CHEMICAL MODIFICATION OF EVERNINOMICINS

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Novel antibiotic everninomicin D is chemically transformed into new biologically active derivatives. Reactions of a nitro group attached to a tertiary carbon center have been investigated. Synthesis and reactions of hydroxylaminoeverninomicin D, aminoeverninomicin D and their derivatives have been discussed.

Everninomicins[1], a novel class of oligosaccharide antibiotics are produced by *Micromonospora carbonaceae*. They are highly active against Gram-positive bacteria and *Neisseria*, including strains resistant to β-lactams, macrolides, lincomycin, tetracycline and rifampicin. The antibiotic complex produced in the above fermentation consists of everninomicin B(1)[2], C(2)[3] and D(3)[4]. Everninomicin D is the major component of the above complex. In earlier publications[2~4] we have reported structures and absolute stereochemistry of the above three antibiotics. Everninomicins possess many unusual features in their structures such as the ortho ester functionalities, an aliphatic methylene dioxy group, a nitro sugar and a fully substituted phenolic ester residue.

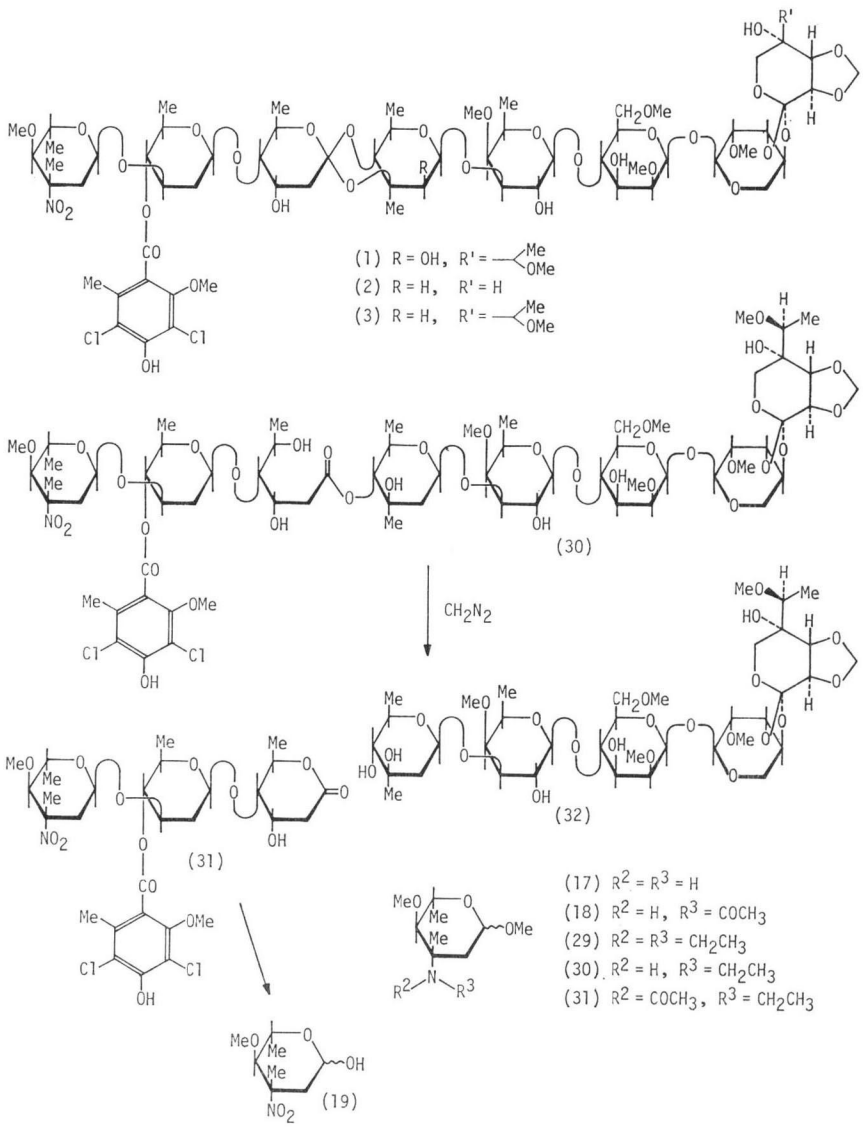
The objective of chemical modification of everninomicin D is to obtain compounds which will maintain the high *in vitro* activity of the parent antibiotic and yet possess improved pharmacokinetic properties.

