PHENAZINES FROM *Streptomyces cinnamonensis*

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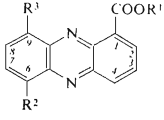
Phenazine-1-carboxylic acid (*Ia*) and 6-(3-methyl-2-butenyl)phenazine-1-carboxylic acid (*IIa*) were isolated from the mycelium of the strain *Streptomyces cinnamonensis* ATCC 15 413. Identification and structure elucidation were performed by spectroscopic methods. Both compounds weakly inhibit the growth of gram-positive bacteria.

Antibiotically active substances belonging to different structural types have been isolated so far from various strains of *Streptomyces cinnamonensis*: yumimycin[1], actithiazic acid[2], monensins A, B, and C (rel. [3]), ensanchomycin[4], and one streptothricin-like compound[5]. Recently, we have obtained two yellow compounds exhibiting a weak inhibitory effect on the gram-positive bacteria[6] from the extract of the mycelium of the strain *S. cinnamonensis* ATCC 15 413 producing monensines A and B. This paper is devoted to the determination of their structures.

The compound of elemental composition $C_{13}H_8N_2O_2$ (high resolution mass spectrum) exhibits in its ^{1}H NMR spectrum peaks due to seven aromatic protons at 7·65–9·00 ppm and a one-proton exchangeable singlet at 15·56 ppm. All signals in the ^{13}C NMR spectrum belong to the sp^2-hybridized carbon atoms only. They consist of seven methines 127·9, 130·0, 130·2, 131·7, 133·1, 135·1 and 137·3 ppm and six quaternary carbons δ_C 125·0, 139·1, 140·0, 143·8, 144·1 and 165·9. A loss of CO_2 from the molecular ion in the mass spectrum leads to the base peak m/z 180. Distinct band at 1 730 and a diffuse one at 2 700 cm^{-1} in the infrared spectrum indicate the presence of a carboxyl group in the molecule. The NMR signals δ_H 15·56 (COOH) and δ_C 165·9 COOH) can be assigned to this group. The chemical shift of the carbon δ_C 125·0 well conforms with the carbon bearing the carboxyl group. From the chemical shifts of the remaining carbon atoms it follows that none CH group lies in the vicinity of nitrogen atom and that no quaternary carbon atom is bonded simultaneously to both nitrogen atoms. Two aromatic protons appear as resolved multiplets at 8·55 ppm (dd, J_{ortho} = 8·8 Hz, J_{meta} = 1·7 Hz) and 8·99 ppm (dd, J_{ortho} = 7·3 Hz, J_{meta} = 1·7 Hz). The only reason for their different chemical shifts in the molecule under discussion might be their location at the same ring as the COOH group. The observed coupling constants indicate that this ring is a six-membered one. Methylation

by diazomethane provides compound $C_{14}H_{10}N_2O_2$. Its ^1H NMR spectrum is lacking the most downfield signal and contains one additional three-proton singlet at 4·12 ppm. The carbonyl carbon signal in the ^{13}C NMR spectrum is shifted 1·2 ppm downfield. There is a strong band at 1 710 cm^{-1} in the infrared spectrum. The molecular ion in the mass spectrum loses CH_3O and $C_2H_2O_2$. Thus, this compound is a methyl ester of the native acid. The ^{13}C NMR spectrum agrees well with the published data[7] for 1-methoxycarbonylphenazine (*Ib*). Also the melting points both the acid *Ia* and its methyl ester *Ib* agree with the literature[8].



