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Pityriazepin and other potent AhR ligands isolated from *Malassezia furfur* yeast

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Abstract

Malassezia furfur yeast strains isolated from diseased human skin preferentially biosynthesize indole alkaloids which can be detected in human skin and are highly potent activators of the aryl hydrocarbon receptor (AhR) and AhR-dependent gene expression. Chemical analysis of an EtOAc extract of a *M. furfur* strain obtained from diseased human skin and grown on *L*-tryptophan agar revealed several known AhR active tryptophan metabolites along with a previously unidentified compound, pityriazepin. While its structure resembled that of the known alkaloid pityriacitrin, the comprised pyridine ring had been transformed into an azepinone. The indoloazepinone scaffold of pityriazepin is extremely rare in nature and has only been reported once previously. Pityriazepin, like the other isolated compounds, was found to be a potent activator of the AhR-dependent reporter gene assays in recombinant cell lines derived from four different species, although significant species differences in relative potency was observed. The ability of pityriazepin to competitively bind to the AhR and directly stimulate AhR DNA binding classified it as a new naturally-occurring potent AhR agonist. *Malassezia furfur* produces an expanded collection of extremely potent naturally occurring AhR agonists, which produce their biological effects in a species-specific manner.¹

Keywords

Malassezia furfur; Aryl-hydrocarbon Receptor; indole metabolites; pityriacitrin; pityriazepin

¹Abbreviations: Aryl hydrocarbon Receptor (AhR), Indolo[3,2-*b*]carbazole (ICZ), 6-formylindolo[3,2-*b*]carbazole (6-FICZ), High Resolution Mass Spectrometry (HRMS), High Pressure Liquid Chromatography (HPLC), CentraalBureau voor Schimmelcultures (CBS).

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Supporting Information Available: Spectroscopic data and isolation experimental details are provided as supporting information.

Introduction

Malassezia is a yeast belonging naturally to the human skin microbiota and it is considered to be the causative agent of several skin diseases such as seborrhoeic dermatitis, pityriasis versicolor, dandruff and psoriasis,¹ although the conditions under which it can become pathogenic are currently only partially understood. Previous studies have shown that *Malassezia furfur* isolates from lesional skin preferentially biosynthesize a variety of indole alkaloids which are among the most potent known activators of the aryl hydrocarbon receptor (AhR).^{2,3} The AhR, or Dioxin Receptor as it is widely known, mediates the expression of a wide variety of genes including the ones that encode CYP1A1, an enzyme responsible for the metabolism of diverse drugs and xenobiotics.⁴ Despite the absence of a known endogenous ligand and limited information on endogenous physiological functions for the AhR, the number of reports demonstrating its involvement with skin homeostasis and skin nosology is constantly increasing. The importance of AhR involvement in skin disease is further supported by our previous work examining the activation of AhR by *Malassezia* extracts² and the detection of AhR-active metabolites like malassezin (1), indolo[3,2-*b*]carbazole (ICZ) (2), pityriacitrin (3), indirubin (4), tryptanthrin (5) and 6-formyl-indolo[3,2-*b*]carbazole (6-FICZ) (6) in *Malassezia furfur* strains isolated from diseased skin as well as in skin scales from patients with seborrhoeic dermatitis and pityriasis versicolor.³ Additionally, the correlation between *Malassezia*-derived indoles and immunomodulation in a recent study,⁵ along with the aforementioned detection of 6-FICZ, one of the most potent AhR activators and a proposed endogenous ligand for the AhR,³ have led to the re-evaluation of the presence of *Malassezia* yeasts in skin microflora and their role in human disease. The potent activation of the AhR and AhR-dependent gene expression by these yeasts,³ suggest that isolation and characterization of the complete metabolome of *Malassezia* will be needed in order to understand the overall mechanism of AhR activation by *Malassezia* produced compounds. Herein is described the isolation and characterization of potent natural AhR agonists, including the identification of a new metabolite, from a *Malassezia furfur* strain grown on *L*-tryptophan agar and demonstrate that each of these products can contribute to the activation of the AhR signaling pathway.