It is known that everninomicin D, when injected into animals subcutaneously, gives poor blood level (*i.e.* the actual amount of antibiotic found in the blood) which in turn results in poor *in vivo* efficacy. The modified everninomicins are hoped to give good *in vivo* protection by giving high blood level when injected into animals. Improved pharmacokinetic properties in this paper refer to better absorption of the compound into blood from the site of the injection and its final elimination after distribution to the various tissues. The desired compound in this series will be one which when administered to animals subcutaneously at 30 mg/kg dose will produce peak blood level of approximately 150 μg/ml of the antibiotic. During the present work many new reactions have been discovered and in this paper a summary of our results is presented. In a separate communication the details of the microbiological activities will be published. We believe some of these reactions described in this paper would find great use in other natural products of similar complexities.

As pointed out above, everninomicin D possesses a nitro sugar moiety evernitrose (19)[5,6]. We, therefore, investigated whether the nitro group could be modified to yield useful intermediates for further manipulations. It should be emphasized that chemical modification of everninomicin D is complicated due to the complexity of its structure, particularly the presence of ortho ester carbons excludes reactions which could only be carried out at acidic pH. The structures of the modified everninomicins reported in this publication have been established by chemical degradations and extensive use of spectroscopic methods. A summary of the most convenient method of degradation of this class of antibiotics, first disclosed by us[3,8], is shown in Chart 1.

Thus, a methylene chloride solution of everninomicin D when treated with aqueous acid yields everninomicin D_1 (30) in which one of the ortho ester linkages is opened to an ester functionality. Evernino-

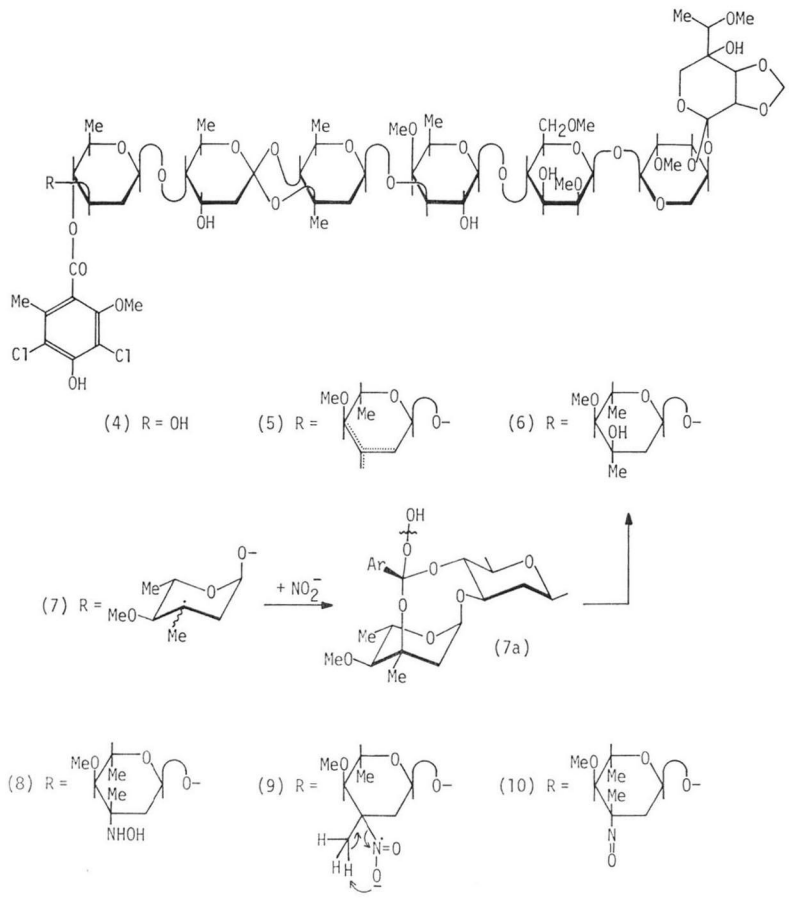
Chart 1.



micin D_1 (30) on treatment with diazomethane undergoes a smooth cleavage to yield the lactone (31) and olgose (32). We have speculated[8] on the possible mechanism of this reaction in an earlier paper. In the present work chemical modification of everninomicin D relates to the changes in the evernitrose portion of the molecule, therefore, when one repeats the above degradation procedure one obtains a modified lactone and olgose (32). The structure of the modified lactone reveals the changes brought about in the chemical modifications.

Recently, we have reported[7] that everninomicin D (3) on electrochemical reduction yields everninomicins 2(4), -3(5), -7(6) and hydroxylaminoeverninomicin D (8) (see Chart 2). Compounds (4), (5) and (6) are produced in non-aqueous media and compound (8) is produced when the electrochemical reduction is carried out in aqueous media. We have proposed[7] that everninomicin D when electrolyzed is

Chart 2.

