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Pyrocoll, an Antibiotic, Antiparasitic and Antitumor Compound Produced by a Novel Alkaliphilic *Streptomyces* Strain†

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A new secondary metabolite was detected in the culture extract of *Streptomyces* sp. AK 409 by HPLC-diode-array screening. The metabolite was identified as pyrocoll, which is known to be a constituent of cigarette smoke. Pyrocoll is known as a synthetic compound, but until now had not been isolated as a natural product from a microorganism. The compound showed biological activity against various *Arthrobacter* strains, filamentous fungi, several pathogenic protozoa, and some human tumor cell lines.

Alkaliphilic and alkalitolerant actinomycetes were included in our screening program to detect novel secondary metabolites by HPLC-diode array analysis (HPLC-DAD). Strain AK 409, which was isolated from steel waste tip soil, became attractive to us because of the appearance of two prominent metabolites in a culture extract which had retention times of 3.8 and 7.9 minutes. These metabolites did not correspond to any of the 700 reference compounds stored in our HPLC-UV-Vis-Database[2].

The compound with a retention time of 3.8 minutes was identified by determining the chemical structure as pyrrole-2-carboxylic acid (**1**), a known natural product[3]. The main compound had a retention time of 7.9 minutes, showed UV maxima at 236, 276, 308 and 320 nm, and was identified as pyrocoll (**2**), *i.e.* the cyclic condensation product of two molecules of **1**.

This report deals with the taxonomy of the producing strain, its fermentation, the isolation and structural elucidation of the metabolites, and some as yet unknown biological properties of pyrocoll.

Materials and Methods

General Experimental Procedures

Melting points were determined on a Reichert-Jung Thermovar hot-plate and are uncorrected. IR spectra were taken on a Perkin-Elmer 1429 spectrophotometer; ^{1}H NMR (600.1 MHz) and ^{13}C NMR (150.9 MHz) spectra were measured on a Bruker DMX 600 instrument using $CDCl_3$ (δ 7.26 and 77.01) and CD_3OD (δ 3.30 and 49.01) as solvents and internal ^{1}H and ^{13}C standards. Proton-detected, heteronuclear correlations were measured using HMQC

† Art. No. 29 in 'Biosynthetic Capacities of Actinomycetes', Part 8 in 'Diversity and Biosynthesis of Secondary Metabolites'. Art. No. 28 and Part 7, respectively: See ref. 1

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(optimized for $^{1}J_{HC}$=145 Hz) and HMBC (optimized for $^{n}J_{HC}$=7 Hz). EIMS and HRMS were determined on Finnigan MAT 8200 and Finnigan MAT 90 instruments (70 eV).

Microorganisms

The producing strain AK 409 was isolated from a steel waste tip soil sample collected at Consett, County Durham, UK. The strain is deposited in the culture collection of the University of Newcastle.

Standard strains for testing the biological activity spectrum and minimal inhibition concentrations were obtained from the ATCC, CBS, DSMZ and the stock collections of our laboratories in Tübingen and Basle.

Taxonomy of the Producing Strain

The organism was inoculated onto oatmeal agar[4] (ISP3), incubated at 25°C for 3 weeks, and examined by eye to determine aerial spore mass colour, substrate mycelium pigmentation and the colour of any diffusible pigments; the colours were recorded using National Bureau of Standards (NBS) Colour Name Charts[5,6]. Plugs of agar taken from the ISP 3 agar plate were used to record spore chain morphology and spore ornamentation by scanning electron microscopy following the procedure described by O'DONNELL *et al.*[7]. The isomeric form of diaminopimelic acid (A_2pm) was determined by TLC of a whole-organism hydrolysates following a standard procedure[8]. 16S rDNA amplification and sequencing were carried out after KIM *et al.*[9].

Fermentation

Batch fermentations of *Streptomyces* AK 409 were carried out in a 10-liter stirred tank fermenter (type Biostat E; B. Braun Melsungen International). The medium consisted of starch 1%, glucose 1%, glycerol 1%, corn steep powder 0.25%, casein peptone 0.5%, yeast extract 0.2%, and NaCl 0.1% in tap water. The fermenter was inoculated with 5 vol.-% of shake cultures, grown in 500-ml Erlenmeyer flasks with one baffle for 48 hours on a rotary shaker at 120 rpm and 27°C using the same medium. The fermentation was carried out for 72 hours at 27°C with an aeration rate of 0.4 vvm and an agitation of 250 rpm. The pH of the culture was kept constant at pH 9 during the cultivation.