Ia, $R^1 = R^2 = R^3 = H$
Ib, $R^1 = CH_3$, $R^2 = R^3 = H$
IIa, $R^1 = R^3 = H$, $R^2 = CH_2CH{=}C(CH_3)_2$
IIb, $R^1 = R^3 = H$, $R^2 = CH_2CH_2CH(CH_3)_2$
IIc, $R^1 = CH_3$, $R^3 = H$, $R^2 = CH_2CH_2CH(CH_3)_2$
IIIa, $R^1 = R^2 = H$, $R^3 = CH_2CH{=}C(CH_3)_2$
IVa, $R^1 = R^2 = H$, $R^3 = CH_3$
IVb, $R^1 = R^3 = CH_3$, $R^2 = H$

The content of the second yellow pigment in the mycelium of our streptomycet is nearly ten times higher than that of *Ia*. This compound has summary formula $C_{18}H_{16}N_2O_2$, according to high resolution mass spectroscopy. The similarity of UV/VIS, ^1H NMR and ^{13}C NMR spectra with those of *Ia* indicates that it is also a phenazine derivative. The presence of a carboxyl group in the molecule is suggested by a diffuse band at 2 700 cm^{-1} and by an another band at 1 732 cm^{-1} in the infrared spectra, by an exchangeable proton signal at 15·60 ppm in ^1H NMR spectrum and by a δ_C 166·1 signal in ^{13}C NMR spectrum. An attempt to prepare the methyl ester was unsuccessful. The compound did react with diazomethane but the purification of the unstable product had failed. However, the dihydro derivative of the natural compound, $C_{18}H_{18}N_2O_2$ (*IIb*), prepared by catalytic hydrogenation of *IIa*, yields a stable methyl ester $C_{19}H_{21}N_2O_2$ (*IIc*).

Double resonance experiments in the ^1H NMR spectrum of native *IIa* picked up the protons of a $(CH_3)_2C{=}CH{-}CH_2{-}$ moiety: 1·80 s (6 H), 4·02 d (2 H, J = = 7·3 Hz), 5·45 t (1 H, J = 7·3 Hz, $J_{H,CH_3} \neq 0$). Peaks of corresponding carbon atoms in the ^{13}C NMR spectrum were located by selective heteronuclear decoupling: 18·1 q, 25·7 q, 135·4 s, 120·3 d, and 29·8 t. A comparison of their chemical shifts with

those of the side chain carbon atoms in 4-(3-methyl-2-butenyl)indole[9] (17·9 q, 25·7 q, 134·0 s, 122·2 d, 32·2 t) exhibits a satisfactory agreement. The remaining six aromatic protons form two ABC systems in the ^{1}H NMR spectrum: 7·82 dd (J_{ortho} = 6·8 Hz, J_{meta} = 1·1 Hz), 7·90 dd (J_{ortho} = 6·8 and 7·3 Hz), 8·18 dd (J_{ortho} = 7·3 Hz, J_{meta} = = 1·1 Hz) and 8·03 dd (J_{ortho} = 7·3 and 8·5 Hz), 8·52 dd (J_{ortho} = 8·5 Hz, J_{meta} = = 1·5 Hz), 8·95 dd (J_{ortho} = 7·3 Hz, J_{meta} = 1·5 Hz). Most sensitive toward the addition of tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octadionato)europium (*III*) are the peaks δ_H 8·95 and 8·52. Therefore, they belong to the protons on the ring carrying the carboxyl group. This deduction is also confirmed by the 4·9 Hz coupling between the proton δ_H 8·95 and the carbonyl carbon δ_C 166·1, proved by low-power selective decoupling. Two of the six sp^2-hybridized methine carbons exhibit in proton-coupled ^{13}C NMR spectrum direct couplings only, three have each one vicinal coupling $^3J_{C,H}$ and one gives a doublet of multiplets arising from the coupling to the side chain protons. The coupling patterns both in ^{1}H NMR andin proton-coupled ^{13}C NMR spectra are evidence for the vicinal proton arrangement in both peripheral phenazine rings. It means that there remain two relative positions of carboxyl and 3-methyl-2-butenyl side chain only: 1,6 (*IIa*) and 1,9 (*IIIa*).

