NEIHUMICIN, A NEW CYTOTOXIC ANTIBIOTIC FROM *MICROMONOSPORA NEIHUENSIS*

II. STRUCTURAL DETERMINATION AND TOTAL SYNTHESIS

LI-MING YANG, RONG-YANG WU[†], ANDREW T. MCPHAIL[††], TOSHIO YOKOI and KUO-HSIUNG LEE*

Natural Products Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27514, U.S.A.
[†]Institute of Botany, Academia Sinica, Taipei, Taiwan, Republic of China
[††]Department of Chemistry, P.M. Gross Chemical Laboratory, Duke University, Durham, North Carolina 27514, U.S.A.

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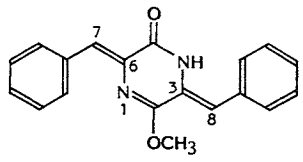
The structure of neihumicin, a new antibiotic isolated from the fermentation broth of *Micromonospora neihuensis* Wu, sp. nov., has been determined as (*Z*)-3,(*Z*)-6-dibenzylidene-2-methoxy-3,6-dihydropyrazin-5-one based upon spectral evidence and X-ray crystallographic analysis. Its total synthesis has also been achieved.

In the preceding paper[1], we have described the isolation of neihumicin, a new antibiotic isolated from the fermentation broth of *Micromonospora neihuensis* Wu, sp. nov., which showed potent cytotoxicity *in vitro* against KB tissue culture cells (ED_{50} 0.94 μg/ml) as well as antifungal activity against *Saccharomyces cerevisiae* ATCC 9763. We wish to report here on the structural determination of neihumicin as well as its total synthesis.

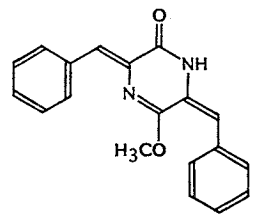
Structure Determination

The structure and stereochemistry of neihumicin were determined based upon the following spectral evidence and single-crystal X-ray analysis.

Neihumicin (**6**) was isolated as yellow crystals. It showed mp 175~176°C and gave a single spot on TLC (silica gel, Rf 0.69, chloroform - acetone (10:1)). It is soluble in DMSO and insoluble in methanol, benzene, *n*-hexane and water. Elemental analysis (calcd for $C_{19}H_{16}N_2O_2$: C 74.98, H 5.30, N 9.21; found: C 75.02, H 5.29, N 9.01) and high resolution mass spectral data indicated the molecular formula $C_{19}H_{16}N_2O_2$ (electron impact (EI)-MS calcd: *m/z* 304.1212; found: *m/z* 304.1211 (M^+)) for **6**. The presence of an extended conjugated double bond system was substantiated by UV absorp-



6 (3*Z*,6*Z*)



6a (3*E*,6*Z*)

tion bands at λ_{max} 364 nm (log ε 4.54) and λ_{max} 243 nm (log ε 3.73). The IR spectrum (KBr) further showed the presence of aromatic ring (1600 and 1580 cm^{-1}) and other kinds of double bonds at 1660 cm^{-1} (C=CC=O or C=CNHC=O). An NH group was suggested by an IR band at 3200 cm^{-1} (NHCO) and by an NMR (200 MHz, DMSO-d_6) signal at δ 10.14, which appeared as a broad singlet and disappeared upon addition of D_2O.

Neihumicin contained a methoxy group which appeared as a three proton singlet at δ 4.01 in the NMR spectrum. The presence of two benzylidene moieties was demonstrated by NMR signals at δ 7.32~7.55 (10H, br m, two aromatic rings) and δ 6.55 (1H, s, vinyl 8-H) and 7.13 (1H, s, vinyl 7-H). The downfield chemical shift of 7-H relative to that of 8-H is due to an anisotropic effect of the adjacent approximately coplanar carbonyl group at C-5 to C-7. This also indicates that the methoxy group should be placed at C-2 instead of at C-5, and that the C-8 aromatic ring is (*Z*)-oriented as shown in **6** rather than (*E*)-oriented (**6a**) in order to prevent very severe steric hindrance between the 2-OCH_3 and *ortho*-hydrogens of the phenyl ring at C-8.

The foregoing evidence led to the assignment of structure **6** for neihumicin. The structure and stereochemistry of **6** were confirmed unequivocally by single crystal X-ray analysis. Neihumicin was recrystallized from dichloromethane - heptane to yield fine yellow needles.