Isolation

Hyphlo Super-cel (2%) was added to the fermentation broth, which was separated by multiple sheet filtration into culture filtrate and mycelium. The culture filtrate (8 liters) was adjusted to pH 9 (5 N NaOH) and extracted three times with 2 liters of cyclohexane (extract I). Extract I, which contained **2**, was concentrated *in vacuo* to dryness. The aqueous layer was adjusted to pH 5 (5 N HCl) and re-extracted three times with EtOAc (extract II). Extract II, which contained compound **1**, was concentrated *in vacuo* to dryness.

Extract I (containing **2**) was dissolved in a small volume of MeOH and purified by preparative reversed-phase HPLC using a stainless steel column (250×16 mm) filled with 10-μm Nucleosil-100 C-18 (Maisch) and linear gradient elution with 0.5% formic acid-MeOH, starting from 40% MeOH to 80% MeOH within 20 minutes at a flow rate of 24 ml/minute. The preparative system consisted of two high pressure pumps (Sepapress HPP-200/100; Kronwald), a gradient unit (Sepacon GCU-311), and a Valco preparative injection valve (Mod. 6UW; VICI) with a 5 ml sample loop. The UV absorbance of the eluate was monitored at 319 and 340 nm using a Gilson spectrophotometer Mod. 116 equipped with a preparative cell. After concentration *in vacuo* to dryness, **2** was obtained as a white powder, which is soluble in acetone and moderately soluble in MeOH.

Extract II (containing **1**) was dissolved in a small volume of MeOH and purified by exclusion chromatography using Fractogel TSK HW-40 F, followed by preparative reversed-phase HPLC using 10-μm Nucleosil-100 C-18 material and linear gradient elution with 0.5% formic acid-MeOH starting from 20% MeOH to 60% MeOH within 20 minutes at a flow rate of 24 ml/minute. The UV absorbance of the eluate was monitored at 260 and 270 nm. After concentration *in vacuo* to dryness, compound **1** was obtained as a white powder, which was found to be soluble in MeOH.

HPLC-DAD Analyses

The chromatographic system consisted of an HP 1090M liquid chromatograph equipped with a diode-array detector and HP Kayak XM 600 ChemStation and HPLC-software revision A.08.03 (Agilent Technologies). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360 and 435 nm. The spectrum measured was from 200 to 600 nm with a 2-nm step and a sampling rate of 640 mseconds.

A 5-ml sample of the fermentation broth was adjusted to pH 5 (1 N HCl) and extracted with the same volume of EtOAc. After centrifugation, the organic layer was concentrated to dryness *in vacuo* and the residue was resuspended in 0.5 ml MeOH. 10 μl of the samples were injected onto an HPLC column (125×4.6 mm), fitted with a guard-column (20×4.6) which were packed with 5-μm

Nucleosil-100 C-18 (Maisch). The samples were analyzed by linear gradient elution using 0.1% *ortho*-phosphoric acid as solvent A and acetonitrile as solvent B at a flow rate of 2 ml/minute. The gradient was from 0% to 100% solvent B in 15 minutes with a 1-minute hold at 100% solvent B, followed by a 5-minute post-time at initial conditions.

Structure Elucidation

Pyrrole-2-carboxylic acid (**1**): All physical and spectroscopic data were identical to those described in the literature[3] and to a sample purchased from Aldrich; colorless crystals (EtOAc-CH_2Cl_2): mp 190°C [ref. 3 (EtOAc-CH_2Cl_2): 190°C]; ^{1}H NMR (600 MHz, CD_3OD) δ 6.17 (1H, dd, J=3.8, 2.6 Hz, 4-H), 6.86 (1H, dd, J=3.8, 1.5 Hz, 3-H), 6.93 (1H, dd, J=2.6, 1.5 Hz, 5-H); ^{13}C NMR (150 MHz, CD_3OD) δ 110.7 (C-4), 116.7 (C-3), 123.8 (C-2), 124.5 (C-5), 164.6 (2-COOH); EIMS *m/z* 112 $[M+1]^+$ (6), 111 $[M]^+$ (100), 94 $[M-OH]^+$ (17), 93 $[M-H_2O]^+$ (81), 66 $[M-COOH]^+$ (20), 65 (34).