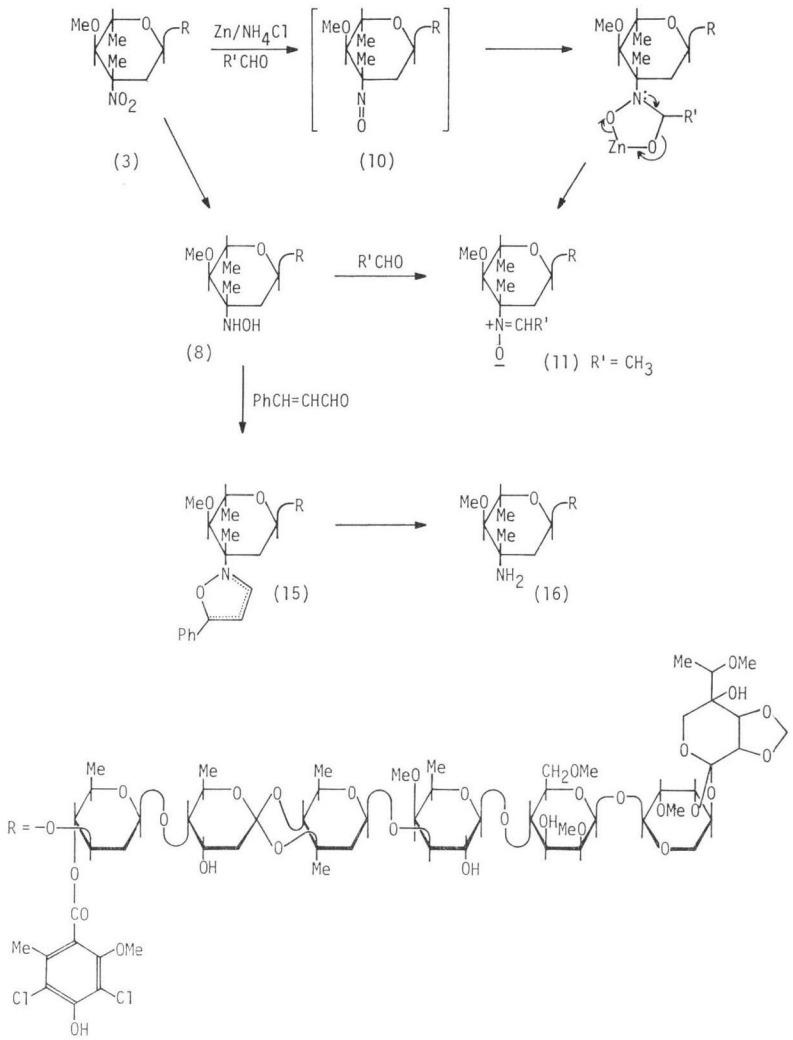


converted to an anion radical (9) which undergoes rearrangement (see arrows) to yield (5) and $HNO_2^{\cdot -}$ (the latter could dissociate to $NO^{\cdot}$ and OH^-). Alternatively the anion radical (9) could dissociate to form the carbon radical (7) and NO_2^-. Owing to the proximity of the aromatic ester carbonyl (as deduced by ^{13}C-analysis of everninomicin D and will be reported in detail elsewhere), the above carbon radical (7) may rearrange to the more stable benzyl carbon radical which in turn could capture oxygen resulting in a structure as (7a). It is expected that (7a) would cleave at the peroxide bond yielding (6).

Hydroxylaminoeverninomicin D (8) is also produced by the reduction of everninomicin D with aluminum amalgam or with zinc in the presence of either ammonium chloride or tetraalkyl ammonium hydroxide in aqueous tetrahydrofuran solution. Compound (8) is highly active against Gram-positive bacteria (mean MIC$\sim$0.05 μg/ml) and when administered to rats subcutaneously (30 mg/kg) it produces higher blood level (235 μg/ml) than the parent antibiotics.* It is also excreted completely within four hours. However, hydroxylaminoeverninomicin D (8) is unsuitable for further use because it undergoes aerial oxidation very easily to the nitroso compound (10). For preparative purposes compound (10) is obtained by sodium hypobromite oxidation of the hydroxylamino derivative (8). Although compound (10) possesses high *in vitro* activity, when compared to (8) its pharmacokinetic behavior is very

* Detailed biology of the modified everninomicins will be published elsewhere.

Chart 3.