Breitmeier and Hollstein[7] have shown using ^{13}C NMR spectra of 1,6- and 1,9--substituted phenazines that the most sensitive to the changes in the relative position of substituents were the signals of quaternary carbon atoms. Their paper also contains data for 9-methylphenazine-1-carboxylic acid (*IVa*) and its methyl ester *IVb*. It can be assumed that the substituent effect of methyl and other alkyl groups is similar. Were the structure *IIIa* correct, the chemical shifts of the quaternary carbon atoms would agree with those in ref.[7] and would be different in the case of *IIa*. The comparison of these chemical shifts between the dihydro derivative of native compound, its methyl ester and compounds *IVa*, *IVb* exhibits a significant difference in each pair (2·6 and 2·3 ppm, respectively). On this base we propose the structure *IIa* for our compound.

6-(3-Methyl-2-butenyl)phenazine-1-carboxylic acid (*IIa*) extends the series of natural phenazines amounting now over 30 compounds. Most of them were isolated from bacteria and actinomycetes[10,11]; dihydrophenazines were found in some green algae[12]. Phenazine-1-carboxylic acid (*Ia*) is a known antibiotic (tubermycin B) active against gram-positive microorganisms, especially against mycobacteria[8,13]. It have been already isolated from *Pseudomonas aeruginosa*[14], *S. aureofaciens*[15,16] and *S. misakiensis*[8]; its occurrence in *S. cinnamonensis* is reported first time.

EXPERIMENTAL

Melting points were measured on a Kofler hot-stage apparatus. UV/VIS spectra were measured in methanol on a Cary 118 C spectrophotometer. Infrared spectra were recorded in KBr pellets on a UR-20 (Zeiss Jena, GDR) spectrometer. Mass spectra were studied on a Varian MAT-311

instrument at the ionization energy 70 eV, ionizing current 1 mA, ion source temperature 200°C, direct inlet temperature 60—110°C. Elemental compcsition of ions was determined by "peak--matching" technique (±5 ppm) using perfluorokerosene standard. Metastable ions in the first field-free region between the magnetic and electrostatic analyser were recorded by voltage scan of the electrostatic analyser. ^{1}H NMR spectra were measured in deuteriochloroform at 25°C on the Jeol FX-60 (59·797 MHz) and Varian XL-200 (200 MHz) instruments working in the FT mode. Chemical shifts were calculated from the digitally obtained address differences with accuracy 0·004 and 0·0003 ppm (0·24 and 0·06 Hz). ^{1}*n*C NMR spectra were measured on a Jeol FX-60 (15·036 MHz) instrument under the same conditions. The accuracy of the chemical shift determinations was 0·04—0·13 ppm (as required). Signals corresponding to more than one carbon atom were identified using the noise decoupled spectra with suppressed nuclear Overhauser effect[17]. Proton-coupled ^{13}C NMR spectra were measured by "gated decoupling" technique (decoupler off during the acquisition)[17] with digital resolution 0 5 Hz.

Isolation of Phenazines

Phenazines were isolated from the mycelium obtained by cultivation of *S. cinnamonensis* strain ATCC 15 413 (ref.[1]). Submerged culture of *S. cinnamonensis* (900 ml) was filtered off, the mycelium was extracted by methanol, the volume of the extract was reduced and redistributed in the solvent system chloroform–methanol–water (2 : 1 : 1). The chloroform portion was subjected to adsorption percolation on silica gel (3 000 g, Spolana CH, 60—100 μm, elution by chloroform) that separated yellow antibiotically active fractions. Activity was monitored by autobiography on agar plates inoculated by *Bacillus subtilis*. The residues of active fractions were dissolved in minimal amount of chloroform and precipitated by hexane. Yellow precipitate (600 mg) was filtered off, washed by hexane and chromatographed on a column (200 g, Spolana CH, 40 to 100 μm, elution by chloroform). This procedure provided first 6-(3-methyl-2-butenyl)phenazine--1-carboxylic acid (*IIa*, 466 mg), then a mixture of three minor yellow pigments and finally phenazine-1-carboxylic acid (*Ia*, 41 mg) as the most polar component.