Pyrocoll (**2**): All physical and spectroscopic data were completely consistent to those described in the literature[10]; mp 270°C [ref. 10: 272~273°C]; IR ν_{max} (NaCl) 3115 (s), 2928 (s), 2864 (m), 1699 (s), 1650 (m), 1627 (m), 1560 (m), 1460 (s), 1412 (m), 1319 (m), 1220 (w), 737 (m) cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.48 (1H, dd, J=3.5, 3.1 Hz, 4-H), 7.37 (1H, dd, J=3.7, 1.5 Hz, 3-H), 7.72 (1H, dd, J=3.0, 1.5 Hz, 5-H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 114.7 (C-4), 123.4 (C-3), 123.7 (C-5), 124.3 (C-2), 150.9 (2-CO); EIMS *m/z* 187 $[M+1]^+$ (11), 186 $[M]^+$ (100), 158 $[M-CO]^+$ (5), 130 $[M-2\times CO]^+$ (13), 93 $[C_5H_3NO]^+$ (49), 65 (19); HRMS: 186.04293 ($C_{10}H_6N_2O_2$ calcd. 186.04294).

Improved synthesis of pyrocoll (**2**): To a solution of 100 mg (0.90 mmol) **1** in 20 ml CH_2Cl_2, 204 mg (0.99 mM) DCC and 10 mg (0.09 mM) DMAP were added carefully, and the reaction mixture was stirred at 25°C for 24 hours. After filtration of the precipitate the solvent was removed *in vacuo*. Purification of the crude product by silica gel column chromatography (petroleum ether-CH_2Cl_2, 2:1) afforded **2** (76.5 mg, 0.41 mM, 91.3%). ^{1}H and ^{13}C NMR spectra were identical with the spectra of an authentic sample of **2**.

Biological Assays

The antimicrobial spectrum of pyrocoll was determined by an agar plate diffusion assay. 20 μl of the samples were added on filter discs (6 mm diameter). The test plates were incubated for 24 hours at the temperature that permitted optimal growth of the test organisms.

For determining the minimal inhibition concentration of pyrocoll the broth dilution method was used. The antibiotic was dissolved in DMSO; the final DMSO concentrations in the cultures did not exceed 5%. The test organisms were grown in a medium consisting of nutrient broth 0.8%, NaCl 0.5% and deionized water. 10^6 cells/ml were used as inoculum, and growth inhibition was evaluated after incubation for 24 and 48 hours at 27°C on a rotary shaker.

The inhibitory activity of **2** on the growth of tumor cells was tested according to NCI guidelines[11], with human cell lines from gastric adenocarcinoma (HMO2), breast carcinoma (MCF 7), and hepatocellular carcinoma (HepG2). Cells were grown in 96-well microtiter plates in RPMI 1640 with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 in air. After 24 hours incubation, compound **2** (0.1~10 μg/ml) was added to the cells. Stock solutions were prepared in DMSO. The final DMSO concentration in the cultures was 0.1%. After a 48-hour incubation period the cells were fixed, and the cell protein determined with sulforhodamine B.

The cytostatic potential of **2** was determined in cell culture assays using modifications of the tetrazolium-formazan assay system[12,13]. L5178y mouse lymphoma cells (ATCC CRL 1722), HeLa S3 human epitheloid carcinoma cells (ATCC CCL 2.2) or PC-12 rat adrenal pheochromocytoma cells (ATCC CRL 1721) were used for the experiments. L5178y; HeLa S3 were grown in RPMI-medium, supplemented with 10% fetal calf serum and PC-12 cells in RPMI-medium enriched with 10% horse serum-3% fetal calf serum. For the dose-response experiments, 200 μl cultures were initiated by inoculation of 5×10^3 cells/ml (L5178y), 30×10^3 cells/ml (HeLa S3) or 50×10^3 cells/ml (PC-12) and the preparations incubated at 37°C for 72 hours in a fully humidified atmosphere of 5% CO_2. Compound **2** was added to the cultures at time zero. At the time of termination, after 72 hours, the cells were still in the logarithmic proliferation phase. They were then treated by the addition of 50 μl of a 2 mg/ml solution of MTT (Sigma no. M-2128); after a further incubation period of 3 hours, 50 μl of aqueous $NaDodSO_4$ (20%) was added and the optical density determined at 595 nm using an ELISA reader. For the statistical evaluation the student's *t*-test was applied; the 50% inhibition of cell growth (ED_{50} value) was calculated by logarithmic regression[14].

