HYDROGENATION DERIVATIVES OF RIFAMPICIN; THEIR TRITIUM LABELLING

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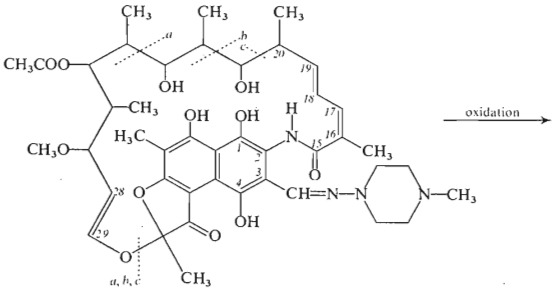
The structures of four rifampicin derivatives obtained by its catalytic hydrogenation were determined by mass and ^{1}H NMR spectroscopy. These are 16,17- and 18,19-dihydrorifampicin, its $\Delta^{17,18}$-isomer and 16,17,18,19-tetrahydrorifampicin. The $\Delta^{18,19}$- and $\Delta^{17,18}$-rifampicins were labelled by tritium.

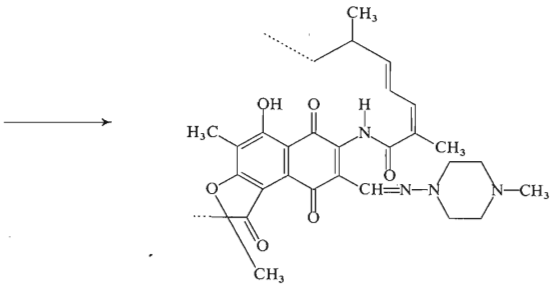
Tritium labelled antibiotic rifampicin with high specific radioactivity was prepared for the basic research of biological effects of its derivatives on the tumor cells[1]. With respect to the structure of rifampicin (*I*), *i.e.* 3-([[(4-methyl-1-piperazinyl)imino]-methyl)rifamycin SV, and the required specific radioactivity, we used for the tritium labelling the catalytic hydrogenation of rifampicin by tritium. We started with an assumption that the partial hydrogenation of *I* causes only a minor structure changes and that the biological activity of the derivative would be similar to that of the starting material. The catalytic hydrogenation of rifampicin S and SV, yielding the corresponding 16,17,18,19-tetrahydro derivative and 16,17,18,19,28,29-hexahydro derivative, respectively, is described in the literature[2,3]. It can be expected that with rifampicin, the hydrogenation of the 16,17- and 18,19-double bonds will be preferred. The aim of this work was to find the conditions of hydrogenation allowing the preparation in good yield and separation of the dihydro- and the tetrahydro derivatives of rifampicin and their labelling by tritium.

Paladium oxide exhibited the most suitable properties in the hydrogenation of *I*. The change of solvent, reaction time or hydrogen pressure, respectively, exerted a marked effect upon the composition of the reaction mixture. Using the thin-layer (TLC) and high-performance liquid chromatography (HPLC), it was found[4] that the reaction mixture besides the starting *I* almost invariantly consists of the five products, designed as B, C, D, E and F in order of their HPLC retention time. The UV and visible spectra of the compounds C, D, E, F are analogous to that of *I*. Therefore, it can be assumed that the chromophor system of benzene rings–conjugated double bond —CH=N— at the position 3 remained unchanged. A different UV and visible spectra exhibit the peak B compounds, which according to HPLC results were iden-

tified[4] as a mixture of the quinone forms of rifampicin and the compounds C, D, E and F. Those forms analogous to the structure *II* are formed by the oxidation of the hydroquinone ring and represent constant congeners of rifampicin and its hydrogenation derivatives.



I



II

The number of hydrogens introduced in the rifampicin molecule by hydrogenation follows from the shift of the M and M-1 ions in the mass spectra (with rifampicin *m/e* 822, 821, with C 826, 825, and with D, E, F 824, 823). Thus, the products are a tetrahydro derivative and three isomeric dihydro derivatives. An intense ion at *m/e* 389, ascribed to the chromophore part including the side chain attached at the

