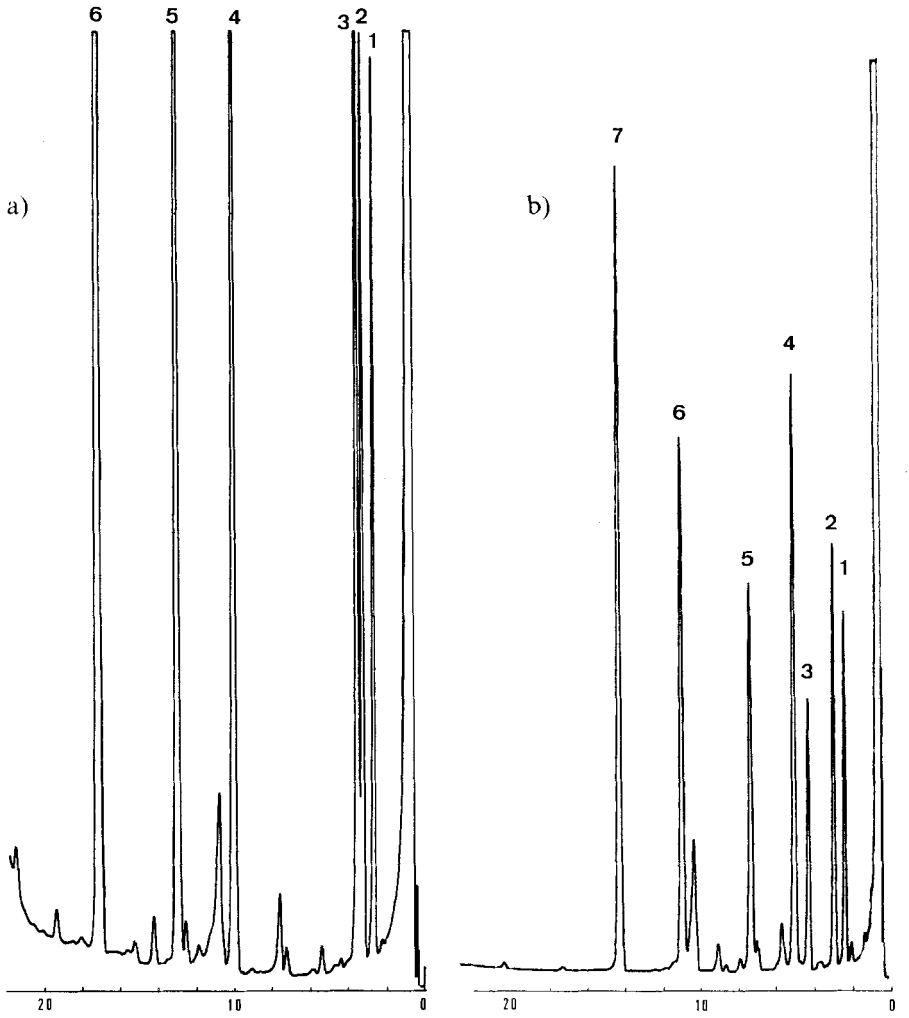


Fig. 3. a) *Gas chromatogram of hydrolysate of virginiamycin* S_5 (retention time expressed in minutes). Amino acids as *N*-trifluoracetyl methyl ester derivatives: 1) alanine, 2) 2-aminobutyric acid, 3) threonine, 4) proline, 5) allo-4-hydroxypipecolic acid, 6) *N*-methyl-phenylalanine. – b) *Gas chromatogram of hydrolysate of viridogrisein II* (retention time expressed in minutes). Amino acids as *N*-trifluoracetyl methyl ester derivatives: 1) alanine, 2) threonine, 3) *N*-methyl-glycine, 4) leucine, 5) *N*-methyl-leucine, 6) 4-hydroxyproline, 7) *N*-methyl-phenylglycine.

$R' = C_5H_{11}$) are observed in viridogrisein I, as opposed to *m/z* 86 ($R = H$, $R' = C_4H_9$) and 100 ($R = CH_3$, $R' = C_4H_9$) in viridogrisein II. As with virginiamycin S_5, a complete amino-acid analysis by GC./MS. (using *N*-trifluoroacetyl methyl esters and GC. conditions as above) confirmed the identity of the inferred 'new' amino acid together with those identified earlier by TLE. *(Fig. 3b)*. In accordance with the EI. data of the intact peptide lactone, the hydrolysate of viridogrisein II contains both leucine and *N*-methylleucine (*ca.* 1:1), identified by co-injection of the derivates prepared from authentic samples.

