STRUCTURE OF THE PRODUCT OF THE ACID HYDROLYSIS OF SIBIROMYCIN

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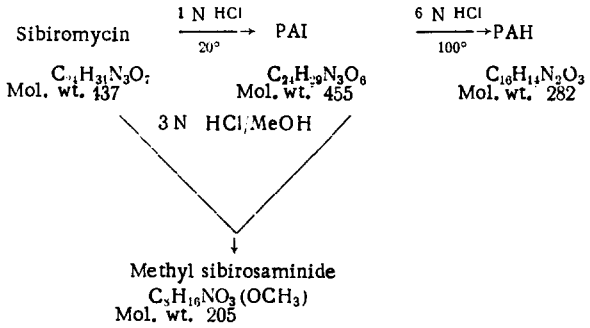
We have previously reported that the antitumoral antibiotic sibiromycin, which is produced by the actinomycete Streptosporangium sibiricum, is rapidly inactivated by the action of dilute mineral acids at room temperature, being converted into a yellow crystalline product which has been called the product of acid inactivation (PAI) [1]. We have established that PAI has the composition $C_{24}H_{29}N_3O_6$ (mol. wt. 455) and, like the initial sibiromycin, is the glycoside of a new amino sugar – sibirosamine [2]. Under the conditions of severe acid hydrolysis, PAI loses the sibirosamine residue and is converted into a bright yellow crystalline product having the composition $C_{16}H_{14}N_2O_3$ called the product of acid hydrolysis (PAH). These transformations can be illustrated by the following scheme:



The present paper gives the results of an investigation of the structure of the PAH of sibiromycin. PAH is an optically inactive yellow crystalline substance with the composition $C_{16}H_{14}N_2O_3$ (mol. wt. 282) which decomposes without melting at temperatures above 270°C; λ_{max} (in methanol), nm; 290, 375, 410, and 435 (ε 49,000, 10,000, 10,150, and 7900); IR spectrum (KBr), cm^{-1}: 3420, 2800-2900, 1680, 1620, 1580. It can be seen from the PMR spectrum that PAH molecule has a $-CH=CH-CH_3$ group (the signal of the methyl group is represented by a doublet at 1.89 ppm and the signal of the olefinic protons by a multiplet at 6.3 ppm), an aromatic methyl group (singlet at 2.16 ppm), and also four protons belonging to an aromatic or heterocyclic system (δ 7.27, 7.51, 8.06, and 8.28 ppm). The values of the chemical shifts (CSs) of the protons of PAH and also of the other compounds considered in this paper are given in Table 1.

The product of acid hydrolysis is moderately soluble in ether and pyridine, sparingly soluble in alcohols and acetone, and practically insoluble in chloroform, benzene, and water ($pH \leq 7$). It is extraordinarily stable to the action of acids; even on prolonged boiling in concentrated hydrochloric acid it underwent practically no change.

On catalytic hydrogenation, PAH absorbed 4 moles of hydrogen, forming octahydro-PAH (IV), $C_{16}H_{22}N_2O_3$, λ_{max} (methanol), nm: 229, 260 (shoulder) and 350 (ε 34,000, 10,000, and 4000); IR spectrum

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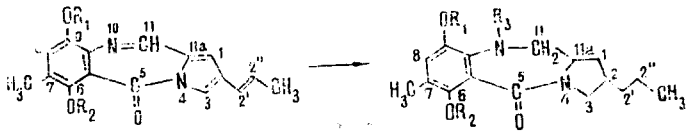
TABLE 1. Chemical Shifts of the Protons of PAH and Its Derivatives, δ, ppm