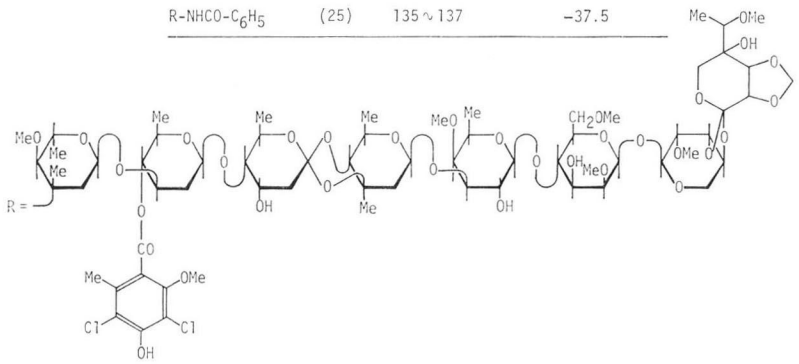
similar to everninomicin D (3).

To improve the chemical stability of (8) while retaining its desirable biological profile, we have modified its structure further. Hydroxylaminoeverninomicin D (8) readily reacts with aliphatic, aromatic and heterocyclic aldehydes to yield nitrones. More conveniently, the nitrones could also be produced in a single step by the reduction of everninomicin D with zinc in the presence of an appropriate aldehyde. It is presumed (see Chart 3) that the above reaction involves reduction of the nitro group into the nitroso equivalent which undergoes further reduction with zinc in the presence of acetaldehyde to form a "zinc complex", which dissociates to yield the nitrone. As expected, the above nitrones are more stable than the parent hydroxylaminoeverninomicin D and possess high activity against Gram-positive bacteria [mean MIC 0.05 μg/ml]. Acetaldehyde nitrone (11), for example, is stable, gives excellent blood level and its pharmacokinetic property is comparable to that of the hydroxylaminoeverninomicin D (8).

Controlled reduction of the above nitrones yield *N*-substituted hydroxylamine derivatives. However, cinnamaldehyde nitrone which exists as the isoxazoline (15) on reduction with zinc yields amino-

Table 1.

Compound		M.p. (°C)	Specific rotation in chloroform $[\alpha]_D^{26}$
R-$\overset{+}{N}$(-O)=CHCH$_3$	(11)	182~184	-72.4
R-$\overset{+}{N}$(-O)=CHC$_6$H$_5$	(12)	168~170	-67
R-$\overset{+}{N}$(-O)=CH-(furyl)	(13)	173~175	-63.2
R-$\overset{+}{N}$(-O)=CH(CH$_2$)$_5$CH$_3$	(14)	159~160	-46
R-NHCOCH$_3$	(20)	189~191	-34.7
R-NHCOCH$_2$CH$_3$	(21)	188~190	-30.6
R-NHCOCH(CH$_3$)$_2$	(22)	190~192	-34.6
R-NHCOCH$_2$C$_6$H$_5$	(23)	120~124	-34
R-NH-CO-(cyclohexyl)	(24)	184~186	-35.9
R-NHCO-C$_6$H$_5$	(25)	135~137	-37.5



everninomicin D (16). This novel method of formation of amines from hydroxylamino derivatives could be of importance where one is restricted in the use of conventional methods of reduction of nitro groups, *e.g.* hydrogenation.

Everninomicin D upon catalytic hydrogenation yields the above aminoeverninomicin D (16). Aminoeverninomicin D could also be prepared by the chemical reduction of everninomicin D with either [$Cr(OAc)_2$ in CH_3OH] or aluminum amalgam in protic solvents. In this connection, it is also of interest to investigate whether the nitroso group in compound (10) would behave like a ketonic function and therefore yield aminoeverninomicin D (16) when treated with hydrazine. In fact, treatment of (10) (see Chart 4) with hydrazine yields (16) in 40% yield. On the other hand, when treated with *t*-butyl hydroperoxide compound (10), not surprisingly, is converted into the corresponding nitro derivative (3) instead of undergoing a BAYER-VILLAGER type of rearrangement.

The structure of aminoeverninomicin D (16) is proven by degradation to the monosaccharide (17) which in turn is converted into the *N*-acetylated derivative (18) which is identical with an authentic sample obtained from evernitrose (19). Because of the presence of the phenolic hydroxyl group in compound (16), the basicity and nucleophilicity of the amine function is considerably weakened. However, the zwitterionic aminoeverninomicin D (16) reacts with acid anhydrides to yield diacyl derivatives. The phenolic acyl group could then be easily hydrolyzed selectively. Following the above procedure, several *N*-acyl derivatives have been prepared (see Table 1). These amides are highly potent

Chart 4.