Plasmodium falciparum: Antiplasmodial activity was determined using the NF54 strain of *P. falciparum* (sensitive to all known drugs) and the K1 strain (resistant to chloroquine and pyrimethamine). A modification of the [^{3}H] hypoxanthine incorporation assay[15] was used[16]. Briefly, infected human red blood cells were exposed to serial drug dilutions in microtiter plates for 48 hours at

37°C in a gas mixture with reduced oxygen and elevated CO_2 concentrations. [^{3}H] Hypoxanthine was added to each well and after further incubation for 24 hours the wells were harvested on glass fiber filters and counted in a liquid scintillation counter. The IC_{50} value was calculated from the sigmoidal inhibition curve. The assays were run in duplicate and repeated at least once.

Trypanosoma cruzi: Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well/100 μl in RPMI 1640 medium with 10% FBS and 2 mM L-glutamine. After 24 hours 5000 trypomastigotes of *T. cruzi* (Tulahuen strain C2C4 containing the galactosidase (Lac Z) gene) were added in 100 μl per well with 2× of a serial drug dilution. The plates were incubated at 37°C in 5% CO_2 for 4 days. The substrate CPRG/Nonidet was added to the wells for measurement of the IC_{50}. The color reaction which developed during the following 2~4 hours was read photometrically at 540 nm. IC_{50} values were calculated from the sigmoidal inhibition curve. Cytotoxicity was assessed in the same assay using non-infected L-6 cells and the same serial drug dilution. The MIC was determined microscopically after 4 days.

Trypanosoma b. rhodesiense: Minimum Essential Medium (50 μl) supplemented according to BALTZ *et al.*[17] with 2-mercaptoethanol and 15% heat-inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were added to the wells followed by 50 μl of a trypanosome suspension (*T. b. rhodesiense* STIB 900) and the plate incubated at 37°C under a 5% CO_2 atmosphere for 72 hours. Alamar Blue (10 μl) was then added to each well and the incubation continued for a further 2~4 hours. The plate was then read using a Millipore Cytofluor 2300 at an excitation wavelength of 530 nm and an emission wavelength of 590 nm[18]. Fluorescence development was expressed as a percentage of the control, and IC_{50} values determined.

Leishmania donovani: Mouse peritoneal macrophages were seeded in RPMI 1640 medium with 10% heat-inactivated FBS in Lab-tek 16-chamber slides. After 24 hours. *L. donovani* amastigotes (strain: MHOM-ET-67/L82) were added at a ratio of 3 : 1 (amastigotes to macrophages). The medium containing free amastigotes was replaced by fresh medium 4 hours later. The next day the medium was replaced by fresh medium containing different drug concentrations. The slides were incubated at 37°C under a 5% CO_2 atmosphere for 96 hours. The medium was then removed, and the slides fixed with MeOH and stained with Giemsa. The ratio of infected to non-infected macrophages was determined microscopically, expressed as a percentage of the control and the IC_{50} value calculated by linear regression.

Results

Taxonomy of the Producing Strain

Strain AK409 produced a white to grey aerial spore mass and a yellow-brown pigmented substrate mycelium on oatmeal agar; diffusible pigments were not formed. Whole-organism hydrolysates were rich in LL-A_2pm. Comparison of the almost complete16S rDNA sequence of the strain with available corresponding sequences of representative actinomycetes showed that it forms a distinct phyletic within the *Streptomyces griseus* 16S rDNA subclade.

Fermentation and Isolation

Batch fermentations were carried out in a 10-liter stirred tank fermenter using a complex medium. The growth of strain AK 409 was significantly increased at alkaline pH values. During pH-static fermentations at pH 9, the culture reached a maximal biomass of 32 vol-% after 40 hours of incubation, and maximal pyrocoll production reaching 23 mg/litre was obtained after 49 hours. The HPLC analysis of a culture extract is shown in Fig. 1.

