NEW VIRGINIAE BUTANOLIDES FROM *STREPTOMYCES VIRGINIAE*

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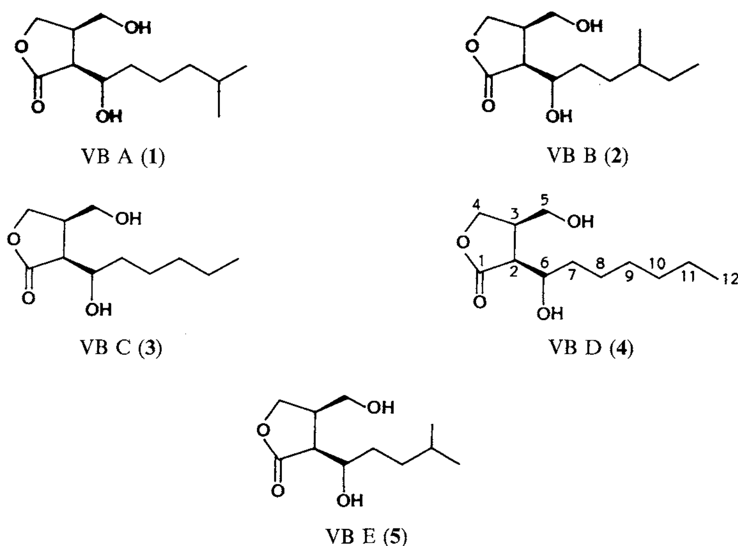
Streptmycetes are widely distributed in nature and are the main producers of biologically active secondary metabolites including many antibiotics. They are also salient in their morphology as prokaryotes forming substrate mycelium, aerial mycelium and spores. In *Streptomyces*, some congenial autoregulators which control cytodifferentiation and secondary metabolite production are already known. They have a common structural feature, 2,3-disubstituted butanolide skeleton[1~4] and are active at nanogram concentrations. We have recently reported the purification and structural elucidation of virginiamycin-inducing factors which were isolated from the culture broth of *Streptomyces virginiae*. We have isolated three of these factors and found that all of them have the butanolide skeleton. We named them virginiae butanolides (VB's) A (**1**), B (**2**) and C (**3**)[4] (Fig. 1). In this report we describe the isolation and structures of two new virginiae butanolides, named VB's D and E, from the culture broth of *S. virginiae*.

Purification of VB's D and E

In the previous purification procedure for VB's A, B and C[4], we have used a strong alkaline hydrolysis step to destroy virginiamycin after partition between *n*-hexane and the MeOH - water layers. In this report, we have omitted alkaline hydrolysis to avoid the hydrolysis of the lactone ring in VB molecules and also changed eluent on aluminum oxide column chromatography as shown in Fig. 2. Fermented broth (1,450 liters)[4] was filtered and extracted with EtOAc (440 liters). The extract (235.3 g) was treated with *n*-hexane and MeOH - H_2O (9:1) to remove nonpolar hydrophobic substances by partition between two layers. The MeOH - H_2O layer which contains the virginiamycin inducing activity was concentrated *in vacuo* and the residue was fractionated on anion-exchanger (Amberlyst A-21, OH^- form, 4,000 g, eluent: methanol). The active fractions were combined and the concentrated residue (131.4 g) was chromatographed on an aluminum oxide column (Aluminium oxide 90, Merck Article No. 1097, 1,200 g). This column separated the active compounds into two fractions, the EtOAc eluate (2.5 liters) and the EtOAc - MeOH (1:1) eluate (1.0 liter). The active fractions in the EtOAc eluate (18.5 g) were further purified by normal phase preparative HPLC (System 500, Waters; column: Prep PAK-500/SILICA, 5 × 30 cm; mobile phase: isocratic elution with *n*-hexane - 2-propanol, 8:2) and then by repeated

Fig. 1. Structures of VB's A (**1**), B (**2**), C (**3**), D (**4**) and E (**5**).



