Tetrahedron Letters No.6, pp. 663-668, 1968. Pergamon Press. Printed in Great Britain.

ISOLATION, CHARACTERIZATION AND DEGRADATION OF NOGALAMYCIN

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(Received in USA 9 October 1967)

Nogalamycin, an antibiotic highly active against gram-positive bacteria and KB cells in vitro (1), was isolated in a crude state by extraction of a filtered fermentation broth with ethyl acetate. Further purification was achieved by repeated extraction from ethyl acetate with dilute acid followed by neutralization and reextraction with ethyl acetate. The final steps in purification were chromatography on silica gel in chloroform-methanol (9:1) and recrystallization from methanol. Pure nogalamycin (I) is an orange-red solid, m.p. 195-196° dec.; $[\alpha]_D^{25°}$ + 479° ($CHCl_3$); pKa 7.45 (60% EtOH); λ_{max}^{EtOH} 236 (ε 52,360), 258 (ε 24,755), 292 (ε 9,890), 390 (ε 4,090), 480 (ε 15,590); $\lambda_{max}^{Alk.EtOH}$ 240 (ε 47,300), 259 (ε 25,150), 290 (ε 9,590), 553 (ε 14,500); ν_{max}^{Nujol} 3390, 3270, 1740, 1670, 1620, 1565, 1325, 1305, 1285, 1255, 1225, 1185, 1150, 1110, 1060, 1015, 1005, 930, 915, 890, 835 and 775 cm^{-1}. Analytical data and degradative studies indicate a molecular formula of $C_{39}H_{49}NO_{17}$ although slightly different formulas are not excluded. The NMR spectrum of nogalamycin in $CDCl_3$ at 60 Mc shows the presence of four CH_3C groups (three singlets at δ 1.38, δ 1.6 and δ 1.67 and a doublet centered at δ 1.39). A signal at δ 2.63 was assigned to a dimethylamino group which was confirmed by isolation of dimethylamine, identified as its p-hydroxyazobenzene-p'-sulfonic acid salt, from base treatment of nogalamycin. Four methoxyl groups were indicated by singlets at δ 3.30, δ 3.57, δ 3.66 and δ 3.75. Two signals appearing as singlets at δ 7.0 and δ 7.1 were assigned to two aromatic protons which must be either isolated or para to each other.

Mild acid hydrolysis of nogalamycin (I) (0.4 N HCl under reflux for one-half hour) formed a mixture of products from which three pure compounds were isolated. Nogalose was removed from the reaction mixture by extraction with chloroform and was purified by sublimation and recrystallization from ethyl acetate. Neutralization of the extracted

aqueous residue gave a red precipitate which was a mixture of several components, the two principal ones being nogalarol (II) and nogalarene. The mixture was separated by countercurrent distribution for 500 transfers using benzene-chloroform-methanol-water (5:5:6:4) as the solvent system. The contents of tubes 65-120 were combined and evaporated under reduced pressure to give nogalarene. Nogalarene was purified for analysis by recrystallization from benzene. Nogalarol was obtained in the same way from tubes 121-233 and was purified further by recrystallization from methanol.

Nogalarol (II) has the molecular formula $C_{29}H_{31}NO_{13}$; melting with dec. about 220°; pK'a 7.15; λ_{max}^{MeOH} 234 (ε 53,250), 258 (ε 24,600), 288 (ε 9,850), 475 (ε 15,400); ν_{max}^{Nujol} 3440, 1725, 1655, 1615, 1565, 1285, 1250, 1215, 1100, 1050 and 1000 cm^{-1}; NMR (100 Mc, CD_3COCD_3) δ 1.52 (3H, s), δ 1.72 (3H, s), δ 1.93 (1H, m), δ 2.49 (1H, m), δ 2.56 (6H, s), δ 2.86 (1H, m), δ 3.66 (1H, d), δ 3.69 (3H, s), δ 3.76 (1H, s), δ 4.16 (1H, m), δ 5.17 (1H, m), δ 5.87 (1H, d), δ 6.60 (1H, s) and δ 7.19 (1H, s).

The molecular formula of nogalarene (III), $C_{29}H_{27}NO_{11}$, is suggestive of a dehydration product of nogalarol. Nogalarene has the following physical properties: decomposes at about 230°; $[\alpha]_D^{25}$ + 843 ($CHCl_3$); pKa' 7.01; λ_{max}^{MeOH} 248 (ε 49,800), 270 (ε 28,880), 290_{sh} (ε 16,300), 303 (ε 14,900), 473 (ε 19,450), 498 (ε 18,900); ν_{max}^{Nujol} 3430, 1725, 1660, 1615, 1570, 1490, 1285, 1255, 1220, 1140, 1120, 1105, 1055 and 1005 cm^{-1}; NMR [100 Mc, $DC^{=O}N(CD_3)_2$] δ 1.70 (3H, s), δ 2.45 (9H, s), δ 2.5-2.75 (1H, m), δ 3.66 (1H, d), δ 4.00 (1H, m), δ 4.07 (3H, s) δ 5.72 (1H, d), δ 7.12 (1H, s), δ 7.12 (1H, d), δ 7.76 (1H, s), δ 8.21 (1H, d).

