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Pityriarubins, Novel Highly Selective Inhibitors of Respiratory Burst from Cultures of the Yeast *Malassezia furfur*: Comparison with the Bisindolylmaleimide Arcyriarubin A

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Dedicated to Prof. Dr. W.-B. Schill on occasion of his 65th birthday

Pityriasis versicolor is the most common skin mycosis in humans worldwide. Yeasts of the genus Malassezia*, particularly* M. furfur*, a saprophyte occurring widely on human skin, are generally regarded as the causative agents.* M. furfur *is able to convert tryptophan into a variety of indole alkaloids, some of them showing biological properties that correlate well with certain clinical features of pityriasis versicolor. This suggests a possible role for these compounds in the pathophysiology of the disease. We here report that the novel pityriarubins A, B and C, isolated from cultures of the yeast, inhibit respiratory burst in human neutrophils, activated by various agents, in a highly selective, unexpected manner. The release of 5-lipoxygenase products after challenge of neutrophils with the calcium ionophore A23187 is also inhibited in a dose-dependent manner. These activities reflect the close structural relationship of pityriarubins to bisindolylmaleimides, which have recently gained great interest as protein kinase inhibitors.*

Introduction

Malassezia furfur is a lipophilic yeast occurring on the human skin as a part of the residential flora. Members of the genus *Malassezia*, currently represented by nine species, are implicated as the pathogenic organisms involved in the skin disease known as pityriasis versicolor.[1,2] This benign but irksome disease, causing mainly cosmetic damage, has several characteristic clinical features. For example, the yellow–green fluorescence observed under UV light at 366 nm is used in diagnosis. Furthermore, the skin depigmentations observed in the course of the disease are accompanied by decreased rather than significantly increased sensitivity towards UV irradiation. The disease occurs with only minimal signs of inflammation and humoral response, despite the high intradermal fungus load found in lesions of the disease.[3–5]

M. furfur produces a great variety of indole derivatives when cultured on a minimal agar medium with tryptophan as sole nitrogen source.[6] Interestingly, *M. furfur* is the only lipid-dependent species of the genus *Malassezia* to posses this ability.[7] Recently, we isolated several new indole alkaloids from the cultural extract, and the biological properties of these compounds correlate well with the clinical features of pityriasis versicolor. Thus, pityriacitrin, a pale yellow solid, could contribute to the decreased UV sensitivity of the depigmented areas in this disease.[8] In solution, pityrialactone exhibits a strong green to blue fluorescence upon irradiation with UV light at 366 nm, and this might contribute to the green fluorescent layer present in affected skin.[9]

Malassezin, a novel type of aryl hydrocarbon receptor agonist, induces apoptosis in human melanocytes and could contribute to the marked depigmentation caused by the disease.[10,11] Furthermore, several new indol alkaloids were found in the culture extract, including several new indolo[3,2-b]carbazole compounds as well as complex structures with unknown biological properties.[12]

The orange to red bis[indolyl]spiran alkaloids pityriarubins A, B and C (Scheme 1[13]) are structurally similar to bisindolylmaleimides like arcyriarubin A, a potent but unspecific inhibitor of

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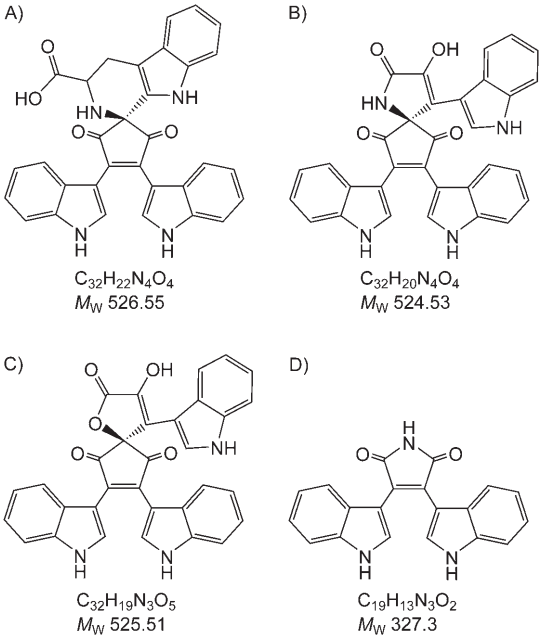

Scheme 1. Structures of the pityriarubins A, B and C; comparison with arcyriarubin A (D).

protein kinase C.[14–20] They can act in vivo as anti-inflammatory agents, thus explaining the modest levels of inflammation observed in affected skin areas. All these phenomena suggest a specific role of the indole metabolites in the pathogenesis of pityriasis versicolor.

Given the structural similarity of the pityriarubins to arcyriarubin A, we investigated the influence of these novel compounds on the release of radical oxygen species [ROS, "burst"] in human neutrophils in response to various agents. Release of ROS is one hallmark of the cellular immune response to various noxious substances like bacteria and fungi[21] and is commonly used as a model to characterise the anti-inflammatory profile of arcyriarubin A and staurosporine analogues.[22–24] In addition, the influence of the pityriarubins on the formation of A23187-induced lipoxygenase products was investigated.