Crystal Data

$C_{19}H_{16}N_2O_2$, M=304.35, monoclinic, a=13.483(2) Å, b=5.453(1) Å, c=24.209(3) Å, β=119.30(1)°, V=1552.2 Å^3, Z=4, D_{calcd}=1.302 gcm^{-3}, μ(CuKα radiation, λ=1.5418 Å)=6.5 cm^{-1}. Space group $Pc(C_s^2)$ or $P2/c(C_{2h}^4)$ from the systematic absences: hOl when l≠2n; shown to be the latter by structure solution and refinement. Sample dimensions: 0.06×0.07×0.90 mm.

Crystallographic Measurements

Preliminary unit-cell parameters and space group information were obtained from oscillation and Weissenberg photographs. Intensity data (hk±l) to θ=67° were recorded on an Enraf-Nonius CAD-4 diffractometer (CuKα radiation, incident-beam graphite monochromotor; ω-2θ scans). From a total of 2773 independent measurements after averaging equivalent forms, only those 1492 reflections with $I>3.0\sigma$ (I) were retained for the structure analysis and corrected for the usual Lorentz and polarization effects. Refined unit-cell parameters were derived by least-squares treatment of the diffractometer setting angles for 25 reflections (35°<θ<55°) widely separated in reciprocal space.

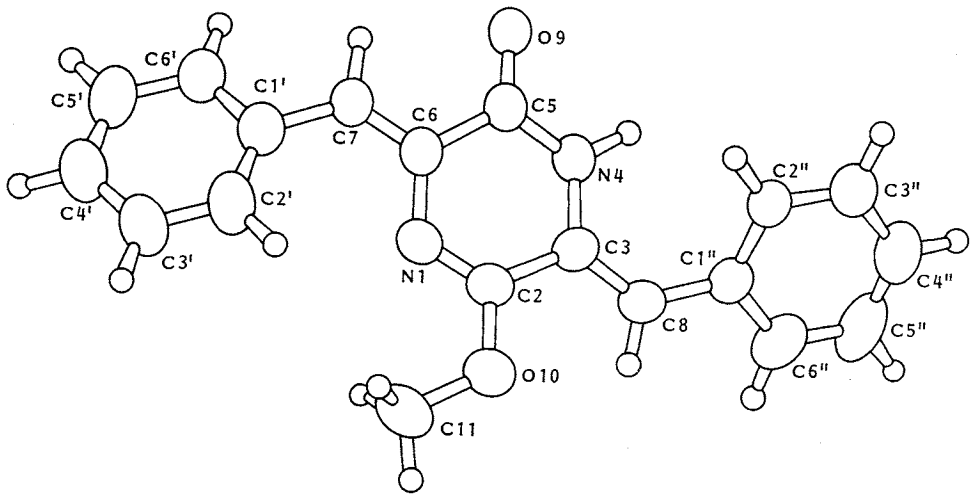
Structure Analysis

The centrosymmetric choice of space group, $P2/c$, was assumed at the outset and the crystal structure was solved by direct methods†. Approximate non-hydrogen atom coordinates were obtained from an *E*-map. Hydrogen atoms, located in a difference Fourier synthesis evaluated following several rounds of least-squares adjustment, of non-hydrogen atom positional and anisotropic temperature factor parameters, were included at their calculated positions in the subsequent iterations which converged at R=0.045 (R_w=0.060) ($R=\Sigma||F_o|-|F_c||/\Sigma|F_o|$; $R_w=[\Sigma w(|F_o|-|F_c|)^2/\Sigma w|F_o|^2]^{1/2}$). Neutral atom scattering factors used in the structure-factor calculations were taken from ref 2. In the least-squares iterations, $\Sigma w\Delta^2[w=1/\sigma^2(|F_o|), \Delta=(|F_o|-|F_c|)]$ was minimized.

A view of structure, with the atom numbering scheme, is presented in Fig. 1 which clearly shows that neihumicin (**6**) is (*Z*)-3,(*Z*)-6-dibenzylidene-2-methoxy-3,6-dihydropyrazin-5-one. The piper-

† Crystallographic calculations were performed on a PDP11/44 computer by use of the Enraf-Nonius SDP suite of programs. The direct methods program MULTAN11/82 was employed.