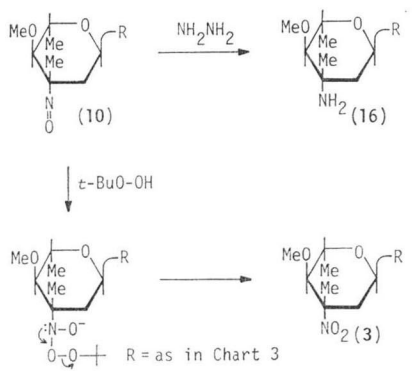


Chart 5.

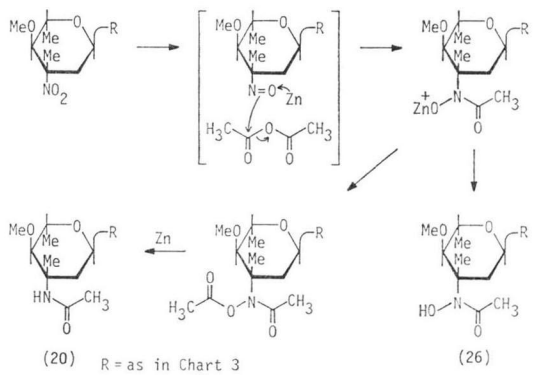
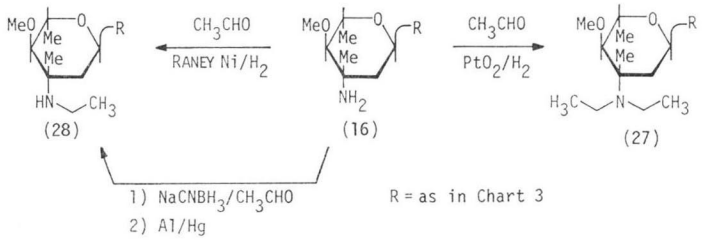


Chart 6.



antibacterials (mean MIC is approximately 0.125~0.5 μg/ml) and depending upon the nature of the *N*-acyl function, the pharmacokinetics of the derivatives could be altered.* Amongst the compounds prepared, the *N*-acetyl derivative (20) possesses high antibacterial activity (mean MIC ~0.25 μg/ml) and when administered to rats (30 mg/kg) it shows peak blood level (151 μg/ml) within half an hour and after four hours only 14 μg/ml of the antibiotic could be detected in the blood. Thus, compound (20) met the objectives of the chemical modification of everninomicin D. Compound (20) could also be prepared in a single step by the reduction of everninomicin D with zinc and acetic anhydride. The by-product (see Chart 5) in the above reaction is *N*-acetyl-*N*-hydroxylamino derivative** (26) which possesses high antibacterial activity.

Aminoeverninomicin D (16) on catalytic hydrogenation (PtO_2 as catalyst) in the presence of acetaldehyde yields the *N*,*N*-diethyl derivative (27) (see Chart 6). However, when the above reaction is carried out in the presence of RANEY nickel as a catalyst, the *N*-ethyl derivative (28) is obtained. Compound (28) may also be prepared by sodium cyanoborohydride reduction of the amine in presence of an aldehyde which gives a very stable boron complex. The free amine is obtained by aluminum amalgam reduction of the boron complex. Both of these classes of compounds possess high antibacterial activity.

When diazotized under buffered condition, aminoeverninomicin D (16) is converted into everninomicin-3(5) and -7(6).

* Detailed biology of the modified everninomicins will be published elsewhere.

** This method appears to be a convenient way of making hydroxamic acids directly from nitro compounds.

Experimental

General

Melting points were determined on a Fisher Johns hot stage melting point apparatus. All melting points are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on XL-100 or T-60 spectrometer. Chemical shifts are reported in parts per million δ-relative to tetramethylsilane as internal standard. Infrared spectra were recorded on Perkin-Elmer 727B spectrometer.

Tetrahydrofuran was distilled from lithium aluminum hydride. Activated zinc dust was made by the procedure given in "Reagents for Organic Synthesis", p. 1276, Vol. 1, by FIESER and FIESER.