reverse phase HPLC (column: Cosmosil $5C_{18}$, 10 × 250 mm, Nakarai Chemical; mobile phase: isocratic elution with MeOH - H_2O, 50 : 50 for the first step, 40 : 60 for second step, 40 : 60 for third step, 35 : 65 for fourth step). Thus, from this alumina eluate, the previously isolated VB's A, B and C (**1**, **2** and **3**) and a new active fraction, named VB D (**4**) (1.34 mg), were obtained (Fig. 2). On reverse phase HPLC, the new active fraction VB D showed the longest retention time. The active EtOAc - MeOH (1 : 1) fraction from alumina column (25.4 g) was applied to reverse phase preparative HPLC (System 500, Waters; column: Prep PAK-500/C_{18}, 5 × 30 cm; mobile phase: isocratic elution with MeOH - H_2O, 7 : 3) and then further purified by the charcoal column (Norit A, Nakarai Chemical, 55 g; stepwise elution with 30% MeOH, MeOH and 80% pyridine) to remove virginiamycin. The active MeOH fractions (150 mg) were finally purified by reverse phase HPLC (column: Cosmosil $5C_{18}$, 10 × 250 mm for first and second steps, 4.6 × 100 mm for fourth step, Nakarai Chemical; mobile phase: gradient elution starting with CH_3CN - 0.1% TFA, 20 : 80, and ending with 80 : 20 for first step, and starting with CH_3CN - 0.1% TFA, 19 : 81, and ending with 31 : 69 for second step, and isocratic elution with CH_3CN - 0.1% TFA, 20 : 80 for fourth step) and normal phase HPLC (column: Unisil Q100-10, 4.6 × 250 mm, Gasukuro Kogyo; mobile phase: isocratic elution with *n*-hexane - 2-propanol, 9 : 1 for third step). From the final HPLC fractions, a new VB which designated VB E (80 μg) was obtained as the most polar VB. The retention time of VB E on reverse phase HPLC was less than that of VB C which was also detected in the EtOAc - MeOH (1 : 1) fraction from alumina column as the main component.

The Structures of VB's D and E

Fast atom bombardment (FAB)-MS of VB D showed the $(M+H)^+$ ion at *m/z* 231 and its electron impact (EI)-MS gave the fragment ions at *m/z* 212 $(M-H_2O)^+$, 181, 145, 116 and 85. The fragmentation pattern of VB D is similar to that of already known VB's and the structures of the characteristic fragments could be assigned as shown in Fig. 3. Thus, these data indicated that VB D has the same skeleton of 2-(1′-hydroxyalkyl)-3-hydroxymethylbutanolide as other VB's. The presence of the skeleton in VB D was confirmed by its ^1H NMR spectrum

Fig. 2. Purification procedure of VB's A, B, C, D and E.