position 3[5] is present in the mass spectra of all five compounds. Also other intense ions in the spectra of the hydrogenation products correspond to the already described[5], fragmentation scheme *a*, *b*, *c*. In the ^{1}H-NMR spectra of the all five compounds there are signals of an isolated —CH=N— type proton from the side chain, H-29 doublet and H-28 quartet in the olefinic proton region. A comparison with the literature[6] shows that these double bonds were not hydrogenated. The tetrahydro derivative C exhibits signals due to the five secondary methyl groups (four signals of this type are observed with rifampicin), three methyl signals in the 2·00–2·40 ppm region (*i.e.* less one olefinic methyl) and only three signals at 4·80–6·00 ppm. From that follows that C is 16,17,18,19-tetrahydrorifampicin. The ^{1}H-NMR spectrum of E contains similarly to the starting rifampicin the signals of four secondary methyl groups and four methyl signals at 2·00–2·40 ppm. From that follows that the double bond at the position 16,17 remains intact. The double resonance experiments indeed confirmed the coupling of the δ 2·06 singlet to the multiplet at δ 5·79 (protons H-30, H-17). The signals of two protons are missing in the region 4·80–6·50 ppm. A comparison of the ^{13}C-NMR spectra of compound E and rifamycin[7] S also shows two sp^2-hybridized carbons missing. The above mentioned facts allows to formulate E as 18,19-dihydrorifampicin. The dihydro derivatives D and F contain each five signals of the secondary methyl groups and one signal of the olefinic methyl less than the starting compound. That indicates the hydrogenation of the 16,17-double bond confirmed by a removal of one olefinic proton. Unfortunately, we were unable on the basis of the 60 and 100 MHz ^{1}H NMR spectra to analyse the structures of the multiplets of both pairs of the mutually coupled olefinic protons. Two compounds come into consideration: $\Delta^{17,18}$ and $\Delta^{18,19}$-derivatives among them the decision can be made using the differences in their mass spectra. The mass spectrum of compound D contains a diagnostic ion m/e 425 whose formation is preferred in the $\Delta^{18,19}$-isomer only in which the breaked bond $C_{(16)}$—$C_{(17)}$ is in the β-position both to the carbonyl $C_{(15)}$ and to the double bond. Therefore, the compound D is 16,17-dihydrorifampicin and the compound F is its $\Delta^{17,18}$-isomer. The value $J_{17,18}$ = 15·6 Hz indicates a *trans*-arrangement.

The cytostatic activity of the compounds C, D, E and F was tested. The derivatives E, F exhibit a significant cytostatic activity on the tumor cells, in contrary to rifampicin[1]. To eliminate the possible effect of the traces Pd present in the hydrogenation products, the compounds E and F were subjected to neutron activation analysis. The content of Pd found was lower than $4 \cdot 10^{-5}$% what permits to exlude its biological effect.

The optimal conditions for the hydrogenation of rifampicin by tritium gas were chosen using the results of the nonradioactive experiments. The tritium labelled mixture of E, F was separated from the reaction mixture by column chromatography on silica gel; the obtained specific radioactivity was 1 000 GBq/mM (27 Ci/mM).

EXPERIMENTAL

The silica gel Silpearl was used for the column chromatography, for TLC were used the Silufol plates (both materials Kavalier, Votice, Czechoslovakia). Systems used: S1 benzene–chloroform–methanol 2 : 2 : 1; S2 chloroform–ethyl acetate–methanol 2 : 2 : 1; S3 benzene–chloroform–aceton–methanol 3 : 1 : 1 : 1; S4: benzen–techrachlormethan–chloroform–ether–methanol 2 : 2 : : 1 : 2 : 1; S5 benzene–tetrachloromethane–chloroform–methanol 2 : 2 : 1 : 1. HPLC was performed on a Varian AG-4100 apparatus with a Micropak-NH_2 column, in the system chloroform–methanol 99 : 1; the eluated were detected at 330 nm. NMR spectra were measured on the instruments Jeol FX-60 and Varian HA-100 (59·7965 and 100 MHz) in deuteriochloroform with tetramethylsilane as an internal standard. Mass spectra were measured using the Varian MAT 311 spectrometer (70 eV, direct inlet at 190—200°C).

Purification of rifampicin: A commercial medical preparate Rifadin for medical purposes (Lepetit, Galenica Beograd, UMB Drugs Factory Bucharest) was used as a starting[8] material. The carriers contained in this medicament were removed by its dissolving in chloroform. The unresolved particles were filtered off and the crude rifampicin was obtained after the evaporation of the solvent. It was used without any further purification for the non-radioactive hydrogenation. For the tritiation, the rifampicin was purified by column chromatography in S1.

Hydrogenation of rifampicin: *a*) PdO (80 mg) was reduced in dimethylformamide (10 ml); *I* (750 mg) was added and hydrogenated at the atmospheric pressure. One equivalent of hydrogen was consumpted after 45 minutes. The reaction was stopped, the catalyst filtered off, the solvent evaporated *in vaccuo* and the residue was separated on the silica gel column (3·5 × 30 cm, S1). This procedure favors the formation of E and F.

b) I (12 g) was dissolved in dimethylformamide (200 ml), PdO (0·5 g) was added and hydrogenated in an autoclave (initial pressure 1·4 MPa). If the reaction was stopped after the consumption of one equivalent of hydrogen, the formation of E and F prevailed. The prolonged reaction time (hydrogen consumption 1·5—2 hydrogen equivalents) yielded predominantly the compounds D and C, respectively. The crude separation of the reaction products was performed as in *a*).