Pyrocoll (**2**) was isolated from the culture filtrate by extraction with cyclohexane at pH 9, and was purified by preparative reversed-phase HPLC using Nucleosil C-18 material and linear gradient elution with 0.5% formic acid-MeOH. After concentration to dryness *in vacuo*, pyrocoll (**2**) was obtained as a white powder which is soluble in Me_2CO and moderately soluble in MeOH.

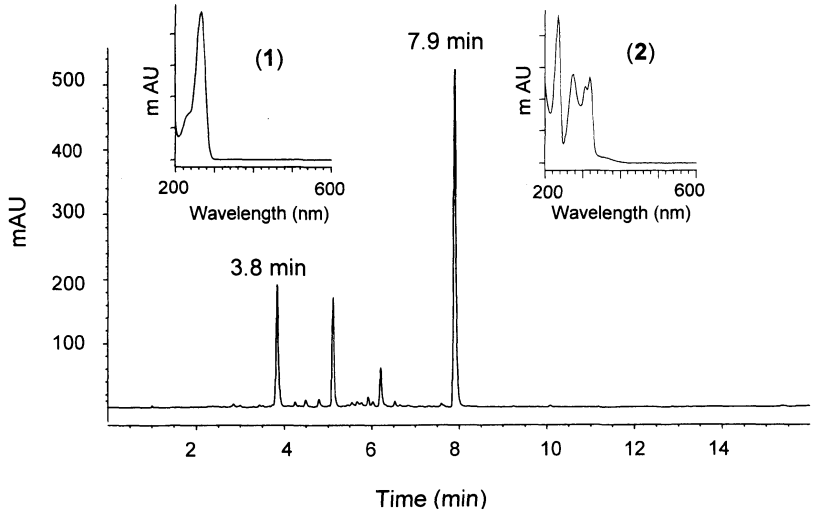
Pyrrole-2-carboxylic acid (**1**) was isolated from the culture filtrate by extraction with EtOAc at pH 5 followed by subsequent purification using size-exclusion chromatography on Fractogel TSK HW-40, and preparative reversed-phase HPLC on Nucleosil C-18 material and linear gradient elution with 0.5% formic acid-MeOH. After concentration to dryness *in vacuo*, pyrrole-2-carboxylic acid (**1**) was obtained as a white, MeOH-soluble powder.

Structural Elucidation

Already by its typical MS fragmentation pattern[19], compound **1** was identified as the well known pyrrole-2-carboxylic acid. All physical and spectroscopic data were identical to those reported in the literature[2] and to a sample purchased from Aldrich.

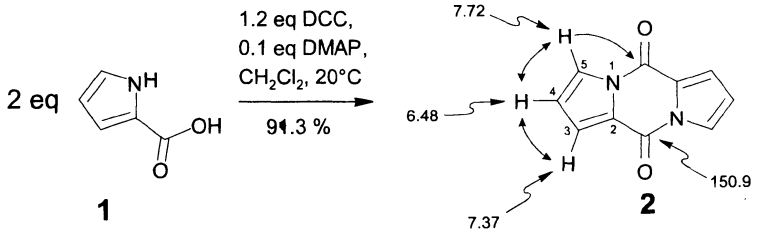
The second compound isolated had the molecular

Fig. 1. HPLC analysis of a culture extract of *Streptomyces* strain AK 409 at a fermentation time of 49 hours, monitored at 280 nm.



3.8 minutes=pyrrole-2-carboxylic acid (**1**); 7.9 minutes=pyrocoll (**2**). Inserts: UV-visible spectra of **1** and **2**.

Fig. 2. Synthesis of pyrocoll (**2**) from pyrrole-2-carboxylic acid (**1**) and selected NMR data of **2**.