6-(3-Methyl-2-butenyl)phenazine-1-carboxylic Acid (*IIa*)

M.p. 168—170°C (chloroform–hexane). Infrared spectrum (KBr): 1 470, 1 530 (aromatic system), 1 732 (C=O), 2 700 cm^{-1} (diffuse band of COOH). UV/VIS spectrum (methanol) λ_{max}, (ε): 255 (65 000), 355 sh (10 500), 367 (14 000) nm. For $C_{18}H_{16}N_2O_2$ (292·1) was calculated: 73·96% C 255 (65 000), 355 sh (10 500), 367 (14 000) nm. For $C_{18}H_{16}N_2O_2$ (292·1) was calculated: 73·96% C, 5·52% H, 9·58% N; found: 73·71% C, 5·50% H, 9·43% N. Mass spectrum *m/z* (% of relative intensity, composition): 292 (97, $C_{18}H_{16}N_2O_2$), 277 (26, $C_{17}H_{13}N_2O_2$), 274 (28. $C_{18}H_{14}N_2O$), 259 (43, $C_{17}H_{11}N_2O$), 249 (43, $C_{15}H_9N_2O_2$), 246 (59, $C_{16}{}^{13}CH_{13}N_2$), 245 (100, $C_{17}H_{13}N_2$), 231 (87, $C_{16}H_{11}N_2$), 205 (68, $C_{14}H_9N_2$). ^{13}C NMR spectrum: 18·1 q. 25·7 q, 29·8 t, 120·3 d, 125·0 s, 128·1 d, 130·1 d, 131·6 d, 131·8 d, 135·0 s, 135·0 d, 135·5 s, 137·1 d. 139·1 s, 139·2 s, 143·0 s, 144·5 s, 166·1 s.

Phenazine-1-carboxylic Acid (*Ia*)

M.p. 240—242°C (lit.[8] 243°C); ultraviolet, visible, and infrared spectra were identical with these published[8]. Mass spectrum *m/z* (% of relative intensity, composition): 224 (2, $C_{13}H_8N_2O_2$, M^+). 180 (100, $C_{12}H_8N_2$, M—CO_2).

Methyl ester Ib was prepared by 2 h action of ethereal solution of diazomethane on *Ia* (20 mg) yielding 18·5 mg of yellow needles m.p. 122—124°C (hexane); lit.[7] 126—128°C. For $C_{14}H_{10}N_2O_2$

(238·3) was calculated: 70·59% C, 4·23% H, 11·76% N; found: 70·49% C, 4·32% H, 12·00% N. Mass spectrum *m/z* (% of relative intensity, composition, *m/z* of daughter ions): 238 (33, $C_{14}H_{10}N_2O_2$, 207, 180), 207 (22, $C_{13}H_7N_2O$, 179), 180 (100, $C_{12}H_8N_2$), 179 (49, $C_{12}H_7N_2$), 152 (14, $C_{11}H_6N$). ^{1}H NMR spectrum: 4·12 s (3 H), 7·72–8·49 mt (7 H). ^{13}C NMR spectrum: 52·6 q, 129·0 d, 130·5 d, 130·9 d, 131·3 d, 133·5 s, 134·0 s, 142·8 s, 143·5 s, 143·8 s, 147·5 s, 167·1 s.

6-(3-Methylbutyl)phenazine-1-carboxylic Acid (*IIb*)