Hydroxylaminoeverninomicin D (8)

To a solution of 150 mg of everninomicin D (3) in 8 ml of peroxide free tetrahydrofuran (THF) was added 500 mg of activated zinc dust, and 2 ml of 2% aqueous solution of tetramethylammonium hydroxide*. The suspension was stirred at room temperature under argon for 15 minutes. The solution was adjusted to pH 6 using 1 M pH 4.5 aqueous phosphate buffer. The product was extracted with 2×25 ml ethyl acetate, and the organic layer was washed twice with brine solution, and once with distilled water. The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. Crystallization under argon from methylene chloride - carbon tetrachloride, 1: 1 afforded hydroxylaminoeverninomicin D (135 mg), as white crystals, mp 185~186°C, $[\alpha]_D^{26}-39.3°$.

Acetaldehyde Nitrone of Hydroxylaminoeverninomicin D (11)

To a solution of 500 mg of everninomicin D in 5 ml dry THF was added 4 ml freshly distilled acetaldehyde and 600 mg of activated zinc dust. The mixture was vigorously stirred under argon. The progress of the reaction was monitored by TLC using 30% (v/v) acetone in toluene as eluent. When the reaction was complete, it was filtered through a sintered funnel, and the solvent was removed under reduced pressure. The product was purified by filtration through silica gel column using acetone - benzene (3: 7). Crystallization from ethanol gave the nitrone** as white crystals, mp 186~188°C, $[\alpha]_D^{26}-72.4°$ (c 0.985, $CHCl_3$).

Benzaldehyde Nitrone of Hydroxylaminoeverninomicin D (12)

To 400 mg of hydroxylaminoeverninomicin D in 15 ml ethanol was added 0.2 ml of freshly distilled benzaldehyde. The solution was kept under nitrogen for 72 hours. Volatile materials were removed under high vacuum at 50°C. The residue was triturated with hexane, and the resultant powder was collected. Product was further purified by preparative thin-layer chromatography: mp 168~170°C; $[\alpha]_D^{26}-76°$ ($CHCl_3$); Bioassay 1309 ν/ml.

Cinnamaldehyde Adduct of Hydroxylaminoeverninomicin D (15)

A solution of 1 g of hydroxylaminoeverninomicin D and 1 ml of freshly distilled cinnamaldehyde in 10 ml dry THF was warmed and kept at 55°C for $\frac{1}{2}$ hour. Solvent was removed under reduced pressure, and the residue was triturated with ether - hexane (1: 1). The precipitate was collected and washed with ether - hexane (1: 1) to get the isoxazoline (15); mp 182~184°C; $[\alpha]_D^{26}-72.4°$ (c 0.764, $CHCl_3$).

Reduction of the Isoxazoline (15), Aminoeverninomicin D (16)

To a solution of 500 mg of the isoxazoline (15) in 25 ml THF was added 600 mg of activated zinc dust and 1.5 g of ammonium chloride in 8 ml water. The mixture was vigorously stirred for 5 minutes. Another 600 mg of zinc dust was added and continued stirring for 1 hour. The product was extracted with 100 ml ethyl acetate. The organic layer was washed twice with water. The extract was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure to get a gelatinous residue. The residue was triturated with ether, and the ether insoluble portion was collected. The relative ratio of the amino function with respect to the phenolic group in the above product was determined by acid-base titration to be 85: 100. The amine was further purified by chromatography through silica gel using acetone - toluene (2: 3). The amine was obtained as a powdered gel, 290 mg; mp 184~186°C; $[\alpha]_D^{26}-21.2°$ (c 0.91, MeOH).

* Instead of tetramethylammonium hydroxide, ammonium chloride (1 g in 5 ml water) may be used.

** The same product was obtained by reacting hydroxylaminoeverninomicin D with acetaldehyde.

Structure Determination of Aminoeverninomicin D

A solution of 700 mg of aminoeverninomicin D in 10 ml dry methanol was stirred in presence of 600 mg of *p*-toluene sulfonic acid for 16 hours. Then it was stirred at 50°C for 1 hour. Methanol was removed under vacuum, and the residue was chromatographed through silica gel using chloroform - ethyl acetate (7: 3) to remove less polar products. The amino component of the mixture was obtained by eluting with 1% ammonia - methanol system. Solvent was removed and the product was dissolved in 0.5 ml acetic anhydride. It was warmed to 60°C for 10 minutes. Excess anhydride was removed under high vacuum. The amide (18) was purified by vacuum sublimation, IR 1690 cm^{-1} ($NHCOCH_3$), M^+ 231.1470 (calcd. 231.1462), M−31 200.1287 (calcd. 200.1283). The product was identical with an authentic sample prepared from reduction of evernitrose and acetylation of the amine.