Results

Human neutrophils release ROS upon stimulation with A23187 (1 μM), IL-3 (20 μg mL^{-1}), formyl peptide *N*-formyl-Met-Leu-Phe [FMLF] (1 μM), 1,2-dioctanoylglycerol (10 μM), phorbol-12-myristate-13 acetate (1 μM) and sodium fluoride (10 μM), as described in the literature.[22–24] In the case of A23187 stimulation, generation of 5-lipoxygenase products was detected by analytical HPLC focused on the simultaneously released leukotriene B_4 [LTB_4] as well as the hydrolysis products 6-*trans*-LTB_4 and 6-*trans*-epi-LTB_4.

The action of these agents is inhibited in a dose-dependent manner by arcyriarubin A, in line with the literature.

Addition of pityriarubin A, B or C only resulted in a similar dose-dependent inhibition of respiratory burst after stimulation with A23187, IL-3 or FMLF, and inhibition of 5-lipoxygenase products after challenge with A23187 was also found. Figures 1, 2 and 3 depict the corresponding experimental values

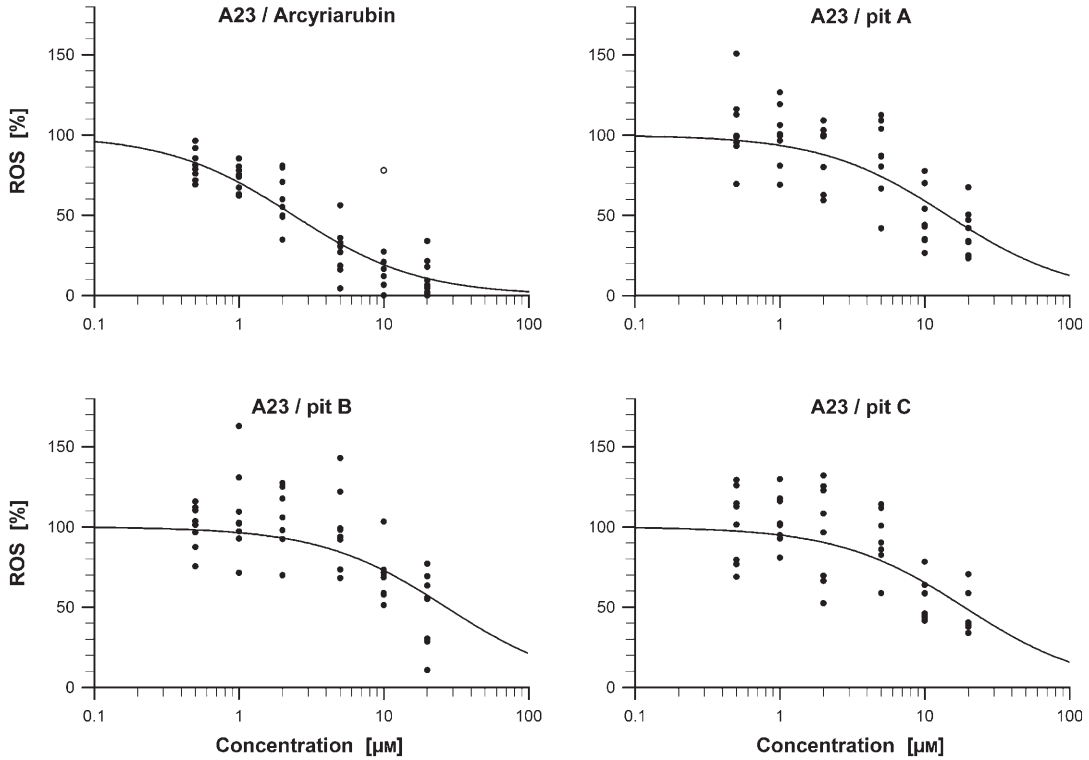

Figure 1. Influence of arcyriarubin A (BIM) and pityriarubins A, B and C on ROS release after challenge of human neutrophils with 1 μM A23187 (Hill Plot). The lines are a guide to the average values.