Materials and Methods

General Experimental Procedures

1D and 2D NMR spectra were acquired in a Bruker UltrashieldTM Plus 600MHz NMR. MS analysis of the obtained compounds was performed with an LTQ ThermoScientific Orbitrap and an Agilent QQQ 6460. All solvents used during the experimental procedure were of analytical grade or redistilled. Additionally, the solvents used for Liquid Chromatography were HPLC grade and purified by filtration through ion- and carbon-exchange resins. The stationary phase for low pressure CC was silicon dioxide (Silica gel 40–63µm/Silica flash). Aluminum and glass plates, coated with silicon dioxide (Silica gel 60 F₂₅₄) were used for TLC and preparative TLC, respectively, and the obtained chromatograms were observed in a CAMAG TLC Visualizer at 254 and 366 nm. For the HPLC analysis, a THERMO Finnigan Spectra System was used, coupled with a PDA UV Detector. The column used was a Lichrosorb RP-18 with dimensions of 5 µm and 250×4 mm.

Extraction and Isolation

The contents of 100 Petri dishes of a *Malassezia furfur* strain (CentraalBureau voor Schimmelcultures (CBS) 1878) cultivation were cut into small pieces and extracted with ethyl acetate (2 L) for 48 hours at room temperature. The solvent was filtered, washed with H₂O (400 mL), and evaporated under reduced pressure. A portion of the solid residue (6.5 g) was dissolved in MeOH (5 mL) and submitted to Column Chromatography using Sephadex LH-20 (30 g) with methanol as the eluent (~750 mL). The first 150 mL corresponding to a fast moving dark brown zone (lipids and macromolecules) were discarded and the latter 600 mL were pooled and evaporated to dryness. A portion of the resulting residue (1 g) was then fractionated with low pressure column chromatography prepared as described above, where the mixture was added as powder absorbed on silica of 70–200 μ m. The eluent consisted of the mixture of CH₂Cl₂ and MeOH (step gradient elution, from 100% CH₂Cl₂ to 100% MeOH). All fractions were then profiled with analytical HPLC, using a gradient system from 100% H₂O to 100% MeOH in 55 min, with flow at 1 mL/min and the UV detector monitoring at 254, 332 and 540 nm.

Pityriacitrin (3)

The alkaloid was isolated from a fraction eluted with 100% CH₂Cl₂. Further isolation was performed with preparative TLC using CH₂Cl₂:Acetone – 97:3 (*R_f* = 0.62). Yellow solid; UV/Vis (MeOH) λ_{max} 287, 314, 378, 389 nm; ¹H-NMR (600MHz, Acetone-d₆): δ 11.45 (brs, 9-NH), 11.15 (brs, 1'-NH), 9.54 (s, H-2'), 8.67 (d, *J*=7.0 Hz, H-4'), 8.59 (d, *J*=4.7 Hz, H-3), 8.35 (d, *J*=4.7 Hz, H-4), 8.31 (d, *J*=7.6 Hz, H-5), 7.95 (d, *J*=7.6 Hz, H-8), 7.64 (t, *J*=7.6 Hz, H-7), 7.59 (d, *J*=7.5 Hz, H-7'), 7.35 (t, *J*=7.6 Hz, H-5'), 7.29 (m, H-6 and H-6'); ¹³C-NMR (600MHz, Acetone-d₆): δ 190.2 (CO), 141.0 (C-8a), 138.1 (C-2'), 138.0 (C-7'a), 137.6 (C-3), 136.4 (C-9a), 132.2 (C-4a), 131.0 (C-1), 129.0 (C-7), 126.4 (C-3'a), 123.4 (C-5'), 123.4 (C-6'), 123.0 (C-4'), 122.2 (C-5), 120.5 (C-6), 118.8 (C-4b), 118.0 (C-4), 114.9 (C-3'), 113.4 (C-8), 112.5 (C-7'); MS (ESI[−]) *m/z* 310 [M-H][−].