The mixture of *IIa* (120 mg) with catalyst (10% Pd/$BaSO_4$, 20 mg) in ethanol (25 ml) was hydrogenated at normal pressure until consumption of 18 ml of hydrogen. The catalyst was filtered off, ethanol was evaporated and the residue was chromatographed on a silica gel column (300 g, Spolana CH, 40–100 μm, elution by chloroform). The yield was 105·0 mg of yellow crystals, m.p. 108–109°C (methanol). Infrared spectrum (KBr): 1 470, 1 530 (aromatic system), 1 732 (C=O), 2 650 cm^{-1} (diffuse band, COOH). UV/VIS spectrum (methanol) $\lambda_{max}(\varepsilon)$: 255 (65 000), 355 sh (10 500), 367 (14 000) nm. For $C_{18}H_{18}N_2O_2$ (294·4) was calculated: 73·45% C, 6·16% H, 9·52% N; found: 73·06% C, 6·21% H, 9·44% N. Mass spectrum *m/z* (% of relative intensity, *m/z* of daughter ions): 294 (29, $C_{18}H_{18}N_2O_2$, 279, 251, 238), 279 (9, $C_{17}H_{15}N_2O_2$, 261), 261 (21, $C_{17}H_{13}N_2O$), 251 (34, $C_{15}H_{11}N_2O_2$, 233), 238 (100, $C_{14}H_{10}N_2O_2$, 220), 233 (55, $C_{15}H_9N_2O$, 205), 220 (99, $C_{14}H_{18}N_2O$, 192), 207 (33, $C_{14}H_{11}N_2$), 205 (47, $C_{14}H_9N_2$), 194 (34, $C_{13}H_{10}N_2$), 192 (35, $C_{13}H_8N_2$). ^{1}H NMR spectrum: 1·06 d (J = 6·1 Hz, 6 H), 1·71 mt (3 H), 3·29 t (J = 7·3 Hz, 2 H), 7·33–8·22 mt (4 H, aromatic protons), 8·46 dd (J_{ortho} = 8·8 Hz, J_{meta} = 1·5 Hz, 1 H), 8·92 dd (J_{ortho} = 6·8 Hz, J_{meta} = 1·5 Hz, 1 H), 15·66 s (exchangeable, 1 H). ^{13}C NMR spectrum: 22·6 q (2 C), 28·4 d, 29·6 t, 39·4 t, 124·9 s, 127·9 d, 130·1 d, 131·7 d (2 C), 134·9 d, 137·0 d, 138·8 s, 139·0 s, 140·6 s, 142·9 s, 144·4 s, 166·0 s.

Methyl ester IIc was prepared from *IIb* (50 mg) by ethereal solution of diazomethane, yield 46·0 mg of yellow crystals, m.p. 54–56°C (hexane). Infrared spectrum (KBr): 1 470, 1 535 (aromatic system), 1 710 (C=O) cm^{-1}. UV/VIS spectrum (methanol) $\lambda_{max}(\varepsilon)$: 254 (65 800), 349 (10 600), 365 (14 100) nm. For $C_{19}H_{20}N_2O_2$ (308·4) was calculated: 74·06% C, 6·54% H, 9·08% N; found: 73·95% C, 6·22% H, 8·90% N. Mass spectrum *m/z* (% of relative intensity, composition, *m/z* of daughter ions): 308 (45, $C_{19}H_{20}N_2O_2$, 293, 277, 265, 252), 293 (18, $C_{18}H_{17}N_2O_2$, 275, 261), 277 (8, $C_{18}H_{17}N_2O$), 275 (6, $C_{18}H_{15}N_2O$), 265 (47, $C_{16}H_{13}N_2O_2$, 233), 261 (25, $C_{17}H_{13}N_2O$, 233), 252 (100, $C_{15}H_{12}N_2O_2$, 220), 233 (75, $C_{15}H_9N_2O$, 205), 220 (88, $C_{14}H_8N_2O$, 192), 205 (45, $C_{14}H_9N_2$), 192 (31, $C_{13}H_8N_2$, 165), 165 (18, $C_{12}H_7N$). ^{1}H NMR spectrum: 1·02 d (J = 6·0 Hz, 6 H), 1·73 mt (3 H), 3·40 t (J = 7·3 Hz, 2 H), 4·11 s (3 H, OCH_3), 7·61 to 8·23 mt (5 H), 8·36 dd (J_{ortho} = 8·3 Hz, J_{meta} = 2·0 Hz, 1 H). ^{13}C NMR spectrum: 22·6 q (2 C), 28·3 d, 29·3 t, 40·0 t, 52·6 q, 127·2 d, 129·0 d, 129·3 d, 129·6 s, 131·0 d, 131·3 d, 132·6 s, 132·8 d, 142·4 s, 142·7 s, 143·5 s, 143·7 s, 168·0 s.

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