Nogalose has a molecular formula of $C_{10}H_{20}O_5$, m.p. 115-121°; $[\alpha]_D^{25}$ - 10.6° (CH_3OH); $[\alpha]_D^{25}$ + 15.5° (H_2O); λ_{max}^{EtOH} only end absorption; ν_{max}^{Nujol} 3400, 1195, 1175, 1155, 1110, 1085, 1060 and 1035 cm^{-1}; NMR [60 Mc, $DC^{=O}N(CD_3)_2$] δ 1.14 (3H, d), δ 1.30 (3H, s), δ 2.98 (1H, d), δ 3.20 (3H, s), δ 3.25 (1H, d), δ 3.40 (3H, s), δ 3.45 (3H, s), δ 3.70 (1H, m), δ 5.15 (1H, m) and δ 6.30 (1H, d).

Methanolysis of nogalamycin with 2N methanolic HCl gave rise to the methyl ether of nogalose and a complex mixture of colored compounds only one of which (O-methylnogalarol) was isolated in a pure state. The colored mixture was separated by a countercurrent distribution of 200 transfers using benzene-chloroform-methanol-water (2:2:3:1) as the solvent system and working up the contents of tubes 33-75 as already described. The residue obtained was purified by recrystallization from a chloroform-methanol mixture.

O-methylnogalarol (III) has a molecular formula of $C_{30}H_{33}NO_{13}$ and the following properties: m.p. 199-202° dec.; $[\alpha]_D^{25}$ + 584° ($CHCl_3$); pKa 6.8; λ_{max}^{MeOH} 236 (ε 55,100), 258 (ε 22,400), 289 (ε 9,050), 468 (ε 15,400); ν_{max}^{Nujol} 3440, 1735, 1725, 1660, 1620, 1565, 1300, 1280, 1250, 1220, 1145, 1115, 1090, 1050, 1015, 1000 and 775 cm^{-1}; NMR [100 Mc, $DC^{=O}N(CD_3)_2$] δ 1.44 (3H, s), δ 1.67 (3H, s), δ 2.0 (1H, m), δ 2.3 (1H, m), δ 2.39 (6H, s), δ 2.6 (1H, m), δ 3.48 (3H, s), δ 3.64 (3H, s), δ 3.7 (1H, m), δ 3.9 (1H, s), δ 4.05 (1H, m), δ 4.71 (1H, m), δ 5.66 (1H, d), δ 7.13 (1H, s) and δ 7.19 (1H, s).

The infrared spectra of nogalamycin, nogalarol, nogalarene and O-methylnogalarol are characteristic of 1-hydroxyanthraquinones in having a strong band at 1615-1625 cm^{-1} due to a carbonyl interacting with a peri hydroxyl group and a weaker carbonyl band at about 1660 cm^{-1} although the values are somewhat lower than those reported (2). The anthraquinone nature of nogalamycin is also indicated by ready reduction of nogalamycin by sodium hydrosulfite to a light yellow solid which was highly unstable to oxygen. The number and position of the hydroxyl groups in hydroxyanthraquinones have a pronounced effect upon their ultraviolet and visible spectra (3). The visible spectra of anthraquinones having only two α-OH groups have been reported to have a single maximum (4) or a single maximum with considerable fine structure (5,6,7). Anthraquinones having more than two α-OH groups have multiple peaks in the visible region (3). In the case of 1,4-dihydroxyanthraquinone the maximum is at 470 mμ in neutral solutions but shifting to 545 mμ in alkaline solutions (4). 1,5- and 1,8-Dihydroxyanthraquinone have maxima at lower wave lengths. Nogalamycin has a single maximum in its visible spectrum which occurs at 480 mμ in acidic or neutral solutions and at 553 mμ in base completely consistent with the reported values for 1,4-dihydroxyanthraquinone. Furthermore it has been argued (5,8,9) that the maxima at these wave lengths are affected very little by β-oxygenation although absorption below 300 mμ may be affected considerably. The NMR spectrum of nogalamycin has a broad low signal at δ 11.2-δ 13.0 representing two hydrogen atoms. Such a chemical shift has been reported for the protons of α-hydroxyl groups in anthraquinones (6). Nogalamycin, II and III have NMR spectra which indicate only two aromatic hydrogen atoms neither ortho nor meta to each other, which necessitates six substituents on the anthraquinone nucleus.