Malassezin (1)

The alkaloid was isolated from a fraction eluted with 100% CH₂Cl₂. Further isolation was performed with Column Chromatography using Sephadex LH-20, with 47–52 mL of MeOH. UV/Vis (ACN) λ_{max} 238, 292 nm; ¹H-NMR (600MHz, Acetone-d₆): δ 10.90 (brs, 1-NH), 10.41 (s, H-9), 10.27 (brs, 1'-NH), 8.20 (d, *J*=8.0 Hz, H-4), 7.51 (d, *J*=7.7 Hz, H-4'), 7.41 (d, *J*=8.2 Hz, H-7), 7.34 (d, *J*=8.1 Hz, H-7'), 7.31 (s, H-2'), 7.16 (m, H-6 and H-6'), 7.10 (t, *J*=8.0 Hz, H-5), 6.98 (t, *J*=7.7 Hz, H-5'), 4.69 (s, 2H, H-8); ¹³C-NMR (600MHz, Acetone-d₆): δ 184.4 (C-9), 151.3 (C-2), 135.4 (C-7'a), 136.2 (C-7a), 126.5 (C-3a), 125.5 (C-3'a), 123.7 (C-2'), 122.6 (C-6), 121.8 (C-5), 121.1 (C-6'), 120.1 (C-4), 118.5 (C-5'), 118.2 (C-4'), 113.0 (C-3), 111.6 (C-7), 111.5 (C-7'), 110.8 (C-3'), 22.0 (C-8); MS (ESI[−]) *m/z* 273 [M-H][−].

6-Formylindolo[3,2-*b*]carbazole (6)

It occurred in a fraction eluted with 100% CH₂Cl₂. Further isolation was performed with preparative TLC using cyclohexane/EtOAc = 6:5 + 1.5% acetic acid (*R_f* = 0.60). UV/Vis (ACN) λ_{max} 223, 248, 386 nm; ¹H-NMR and ¹³C-NMR data were identical with literature values;⁶ MS (ESI[−]) *m/z* 283 [M-H][−].

Indolo[3,2-*b*]carbazole (2)

It was contained in a fraction eluted with 100% CH₂Cl₂. Further isolation was performed with column chromatography using Sephadex LH-20, with 102–109 mL of MeOH. UV/Vis (ACN) λ_{max} 271, 333, 398 nm; ¹H-NMR and ¹³C-NMR data were identical with literature values;⁷ MS (ESI[−]) *m/z* 255 [M-H][−].

Tryptanthrin (5)

It was isolated from a fraction eluted with CH₂Cl₂:MeOH – 99:1. Further isolation was performed with a second Column Chromatography from which it was eluted with CH₂Cl₂ 100%. UV/Vis (ACN) λ_{max} 248, 332, 390 nm; ¹H-NMR and ¹³C-NMR data were identical with literature values;⁸ MS (ESI[−]) *m/z* 247 [M-H][−].

Pityriazepin (8)

It was isolated from a fraction eluted with CH₂Cl₂:MeOH – 98:2. Further isolation was performed with preparative TLC using cyclohexane:EtOAc – 70:30 (R_f = 0.24). Yellow solid; UV/Vis (MeOH) λ_{max} 287, 313, 388 nm; ¹H-NMR (600MHz, Acetone-d₆): δ 12.04 (brs, 6-NH), 11.80 (brs, 1'-NH), 9.05 (d, *J*=6.0Hz, H-1), 8.94 (d, *J*=6.0 Hz, H-2), 8.63 (d, *J*=8.2 Hz, H-10), 8.52 (d, *J*=5.0 Hz, H-2'), 8.40 (dd, *J*=8.8, 3.7 Hz, H-4'), 7.92 (d, *J*=8.2 Hz, H-7), 7.89 (t, *J*=7.5 Hz, H-8), 7.58 (t, *J*=7.5 Hz, H-9), 7.68 (dd, *J*=8.8, 3.7 Hz, H-7'), 7.40 (m, H-5' and H-6'); ¹³C-NMR (600MHz, Acetone-d₆): δ 173.2 (C-5), 145.9 (C-6a), 138.9 (C-2'), 138.4 (C-4), 138.2 (C-7'a), 134.7 (C-5a), 133.4 (C-10b), 133.4 (C-8), 130.9 (C-2), 127.2 (C-3'a), 125.1 (C-6'), 124.0 (C-10), 123.8 (C-5'), 122.9 (C-9), 122.2 (C-4'), 120.7 (C-10a), 119.2 (C-1), 115.8 (C-3'), 113.8 (C-7), 113.3 (C-7'); HRMS (ESI) *m/z* 312.1125 [M + H⁺], calculated for C₂₀H₁₃N₃O, 311.3367.