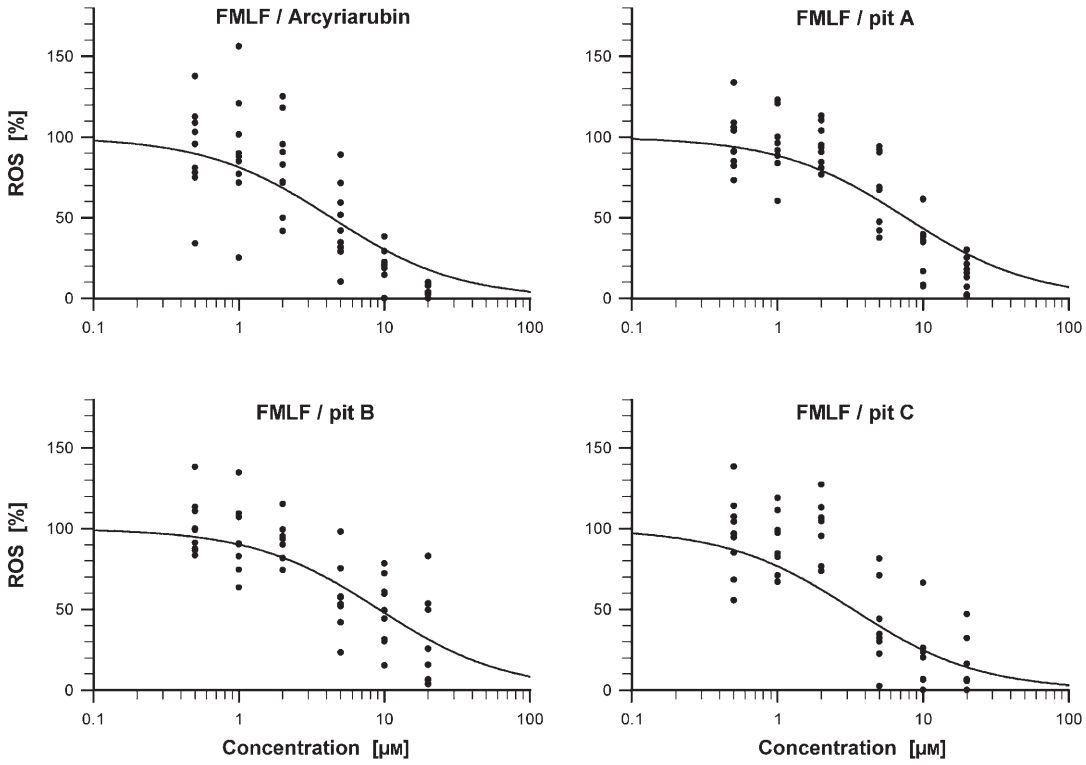

Figure 2. Influence of arcyriarubin A and pityriarubins A, B and C on ROS release after challenge of human neutrophils with 1 µM *N*-formyl-Met-Leu-Phe (FMLF) after priming with cytochalasin B 5 µg mL^{-1} (Hill Plot). The lines are a guide to the average values

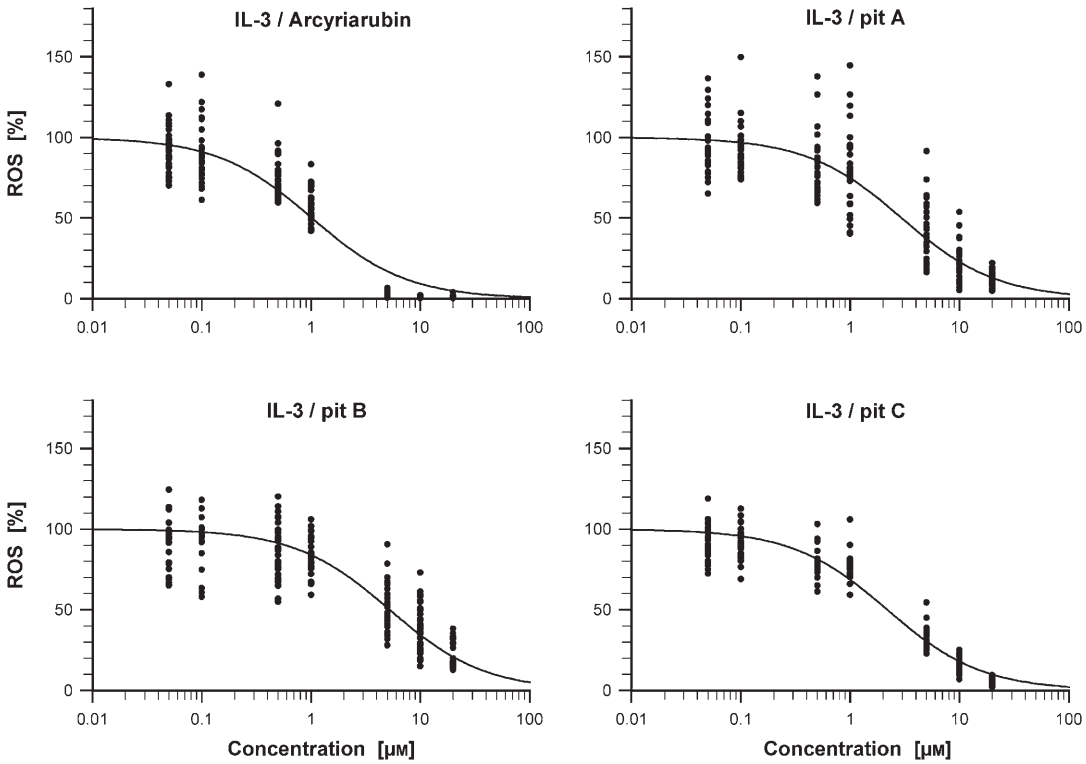

Figure 3. Influence of arcyriarubin A and pityriarubins A, B and C on ROS release after challenge of human neutrophils with 20 µg mL^{-1} IL-3 (Hill Plot). The lines are a guide to the average values.

and Hill plots for ROS release. No influence of the other agents on the release of ROS was detected. In Figure 4, the results for challenge with phorbol-12-myristate-13 acetate ester are given as an example of noninhibition. In all cases of inhibition, pityriarubin C had the most prominent effect.