Both procedures yield the reaction mixture containing in different proportions the starting *I* and its derivatives C, D, E, F and from them derived quinones, respectively. When working with a non-purified Rifadin, some other coloured impurities are detected by chromatography. They evidently come from the preparation of rifampicin; one of them is 3-formylrifamycin SV. For the separation of hydrogenated compounds it is recomended to use shorter columns of larger diameters. If the column is too long, the zones are diffuse out and the separation is worse.

Isolation of E and F: Roughly purified mixture of E and F from the column chromatography (S1) was purified using S4 and S5. The samples of E and F for the mass and NMR spectra were separated and finally purified by HPLC. ^{1}H NMR of E: —0·28 d (6·7 Hz, 3 H), 0·52 d (7·4 Hz, 3 H), 0·64 d (6·7 Hz, 3 H), 0·95 d (7·3 Hz, 3 H), 1·79 s (3 J, H-13), 2·05 s (3 H),2·06 s (3 H), 2·23 s (3 H), 2·41 s (3 H), 3·04 s (3 H, $COOCH_3$), 4·99 d (11·0 Hz, H-25), 5·18 dd (6·1 and 12·2 Hz, H-28), 5·79 mt (1 H, H-17), 6·29 d (12·2 Hz, H-29), 8·27 s (1 H), 12·05 s (1 H), 12·80 s (1 H). F: —0·25 d (6·7 Hz, 3 H), 0·51 d (6·7 Hz, 3 H), 0·80 d (6·9 Hz, 3 H), 0·97 d (7·3 Hz, 3 H), 1·33 d (7·3 Hz, 3 H), 1·78 s (3 H, H-13), 2·04 s (3 H), 2·23 s (3 H), 2·38 s (3 H), 3·05 s (3 H, $COOCH_3$), 4·92 d (10·4 Hz, H-25), 5·24 dd (12·8 and 7·3 Hz, H-28), 5·38 mt (1 H), 5·84 dd (15·6 and 4·3 Hz, 1 H), 6·30 d (12·8 Hz, H-29), 8·27 s (1 H), 12·03 s (1 H), 12·61 s (1 H), 12·87 br s (1 H).

Isolation of C: The crude derivative C obtained by separation in S1 was repeatedly purified in S2 (checked by HPLC). ^{1}H-NMR: —0·33 d (7·3 Hz, 3 H), 0·53 d (7·3 Hz, 3 H), 0·73 d (7·3 Hz, 3 H), 0·95 d (6·1 Hz, 3 H), 1·30 d (6·1 Hz, 3 H), 1·78 s (3 H, H-13), 2·06 s (3 H), 2·23 s (3 H), 2·37 s (3 H), 3·06 s (3 H, $COOCH_3$), 3·82 d (8·5 Hz, 1 H), 4·94 d (9·8 Hz, H-25), 5·14 dd (7·3

and 12·2 Hz, H-28), 6·26 d (12·2 Hz, H-29), 8·27 s (1 H), 12·07 s (1 H), 12·74 s (1 H), 12·74 s (1 H), 13·17 br s (1 H).

Isolation of D: For the final purification the systems S4 and S2 were used (HPLC checked). ^{1}H NMR: −0·42 d (6·8 Hz, 3 H), 0·42 d (6·8 Hz, 3 H), 0·72 d (6·4 Hz, 3 H), 0·96 d (6·8 Hz, 3 H), 1·79 s (3 H, H-13), 2·06 s (3 H), 2·23 s (3 H), 2·36 s (3 H), 3·03 s (3 H, $COOCH_3$), 4·90 d (10·7 Hz, H-25), 5·10 dd (12·7 and 8·3 Hz, H-28), 5·57 mt (2 H), 6·31 d (12·7 Hz, H-29), 8·22 s (1 H), 12·02 s (1 H), 12·93 s (1 H), 13·14 br s (1 H).

Tritiation of the mixture of E and F: Rifampicin purified by silica gel column chromatography (10—50 mg) was dissolved in dimethylformamide (1 ml) and hydrogenated over the pre-reduced PdO (about 20 mg) at the pressure 93 kPa using the tritium gas without any carrier. The tritium consumption stopped after taking one equivalent. Dimethylformamide was lyophylized, the residue dissolved in chloroform and tert-butanol mixture (1 : 1), the catalyst filtered off and the solvents were again removed by lyophylization. This procedure was repeated twice to get off the labile radioactivity. The reaction mixture was chromatographed on a silica gel column (2 × 12 cm, S3) with continuous monitoring the radioactivity of eluates. The fraction containing unseparated E and F was picked up. Its radiochemical purity was checked by TLC (Silufol, S3), and was higher than 95%. The total radioactivity was 17 GBq, specific radioactivity about 1 TBq/mM.

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