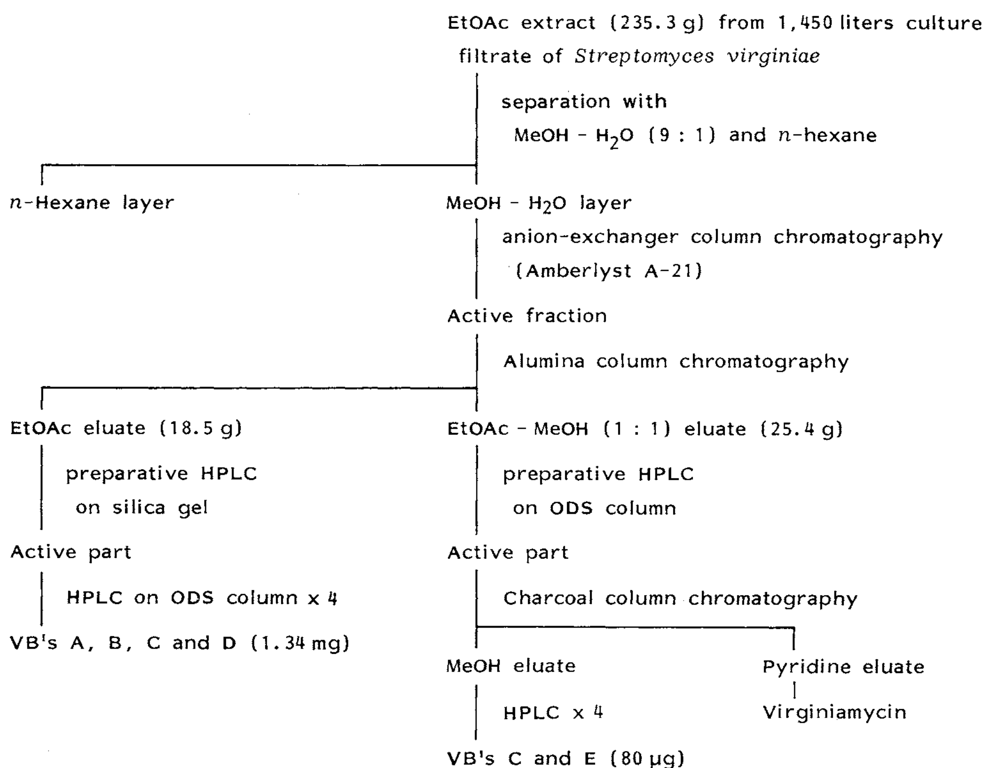
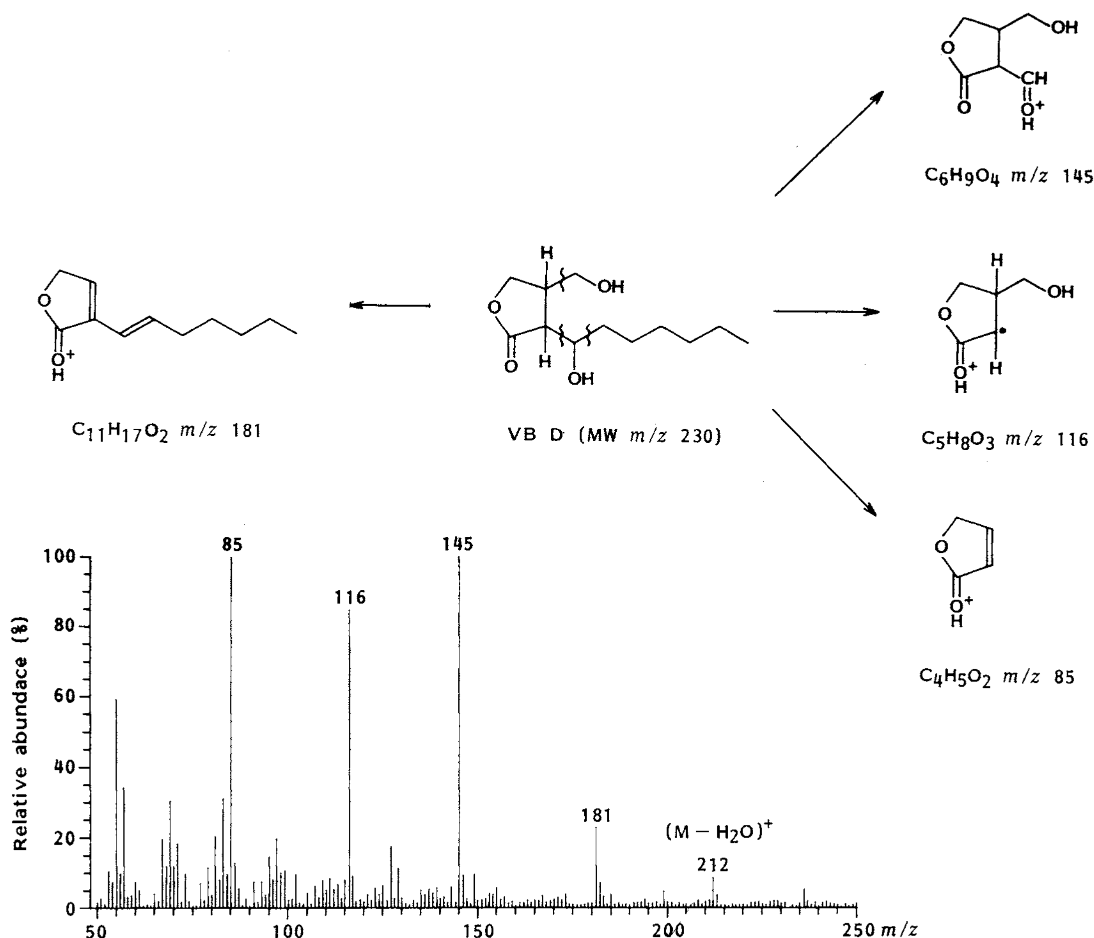


Fig. 3. EI-MS analysis of VB D.