I, II, III IV, V, VI

Substance	$C_{2''}-CH_3$	C_7-CH_3	H_1	H_2	$H_{2'}$	$H_{2''}$	H_3	H_{11}	H_{11a}	H_8	R_1, R_2	R_3	Solvent
PAH (I) $R_1=R_2=H$	1,89	2,16	7,27	—	←6,31→		8,06	8,28	—	7,51			DMSO
Diacetate of (I) (II) $R_1=R_2=COCH_3$	1,89	2,15	7,07	—	←6,27→		8,07	8,37	—	8,24	2,34 2,41		$CDCl_3$
Dimethyl ether of (I) (III) $R_1=R_2=CH_3$	1,88	2,29	7,07	—	←6,30→		8,07	8,48	—	7,88	3,89 3,96		$CDCl_3$
Octahydro-I (IV) $R_1=R_2=R_3=H$	0,87	1,97	←range 1,29→				←2,80–3,90→			6,92			DMSO
Triacetate of (IV)(V) $R_1=R_2=R_3=COCH_3$	0,90	2,05	←range 1,36→				←2,80–4,50→			7,40	2,29 2,33	1,80	$CDCl_3$
N-Acetate of dimethyl-IV (VI) $R_1=R_2=CH_3$, $R_3=COCH_3$	1,01	2,26	←range 1,35→				←2,80–4,50→			7,15	3,74 3,92	1,86	$CDCl_3$

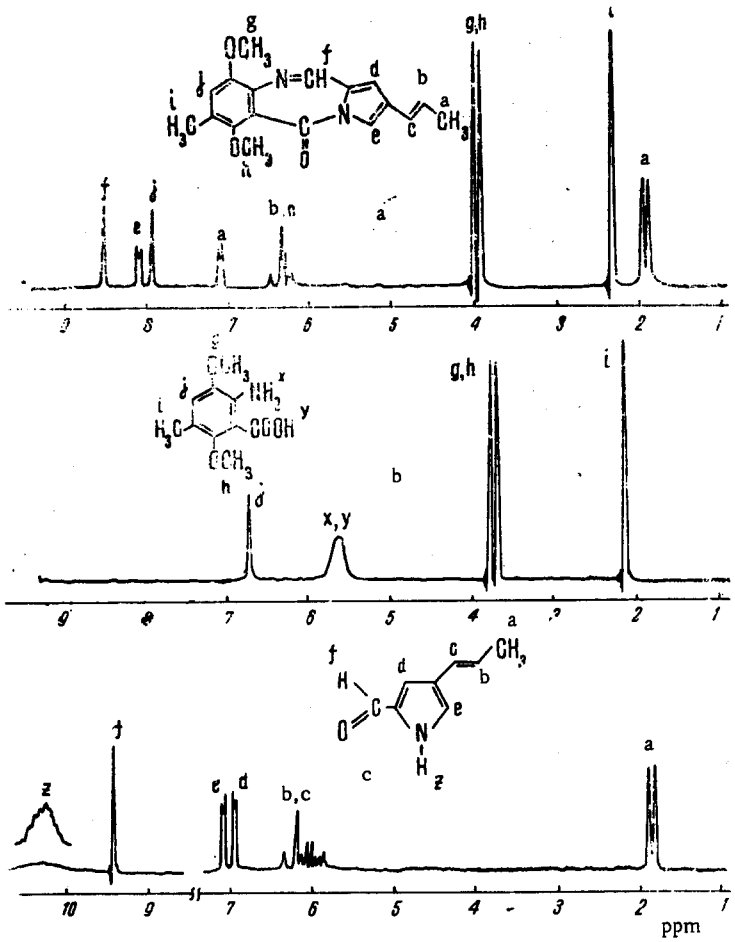


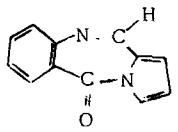
Fig. 1. PMR spectra of the dimethyl ether of PAH (a), 3,6-dimethoxy-5-methylanthranilic acid (b), and 4-(prop-1-enyl)pyrrole-2-carboxaldehyde (c) (100 MHz).