Fig. 1. Atom numbering scheme and solid-state conformation of neihumicin; small circles denote hydrogen atoms.



azine ring (root-mean-square atom displacement=0.009 Å) is essentially planar and its directly-bonded carbon and oxygen atoms lie close (Δ_{max}=0.069 Å) to the ring plane. Severe overcrowding of phenyl ring *ortho*-hydrogen atoms and the piperazine atoms, which would be present in a completely planar structure, is relieved by a combination of out-of-plane bending of C-7 and C-8 (Δ 0.069 Å and 0.042 Å, respectively) to opposite sides of the piperazine ring plane, bond angle deformation (C-7 – C-1′ – C-2′=124.1 (2)°>C-7 – C-1′ – C-6′=117.9 (3)°; C-8 – C-1″ – C-2″=123.7 (3)°>C-8 – C-1″ – C-6″=118.5 (3)°), and rotation of the phenyl rings about the benzylidene C-C bonds (dihedral angles between least-squares planes: N-1 – C-6/C-1′ – C-6′=24.9°; N-1 – C-6/C-1″ – C-6″=31.9°). Molecules of **6** are associated as dimers in the solid state by N-H···O (N-H···O-9 2.897(3) Å) hydrogen bonds between molecules related by crystallographic centers of symmetry.

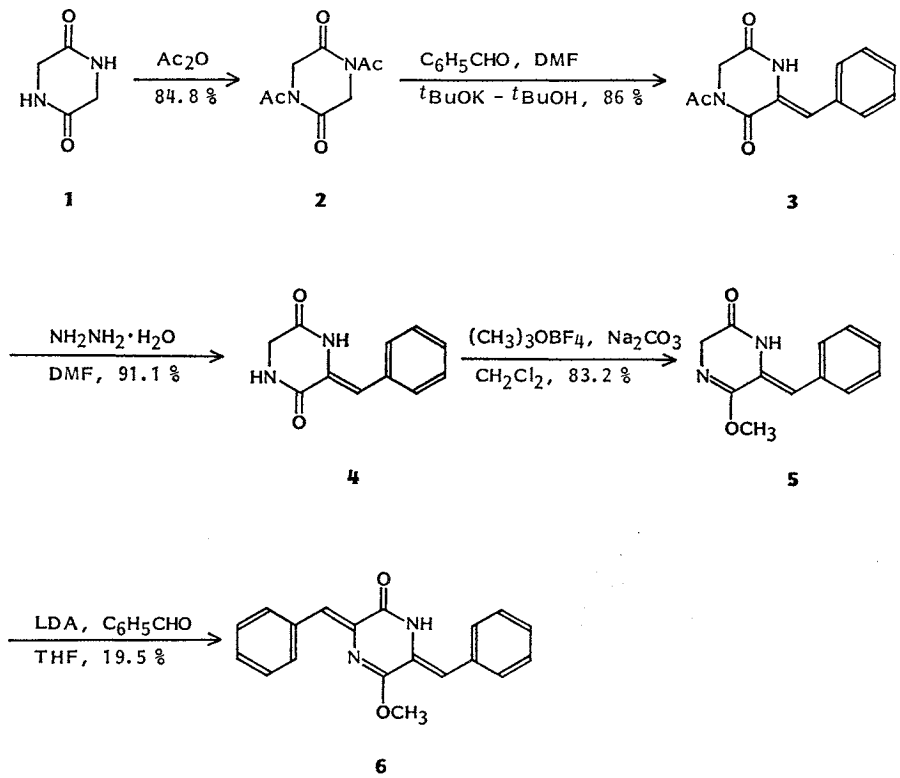
It is noteworthy that the isolation of **6** provides the first example that the production of a 3,6-dihydropyrazin-5-one type antifungal cytotoxic agent from *Micromonospora* species. The previously reported compound albonoursin, isolated from *Streptomyces noursei*[3], *Streptomyces thioluteus*[4] and *Actinomyces tumemacerans*[5], is a piperazine-2,5-dione type antitumor antibiotic.

Total Synthesis of Neihumicin

Although there were several reports[6~15] describing the synthesis of piperazinedione derivatives, no total synthesis of neihumicin had been reported. Neihumicin (**6**) was synthesized in five steps from glycine anhydride (**1**) by the reaction sequence outlined in Fig. 2.