($CDCl_3$, 600 MHz, Brucker AM600 belonging to Faculty of Engineering, Osaka University), which showed signals at 4.41 (1H, dd, $J_{4a,4b}=9.0$ Hz, $J_{4a,3}=8.7$ Hz, 4-H_a), 4.12 (1H, m, 6-H), 4.09 (1H, dd, $J_{4b,3}=6.9$ Hz, 4-H_b), 3.75 (1H, dd, $J_{5a,5b}=10.5$ Hz, $J_{5a,3}=5.5$ Hz, 5-H_a), 3.70 (1H, dd, $J_{5b,3}=6.2$ Hz, 5-H_b), 2.85 (1H, m, 3-H), 2.56 (1H, dd, $J_{2,3}=7.3$ Hz, $J_{2,6}=3.7$ Hz, 2-H), 1.65~1.25 (10H, m, 7~11-H_2) and 0.88 (3H, t, $J_{12,11}=6.9$ Hz, 12-H_3). Since the spectrum was almost the same as VB C but the molecular weight of VB D was increased by 14 mass units, we determined the substituent of C-2 as 1′-hydroxy-*n*-heptyl group. The coupling constant between 2-H and 3-H was 7.3 Hz and this value suggested that the configuration of two substituents on butanolide ring is *cis*[4]. Thus the structure of VB D was assigned as *cis*-2-(1′-hydroxyheptyl)-3-hydroxymethylbutanolide (**4**).

The mass and ^{1}H NMR spectrum data of VB E: FAB-MS *m/z* 217 $(M+H)^+$; EI-MS *m/z* 198 $(M-H_2O)^+$, 167, 145, 116, 85; ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.40 (1H, dd, $J_{4a,4b}=9.0$ Hz, $J_{4a,3}=8.7$ Hz, 4-H_a), 4.08 (1H, dd, $J_{4b,3}=6.9$ Hz, 4-H_b), 4.08 (1H, m, 6-H), 3.74 (1H, dd, $J_{5a,5b}=10.5$ Hz, $J_{5a,3}=5.4$ Hz, 5-H_a), 3.70 (1H, dd, $J_{5b,3}=6.3$ Hz, 5-H_b), 2.85 (1H, m, 3-H), 2.55 (1H, dd, $J_{2,3}=7.5$ Hz, $J_{2,6}=3.6$ Hz, 2-H), 1.6~1.2 (5H, m, 7, 8-H_2 and 9-H), 0.89 (3H, d, $J_{9,10}=6.6$ Hz, 10-H_3), 0.89 (3H, d, $J_{9,11}=6.6$ Hz, 11-H_3), showed that VB E also has the same skeleton as other VB's. The ^{1}H NMR spectrum of VB E was very similar to that of VB A, whose molecular weight is 14 mass units larger than VB E. Thus we determined the substituent at C-2 of VB E as 1′-hydroxy-4′-methylpentyl group. Since the value of $J_{2,3}$ (7.5 Hz) suggested the *cis* configuration between 2-H and 3-H, the structure of VB E was assigned as *cis*-2-(1′-hydroxy-5′-methylpentyl)-3-hydroxymethylbutanolide (**5**).

The virginiamycin inducing activity of VB D is a little weaker than VB A, which has the highest

activity among the known natural VB's[4,5]; values of minimum effective concentration were 1.0 and 0.6 ng/ml, respectively. The activity of VB E is about only 10% of that of VB A, 25% of that of VB C and about the same as that of VB B.

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