Statistics were obtained by nonparametric methods that do not rely on the assumption of a normal distribution of the variable of interest. Basically, we used two statistical approaches.

Firstly, numeric approximation of the nonaveraged single dose-response curve by mixed exponential curves with four parameters was used when possible to obtain the IC_{50} values of all four substances for ROS release and generation of 5-lipoxygenase products (Figure 5). Secondly, as estimation of the parameter of the dose-response curves was not possible for all experimental data due to aberrant curve shape. Statistical analysis and testing for distribution-free multiple comparison based on Kruskal–Wallis rank scenes of the highest concentration data were performed, assuming monotonic falling relationships for the dose-response curves. The same tests were used to compare the obtained IC_{50} values. In both approaches, no indication of relevant difference was detected between the inhibitors arcyriarubin A and pityriarubin C by using FMLF and IL-3; this suggests similar inhibitory potencies for both compounds.

Any toxic effects of the pityriarubins/arcyriarubin A as well as of the solvent DMSO (0.2%, maximum content in the experiments always $< 0.1\%$) could be excluded by measurement of intracellular ATP in HaCaT cells even after an incubation period of 48 h (Figure 6).

Discussion

The biological activities of the known indole metabolites from *Malassezia furfur* seem to be well related to certain clinical features of pityriasis versicolor, although we have not yet succeeded in their direct detection in lesions or scales from pityriasis versicolor due to the complex nature of these materials. A typical feature of the disease is the relative lack of inflammatory signs compared to other dermal mycoses.[3,4] Moreover, the humoural and cellular immune responses to *Malassezia*-yeasts are strongly diminished in patients with pityriasis versicolor.[5,25–28]

Malassezia furfur is able to convert tryptophan into a variety of complex indole compounds, most of them having new chemical structures. One family of the complex alkaloids that we isolated recently comprises the bright red pityriarubins A, B and C, all of them containing a bisindolyl-spirane moiety. We were able to document the close similarity of the pityriarubins to unspecific PKC inhibitors of the arcyriarubin A type by a similar dose-dependent inhibition of release of reactive oxygen species from neutrophils caused by several agents, as well as an inhibition of the production of 5-lipoxygenase products after challenge with calcium ionophore A23187. Direct inhibition of NADPH oxidase and radical scavenging could be excluded by the unaltered burst in cases of noninhibition. Toxic effects of the substances were negligible over the concentration range used for the experiments. Nevertheless, the pityriarubins exhibited a marked selectivity with respect to the agents used, with a restricted, specific ability for inhibition.

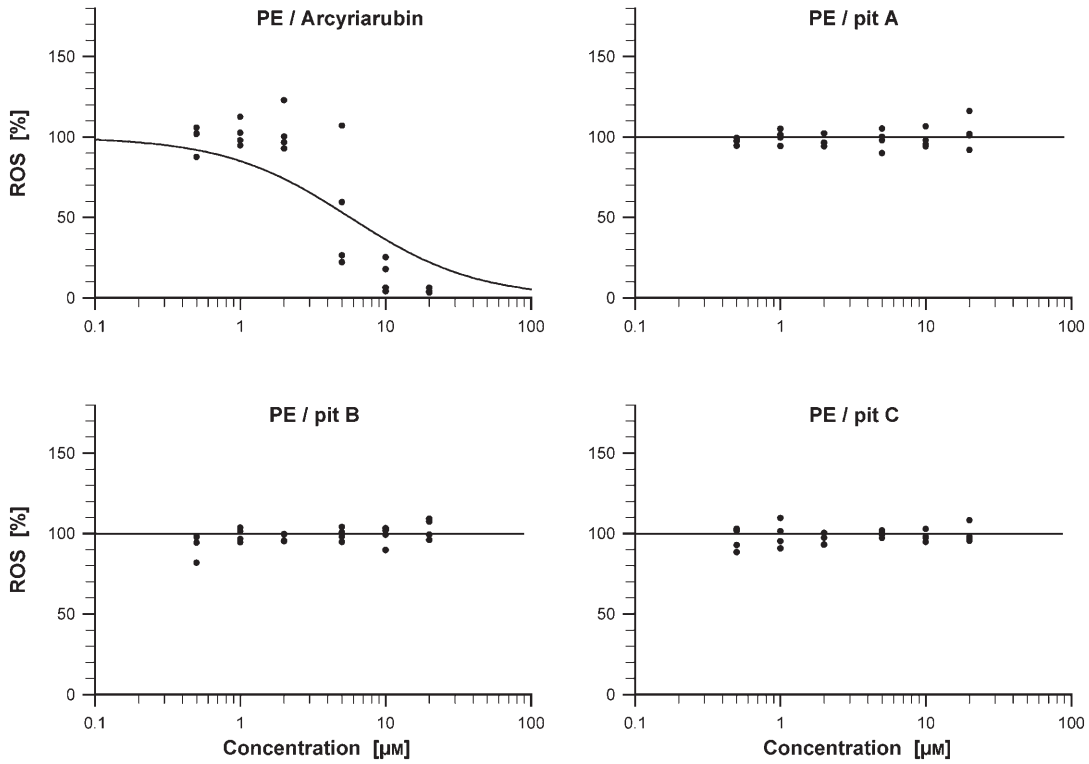

Figure 4. Influence of arcyriarubin A and pityriarubins A, B and C on ROS release after challenge of human neutrophils with 1 µM phorbol-12-myristate-13 acetate (PE, Hill Plot).