(KBr), cm^{-1}: 3420-3300, 2970-2860, 1590, 1545, and 1510. In the PMR spectrum of (IV) the signals of the olefinic protons have disappeared, the signal of the aliphatic methyl group has shifted upfield by 1 ppm and become a triplet, and in the weak-field region of the spectrum only the signal of one proton (δ 6.92 ppm) remains (see Table 1). In contrast to PAH, octahydro-PAH is readily soluble in acid and alcoholic solutions of acids and gives a stable hydrochloride which is soluble in ethanol and insoluble in ether. Neutral or weakly alkaline solutions of (IV) in ethanol rapidly darken in the air. The acylation of (IV) with acetic anhydride in pyridine formed a triacetate (V), which was obtained in the form of a white crystalline substance having the composition $C_{22}H_{28}N_2O_6$, mp 131-133°C, IR spectrum (chloroform), cm^{-1}: 1780, 1680-1665, 1208. The PMR spectrum of (V) shows the signals of two $O-COCH_3$ groups (δ 2.29 and 2.33 ppm) and the signal of a $N-COCH_3$ group (δ 1.80 ppm). Octahydro-PAH is methylated by diazomethane, forming a di-O-methyl ether, the subsequent acylation of which with acetic anhydride in pyridine led to the N-acetate of the dimethyl ether (VI), which was isolated in the form of a white crystalline substance with the composition $C_{20}H_{28}N_2O_4$, mp 161-163°C. The PMR spectrum of (VI) shows the signals of two OCH_3 groups (δ 3.74 and 3.92 ppm) and the signal of a $N-COCH_3$ group (δ 1.86 ppm).

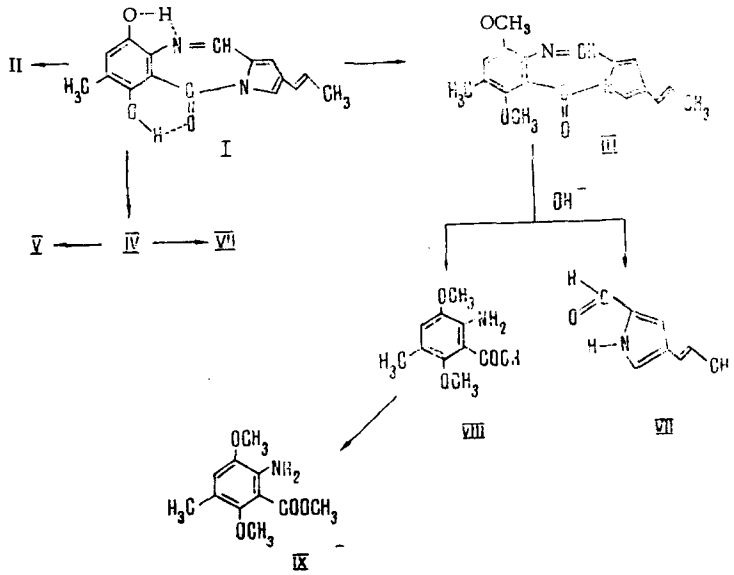
In contrast to octahydro-PAH, the initial PAH is not methylated by diazomethane, which shows that both the phenolic hydroxyls present in the PAH molecule participate in the formation of strong hydrogen bonds. PAH is acylated by acetic anhydride in pyridine, giving a yellow crystalline diacetate (II), $C_{20}H_{18}N_2O_5$, mp 225-226°C; IR spectra (chloroform), cm^{-1}: 1775, 1680, 1630, 1205. When (II) is heated with 2 N HCl, PAH is re-formed. The phenolic hydroxyls in the PAH molecule are methylated when the substance is boiled with dimethyl sulfate in acetone in the presence of K_2CO_3. The methylation of PAH for 1 h gives a quantitative yield of the monomethyl ether of PAH, $C_{17}H_{16}N_2O_3$ (mol. wt. 296), with mp 200-201°C, δ 3.96 ppm [its IR spectrum shows an absorption band at 3250 cm^{-1} (OH)]; on methylation for 48 h, however, the PAH is

converted into the dimethyl ether (III), $C_{18}H_{18}N_2O_3$ (mol. wt. 310) with mp 192-193°C. The IR spectrum of (III) lacks absorption in the 3200-3600-cm^{-1} region. In the PMR spectrum (Table 1 and Figure 1a) the signals of the OCH_3 groups appear in the form of two three-proton singlets at 3.90 and 3.96 ppm.

