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Oxaspirodion, a New Inhibitor of Inducible TNF-α Expression from the Ascomycete *Chaetomium subspirale*

Production, Isolation and Structure Elucidation

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In a search for new inhibitors of the TNF-α promoter activity, a new spiro-compound, designated oxaspirodion, was obtained as a mixture of four isomers from fermentations of the ascomycete *Chaetomium subspirale*. The structure was determined by a combination of spectroscopic techniques.

TNF-α is the main pro-inflammatory cytokine in inflammatory diseases like septic shock, rheumatoid arthritis and Crohn's disease. Therefore, low molecular weight compounds that interfere with the regulation of TNF-α expression may represent novel therapeutic strategies to manipulate TNF-α levels in immunopathological processes in which TNF-α plays a destructive role[1,2]. In order to search for inhibitors of the inducible TNF-α expression, we developed a cell-based screening system by using a human TNF-α promoter driven transcriptional reporter in Jurkat T-cells. A screening of 1700 mycelial cultures of basidiomycetes, ascomyctes and imperfect fungi for the production of compounds inhibiting the inducible expression of the hTNF-α driven luciferase reporter gene construct resulted in the isolation of oxaspirodion (**1**) from fermentations of the ascomycete *Chaetomium subspirale*. As described in the following paper, oxaspirodion inhibited the TNF-α driven luciferase reporter gene expression with an IC_{50}-value of 2.5 μg/ml (10 μM) in TPA/ionomycin stimulated Jurkat T-cells by interfering with signal transduction pathways involved in the inducible expression of many pro-inflammatory genes[3]. In this paper we report the isolation and structural elucidation of oxaspirodion (**1**).

Material and Methods

Producing Organism, Fermentation and Isolation of Oxaspirodion

The ascomycete strain D99007 was isolated from deer-dung and identified as *Chaetomium subspirale* according to AMES[4] and DOMSCH *et al.*[5]. The strain is deposited in the culture collection of the Dept. of Biotechnology, University of Kaiserslautern.

For maintenance on agar slants the strain was kept on YMG medium (10 g/liter malt extract; 4 g/liter glucose; 4 g/liter yeast extract and 1.5% agar for solid media; pH 5.5). For submerged cultivation, strain D99007 was grown in YMG medium (pH 5.5). A well-grown seed culture of *C. subspirale* D9907 (200 ml YMG medium) was used to inoculate a Biolafitte C-6 fermenter containing 20 liters of YMG medium with aeration (3 liters air/minute) and agitation (120 rpm) at 22°C. The production of oxaspirodion (**1**) was followed by the inhibitory effect of various concentrations of a crude extract of the culture fluid on the TNF-α promoter activity as described[6]. After 50 hours, the culture fluid was separated by filtration and extracted with EtOAc (15 liters). The solvent was evaporated and the crude product (950 mg) was separated

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by chromatography on Sephadex LH-20 with MeOH as eluent resulting in 405 mg of an enriched product. Preparative HPLC (LiChrosorb RP18, 2.5×25 cm column) with H_2O : acetonitril (40 : 60) as eluant yielded 16.1 mg of pure oxaspirodion (**1**).

Spectroscopy

Spectral data were recorded with the following instruments: ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probe head equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine-shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH}$=145 Hz and $^nJ_{CH}$=10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). FAB mass spectra were recorded with a Jeol SX102 spectrometer, while the UV and the IR spectra were recorded with a Perkin-Elmer λ 16 and a Bruker IFS 48 spectrometer. The optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22°C.

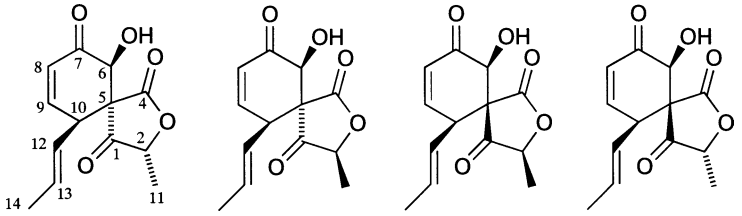
Oxaspirodion

Oxaspirodion (**1**) was obtained as brown oil. It consists of a mixture of four isomers which could not be separated from each other (see discussion), in the following proportions (according to the integrals of the 11-H_3 signals in the ^{1}H NMR spectrum): 34, 28, 22 and 16%. UV (MeOH) λ_{max} nm (log ε) 357 (3.00) and 242 (3.57). IR (KBr) cm^{-1} 3440, 2925, 1800, 1755, 1700, 1450, 1375, 1340, 1260, 1085, 975, 875 and 745. ^{1}H NMR at 500 MHz in $CDCl_3$ (δ, mult., J in Hz) 6.73, 6.67, 6.67 and 6.62, dd, 2.4 and 10.2, 9-H; 6.28, m, 8-H; 5.83, 5.79, 5.73 and 5.70, dqd, 0.7, 6.5 and 15.3, 13-H; 5.36, 5.35, 5.29 and 5.25, qdd, 1.7, 9.2 and 15.3, 12-H; 4.80, 4.62, 4.60 and 4.49, q, 7.1, 2-H; 4.60, 4.59, 4.51 and 4.49, s, 6-H; 3.86, 3.84, 3.74 and 3.68, m, 10-H; 1.70, m, 14-H_3; 1.53, 1.45, 1.41 and 1.37, d, 7.1, 11-H_3. ^{13}C NMR at 125 MHz in $CDCl_3$ (δ) 209.5, 209.4, 209.1 and 208.8, C-1; 195.1, 194.7, 194.7 and 194.3, C-7; 172.8, 172.7, 171.5 and 171.4, C-4; 148.9, 148.8, 148.0 and 147.8, C-9; 134.4, 133.9, 133.9 and 133.9, C-13; 126.4, 126.4, 126.1 and 126.1, C-8; 125.6, 125.3, 124.6 and 124.0, C-12; 81.5, 81.4, 81.2 and 81.1, C-2; 73.7, 73.5, 72.7 and 72.4, C-6; 61.8, 61.6, 61.0 and 61.0, C-5; 45.2, 45.2, 44.0 and 44.0, C-10; 18.0, 17.9, 17.9 and 17.8, C-14; 15.6, 15.4, 15.2 and 15.2, C-11. HRFABMS $[M+H]^+$ m/z 251.0929 (required for $C_{13}H_{15}O_5$, 251.0920).