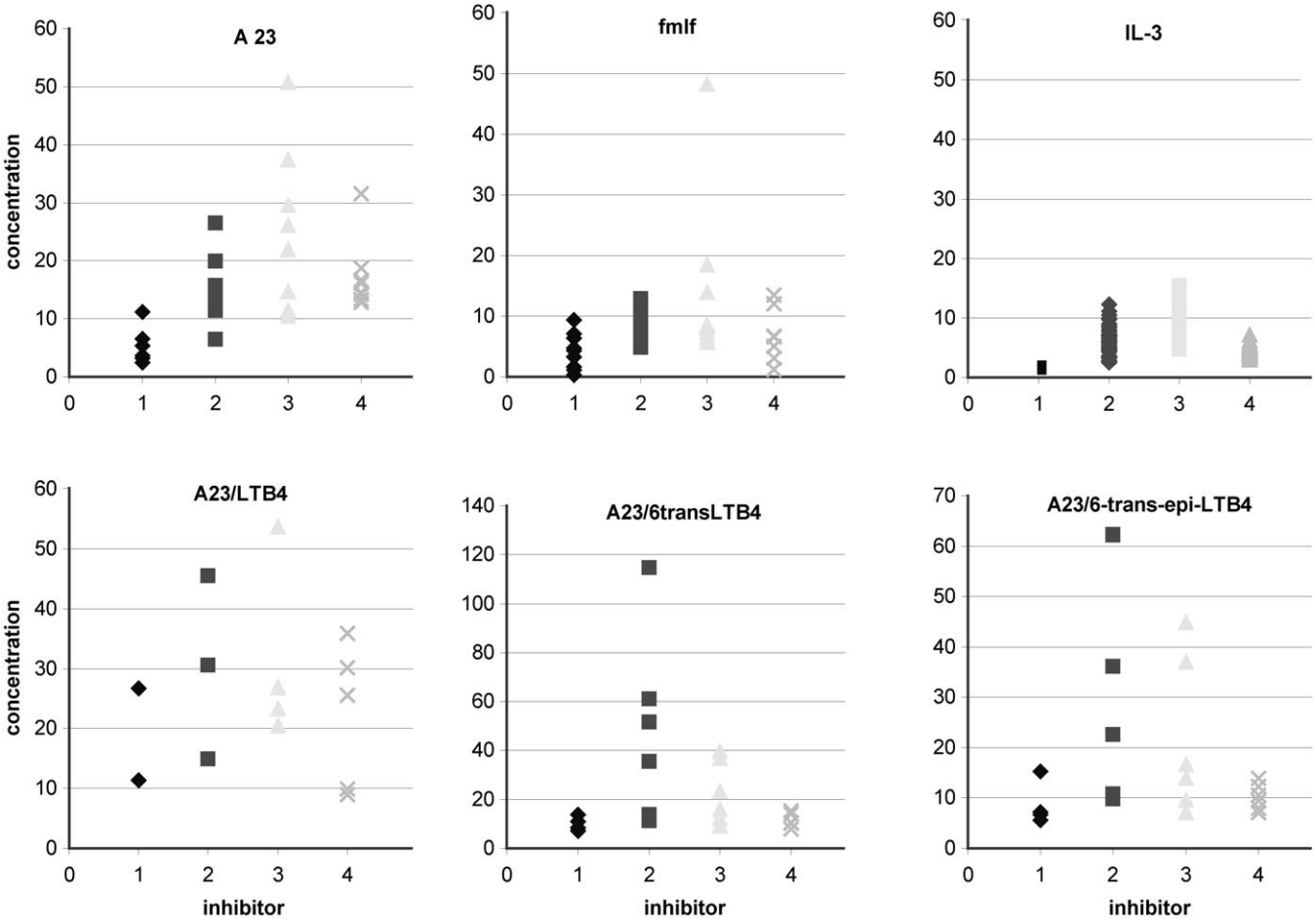

Figure 5. IC_{50} values obtained after numeric exponential approximation and calculating the concentration of half-maximal inhibition: 1) arcyriarubin A, 2) pit. A, 3) pit. B, 4) pit. C.

