NEW METABOLITES OF *Streptomyces globisporus* AND *Streptomyces griseus*

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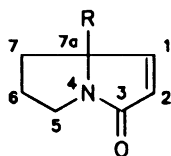
5,6,7,7a-Tetrahydro-3*H*-pyrrolizin-7a-ol-3-one (*VI*) and N-[2-(1-hydroxy-4-methylpentyl)]acetamide (*VII*) were isolated from submerged cultures of the macrotetrolide-producing strains *Streptomyces globisporus* and *S. griseus*. Proposed structures are based on spectroscopic measurements.

5,6,7,7a-Tetrahydro-3*H*-pyrrolizin-3-one (*I*), an Ehrlich-positive compound exhibiting no antimicrobial activity, was described first as a metabolite of *Streptomyces globisporus* 0234 (ref.[1]) and *S. griseus* LKS-1 (ref.[2]) co-produced with macrotetrolide antibiotics[1 – 4] and nonactic acids[1,2,5]. Later the same compound (named pyrrolam A) was isolated from culture broth of *S. olivaceus* Tü 3082 together with its derivatives lacking the 1,2-double bond (pyrrolams B *IIa*, C *IIb* and D *IIc*); in addition, two aliphatic hydroxy ketones (4-hydroxy-4-methyl-2-pentanone (*III*) and 7-hydroxy-4,7-dimethyl-3-hepten-2-one (*IV*)) were found. Compound *IIb* was obtained also from *IIa* by a slow conversion in solutions containing traces of water, whereas purified crystalline *I* (exposed to air for a longer time) formed *V* spontaneously in considerable yields[6].

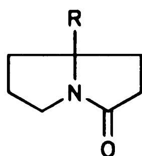
From cultures of both *S. globisporus* and *S. griseus*, a mixture of at least five Ehrlich-positive metabolites (fraction A) was obtained, besides *I*, by column chromatography. The isolation and identification of the major component of this complex (detectable in UV light) is reported in the present communication.

This compound exhibits a molecular ion *m/z* 139 ($C_7H_9NO_2$). According to ^{13}C NMR, eight hydrogen atoms are attached to carbons, the remaining one has to be bonded to a heteroatom. They form three aliphatic methylene and two olefinic methine groups. Mutual coupling between the olefinic protons (5.7 Hz) is diagnostic for a five-membered ring. Large differences between the proton and carbon chemical shifts of the olefinic atoms suggest conjugation of a carbonyl group to this double bond. However, a conjugated ketone moiety alone cannot explain the observed chemical shift of 175.54 ppm (carbonyl in 2-cyclopentenone resonates at 208.1 ppm, ref.[7]) so that an α,β-unsaturated amide must be assumed. Absence of coupling of the olefinic protons with the remaining protons requires presence of an unsubstituted carbon atom in its allylic position. COSY and decoupling experiments allow us to formulate a partial structure

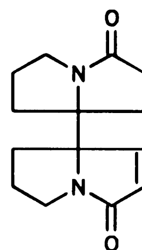
$N-CH_2-CH_2-CH_2$ ending with a quaternary carbon atom. The carbon chemical shift (99.92 ppm) suggests that two electronegative atoms are attached to it. There are only two atoms so far unassigned, logically forming an OH group that has to be placed at the quaternary carbon atom under discussion. The structure 5,6,7,7a-tetrahydro-3*H*-pyrrolizin-7a-ol-3-one (*VI*) is obtained. Losses of OH and C_2H_2 observed in the mass spectrum support this deduction. The compound did not show any measurable optical rotation; shift agents were not used with regard to the character of ^1H NMR spectrum.



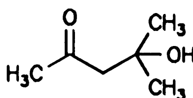
I, R = H
VI, R = OH



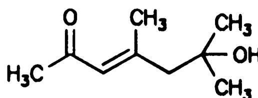
IIa, R = OCH_3
IIb, R = OH
IIc, R = $OCH(CH_3)OC_2H_5$