^1H and ^{13}C NMR shifts (δ values in ppm), as well as HMBC (single arrow) and H,H-COSY (double arrows) interactions.

formula $C_{10}H_6N_2O_2$, as deduced by HRMS of the $[M]^+$ peak. The ^1H NMR spectrum showed the signals of three neighboring aromatic hydrogens (δ 6.48, 7.37, and 7.72) (see Fig. 4). The ^{13}C NMR spectrum revealed resonances corresponding to a carbonylic carbon belonging to an amide function (δ 150.9), three tertiary (δ 114.7, 123.4, 123.7) and one quaternary (δ 124.3) aromatic carbons. The resulting molecular formula (C_5H_3NO) compared to the one mentioned above, as indicated by HRMS, suggested that the molecule was symmetric. A first hint at the structure of the molecular moiety was given by the small coupling constants of the aromatic protons, indicating a pyrrole-system. The cyclic structure was confirmed by the HMBC experiment, because every hydrogen showed interactions to all of the carbon atoms. Because of the spin system of three neighboring protons the carbonyl had to be located at C-2 of the pyrrole system. The two molecular moieties had to be coupled by linkages between the carbonyl C-atom of the one and the pyrrole nitrogen of the other portion for the 'dimer' to attain the afore-mentioned symmetry and to explain the HMBC interaction between 5-H and the carbonyl-carbon. Thus, the second compound had to have structure **2** (Fig. 2). This diketopiperazine has previously been described as a synthetic product[10] and as a constituent

Table 1. Antibacterial and antifungal spectrum of pyrocoll (**2**) determined by the agar plate diffusion assay at various concentrations.

Organism	Inhibition zone (mm) 1 mg/ml	0.3 mg/ml	0.1 mg/ml
Arthrobacter aurescens DSM 20166	9	7	–
Arthrobacter globiformis DSM 20124	15	11	8
Arthrobacter oxydans DSM 6612	14	7	–
Arthrobacter pascens DSM 20545	14	9	7
Rhodococcus erythropolis DSM 1069	10	7	–
Aspergillus viridi nutans CBS 12756	11	–	–
Botrytis cinerea Tü 157	21	13	–
Paecilomyces variotii Tü 137	9	7	–

of cigarette smoke[20] (named pyrocoll), but has never been reported previously as a natural product. All physical and spectroscopic data were completely consistent to those described in the literature. For additional proof of structure, compound **2** was 'biomimetically' synthesized from **1** in one step with a 91.3% yield. For previous preparations of **2** by flash vacuum pyrolysis of **1** or *via* the pyrrolecarbonyl chloride, see refs. 10 and 21. Given the presence of **1** in the culture broth, a possible spontaneous formation of **2** from **1** was excluded by identifying **2** as a genuine product already present in the culture filtrate by HPLC.

Biological Properties

A wide variety of Gram-positive and Gram-negative bacteria and fungi were tested using the agar plate diffusion assay and broth dilution method. Pyrocoll (**2**) showed a good antibacterial activity in the agar plate diffusion assay especially against various *Arthrobacter* strains in the agar plate diffusion assay, wheras filamentous fungi like *Botrytis cinerea*, *Aspergillus viridi nutans* and *Paecilomyces variotii* were less sensitive, as shown in Table 1. The minimal inhibitory concentrations detected in the broth dilution assay are summarized in Table 2. Yeasts such as *Saccharomyces cerevisiae*, *Rhodotorula rubra* and *Candida albicans* were not sensitive in either assays.

Pyrocoll (**2**) caused concentration-dependent inhibition of cell growth in HM02, HepG2 and MCF 7 cells with

Table 2. Minimal inhibition concentration (MIC) of pyrocoll (**2**) determined by the broth dilution method.

Organism	MIC (μg/ml)
Arthrobacter aurescens DSM 20166	10
Arthrobacter globiformis DSM 20124	1
Arthrobacter oxydans DSM 6612	10
Arthrobacter pascens DSM 20545	3
Rhodococcus erythropolis DSM 1069	10

IC_{50}-values of 0.28, 0.42 and 2.2 μg/ml, respectively. **2** (10 μg/ml) did not reduce the cell count present at time point zero (Table 3). **2** displayed a strong inhibitory potency on the proliferation of L5178y lymphoma cells; an ED_{50}-value of 0.65±0.09 μg/ml was determined. Less pronounced was this effect on HeLa S3 cells; their proliferation was inhibited by 50% at 2.1±0.3 μg/ml. Inhibition by pyrocoll was not recorded in the assays using PC-12 cells below a concentration of 20 μg/ml.