Sequence information for the two cyclic molecules can be derived from several series of ions in their EI.-MS. spectra, resulting from fragmentation of specific open-chain intermediates after ring opening of the molecular ion radical. For virginiamycin S_5 (*Fig. 2a* and *Scheme*, path *a*) ions A_1 (*m/z* 205), A_2 (*m/z* 290), A_3 (*m/z* 387) and A_4 (*m/z* 548), all present also in virginiamycin S_1 [4b], reflect an identical partial sequence (HO)Pic-Thr-2-Abu-Pro-*N*-MePhe. In analogy to S_1, this sequence should proceed in the order (HO)Pipec-Ala- rather than the reverse; this is confirmed by another ion series derived from the prominent B_2-ion (*Scheme*, path *b*). Since B_2 (*m/z* 621) decomposes through successive 'C-terminal' losses of *N*-MePhe ($B_{2,3}$, *m/z* 460), Pro ($B_{2,2}$, *m/z* 363) and 2-Abu ($B_{2,1}$, *m/z* 278) residues, while retaining the alanine residue throughout, the latter amino acid ought to be linked directly to threonine and thus to represent the lactone-forming constituent. It is noteworthy that analogous $B_{2,n}$ ion series are present in virginiamycin S_1 (*m/z* 683, 522, 425 and 340) [4b] and in its *trans*- and *allo*-dihydro derivatives. Similar reasoning also applies to the sequence verification of viridogrisein II from its EI.-MS. spectrum *(Fig. 2b)*.

The chirality of the amino acids in the two new components was determined enzymatically with D-amino-acid oxidase. Virginiamycin S_5 was found to contain D-aminobutyric acid, and viridogrisein II contained D-leucine and D-hydroxyproline. Following TLE. experiments on cellulose plates, only these amino acids were oxidized in the hydrolysate upon addition of D-amino-acid oxidase. The structures shown in *Fig. 1* are therefore assigned to the new natural compounds virginiamycin S_5 and viridogrisein II.

We are indebted to Mr. *W. Blum, Ciba-Geigy AG*, Basel, for performing CI. mass spectrometry. We thank *Smith Kline*, Brussels, for supplying virginiamycin. We thank *Parke, Davies & Co.*, Detroit, and *Bristol Lab. Inc.*, Syracuse, for viridogrisein.

Experimental Part

Methods. TLE.: To characterize the amino-acid constituents of the antibiotics, total hydrolyses were performed in 6N HCl at 120° for 20 h. After evaporation, the residues were twice redissolved in water and dried (solid KOH), under reduced pressure. The amino-acid identification was based on electrophoresis (*Camag* chamber and power supply) on thin cellulose plates *(Merck)* with a buffer system of pH 1.94 (formic acid/acetic acid/water, 12.5:34.5:900 *v/v*) at a voltage of 50 V/cm. The amino acids were visible after spraying the plates with the ninhydrin-copper nitrate reagent [10]. In order to analyze the chirality of the amino acids, the hydrolysate was incubated with D-amino-acid oxidase under conditions similar to [11], and subsequently characterized by TLE. – NMR.: ^{1}H-NMR. spectra were measured at 270 MHz on a *Bruker* WH 270 spectrometer. – MS.: for the MS. analyses

of the intact antibiotics, a *Finnigan-Mat CH 7* mass spectrometer was employed (70 eV ionization energy, 220° ion source temperature, direct sample insertion). Mass spectra were obtained at probe temperatures in the 200–250° range. GC./MS.: total hydrolyses of the antibiotics were effected by treatment with 6N HCl at 120° for 20 h. Each dry residue (about 1 mg) was dissolved in 2 ml 1.25N HCl/CH_3OH and kept in sealed vials at 100° for 1 h. Subsequently, the residues (amino-acid methyl ester hydrochlorides) obtained *in vacuo* were treated with a mixture of 0.5 ml trifluoroacetic anhydride and 1 ml CH_2Cl_2 at 110° for 1 h. After removal of excess reagent *in vacuo* the trifluoroacetyl methyl esters were redissolved in 100 µl ethyl acetate of which solution 1 µl portions were subjected to GC./MS. analysis. The GC./MS. system used for the analyses of the hydrolysates consisted of a *Varian 1400* gas chromatograph equipped with a packed column (2 mm i.d. × 2 m, 10% OV-17 on *Gas Chrom Q*), and a *Finnigan 1015 C* quadrupole mass spectrometer equipped with an EI. ion source. He was used as the GC. carrier gas, and the column temperature was raised from 100 to 250° at a rate of 6°/min. The mass spectrometer was operated in the customary 70 eV EI. mode.