Acetylation of glycine anhydride (**1**) according to the procedure of MARCUCCIO and ELIX[16] with acetic anhydride gave 1,4-diacetylpiperazine-2,5-dione (**2**) in 84.8% yield. Condensation of **2** with benzaldehyde in the presence of dimethylformamide and potassium *tert*-butoxide in *tert*-butanol according to GALLINA and LIBERATORI[17] afforded 1-acetyl-3-benzylidenepiperazine-2,5-dione (**3**) in 86% yield. Deacetylation of **3** by the method of GALLINA and LIBERATORI[18] with hydrazine hydrate in dimethylformamide furnished 3-benzylidenepiperazine-2,5-dione (**4**) in 91.1% yield. The target compound (**6**) was obtained by methylation[19] of **4** with trimethyloxonium tetrafluoroborate in dichloromethane to give 83.2% yield of 3-benzylidene-2-methoxy-3,4,5,6-tetrahydropyrazin-5-one (**5**),

Fig. 2. The total synthesis of neihumicin.



1 2 3 4 5 6

which was condensed with benzaldehyde in the presence of lithium diisopropylamide (LDA)[20] in anhydrous tetrahydrofuran at −78°C to yield 19.5% of **6**. The identity of this synthetic compound (**6**) with an authentic sample of neihumicin, isolated from *M. neihuensis* Wu, sp. nov. described above, was established by mixed mp determination, and superimposable TLC, UV, IR and ^{1}H NMR spectra.

Experimental

General

All mp were determined in open-ended capillaries on a Thomas-Hoover mp apparatus and are uncorrected. UV spectra were recorded on a Varian-Cary model 2200 instrument. IR spectra were obtained using KBr-pellets on a Perkin-Elmer spectrophotometer model 1320. All absorption bands are reported in wave numbers (cm^{-1}). MS were measured on a V.G. micromass model 70-70F instrument at 70 eV with an inlet system. ^{1}H NMR spectra were recorded in $CDCl_3$ with TMS as an internal standard, on either a JECO-FX 60 MHz, or a Bruker AC-200 MHz spectrometer unless stated otherwise. All chemical shifts are reported in ppm. ^{1}H NMR data are presented as follows: Chemical shift (number of protons, multiplicity, structural assignment). The abbreviations br, s and m refer to broad, singlet and multiplet, respectively. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

TLC was accomplished on 0.25 mm precoated plates of Silica gel $60F_{254}$ (Merck) eluted with $CHCl_3$ - acetone (10 : 1). Developed plates were either visualized with UV light (254 and 366 nm) or by development in an iodine crystal chamber. Silica gel 60 (Merck 70~230 mesh) was used for preparative column chromatography.

1,4-Diacetylpiperazine-2,5-dione (**2**)

A mixture of glycine anhydride (11.4 g, 0.1 mol) in acetic anhydride (50 ml) was stirred under reflux for 7 hours. The solvent was removed by azeotropic distillation with methanol and toluene under reduced pressure. The residue was crystallized from ethyl acetate - ether to yield **2** (16.8 g, 84.8%) as colorless needles: MP 98~99°C (literature[16] 99.5~100.5°C); IR (KBr) cm^{-1} 1700; NMR (60 MHz) δ 2.60 (6H, s, 2×Ac), 4.66 (4H, s, 2×CH_2).

1-Acetyl-3-benzylidenepiperazine-2,5-dione (**3**)

Compound **2** (11.7 g, 0.05 mol) was dissolved in DMF (118 ml) under argon. To this solution was added dropwise benzaldehyde (24 ml, 0.24 mol) and 0.5 N potassium *tert*-butoxide in *tert*-butanol (118 ml) over 20 minutes at 0°C. After the pale yellow solution had been stirred at room temperature for 6 hours, it was neutralized with acetic acid (148 ml) and poured into water. The product was extracted with brine, water, dried over anhydrous sodium sulfate, and concentrated in vacuum. The residue was crystallized from ethyl acetate to yield **3** (12.4 g, 86%) as colorless crystals: MP 197~198°C (literature[17] 200~201°C); IR (KBr) cm^{-1} 3270, 1695, 1675, 1625, 1360, 1100; UV λ_{max}^{EtOH} nm (log ε) 312 (4.22), 228 (4.14); NMR (60 MHz) δ 2.66 (3H, s, NAc), 7.19 (1H, s, vinyl H), 7.43 (5H, s, ArH), 7.98 (1H, br, NH).