Decisive information on the structure of PAH was obtained by studying the products of the alkaline hydrolysis of its dimethyl ether (III). We have reported previously that the alkaline hydrolysis of PAH itself forms a derivative of pyrrole-α-carboxaldehyde with an unsaturated side chain [1]. We have established that the alkaline hydrolysis of PAH is accompanied by the formation, in addition to the pyrrole carboxaldehyde derivative, of several readily oxidized compounds possessing acidic properties. The reaction mixture in the alkaline hydrolysis of PAH has a dark violet color which disappears on the addition of zinc dust or sodium hydrosulfite. After we had succeeded in obtaining the dimethyl ether of PAH, we performed the alkaline hydrolysis of this substance and found that in this case, together with the pyrrolecarboxaldehyde (VII), an anthranilic acid derivative (VIII), $C_{10}H_{13}NO_4$ (mol. wt. 211), mp 178°C, was formed. The acid (VIII) precipitated in the form of white crystals readily soluble in alcohols, ether, chloroform, and alkalis, and insoluble in acids. Solutions of the acid (VIII) possess a strong blue fluorescence in UV light. The IR spectrum of the acid (VIII) is practically identical with that of anthranilic acid in the 3500-1550-cm^{-1} region and differs from it only in the fingerprint region. The UV spectra of (VIII) and of anthranilic acid are also extremely similar in nature and intensity. In the PMR spectrum of (VIII) there are the signals of two OCH_3 groups (δ 3.70 and 3.77 ppm), of an aromatic CH_3 group (δ 2.14 ppm), of three mobile protons (broad signal in the 5.5-ppm region) and of an aromatic proton (δ 6.73 ppm).



The methylation of the acid (VIII) with diazomethane formed an ester (IX), $C_{11}H_{15}NO_4$ (mol. wt. 225), mp 72-73°C. The PMR spectrum of (IX) showed the signal of a $-COOCH_3$ group (δ 3.84 ppm) and the signals of two mobile protons in the 5.58-ppm region.



A comparison of the spectral properties of the dimethyl ether of PAH, the pyrrolecarboxaldehyde (VII), and the acid (VIII) (see Fig. 1a, b, c) permits the conclusion that in the molecule of (III) fragments (VII) and (VIII) are joined, in the first place by a $-C=N-$ bond, which arises in the condensation of the amino group of the acid (VIII) with the aldehyde of the pyrrole derivative (VII), and, in the second place, by an amide bond $-CO-N-$ formed through the condensation of the carboxy group of the acid (VIII) and the HN group of the pyrrole fragment. Thus, the heterocyclic skeleton of the molecule of PAH can be represented as a pyrrolo-1,4-benzodiazepine system (see formula above).

The positions of the two OCH_3 groups in the acid (VIII) follow from the participation mentioned above of the corresponding unsubstituted hydroxy groups in the formation of strong hydrogen bonds in the PAH molecule. The position of the aromatic methyl group at the C_5 atom of the acid (VIII) was established on the basis of an estimate of the influence of substituents in the benzene nucleus on the CS of an aromatic proton. For the ester (IX) the calculation, performed by an additive scheme on the basis of literature information [3], gives for the C_5–H proton (CH_3 group in the C_4 position) a CS value of 6.04 ppm and for the C_4–H proton (CH_3 group in position C_5) one of 6.51 ppm. The experimentally obtained CS of the aromatic proton in the PMR spectrum of the ester (IX) is 6.92 ppm.

Thus, the structure of 3,6-dimethoxy-5-methylanthranilic acid may be put forward for the acid (VIII) and, consequently, the PAH of sibiromycin will have structure (I).