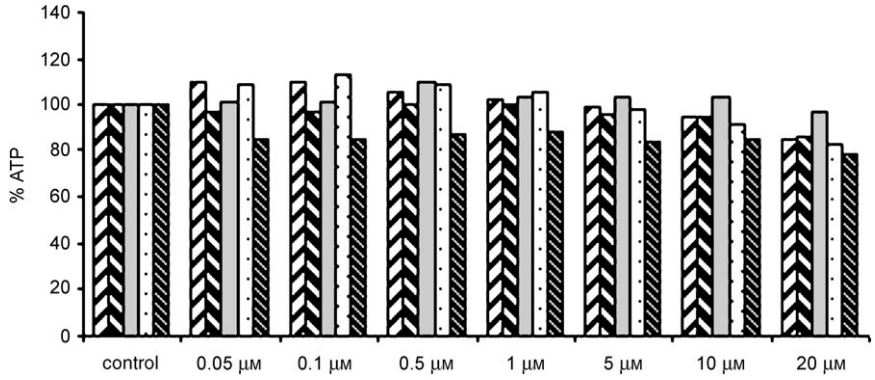

Figure 6. Influence of, from left to right, pityriarubins A, B and C, arcyriarubin and solvent (0.2% DMSO) on the ATP level of HaCaT cells at the concentrations given (48 h, $n = 32$).

There seems to be a remarkable difference in the signalling pathways inhibited by these agents and those inhibited by the classical arcyriarubin A-type PKC inhibitors. As no inhibition of PKC activation by 1,2-dioctanoylglycerol, phorbol-12-myristate-13 acetate and sodium fluoride was detected, this target could be excluded, although it is involved in the activation of NADPH oxidases and release of ROS.[29,30]

Furthermore, it can be concluded from the inhibitory profile that the target should be a common element of the signalling pathways of A23847, FMLF and IL-3. The last two stimuli are the most promising for searching for such common elements, while A23847 is an unspecific elevator of intracellular Ca^{2+}, with consecutive activation of various Ca^{2+}-dependent processes, including nucleotide-dependent protein kinases. FMLF and IL-3 are naturally occurring activators in inflammatory processes and the release of reactive oxygen species, and both are receptor agonists on neutrophils.[31–33]

In the literature on the signalling pathways of both compounds, several elements in the activation of neutrophils have recently been described: p38 Map kinases (mitogen-activated protein kinases), ERK2 (extracellular signal-regulated kinase)[32,34] and PI3Kδ[35] are all involved in the phosphorylation of the NADPH oxidase component, $p47^{phox}$, with subsequent production of ROS. A recently discovered activation pathway includes the interaction of H-ferritin with the cytokine receptor.[36]

It is remarkable that, in the search for more specific derivatives, variations of the structural lead for this kind of inhibitor provided by the natural compounds indolocarbazoles, staurosporine and K252a were made mostly at the indolyl hydrogens.[17–20,22,23,37–39] Furthermore, substitution of the imido hydrogen by methyl, hydroxyl or hydroxymethyl residues resulted in partial to total loss of PKC inhibitory activity.[17,18,22] Surprisingly, substitution of the maleimide moiety by the spirane structure provides highly specific and potent inhibitors with low cellular toxicity. None of the previously reported analogues of arcyriarubin A presented a selectivity comparable to that of the pityriarubins, thus it is reasonable, especially from a spatial point of view, to postulate a still unknown common target in the course of signalling of FMLF and IL-3 and activation by A23187. As a result of the unspecific activation by A23187, the inhibition of ROS release is suppressed only in a limited manner over the concentration range used, which was limited to 20 μM by solubility. In the cases of FMLF and IL-3 challenge, pityriarubin C was practically as capable of suppressing ROS release as arcyriarubin A; this suggests a nanomolar activity at the target. It seems likely that the unexpected structural modifications presented by the pityriarubins could give new impetus to the search for kinase inhibitors and their targets.

Finally, it could be assumed that the pityriarubins, especially pityriarubin C, due to its high potency comparable to that of arcyriarubin A, might act in vivo as immunomodulators in the course of pityriasis versicolor, expressing a highly adaptive strategy of *M. furfur* to encounter with the host's defense mechanisms and contributing to the comparably low incidence of inflammatory signs in affected skin lesion.

Experimental Section

Organism and pigment production: The *M. furfur* reference strain CBS 1878 was used for pigment production according to a previously published procedure.[40] The strain was maintained at 32 °C on modified Dixon agar (mDixon), as described.[6]

Growth medium: The medium for inducing pigment synthesis was used with modifications previously described.[6] It consisted of (for 1 L medium) Tween® 80 (30 mL; Sigma, St. Louis, USA) and Agar (20 g; Merck, Darmstadt, Germany). After this had been sterilised and cooled to 50 °C, sterile filtered L-tryptophan (15 mmol; Sigma) was added and aliquots (10 mL) of the medium were poured into sterile Petri dishes 10 cm in diameter.