Indirubin (5)

It was isolated from a fraction eluted with CH₂Cl₂:MeOH – 99:1. UV/Vis (ACN) λ_{max} 288, 362, 540 nm; ¹H-NMR and ¹³C-NMR data were identical with literature values;⁹ MS (ESI[−]) *m/z* 261 [M-H][−].

Reporter gene assays

The isolated alkaloids were evaluated for their ability to activate AhR-dependent reporter gene expression in four different cell lines (human hepatoma (HG2L7.5c1), mouse hepatoma (H1L7.5c3), rat hepatoma (H4L7.5c2), and guinea pig intestinal adenocarcinoma (G16L7.5c8)) stably transfected with an AhR-responsive luciferase reporter gene. Based on the concentration-luciferase induction response curves, the EC₅₀ values (nM/pM) were calculated for each compound and compared to those of dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)), the prototypical AhR agonist. Preparation, treatment, incubation and luciferase analysis of these recombinant cell lines was carried out as previously described.¹⁰ Briefly, the recombinant cell lines were grown in 100-mm cell culture plates (Corning Glass Works; Corning, NY) using sterile technique, maintained in alpha-minimum essential media (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and G418 (1mg/mL) and incubated at >80% humidity and 37°C. For luciferase activity analysis, plates of stable cell clones (approximately 80–100% confluent) were trypsinized and resuspended in 20 mL α-

MEM. An aliquot (100 μ L) of the indicated cell suspension was added into each well of sterile 96-well tissue culture plates (Corning) and plates were incubated for 24h prior to ligand exposure, to allow cells to attach and reach confluence. Prior to ligand addition, wells were washed with 1X phosphate-buffered saline (PBS), and then cells were incubated with the indicated concentration of DMSO (10 μ L/mL) or corresponding solutions of TCDD or tested compounds in DMSO for 6h at 37°C. After incubation, cells were washed twice with PBS, 100 μ L of 1X Lysis buffer (Promega; Madison, WI) was added to each well and plates were placed on a plate shaker at room temperature until cells were lysed (approximately 20min). Luciferase activity was measured using an automated microplate luminometer (Anthos Lucy2, Austria) in enhanced flash mode with the automatic injection of 50 μ L of Promega stabilized luciferase reagent as previously described.¹⁰

***In vitro* analysis of AhR ligand/agonist properties of Pityriazepin**

Pityriazepin was analysed for ligand binding to the AhR using guinea pig hepatic cytosol and the hydroxyapatite binding method as previously described.¹¹ In this protocol, aliquots of diluted guinea pig cytosol were incubated in the presence of indicated concentrations of pityriazepin or solvent control DMSO (1% v/v) for 1h at RT in the presence of 2 nM [³H]TCDD (kindly provided by Dr S. Safe, Texas A&M University). Aliquots were analysed for [³H]TCDD specific binding by the hydroxyapatite ligand binding assay. Specific [³H]TCDD binding was determined by subtracting non-specific [³H]TCDD binding values (reactions performed in the presence of [³H]TCDD and 200 nM TCDF) from those of total [³H]TCDD binding (reactions performed with [³H]TCDD). The ability of pityriazepin to competitively displace [³H]TCDD in a concentration-dependent manner indicates that is an AhR ligand.

Determination of the ability of pityriazepin to directly stimulate transformation (i.e. conversion of the AhR into its high affinity DNA binding form) and DNA binding of the AhR, measured by the gel retardation assay, was performed exactly as previously described in detail.¹²

Results and Discussion

The study of the metabolites was performed on the *M. furfur* strain CBS1878 cultured with *L*-tryptophan as single nitrogen source. The EtOAc extract, after being subjected to molecular exclusion chromatography to eliminate several lipids, was fractionated with low pressure column chromatography (STable 1) and the resulting fractions were analyzed with High Pressure Liquid Chromatography – UV. This process offered the profile of each fraction and led us to targeted isolation of known, as well as yet unidentified, metabolites using semi-preparative HPLC and preparative TLC. The isolation of the alkaloids was followed by NMR and MS assisted structure elucidation.