Chemicals. – *General.* All chemicals used were of analytical grade *(Merck, Fluka).* D-amino-acid oxidase was obtained from *Boehringer,* Mannheim.

Virginiamycin. Crude virginiamycin contained (TLC., ^{1}H-NMR.) about 17% virginiamycin S_1, 2% virginiamycin S_4 and at least 2 minor (< 0.5%) components. One of them was identified as virginiamycin S_2 by MS., the other was a hitherto unknown component which we denoted virginiamycin S_5.

Virginiamycin S_1 was isolated from crude virginiamycin similarly to [3]. The isolated component was recrystallized from methanol, m.p. 162–170°; 240–246° (dec.); after drying (12 h at 130° and 0.01 Torr) m.p. 240–246° (dec.). – TLC.: Rf 0.62 in $CHCl_3/CH_3OH$/acetic acid (100:3:12 *v/v*). – UV. (methanol): 303 (7500), 366 (590). – ^{1}H-NMR. ($CDCl_3$): uniform spectrum; 11.63 (*s*, phenolic OH).

$C_{43}H_{49}N_7O_{10}$ (824) Calc. C 62.68 H 5.99 N 11.90% Found C 62.46 H 5.94 N 11.87%

Virginiamycin S_5 ($C_{38}H_{49}N_7O_{10}$ (763)). Starting with 50 g of the crude mixture of antibiotics, 18.1 g of the A component were removed by fractional crystallization from ethyl acetate (freshly distilled). From the remaining material a fraction (370 mg) enriched in virginiamycin S_5 was obtained by repeated column chromatography on silica gel (reinst, *Merck*) with benzene/CH_3OH/acetic acid (100:11:14 *v/v*). From this fraction, 30 mg of pure virginiamycin S_5 were isolated by preparative TLC. with $CHCl_3/CH_3OH$/acetic acid (100:3:12 *v/v*) [12]: m.p. dec. above 122°. – TLC.: Rf 0.45 in $CHCl_3/CH_3OH$/acetic acid (100:3:12 *v/v*). – UV. (CH_3OH): 304 (5250), 358 (460). – ^{1}H-NMR. ($CDCl_3$): uniform spectrum; 11,72 (*s*, phenolic OH).

Viridogrisein. The material from both *Parke, Davies* and *Bristol Lab.* contained several minor components besides the main component viridogrisein I. The bulk of the minor components consisted of an unknown compound denoted viridogrisein II.

Viridogrisein I was obtained from crude viridogrisein by repeated column chromatography on silica gel in benzene/CH_3OH/acetic acid (100:10:8 *v/v*) [12], yield 56%; m.p. 179–182°; 258° (dec.). – TLC.: Rf 0.41 in benzene/CH_3OH/acetic acid (100:10:8 *v/v*). – UV. (CH_3OH): 307 (9000), 345 (375)). – ^{1}H-NMR. ($CDCl_3$): superposition of three similar spectra due to the existence of three slowly interconverting conformers; 11.82–11.71 (3 signals for 1 phenolic OH).

$C_{44}H_{62}N_8O_{11} \cdot H_2O$ (879 + 18) Calc. C 58.91 H 7.19 N 12.49% Found C 59.02 H 7.11 12.78%

Viridogrisein II was isolated from crude viridogrisein by repeated column chromatography on silica gel in benzene/CH_3OH/acetic acid (100:10:8 *v/v*) [12], yield 4.5%, relative to that of component I; m.p. 130°; 163–167° (dec.). – TLC.: Rf 0.34 in benzene/CH_3OH/acetic acid (100:10:8 *v/v*). – UV. (H_2O): 305 (3600); 349 (5900). – ^{1}H-NMR. ($CDCl_3$): superposition of two similar spectra due to the existence of at least two slowly interconverting conformers; 11.74 and 11.64 (two signals for 1 phenolic OH).

$C_{43}H_{60}N_8O_{11}$ (865) Calc. C 59.71 H 6.99 N 12.95% Found C 59.39 H 7.17 N 12.98%

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