Preparation and separation of the crude extract: Suspensions of CBS 1878 were smeared onto the agar medium with a swab. After 14 days of incubation, the contents of 200 Petri dishes were puréed and extracted with ethyl acetate (Merck) for 12 h. The extract was filtered through glass wool, the filtrate was evaporated to dryness, and the residue was dissolved in methanol. The solution was separated under UV light (254 nm) into seven fractions by chromatography on Sephadex LH-20 (Sigma) with methanol as eluent. Thin layer chromatography was performed on silica gel 60 plates (Merck) with toluene/ethyl formate/formic acid (TEF; 10:5:3, *v/v/v*) elution.[35] After the plates had been dried, the orange bands with R_f values of 0.14 (Pit. A), 0.27 (Pit. B) and 0.38 (Pit. C) were scraped out and partitioned between H_2O and EtOAc. The EtOAc layer was dried over anhydrous Na_2SO_4, evaporated and dissolved again in acetonitrile. Further purification of the fractions was achieved by preparative HPLC over a gradient with a LiChrospher-RP-8 column (30×250 mm; Merck) and a LiChrosorb RP-18 precolumn. An M305 master pump combined with a 302 model pump (Gilson, Villiers le Bel, France) was used, each with a preparative 50 mL min^{-1} pump head, controlled by a Gilson 802 module. Detection was made by a UV detector (Holochrome, Gilson) at 220 nm. A linear gradient of acetonitrile/water (0:100–100:0% over 180 min) was used (chromatography grade solvents; Merck), the flow rate was 5 mL min^{-1}. The compounds were collected with a fraction collector (Super Frac, Pharmacia Biotech, Uppsala, Sweden; 180 fractions, 5 mL each), combined according to the resulting peaks (~10 fractions), and then lyophilised by using a Lyovac GT2 machine (Leybold–Heraeus, Hanau, Germany). The orange pityriarubins eluted in fractions number 107–120 (Pit. A), 112–129 (Pit. B) and 112–120 (Pit C) from the preparative column. They were repurified by means of an extended gradient (40–70% acetonitrile and 60–0% water, 180 fractions of 5 mL each). Elution occurred in fractions 18–25 (Pit A), 55–65 (Pit. B) and 55–68 (Pit. C). The purity of the freeze-dried dark red compounds was checked by analytical HPLC, consisting of a reversed-phase column (RP-18, 4×250 mm, stationary-phase Shandon ODS Hypersil 3 μm, Life Science International Ltd., Cheshire, England). Elution occurred with a high-pressure gradient system (Gynkotek Gradientenpumpe 480, Gynkotek, Germering, Germany). Mobile phase: linear gradient of acetonitrile/water (0:100–100:0% over 100 min, flow rate 1 mL min^{-1}). Detection of the components was achieved at 220 nm (UV detector 785, Bai, Bensheim, Germany), chromatographic monitoring being carried out by using an integrator C-R 6 A Chromatopac from Gynkotek (Germering). Application of specimens was achieved by using an Alcott Modell 738 autosampler from Bischoff Analysentechnik (Leonberg, Germany) at a volume of 100 μL. The purity was about 99.5% in all samples.

Release of ROS from human neutrophils: Isolation of neutrophils from whole blood and measurement of ROS release were performed according to the literature.[41]

Peripheral venous blood was taken from healthy volunteers (600 mL) after informed consent. EDTA blood samples were immediately processed for isolation of polymorphonuclear neutrophils (PMN). The blood was centrifuged in a Ficoll–Paque gradient (Pharmacia, Uppsala, Sweden), erythrocytes were sedimented by using polyvinyl alcohol (Merck–Schuchardt, Hohenbrunn, Germany), and the remaining erythrocytes in the cell pellet were eliminated by hypotonic lysis (distilled water, 30 s). Cells were centrifuged (150 *g*, 10 min, 4 °C), washed twice in phosphate buffer (PBS, 298 mM with Ca^{2+} and Mg^{2+}) and resuspended in PBS at a concentration of 5×10^6 cells per mL. In the case of FMLF stimulation, additional preincubation of the total cell suspension with cytochalasin B (5 μg mL^{-1}) for 30 min was required. Cell purity was generally higher than 98% (Pappenheim stain), cell vitality was higher than 96% as shown by trypan blue staining. After preincubation with PBS (500 μL), with and without the given concentrations of inhibitors, and cytochrome C (75 μM), with and without superoxide dis-

mutase (SOD, 100 μg per sample) in PBS (100 μL), isolated neutrophils (300 μL of the above mentioned suspension) were stimulated for superoxide release [O_2^-] by adding calcium ionophore A23187 (100 μL in PBS, final concentration 1 μM). The release of O_2^- was measured by the reduction of cytochrome C at 546 nm (incubation for 10 min, then stopping with ice for 5 min and subsequent centrifugation for 3 min at 13000 *g* for removal of cells). The same preparation with SOD as additive to prevent reduction of cytochrome C was carried out as reference. The difference of the extinctions of both preparations is a measure for the production of superoxide anions. Experiments without inhibitors were performed as controls for maximum release (100%); in order to control the spontaneous burst, experiments on preparations without inhibitors and stimulators were carried out for each assay. Data from the experiments were adjusted by subtraction of the low spontaneous activity (< 5%) and ratios (in percent) of the maximal values were calculated from the extinctions of the experiments with inhibitors.