This analysis allowed the detection and isolation of a series of known AhR-active metabolites (Fig. 1), including: malassezin (1), indolo[3,2-*b*]carbazole (ICZ) (2), pityriacitrin (3), indirubin (4), tryptanthrin (5) and 6-formyl-indolo[3,2-*b*]carbazole (6-FICZ) (6). Further investigation of the HPLC results, revealed several peaks corresponding to unknown metabolites. One of the peaks (SFig. 1) appeared to be an uncharacterized

yellow compound with UV absorption bands similar to those of the pityriacitrin (**3**) spectrum, which suggested the presence of at least an indole ring system in the molecule. Further purification of the corresponding fraction by preparative TLC led to the isolation of the unknown compound and its structure was elucidated by NMR and MS analysis.

The isolated alkaloid was examined in 1D and 2D NMR experiments (^1H , ^{13}C , COSY, HMBC, HSQC). According to its ^1H spectrum, the new molecule had eleven aromatic hydrogen atoms suggestive of a bisindolic scaffold. Additionally, two of them belonged to a pyridine ring and were placed next to the nitrogen atom, since their coupling constant was 6.0 Hz. Further investigation of this spectrum revealed many similarities (SFig. 2), as well as significant differences, with the ^1H -NMR spectra of pityriacitrin (**3**) and pityriacitrin B (**7**), two previously known alkaloids that were isolated from extracts of *M. furfur*. This finding provided further support to the structural similarity of these compounds whilst the collected dataset suggested that this compound had a skeleton quite similar to that of pityriacitrin (**3**). This was confirmed by HRMS analysis, since the molecular mass of the new product was identical to that of pityriacitrin (**3**) with the structural formula $\text{C}_{20}\text{H}_{13}\text{N}_3\text{O}$. However in the HMBC spectrum (SFig. 3) it was not possible to detect any coupling related to the carbonyl group. An explanation could be that the carbonyl group was included in the molecule but in a position where no proton was found at a J^3 distance. The only possible structure to meet these requirements would contain a pyridine ring transformed to a seven-member azepinone ring, with the carbonyl group incorporated in it (Fig. 2). This structure was further supported by the correlation of H-2' with C-4 in the HMBC spectrum. The compound was named pityriazepin (**8**). The fusion of the indole ring with the azepine ring leads to an indoloazepine skeleton that has been previously found only once in nature in the structure of chromoazepinone B from *Chromobacterium violaceum*.¹³ The indoloazepine ring is biosynthetically formed *via* coupling of two tryptophan molecules in a manner similar to that of pityriacitrins.¹³

It should also be noted that *Malassezia furfur* is the first living organism from which 6-FICZ (**6**) has been isolated as a metabolite from L-tryptophan and not the product of photochemical reactions. 6-FICZ together with indirubin have been proposed to be the physiological natural ligands of AhR and their simultaneous isolation from a single microorganism is noteworthy.

Malassezia furfur-derived alkaloids are agonists of the AhR signaling pathway

Pityriazepin was analysed for ligand binding to the guinea pig hepatic cytosolic AhR using and the hydroxyapatite binding method (Fig. 3). The ability of pityriazepin to competitively displace [^3H]TCDD in a concentration-dependent manner indicated that it is an AhR ligand.

In addition, each of the isolated compounds were evaluated in recombinant human, rat and mouse hepatoma and guinea pig intestinal carcinoma cell lines for their potency as activators of AhR-dependent gene expression. These cell lines contain a stably transfected luciferase reporter gene plasmid (pGudLuc7.5) that responds to activators of the AhR in a time-, concentration-, AhR- and chemical-dependent manner with the induction of luciferase