Reaction of Hydrazine with Nitrosoeverninomicin D

To a solution of 300 mg of the blue nitrosoeverninomicin D in 20 ml ethanol at room temperature was added 0.1 ml (90%) hydrazine hydrate. There was immediate de-colorization accompanied by evolution of N_2 gas. The solution was adjusted to pH 6.8 using 1 M pH 4.5 phosphate buffer, and the product was extracted with 75 ml ethyl acetate. The solution was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to get a gelatinous solid. It was chromatographed through a silica gel column using acetone - toluene (2: 3) as eluent to get the amine (120 mg).

Aminoeverninomicin D by Chromous Acetate Reduction

One g of everninomicin D (3) in 25 ml ethanol was stirred in presence of 1.2 g of chromous acetate and 3 ml of mercaptoethanol under N_2 gas for 20 hours. All the volatile materials were then removed under high vacuum at 55°C to get a solid mass. It was vigorously stirred with 2 g *N*-methylglucamine in 20 ml water for a half hour. The solution was adjusted to pH 6.5 using 1 M pH 4.2 phosphate buffer, and the product was extracted with 2×100 ml ethyl acetate. Solution was dried over anhydrous sodium sulfate, and solvent was removed. Filtration through silica gel column using acetone - toluene, 1: 1 gave the amine (700 mg).

Aluminum Amalgam Reduction of Everninomicin D

To a solution of 500 mg of everninomicin D (3) in 25 ml ethanol and 25 ml THF was added a suspension of 2.5 g of Al/Hg. The mixture was stirred at 30~35°C for 2 hours. A further 1 g of Al/Hg was added to complete the reaction. After a total of 3 hours, 1 M pH 7 phosphate buffer (3 ml) was added to the stirred reaction mixture. It was filtered, and the solid mass was washed down with 20 ml THF. The clear filtrate was then concentrated under reduced pressure. The solid so obtained was taken up in a mixture of 100 ml ethyl acetate and 20 ml THF. The solution was washed with brine solution, and finally with water. Solvent was removed, and the product was filtered through a silica gel column using acetone - toluene (1: 1) to get aminoeverninomicin D (330 mg).

Hydrogenation of Everninomicin D

One g of everninomicin D and 200 mg of *N*-methylglucamine in 60 ml methoxyethanol was hydrogenated in presence of 1.5 ml of active RANEY nickel at 2.1 Kg/cm^2 for 16 hours. It was filtered under nitrogen to get a clear solution. Solvent was removed under vacuum to get the *N*-methylglucamine salt of aminoeverninomicin D (1.1 g).

Amides of Aminoeverninomicin D

General Method: Method (A). 500 mg of aminoeverninomicin D was dissolved in acid anhydride (20~50 equivalents). It was stirred in presence of calcium carbonate (600 mg~1 g). The progress of acylation was monitored by TLC using acetone - toluene, 3: 7, as eluent. Excess anhydride was removed (if volatile) by high vacuum. Alternatively, excess anhydride was removed by stirring with aqueous sodium bicarbonate solution. The product was then extracted with ethyl acetate. The solution was dried over anhydrous sodium sulfate, and the solvent was removed. The residue was dissolved in 10 ml of methanol and stirred in presence of 10 ml, 20% tetramethylammonium hydroxide solution. Progress of hydrolysis of phenolic ester was monitored by TLC using 30% acetone in toluene. The solution was adjusted to pH 6.5 using pH 4.2 phosphate buffer. Product was extracted with ethyl acetate, and the solution was dried over anhydrous sodium sulfate. Solvent was removed to get a colorless foam. This

method was used for the preparation of (20), (21) and (22).

Amides of Everninomicin D

Method (B): A solution of 1.3 g of cyclohexane carboxylic acid and 1.05 g of triethylamine in 20 ml dry THF was cooled to 0°C. 1.1 g of ethylchloroformate in 5 ml of THF was added and stirred for 10 minutes. The resulting slurry was quickly filtered through a bed of celite under N_2 gas, and the solution was directly used as acylating agent by adding 600 mg of aminoeverninomicin D in 10 ml THF. After 2 hours, it was basified with aqueous ammonium hydroxide and stirred for 1 hour, maintaining the pH at 9~10. The solution (two layers) was adjusted to pH 6.5 using 1 M pH 4.2 phosphate buffer. Product was extracted with 100 ml ethyl acetate. The solution was dried over anhydrous sodium sulfate. Removal of the solvent afforded a white foam. The amide (24) was crystallized from ethanol solution; mp 184~186°C; $[\alpha]_D^{26}$ −35.9° (*c* 0.985, $CHCl_3$); IR 1660, 1725 cm^{-1}.

This method was employed for the preparation of (23) and (25).