EXPERIMENTAL

The IR spectra were taken on a UR-10 instrument, the UV spectra on a Unicam SP-800 instrument, and the PMR spectra on a JNM-4H 100-MHz spectrometer (with TMS as internal standard). The molecular weights of the compounds studied were determined mass spectrometrically on an MKh-1303 instrument at an ionizing voltage of 50 eV. Silica gel of the "aqueous silicic acid" type was used for the chromatographic purification of the compounds obtained. The analyses of all the compounds corresponded to the calculated figures.

PAH of Sibiromycin (I). A solution of 10 g of sibiromycin in 100 ml of n-butanol was treated with 10 ml of concentrated hydrochloric acid. After 10 min, a yellow precipitate began to deposit from the solution. The reaction mixture was kept at room temperature for 1 h and then at 0-2°C for another 20 h.

The precipitate was separated off, washed with butanol and petroleum ether, and dried in vacuum over KOH (60°C/1 mm Hg). The dried product was dissolved in 50 ml of methanol and, with stirring, a 1 N solution of ammonia in methanol was added by drops to pH 7.0-7.5. A light-yellow precipitate of the free "product of acid inactivation" (PAI) deposited. The precipitate was separated off, washed with ether, and dried in vacuum at 60-70°C. Then it was recrystallized from n-butanol, giving 4-4.6 g of the pure PAI.

A suspension of 4 g of PAI in 100 ml of 6 N HCl was heated with stirring in the boiling water bath for 1 h. The whole of the PAI gradually passed into solution and simultaneously the separation of a crystalline precipitate of PAH (I) began. The reaction mixture was cooled and the precipitate was separated off and was washed with 50% aqueous pyridine solution and then water. It was dried in vacuum over KOH at 80°C. The dry product was charged into a Soxhlet apparatus and extracted with diethyl ether. The ether was distilled off and the residue was recrystallized from n-butanol. This gave about 2 g of pure PAH in the form of a bright-yellow substance with a decomposition temperature above 270°C.

Diacetate of PAH (II). To a suspension of 100 mg of PAH in 5 ml of pyridine was added 5 ml of acetic anhydride, and the reaction mixture was stirred at room temperature for 1 h. After some minutes, the whole of the PAH had dissolved, and then a light-yellow crystalline precipitate began to separate out from the solution. The precipitate was separated off, washed with water, and dried in vacuum over P_2O_5. After recrystallization from benzene, about 100 mg of pure (II) was obtained with mp 225-226°C.

Dimethyl Ether of PAH (III). Over 48 h, 500 mg of K_2CO_3 and 300 mg of dimethyl sulfate was added (in five portions) to a boiling solution of 200 mg of PAH in 50 ml of acetone. After only an hour, the whole of the PAH had been converted into the monomethyl ether (detected by means of TLC on silica gel). After the whole of the monomethyl ether had been converted into the dimethyl ether (III) (checked by TLC), the hot solution was separated from the precipitate of salts and was cooled. A light-yellow crystalline substance (III) separated out. After recrystallization from acetone, about 200 mg of chromatographically homogeneous (III) with mp 192-193°C was obtained.

Octahydro-PAH (IV). The hydrogenation of 500 mg of PAH was carried out in 25 ml of acetic acid in the presence of 250 mg of Pd/C (10%). As it was hydrogenated, the PAH passed into solution, which acquired an intense blue fluorescence. Over 3 h, about 160 ml of hydrogen (4 moles of H_2 per mole of PAH) had been absorbed. The reaction mixture was filtered through a layer of Celite to eliminate the catalyst. Then the solvent was driven off in vacuum and the residue was dried over KOH in vacuum at room temperature. The dried product was dissolved in the minimum amount of methanol and the (IV) was precipitated with diethyl ether. This gave practically pure (IV) in the form of a light-gray powder which darkened on storage; mol. wt. 290; λ_{max} (methanol), nm: 229, 260 (shoulder), and 350.