Nogalarol and O-methylnogalarol, on the basis of spectral data, must have the same anthraquinone system as does nogalamycin. However, nogalarene has quite a different

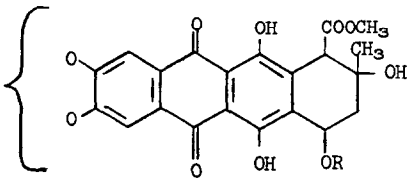
ultraviolet and visible spectrum, two more aromatic hydrogen atoms ortho to each other (NMR) and a molecular formula indicating that it is a dehydration product of nogalarol. In fact O-methylnogalarol is readily converted to nogalarene by acid treatment. These data are indicative of the presence of a fourth ring, which is hydroaromatic and readily aromatized, attached to the anthraquinone system of nogalamycin. That the fourth ring is attached linearly is indicated by the spectra of the aromatized products obtained from nogalamycin. The similarity of the ultraviolet spectrum of nogalarene to the spectra of the various bis-anhydroanthracyclinones (6,10) is quite striking. Nogalarene's visible spectrum has maxima at 473 mμ and 498 mμ which is quite similar to the maxima in the visible spectrum of 6,11-dihydroxytetracenequinone (5). Nogalamycin, upon pyrolysis with zinc dust, gave a product in very low yield, which was not obtained crystalline, but whose visible spectrum was identical with that of tetracene (3). Possessing, then, such a ring system, nogalamycin must be a member of the anthracyclinone family (6) and is identical with or very closely related to ruticulomycin A (11) on the basis of direct comparison.

The presence of two phenolic hydroxylic groups is indicated by the two protons giving NMR signals very far downfield and by the ultraviolet and infrared spectra. Furthermore, both of the benzenoid anthraquinone rings must be oxygenated since permanganate oxidation of nogalarene forms only one aromatic product, benzene-1,2,3,4-tetracarboxylic acid. The ready aromatization of the hydroaromatic ring indicates that there must be two oxygen substituents, either hydroxyl or ether, in that ring. One of these must be on a carbon atom bearing a methyl group since an unsplit aliphatic CH_3C signal in nogalarol appears as an aromatic CH_3C signal in nogalarene. There is both spectral and chemical evidence that the second oxygen is in a benzylic position and is the site of attachment of nogalose. Catalytic reduction of nogalamycin removes nogalose, and nogalarol (II) in methanolic HCl forms O-methylnogalarol (III). A doublet of doublets centered at δ 5.17 in the NMR spectrum of nogalarol is indicative of a benzylic hydrogen adjacent to a methylene group (δ 1.93 and δ 2.49) and attached to a carbon atom substituted by oxygen.

The band appearing at 1725-1740 cm^{-1} in nogalamycin and its degradation products indicates the presence of an ester or ketone. No evidence for a ketone was found and, in all the mentioned compounds, there is an NMR signal due to CH_3O indicative of a methyl ester. Furthermore, an NMR signal at δ 3.76 in nogalarol and δ 3.94 in O-methylnogalarol

suggests a carbalkoxy substituent in a benzylic position with no neighboring hydrogen. In view of these data and the results of permanganate oxidation a CH_3C group must be adjacent to the carbomethoxy group. Many of the anthracyclinone antibiotics have a benzylic carbomethoxy flanked by an alkyl substituent (6) although in only one case is the alkyl group methyl (12).

These data indicate the following partial structures for nogalamycin and some of its degradation products. In nogalarene the right hand ring is aromatized with loss of ROH and H_2O.

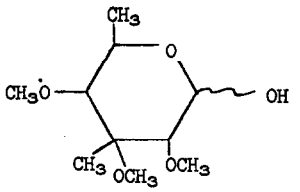
$C_8H_{15}NO_3$ 

I. Nogalamycin: R = nogalosyl

II. Nogalarol: R = H

III. O-Methylnogalarol: R = CH_3

Although nogalose is only weakly reducing, its infrared spectrum, analysis and ready formation of a methyl ether in methanolic HCl indicate that it is a sugar. This was confirmed by oxidation with Jones reagent to nogalolactone, b.p. 76°/0.1 mm; $[\alpha]_D^{25}$ + 6.7° (CH_3OH); ν_{max}^{Neat} 1765 cm^{-1}. The NMR spectrum of nogalose indicates that it has two CH_3C groups and three CH_3O groups. The anomeric hydrogen (δ 5.15) gives rise to a doublet of doublets and is coupled with an exchangeable hydrogen (δ 6.30, J = 4 cps.) and a hydrogen on carbon bearing methoxyl (δ 3.25, J = 2 cps.) which is not adjacent to another hydrogen. The CH_3C signal which is split is attached to a carbon bearing one hydrogen (multiplet centered at δ 3.7, J = 6 cps.) which is in turn coupled with a single hydrogen (δ 2.98, J = 9 cps.) in a diaxial relationship. The hydrogen giving rise to the doublet at δ 2.98 is adjacent to only one hydrogen. Between these two systems there must be a carbon atom substituted by the remaining CH_3C group and a methoxyl group. In nogalolactone the multiplet due to hydrogen attached to carbon substituted by methyl has moved downfield to δ 4.2 indicating that nogalose is a pyranose. The structure of nogalose exclusive of stereochemistry must be as shown on the following page.



The above discussion leaves a moiety of $C_8H_{15}NO_3$ unaccounted for. Analogy with other anthracyclinone antibiotics suggests that this moiety represents an aminosugar. The NMR spectra of the various compounds already mentioned and the isolation of dimethylamine from nogalamycin are consistent with this interpretation. However, the aminosugar in nogalamycin is a great deal more resistant to hydrolytic cleavage than is the case in other antibiotics of this class and has not been isolated intact.

ACKNOWLEDGMENT

This work was supported by contract PH43-62-168, Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda, Maryland.

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