activity.¹⁴ The relative potency of each compound as an activator of AhR-dependent luciferase gene induction (EC₅₀) was determined from the results of concentration response analysis studies and are presented in Table 1. In a previous study³ we showed that in the recombinant human cell line HG2L7.5c1, that indirubin was the most potent AhR activator ever reported in human cells with an EC₅₀ of 26 pM, 20 times lower than the prototypical ligand TCDD and 10 times lower than that of 6-FICZ. While the results of these analyses revealed that all of the isolated compounds exhibited potent inducing activity, in some cases, their relative potency was varied significantly between cell lines from different species. For example, while the relative inducing potency differences between the human and mouse cell lines for indirubin were about 1550-fold (0.026 nM versus 40.3 nM, respectively), differences in EC₅₀ between the mouse and rat cell lines for ICZ were ~109-fold (9 pM versus 980 pM, respectively) and differences for malassezin were ~26 fold more potent in human hepatoma cells than in mouse hepatoma cells (11.1 nM versus 291 nM, respectively). Pityriacitrin and Pityriazepin showed the lowest degree of difference in relative potency among the cell lines studied.

The observed differences in relative potency demonstrate that the mouse cell line which is routinely used for AhR screening is not necessarily the most appropriate for examining the AhR response to indole metabolites. Despite its lower potency for TCDD, the human cell line, for reasons not yet understood, exhibits higher sensitivity to these indole-derived compounds, especially indirubin and should be preferentially used in experiments that are attempting to identify new AhR ligands of indolic origin. While the explanation for the dramatic variation in inducing potency of many of these compounds among cell lines from different species remains to be elucidated, increased understanding of these differential responses may provide critical insights into the reported variation in species sensitivity and differential response reported for many other AhR ligands.^{4,15} Interestingly, indoloazepinone-containing derivatives originating from a marine natural product (hymenialdisine) have been shown to exert antiproliferative effects on cancer cells. While these compounds are suggested to represent novel lead structures for anticancer drug discovery, the mechanism of the antiproliferative effect is unclear and appears result in part from the inhibition of cytokine production or kinase inhibition.^{16,17} The documented ability of the AhR to mediate some of the antiproliferative effects of a variety of chemicals^{4,18}, coupled with the ability of pityriazepin to bind to and activate the AhR and AhR signaling pathway, suggests that the AhR may also play a role in the biological action of other indoloazepinone-containing compounds. Thus, future studies into the role of the AhR may play in these and other responses and the suggested species specificity of these compounds are areas for in-depth analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The structures of the isolated and tested compounds

Figure 2.
The structure of Pityriazepin (**8**).

Figure 3.

Pityriazepin is an AhR agonist. A. Pityriazepin displaced [³H]TCDD from guinea pig hepatic cytosol in a concentration-dependent manner. Indicated concentrations of compound were incubated with guinea pig hepatic cytosol in the presence of 2 nM [³H]TCDD for 1 h at room temperature followed by hydroxyapatite ligand binding assay. Values are the means $\pm$ standard deviations of three replicate reactions. An asterisk indicates those values statistically different from no competitor ('no comp') reaction at $P < 0.05$ as determined by the Student's t-test. B. Pityriazepin stimulated DNA binding of the guinea pig hepatic cytosolic AhR complex. Cytosol was incubated in the presence of increasing concentrations of pityriazepin for 1.5 h at room temperature, and binding of the AhR complex to a ³²P-labeled DRE-containing oligonucleotide was analyzed using the gel retardation assay as described in the Experimental Section. Values represent the means $\pm$ standard deviations of three replicate reactions. Non-linear regression analysis was performed in SigmaPlot. A, B. Results are representative of two-three independent experiments.

Relative potency (EC_{50}) of alkaloids isolated from *Malassezia furfur* as activators of AhR-dependent reporter gene expression in recombinant cells from various species.

Table 1

Cell Line	Indirubin	FICZ	ICZ	Pityriacitrin	Malassezin	Tryptanthrin	Pityriazepin	TCDD
Human	0.026 ^a	0.35	0.60	4.9	11.1	107	5.0	0.53
Mouse	40.3	0.48	0.98	7.6	291	35	7.1	0.073
Rat	0.94	0.078	0.009	4.3	56.0	946	4.5	0.10
Guinea pig	0.95	0.58	0.17	4.3	276	181	4.7	0.10

^aValues represent the mean EC_{50} (nM) determined from triplicate concentration response analysis