Zinc Reduction of Everninomicin D in Presence of Acid Anhydride

Preparation of *N*-Hydroxy-*N*-acetylaminoeverninomicin D (26): A suspension of 1.6 g activated zinc dust, 1 g calcium carbonate and 1 g of everninomicin D in 5 ml acetic anhydride was stirred at 0°C for 1 hour. Progress of reaction was followed by monitoring the disappearance of everninomicin D. Excess anhydride was removed under high vacuum at 30°C. The residue was stirred with 20 ml 1% sodium bicarbonate solution and 75 ml ethyl acetate. Organic layer was separated and washed once with 50 ml water. Ethyl acetate solution was dried over sodium sulfate. Solvent was removed and the residue was dissolved in 25 ml THF. Ten ml of tetraethylammonium hydroxide (10% solution) was added and stirred for 3 hours. The solution was adjusted to pH 6.5 using pH 4.2 phosphate buffer. Product was extracted with 100 ml ethyl acetate, and washed once with 50 ml water. Solvent was removed, and the residue was chromatographed through silica gel using acetone - toluene, 3: 7. Two components were separated: 1) *N*-acetyl-*N*-hydroxyaminoeverninomicin D (26), 690 mg, mp 176~178°C, $[\alpha]_D^{26}$ −34° (*c* 0.735, $CHCl_3$); IR 1690, 1725 cm^{-1}. 2) *N*-acetylaminoeverninomicin D, 280 mg.

N-Ethylaminoeverninomicin D (28)

Method (A): To a solution of 200 mg of aminoeverninomicin D in 10 ml methanol was added 0.5ml acetaldehyde and 70 mg of sodium cyanoborohydride, at 10~15°C. After 0.5 hour the solution was diluted with 75 ml ethyl acetate, and washed twice with water. Solution was dried over anhydrous sodium sulfate. Solvent was removed under reduced pressure to get a white foamy residue. Boron 0.68% NMR in $CDCl_3$/DMSO, δ 4.6, 2H, q; 1.45 3H, t. (O–B–O–CH_2CH_3).

—NH

The above boron complex was dissolved in 20 ml methanol and 5 ml water. Aluminum amalgam (800 mg) was added and stirred for 6 hours.

The solution was filtered through celite bed, and celite was washed with THF and the solution was collected. Removal of the solvent gave *N*-ethylaminoeverninomicin D 180 mg*. It was crystallized from acetone. mp 184~186°C; $[\alpha]_D^{26}$ −18° (*c* 1.05, MeOH).

N-Ethylaminoeverninomicin D (28)

Method (B): A solution of 1 g of aminoeverninomicin D in 75 ml methoxyethanol and 10 ml freshly distilled acetaldehyde was stirred under hydrogen in presence of active RANEY nickel (3.5 ml) at 2.1 Kg/cm^2 for 30 hours. Catalyst was filtered off and the solvent was removed under reduced pressure to get *N*-ethylaminoeverninomicin D, 990 mg.

N,N-Diethylaminoeverninomicin D (27)

To a solution of 500 mg of aminoeverninomicin D in 15 ml ethyl alcohol was added 3 ml acetaldehyde and 200 mg PtO_2. The mixture was stirred under hydrogen at atmospheric pressure for 30 hours. Catalyst was filtered off, and the solution was concentrated under reduced pressure. The residue was crystallized from ethanol solution to get the amine**; mp 173~175°C; $[\alpha]_D^{26}$ −21.6° (*c* 0.233, MeOH).

* Compound (30) M^+=217.1651 (calcd. 217.1678); M−31=186. Compound (31) IR 1690 cm^{-1}; NMR 1.35, 3H (t); 2.1, 3H ($COCH_3$); 3.4, 2H (q); 3.35, 3H; 3.2 and 3.5, 3H (two peaks - anomeric -OCH_3).

** Compound (29) M^+=245; M−31=214; NMR 1.4, 6H (t); 2.75, 4H (m).

Hydroxyeverninomicin D (6) (Everninomicin-7)

A two phase solution of 150 mg of aminoeverninomicin D in 20 ml chloroform and 50 mg sodium nitrite in pH 4.5 sodium dihydrogen phosphate buffer solution (5 ml) was stirred at 0°C for 3 hours. The chloroform layer was separated and washed with cold water. Solution was dried over anhydrous sodium sulfate. Solvent was removed and the residue was chromatographed through silica gel column using acetone - toluene, 1 : 4. Hydroxyeverninomicin (6)[7] was obtained in 24% yield. The other product was everninomicin-3 (5)[7], 76%.

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