Pyrocoll (**2**) exhibits moderate activity against *Plasmodium falciparum*, the pathogenic agent of malaria[22]. An IC_{50}-values of 1.19 μg/ml (standard chloroquine

Table 3. Activity (μg/ml) of pyrocoll (**2**) against selected human tumor cell lines.

Cell line	GI_{50}[a]	TGI[b]	LC_{50}[c]
HMO2	0.28 ± 0.02	5.1 ± 1.3	>10
HepG2	0.42 ± 0.04	>10[d]	>10
MCF 7	2.2 ± 0.35	>10[e]	>10

[a] Drug concentration causing 50% growth inhibition
[b] Drug concentration causing 100% growth inhibition
[c] Drug concentration causing 50% reduction of the cells present at time point zero, *i.e.* at 24 hours
[d] **2** caused 77% growth inhibition at 10 μg/ml
[e] **2** caused 70% growth inhibition at 10 μg/ml

0.078 μg/ml) was recorded against the chloroquine resistant K1 strain. *Leishmania donovani* is the pathogen of visceral leishmaniasis ('Kala Azar'); related protozoan parasites are *Trypanosoma cruzi*, the causative agent of Chagas disease, and *T. brucei rhodesiense*, the pathogen of African sleeping sickness. **2** displayed only moderate activity against *T. b. rhodesiense* (IC_{50} 1.97 μg/ml relative to the standard melarsoprol IC_{50} of 0.001 μg/ml) and against *T. cruzi* (IC_{50} 17.6 μg/ml compared to the standard benznidazole IC_{50} 1.27 μg/ml). Due to the cytotoxic effect of **2** on the host cells of *Leishmania donovani*, murine macrophages, it was not possible to determine the antileishmanial activity. The cytotoxic effects of pyrocoll on mammalian cells is supported by the IC_{50} value of 7.1 μg/ml for L-6 cells.

Discussion

The chemical and morphological properties shown by the producing strain AK 409 are consistent with its classification in the genus *Streptomyces*[23,24]. Its assignment to this genus was underpinned by the 16S rDNA data which showed that the strain forms a distinct branch in the evolutionary radiation encompassed by *Streptomyces griseus* and related species[25].

The structure of the secondary metabolite pyrocoll (**2**) shows two characteristic features: the pyrrole ring and the central diketopiperazine core. In searches for other anticancerogenic, antifungal, or antibacterial metabolites with one of these two structural elements we have found pyrrole ring and diketopiperazine ring containing structures. Examples are the pyrrolic compound reductiline[26], and the diketopiperazine antibiotic 593A[27] with antifungal properties. Looking for a structurally similar compound we found the metabolite PD 125375[28], an antibiotic having three rings like in pyrocoll, but without the diketopiperazine structure. Interestingly, this compound did not show antibacterial or antitumoural activities. This fact indicates a correlation between biological activity and diketopiperazine structure. It would be worthwhile synthesizing pyrocoll derivatives for structure-activity relationships.

Both, the symmetric pyrocoll molecule and the occurrence of pyrrole-2-carboxylic acid (**1**) in the culture broth of strain AK 409 stimulate speculations regarding the biosynthesis. It is feasible that as for the simple 1-step synthesis described above, two molecules of **1** acid react with dehydration to form pyrocoll (**2**). **1** is known to be derived from L-proline metabolism[29].

Pyrocoll (**2**) inhibits cell growth in the human tumor cell lines HM02, HepG2 and MCF 7 but does not exert a cytotoxic effect. The effect of **2** on the growth of mammalian cells was further determined using L5178y cells (ED_{50}: 0.65±0.09 μg/ml), HeLa S3 cells (2.1±0.3 μg/ml) and PC-12 cells (>20 μg/ml). This effect is attributed to the different proliferation capacity of the cells. While a high inhibition is seen with rapidly growing cells (the generation time of L5178y cells is 12±2 hours), almost no effect is caused by the compound in assays using PC-12 cells (generation time of 24±4 hours). An intermediate effect is caused by the compound with HeLa S3 cells, which show a generation time of 13±3 hours. Therefore we conclude that **2** is a compound with a specific effect restricted to processes proceeding during the S-phase.

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