V



III



IV

HPLC of the fraction A yielded one Ehrlich-negative compound (fraction B). This compound of intermediate mobility displays a molecular ion at *m/z* 160 of weak intensity and several nitrogen-containing fragments (*m/z* 128, $C_7H_{14}NO$; 102, $C_4H_8NO_2$; 86, $C_5H_{12}N$) in its electron impact mass spectrum. Eight signals are observed in the ^{13}C NMR spectrum corresponding to three methyl, two methylene, two methine, and one carbonyl groups. Sixteen protons are observed in ^1H NMR. Thus, we arrive to the molecular formula $C_8H_{17}NO_2$. The presence of an acetyl group can be inferred from ^1H NMR and ^{13}C NMR spectra: a three-proton singlet at 2.016 ppm and resonances at 23.39 (q) and 171.04 (s) ppm. Signal at 66.32 (t) suggests a CH_2OH group (OH signal missing in ^1H NMR spectrum). However, a doublet (J = 6.9 Hz) at 5.666 ppm may be explained only by an $NHCOCH_3$ group. COSY and decoupling experiments provide unambiguously the structure *VII*: N-[2-(1-hydroxy-4-methylpentyl)]acetamide. This structure allows an easy rationalization of the MS

fragmentation. Ions *m/z* 128, 102, and 60 arrise by splitting the bonds α to nitrogen (in the later case with hydrogen transfer and protonation). The base peak is due to elimination of a CH_2OH group and ketene.

The structures of the compounds *VI* and *VII* described in the present work differ from those found previously for metabolites of *S. olivaceus* Tü 3082 (ref.[6]).

The other (minor) components of the fraction A (Ehrlich-positive, but – in contrast with *I* and *VI* – undetectable in UV light) were rather unstable. Each compound isolated by preparative TLC always returned into the initial mixture. In alkaline solution, the mixture was transformed to one chromatographically pure compound *VIII* (Ehrlich-positive, undetectable in UV light) with mobility different from that of *I* and *VI*. Absence of olefinic protons in ^{1}H NMR spectrum of *VIII* and distribution of carbon signals in ^{13}C NMR (83.81, 77.86, 61.36, 50.17, 38.45, 35.76, 32.58, 31.55, 30.98, 30.18, 23.18) point out to a deep degradation. Undetectability of the minor components of the fraction A in UV light suggests that these compounds, in contrast to *I* and *VI* lack the 1,2-double bond. Some of them may be identical with *IIa*, *IIb* and *IIc* (ref.[6]), but their structures could not be determined due to the above mentioned unstability.

Our additional studies proved the unstability of *I*. In contrast with previous data[6], however, *I* was transformed to *VI* (not to *V*) and, on the other hand, it originated from *VI* at the same rate, the resulting ratio being approximately 1 : 2 in both cases; the spontaneous reactions took place even in dry samples stored for a longer time, but it was accelerated considerably in solutions. Similar ratio of *I* and *VI* was found in culture broth of *S. globisporus* or *S. griseus*, however, it could be changed by both genetic and physiological manipulations with the respective strains[8]. This suggests that both non-enzymatic (spontaneous) and enzymatic (biosynthetic) reactions can participate in the formation of these compounds in the two strains. A possible relation of *VII* to the biosynthesis of *I* and *VI* still remains[5] to be studied.

Some preliminary findings on biological activity of *I* and *VI* were published elsewhere[9].

EXPERIMENTAL

NMR spectra (δ, ppm; *J*, Hz) were measured on a Varian VXR-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively) in $CDCl_3$ at 25 °C using tetramethylsilane as an internal standard. The assignment is based on COSY and APT experiments. Mass spectra were measured with a Finnigan MAT-90 instrument (positive ions, electron impact, 70 eV, direct inlet at 150 °C); perfluorokerosene was used as a reference for high resolution measurements (± 5 ppm).

The column chromatography was accomplished on silica gel L (100 – 160 mesh, Lachema, The Czech Republic) in the system chloroform–methanol 95 : 5 (S1). The TLC analysis and preparative chromatography were performed on silica gel coated plates (Silufol R[20], Kavalier, The Czech Republic), in ethyl acetate–methanol–water–ammonia 90 : 8 : 0.5 : 0.3 (S2) and chloroform–methanol–ammonia 92 : 8 : 0.03 (S3). The R_F values for *I* and *VI* were 0.38 and 0.22, respectively, in the system S2. Preparative HPLC was carried out on a Spectra Physics system SP 8000B using SF 770 variable wavelength detector at 215 nm.

Sample (50 mg/100 μl sample loop) was injected on a Chrompack Lichrosorb SI 60-5 column (250 × 22.5 mm, Merck) at 35 °C using hexane–isopropyl alcohol (2% water) 80 : 20 (S4) at 10 ml/min.

Fermentation

Streptomyces globisporus 0234 refs[1,3] and *S. griseus* LKS-1 refs[2,4] were grown aerobically at 28 °C for 4 days in a medium containing starch, soybean flour, distiller's solubles, molasses and $CaCO_3$; the second vegetative generation was inoculated with a 24 h-old seed culture[3].