Results and Discussion

The structure of oxaspirodion was determined by a combination of spectroscopic techniques. High resolution MS experiments indicated that the composition of the metabolite is $C_{13}H_{14}O_5$, and although there are many more than 13 signals in the ^{13}C NMR spectrum, it is obvious that there are 13 sets of distinct signals, each of which are quadruple. This suggests that oxaspirodion was obtained as a mixture of non-separable isomers, and extensive analysis of the NMR data confirmed that this is the case. The unsaturation index for the compound is 7, and as the ^{13}C NMR data suggest that it contains three carbonyl groups and two carbon–carbon double bonds there must consequently be two rings. The ^{1}H NMR signals for 11-H_3 were four well separated doublets, and the COSY coupling to 2-H as well as HMBC coupling to C-2 and C-1 demonstrate the link from C-11 *via* C-2 to C-1. 2-H, appearing as four quartets with the corresponding coupling constants in the ^{1}H NMR spectrum, also gives HMBC coupling to C-4, indicating that C-2 is linked to an acyl group in an ester or a lactone. This is supported by the chemical shifts of 2-H, C-2 and C-4. COSY couplings from the second methyl group (C-14) show that it is attached to the C-13/C-12 double bond, which in turn is connected to C-10. The *E* configuration of the C-13/C-12 double bond is established in all four isomers by the large 12-H/13-H ^{1}H-^{1}H coupling constant (15.3 Hz). C-10 is attached to the C-8/C-9 double bond: both 8-H and 9-H give COSY correlations with 10-H, and 9-H gives a HMBC correlation to C-12 while 12-H gives an HMBC correlation to C-9. The C-8/C-9 double bond is *Z*, as indicated by the 8-H/9-H ^{1}H-^{1}H coupling constant (10.2 Hz). 10-H consequently gives HMBC correlations to C-8, C-9, C-12 and C-13, and in addition to C-1, C-4 and C-5. 9-H gives an HMBC correlation to C-7, suggesting that C-7/C-8/C-9 constitutes a conjugated system, and this is supported by the chemical shifts of the protons and carbons involved. In addition, 9-H gives HMBC correlations to C-10 and C-5, suggesting that C-5 is the third substituent on C-10. 8-H gives HMBC correlations to C-10, as expected, and to C-6, an oxidized carbon, whose single proton appears as a singlet in the ^{1}H NMR spectrum. 6-H gives HMBC correlations to C-5 and C-7, confirming the position of C-6 next to C-7 and closing the six membered ring with the connection C-6/C-5/C-10.

Fig. 1. Structure of the stereoisomers of oxaspirodion (**1**) .



The second ring is formed by connecting C-1 and C-4 to C-5.

The analysis shows that all four isomers have the same planar structure, and they should therefore have different configurations. Both carbon–carbon double bonds are the same for all, as discussed above. The molecule contains four stereocenters, C-2, C-5, C-6 and C-10, and of these the two latter appear to have the same relative configuration as a strong NOE can be observed between 6-H and 10-H in all four isomers in the NOESY spectrum. This also suggests that both 6-OH and 10-propenyl are equatorial. The four isomers must then be the result of a mixture of epimers at the spiro center and at C-2. There is a strong NOE between 13-H and 10-H and a weak NOE between 12-H and 10-H, suggesting that the C-12/C-13 double bond is perpendicular to the six membered ring with 13-H pointing in the same direction as 10-H in the predominant conformation. In one of the isomers (the minor) an NOE is observed from 11-H_3 to 12-H, while in another (the second largest) NOEs are observed to both 12-H and 13-H, supporting the proposal that oxaspirodion is a mixture of the four diastereomers indicated in Figure 1. However, it is hazardous to assign a relative stereostructure to each isomer. The isolate has optical activity ($[\alpha]_D^{22}$ +19°, *c* 0.5 $CHCl_3$), suggesting that the diastereomers are not racemic mixtures, although it has not been possible to determine the absolute configuration of C-6/C-10.

The unusual 2-oxaspiro[4,5]decane skeleton of oxaspirodion has been reported for several fungal metabolites including the antibacterial oxaspirol A from *Rhodutorula glutinis*[7], the cytotoxic mycosporulone from *Coniothyrium sporulosum*[8], the arthropsolides from *Arthropsis truncata*[9] and the recently isolated massarigenins from the aquatic fungus *Massarina tunicata*[10].

Oxaspirodion exhibited no antibacterial and antifungal activities up to 100 μg/disk in the standard disk assay. Only weak cytotoxic properties against tumor cells could be observed starting form 50 μg/ml oxaspirodion.

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