Determination of 5-lipoxygenase (5-LO) products after challenge with A23187: Arachidonic acid-related 5-LO products were determined in cell supernatants after challenge with A23187 (1 μM) by solid-phase extraction and subsequent reversed-phase HPLC/UV detection at 230/270 nm as described.[40] Briefly, 5×10^6 cells in Hank's HEPES buffer with Ca^{2+} and Mg^{2+} (400 μL; HH++, Gibco, Karlsruhe, Germany) were equilibrated for 10 min at 37 °C, then a solution of A23187 (1 μM) in DMSO (5 μL) was added. After 10 min at 37 °C, the reaction was stopped by immersion in crushed ice and centrifugation at 4 °C (1200 *g*, 4 min.). Supernatants were stored at −20 °C for final analysis. Solid-phase extraction was performed after conditioning the columns with methanol (2×1 mL) and distilled water (2×1 mL). Cell supernatants were added to the columns, and, after passing through the columns, these were washed twice with distilled water (1 mL). Additionally, trichloromethane (100 μL) was used to elute nonpolar lipophilic compounds and replace water residuals on the columns. Elution of 5-LO-products was achieved by adding methanol (250 μL), which was applied by gentle suction. Eluates were evaporated by a continuous nitrogen stream and stored at −20 °C for HPLC analysis. Samples were dissolved in methanol (50 μL), of which solution 30 μL were used for HPLC analysis. The chromatographic device consisted of a reversed-phase column ((RP-18, 4×250 mm, stationary-phase Shandon ODS Hypersil 3 μm; Life Science International LTD, Cheshire, England), mounted in a Kontron HPLC analysis system (HPLC Pump 420, HPLC detector 430, HPLC Autosampler 465, Kontron, Munich, Germany)). Mobile phase: methanol/water/acetic acid (78:28:0.16, *v*/*v*/*v*, pH 4.9) adjusted with ammonia and degassed by membrane filtration), the flow rate was 1.3 mL min^{-1}. The peak heights were taken as signals for quantification, known amounts of the analytes were used as references. Recoveries were determined by separate recovery experiments comprising solid-phase extraction, evaporation and subsequent HPLC analysis with known amounts of standards. The values obtained from cell supernatants were corrected by the recovery, which was > 70% in all cases.

Experimental methods used in IL-3 stimulation

Isolation of neutrophils: The isolation of neutrophils was performed by means of the density gradient method.[42] EDTA blood (8 mL) was mixed with dextran (2 mL). After 90 min of sedimentation and removal of the supernatant, the suspension was centrifuged for 15 min at 900 rpm. Resuspension of the cell pellets, addition of aqueous NH_4Cl and incubation at 4 °C were used for the lysis of erythrocytes. The leukocyte cell pellets obtained after another centrifugation were mixed with CAST stimulation buffer (10 mL), containing IL-3 (20 μg mL^{-1}; human recombinant, Miltenia Biotec, Bad Nauheim, Germany).

The cell number was determined by means of the cell counter Casy 1 (Schärfesystem, Germany) with the cell suspension (20 μL) in Casyton (10 mL). The adjustment of the used cell number (1×10^6 mL) was carried out by dilution with stimulation buffer.

Chemiluminescence measurements: The chemiluminescence measurements were carried out by means of the microplate luminometer "LUMIstar Galaxy" (BMG LabTechnologies GmbH, Offenbach, Germany). In each well of a white 96-well-plate (Nunc Maxisorp, Wiesbaden, Germany). the cell suspension (50 μL) and solutions (50 μL) of pityriarubin A, B or C or arcyriarubin A in Dulbecco's modified Eagle's medium (DMEM, Seromed-Biochrom, Berlin, Germany) were added to achieve final concentrations of 0.05, 0.1, 0.5, 1, 5, 10 and 20 μM. Lucigenin solution (100 μL, 0.0001 mol L) was injected directly into the wells of the microplate by means of a reagent injector at 37 °C. For the measurement, we used the slow kinetic method (plate mode).The results are mean values of accumulated single readings over a period of 24 min.

Cytotoxicity: Human HaCaT keratinocytes were cultured in DMEM according to ref. [43], supplemented with 1% antibiotic–antimycotic solution (Gibco BRL) and 10% foetal calf serum (Seromed-Biochrom). Cell culture was done in 200 mL culture flasks (Greiner Bio-One, Kremsmünster, Austria) for 5–7 days at 37 °C under 5% CO_2.

Cells were then mobilised by means of trypsin EDTA (Gibco BRL), disseminated in 96-well microtiter plates (Greiner) at a density of 20–40000 cells per cm^2 and cultured for 48 h in medium (100 μL), as described. Incubation for 24 h and 48 h with pityriarubins A, B and C as well as arcyriarubin A was done thereafter by addition of solutions in DMEM to achieve final concentrations of 0.05, 0.1, 0.5, 1, 5, 10 and 20 μM.

ATP bioluminescence: Intracellular ATP was determined by use of ATPLite-M (Packard Bioscience B.V., Groningen, The Netherlands). This assay is based upon the ATP-dependent reaction of luciferase and D-luciferin. The emitted light is directly proportional to the ATP concentration. lysis buffer (50 μL) was added to each well after the incubation period, followed by shaking for 2 min at 700 rpm in an orbital shaker. Substrate solution (luciferin/luciferase; 50 μL) was added to each well. After being shaken again for 1 min, the plate was kept in the dark for 10 min. Luminescence was measured in a microplate luminometer LUMIstar Galaxy (BMG LabTechnologies GmbH, Offenburg, Germany). ATP concentrations were determined according to a standard curve.

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Keywords: amino acids · granulocytes · *Malassezia furfur* · natural products · signalling

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