3-Benzylidenepiperazine-2,5-dione (**4**)

A solution of **3** (10.9 g, 145 mmol) in hydrazine hydrate (4.4 ml) and DMF (135 ml) was stirred at room temperature for 2 hours. The reaction mixture was poured into ice-water and the crystals that separated were collected, washed with water, and recrystallized from boiling acetic acid - ether to give **4** as colorless crystals (8.28 g, 91.1%): MP 266~268°C; IR (KBr) cm^{-1} 3195, 1665, 1615; UV λ_{max}^{EtOH} nm (log ε) 296 (4.26), 224 (4.07); NMR (60 MHz, DMSO-d_6) δ 4.03 (2H, s, CH_2), 6.68 (1H, s, vinyl H), 7.44 (5H, m, ArH), 8.34 (1H, br, NH), 9.96 (1H, br, NH).

3-Benzylidene-2-methoxy-3,4,5,6-tetrahydropyrazin-5-one (**5**)

The key intermediate **5** was prepared by a modification of the method of FUKUYAMA *et al.*[18]. A mixture of **4** (8.06 g), trimethyloxonium tetrafluoroborate (32 g), and anhydrous sodium carbonate (32 g) was stirred in dichloromethane for 7 hours at room temperature. The combined extracts were washed with brine, water, dried over anhydrous sodium sulfate and evaporated under vacuum to give **5** as pale yellow crystals (7.2 g, 83.2%) after crystallization from benzene: MP 119~120°C; IR (KBr) cm^{-1} 3170, 1665, 1620; UV λ_{max}^{EtOH} nm (log ε) 288 (4.21), 233 (3.89), 210 (3.92); NMR (200 MHz) δ 3.84 (3H, s, OCH_3), 4.43 (2H, s, NCH_2CO), 6.54 (1H, s, vinyl H), 7.26~7.39 (5H, m, ArH), 7.82 (1H, br, NH); *Anal* Calcd for $C_{12}H_{12}N_2O_2$: C 66.65, H 5.59, N 12.96; Found: C 66.77, H 5.64, N 12.91.

3,6-Dibenzylidene-2-methoxy-3,6-dihydropyrazin-5-one (**6**)

A solution of diisopropylamine (0.31 ml, 2.2 mmol) (freshly distilled from calcium hydride) in anhydrous THF (freshly distilled from $LiAlH_4$) (1.3 ml) was dropped by injection under argon to a solution of *n*-butyllithium in hexane (1.3 ml) at −78°C. After the addition was complete, the solution was allowed to reach 0°C for 10 minutes, and then re-cooled to −78°C. To this solution, a solution of **5** (216 mg, 2 mmol) in anhydrous THF was added dropwise. After the yellow solution has been stirred at −78°C for 15 minutes, benzaldehyde (0.2 ml, 2 mmol) was added, and the mixture was further allowed to stir at −78°C for 20 minutes and at 0°C for 30 minutes. The reaction solution was neutralized with 0.1 M phosphate buffer and extracted with ethyl acetate (3×30 ml). The combined organic phases were washed with brine, water, dried over anhydrous sodium sulfate, and evaporated under vacuum to afford 5.91 mg (19.5%) of **6**, which was recrystallized from ethanol to yield pure **6** as yellow crystals: MP 175~177°C (natural neihumicin: 175~176°C); IR (KBr) cm^{-1} 3200, 1660, 1630, 1600, 1585; UV λ_{max}^{EtOH} nm (log ε) 364 (4.54), 243 (3.73); NMR (200 MHz, DMSO-d_6) δ 3.99 (3H, s, OCH_3), 6.53 (1H, s, vinyl H), 7.11 (1H, s, vinyl H), 7.29~7.53 (10H, m, ArH), 10.11 (1H, br, NH, exchangeable with D_2O); *Anal* calcd for $C_{19}H_{16}N_2O_2$: C 74.98, H 5.30, N 9.21; found: C 74.85, H 5.47, N 9.20.

This synthetic compound (**6**) was identical with naturally occurring neihumicin by a direct comparison (mixed mp, TLC, UV, IR and NMR spectra).

Acknowledgments

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