Isolation of Metabolites

Cultures of both strains were processed in the same way and similar results were obtained in both cases. As an example, quantitative data on *S. griseus* are reported. Mycelium (950 g) separated from fermentation broth (11 l) was extracted with methanol (3 × 1 000 ml) at 25 °C. Filtrate of the broth was lyophilized and extracted three times with ethyl acetate–methanol–acetone (4 : 1 : 4). Both extracts of mycelium and broth were combined and evaporated to dryness. The residue was dissolved in water and pH adjusted with 0.1 M NaOH to 8.5 to remove nonactic acids[5] interfering in the chromatography and isolation of pyrrolizinones. The solution was extracted three times with ethyl acetate, combined extracts were dried with sodium sulfate and evaporated. The resulting oily residue (7.5 g) was processed by two procedures as follows:

a) The residue (3 g) was subjected to column chromatography in system S1. The fraction A (0.7 g) containing mainly the mixture of *I* and *VI* was purified by preparative TLC in S2. Compounds *I* and *VI* were separated and *VI* was re-purified by preparative TLC (with double developing) in S3. Colourless oil (46 mg) was obtained. After separating *I* and *VI*, the remaining parts of the fraction A were combined and treated with 0.5 M NaOH at 95 °C for 2 h. Reaction mixture was evaporated to dryness and analyzed by TLC in S2. It contained only one Ehrlich-positive compound *VIII* (R_F 0.32).

b) The residue (200 mg) was subjected to preparative HPLC. In addition to the fractions containing *I*, *VI* and other Ehrlich-positive components, 10.5 mg of *VII* (oil) were isolated.

5,6,7,7a-Tetrahydro-3*H*-pyrrolizin-7a-ol-3-one (*VI*)

Mass spectrum (*m/z*, rel. intensity): 139 (100, $C_7H_9NO_2$, M^+), 122 (11), 111 (71, $C_5H_5NO_2$), 98 (1), 83 (46, C_4H_5NO), 55 (11), 40 (10), 27 (8). ^1H NMR spectrum: 1.572 ddd, 1 H, *J* = 12.8, *J* = 11.6, *J* = 8.3; 2.122 ddd, 1 H, *J* = 12.8, *J* = 7.1, *J* = 2.0; 2.272 mt, 1 H, *J* = 12.8, *J* = 8.3, *J* = 8.3, *J* = 2.9, *J* = 2.0; 2.570 mt, 1 H, *J* = 12.8, *J* = 11.6, *J* = 8.8, *J* = 8.7, *J* = 7.1; 3.329 ddd, 1 H, *J* = 11.2, *J* = 8.8, *J* = 2.9; 3.487 ddd, 1 H, *J* = 11.2, *J* = 8.7, *J* = 8.3; 5.946 d, 1 H, *J* = 5.7; 7.102 d, 1 H, *J* = 5.7. ^{13}C NMR spectrum: 28.82 t, 33.94 t, 41.99 t, 99.92 s, 126.90 d, 149.73 d, 175.43 s. For $C_7H_9NO_2$ (139.2) calculated: 60.42% C, 9.07% H, 14.01% N; found: 60.88% C, 9.32% H, 14.13% N.

N-[2-(1-hydroxy-4-methylpentyl)]acetamide (*VII*)

Mass spectrum (*m/z*, rel. intensity): 160 (<1, M + 1), 128 (44, $C_7H_{14}NO$, M – CH_2OH), 102 (18, $C_4H_8NO_2$), 86 (100, $C_5H_{12}N$), 60 (21, C_2H_6NO), 44 (20), 43 (19), 30 (22). ^1H NMR spectrum: 0.929 d, 3 H, *J* = 6.6; 0.940 d, 3 H, *J* = 6.6; 1.357 mt, 2 H; 1.637 mt, 1 H; 2.016 s, 3 H; 3.531 dd, 1 H, *J* = 5.8, *J* = 11.0; 3.686 dd, 1 H, *J* = 3.5, *J* = 11.0; 4.045 mt, 1 H; 5.666 d, 1 H, *J* = 6.9. ^{13}C NMR spectrum: 22.19 q, 22.97 q, 23.39 q, 24.91 d, 40.21 t, 50.16 d, 66.32 t, 171.04 s. For $C_8H_{17}NO_2$ (159.3) calculated: 60.35% C, 10.78% H, 8.79% N; found: 60.47% C, 10.52% H, 8.58% N.

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