The substance (IV) (100 mg) was dissolved in 1 ml of 3 N HCl/MeOH, and 5 ml of ether was added. A white precipitate of the hydrochloride of (IV) deposited. Its chlorine content was about 11%.

Triacetate of (IV) (V). The acetylation of 200 mg of (IV) was performed in 10 ml of a mixture of acetic anhydride and pyridine (1 : 1) at room temperature for 48 h. The solvent was driven off in vacuum at 30°C and the residue was purified by chromatography on silica gel, the substance being eluted from the column with ethyl acetate–hexane (the amount of ethyl acetate in the mixture was increased gradually from 0 to 50%). The fractions of the eluate containing the pure triacetate (V) were combined and evaporated in vacuum to dryness, and the residue was recrystallized from ether–hexane (1 : 5). This gave (V) in the form of a white crystalline powder with mp 131-133°C.

N-Acetate of Dimethyl-IV (VI). An excess of an ethereal solution of diazomethane was added to a suspension of 100 mg of (IV) in 10 ml of methanol. After some minutes, the whole of the octahydro-PAH passed into solution. The reaction mixture was left in the dark at room temperature for 20 h, and then the solvent was driven off in vacuum at 25°C and the residue was acylated in 5 ml of acetic anhydride–pyridine (1 : 1) at room temperature for 20 h. The isolation and purification of the (VI) were performed in the same way as in the preceding experiment. After recrystallization from ether–n-hexane (1 : 20), (VI) was obtained in the form of a white crystalline powder with mp 161-163°C.

Alkaline Hydrolysis of the Dimethyl Ether of PAH. A tube was charged with 200 mg of the dimethyl ether of PAH (III), 20 ml of 0.15 N NaOH, and 10 ml of CH_3OH. The mixture was heated in the boiling water bath for 20 min, whereupon the yellow solid (III) gradually dissolved, the final solution being colorless. Then the reaction mixture was cooled. A crystalline precipitate of the pyrrolecarboxaldehyde (VII) deposited. The methanol was evaporated off in vacuum and the residue of (VII) was separated, dried over P_2O_5, and recrystallized from n-hexane. This gave 80 mg (more than 90%) of pure (VII) in the form of white acicular crystals with mp 115-116°C; λ_{max} (in ethanol), nm: 242, 250 (shoulder) and 325 (ε 19,000, 15,000, and 9800).

The filtrate after the separation of the (VII) was acidified with 2 N HCl to pH 5.0. The white crystalline precipitate of the acid (VIII) deposited. The precipitate was separated off, dried over P_2O_5, and recrystallized from n-hexane. This gave about 100 mg (~75%) of the pure acid (VIII) in the form of a white crystalline powder with mp 178°C; λ_{max} (in methanol), nm: 229, 250 (shoulder) and 350; λ_{max} (in 0.1 N HCl), nm: 220, 250, and 298; λ_{max} (in 0.1 N NaOH), nm: 228, 245 (shoulder), and 330.

Methyl Ester of the Acid (VIII) (IX). An excess of an ethereal solution of diazomethane was added to a solution of 50 mg of the acid (VIII) in 5 ml of ether. After an hour, the ether was distilled off and the residue was dissolved in a fresh portion of ether and the solution was extracted with 0.2 N NaOH to eliminate impurities of an acid nature. The ethereal solution was dried with Na_2SO_4, the solvent was driven off, and the residue was recrystallized from n-hexane. The yield was about 45 mg (~88%) of a white crystalline substance with mp 72-73°C.

SUMMARY

1. The acid hydrolysis of the dimethyl ether of the "product of acid hydrolysis" of the antitumoral antibiotic sibiromycin forms 4-(prop-1-enyl)pyrrole-2-carboxaldehyde and 3,6-dimethoxy-5-methylanthranilic acid.

2. The "product of acid hydrolysis" of sibiromycin is a derivative of pyrrolo-1,4-benzodiazepine.

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