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Chem. Res. Toxicol., **Just Accepted Manuscript** • DOI: 10.1021/acs.chemrestox.9b00270 • Publication Date (Web): 24 Oct 2019

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

A biomimetic, one-step transformation of simple indolic compounds to *Malassezia*-related alkaloids with high AhR potency and efficacy

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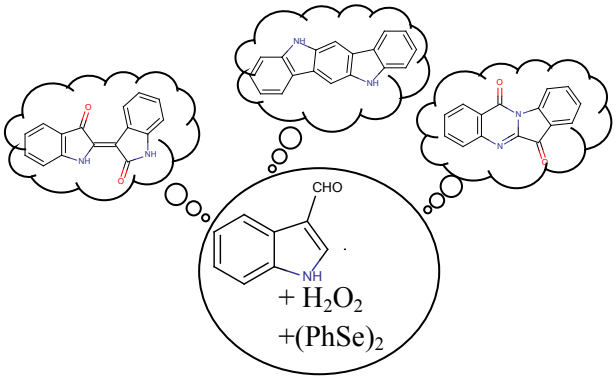

ABSTRACT

Malassezia furfur isolates from diseased skin preferentially biosynthesize compounds which are among the most active known Aryl-hydrocarbon Receptor (AhR) inducers, such as indirubin, tryptanthrin, indolo[3,2-*b*]carbazole and 6-formylindolo[3,2-*b*]carbazole. In our effort to study their production from *Malassezia* spp., we investigated the role of indole-3-carbaldehyde (I3A), the most abundant metabolite of *Malassezia* when grown on tryptophan agar, as a possible starting material for the biosynthesis of the alkaloids. Treatment of I3A with H_2O_2 and use of catalysts like diphenyldiselenide resulted in the simultaneous one-step transformation of I3A to indirubin and tryptanthrin in good yields. The same reaction was first applied on simple indole and then on substituted indoles and indole-3-carbaldehydes, leading to a series of mono- and bi-substituted indirubins and tryptanthrins bearing halogens, alkyl or carbomethoxy groups. Afterwards, they were evaluated for their AhR agonist activity in recombinant human and mouse hepatoma cell lines containing a stably transfected AhR-response luciferase reporter gene. Among them, 3,9-dibromotryptanthrin was found to be equipotent to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as an AhR agonist and 3-bromotryptanthrin was 10-times more potent than TCDD in the human HG2L7.5c1 cell line. In contrast, 3,9-dibromotryptanthrin and 3-bromotryptanthrin were ~4,000- and >10,000-times less potent than TCDD in the mouse H1L7.5c3 cell line, respectively, demonstrating that they are species-specific AhR agonists. Involvement of the AhR in the action of 3-bromotryptanthrin was confirmed by the ability of the AhR antagonists CH223191 and SR1 to inhibit 3-bromotryptanthrin-dependent reporter gene induction in human HG2L7.5c1 cells. In conclusion, I3A can be the starting material used by *Malassezia* for the production of both indirubin and tryptanthrin through an oxidation mechanism and modification of these compounds can produce some highly potent, efficacious and species-selective AhR agonists.

INTRODUCTION

Malassezia yeasts are part of the human skin microbiota that can become pathogenic and cause serious infections such as seborrhoeic dermatitis, pytiriasis versicolor, dandruff and psoriasis.[1] Extensive studies have revealed the preferential biosynthesis of indole derivatives by *Malassezia furfur* isolates from lessional skin which are among the most potent Aryl-hydrocarbon Receptor (AhR) activators known, e.g. indirubin, tryptanthrin, indolo[3,2-*b*]carbazole (ICZ) and 6-formylindolo[3,2-*b*]carbazole (6-FICZ).[2-4] Although the production of the above AhR activators seems to be a general characteristic of the genus, specific *Malasezia* species and strains are able to overproduce them and consequently their constant presence in the skin at elevated concentrations has been proposed to be implicated in the development of skin diseases.[2,3] However up today there is no available data about the biosynthetic origin of those compounds except that they are related with tryptophan.

Having as ultimate target of future works the inhibition of their overproduction and possibly the inhibition of their toxic effects for skin, we considered as a very important first step to investigate the role of potential biosynthetic precursors and the chemical mechanism that could lead to their production, mimicking what is potentially happening with *Malassezia*.The first compound that was investigated as a possible starting material for the biosynthesis of the alkaloids was indolo-3-carbaldehyde, I3A (**1**), the most abundant metabolite of all studied *Malassezia* strains when cultured with *L*-tryptophan as single nitrogen source.[5, 6]

During early trials, the treatment of I3A (**1**) with H_2O_2 and acetic acid resulted in intensively colored solutions, suggesting thus the formation of indigoids. The major product was indigo but other indole derivatives such as indirubin (**2**) could be detected in traces by NMR spectroscopy. Further trials under Baeyer-Villiger oxidation conditions led to increased yield of indirubin (**2**) . This type of reaction is commonly used for the transformation of aromatic ketones and aldehydes to the corresponding esters[7] but in this case it led to more complicated structures. For realizing this reaction we chose, among the available oxidants, hydrogen peroxide due to its eco-friendly nature, its natural presence and that exposure to H_2O_2 occurs in most human cells.[8] We also investigated the use of a potential catalyst in order to promote the reaction and enhance the yield of the desirable compounds. A thorough investigation of several parameters led to the discovery of a biomimetic reaction that can possibly explain the common presence of indirubin (**2**) and tryptanthrin (**3**) in *Malassezia* extracts.

Complementary to this assumption is a recent publication that indicates the synthesis, from indole-3-acetaldehyde upon treatment with H_2O_2, of 6-FICZ, another *Malassezia* metabolite.[9] Although we were unable to detect indole-3-acetaldehyde in the extracts of *Malassezia* species, it is biosynthetically a probable product of tryptophan metabolism.[10]

At a next step, the above described reaction was applied to several substituted indolic monomers for the preparation of a series of new or known indirubin and tryptanthrin derivatives, having as target the investigation of the structure-activity relationships concerning the AhR activation. Finally, all the synthesized analogues were evaluated for their stimulate AhR-dependent gene expression, leading to the identification of some compounds with significantly increased potency and efficacy.

EXPERIMENTAL PROCEDURES

Materials and Equipment

All the solvents used during the experimental procedure were of analytical grade or distilled. Aluminum and glass plates, covered with silicon dioxide (Silica gel 60 F_{254}) were used for TLC and preparative TLC respectively and the obtained chromatograms were observed in a CAMAG TLC Visualizer at 254 and 366 nm. All the one- and two-dimensional NMR spectra of the purified compounds, as well as the calculations of the reaction yields, were acquired in a Bruker UltrashieldTM Plus 600MHz NMR.

Initial methodology of the reaction on I3A (1)

0.34 mmol of I3A (**1**) were dissolved in 1.0 mL distilled DCM while 0.1 mL H_2O_2 10% and 0.05 mmol of $(PhSe)_2$ were then added in the reaction mixture. The flask was covered with aluminum foil to prevent occurrence of any light-induced side-reactions and the solution was stirred for 1.5 hours at room temperature (rt). Then, the same quantities of catalyst and oxidative reagent were added again and the reaction remained under mechanical stirring overnight at rt. After the formation of a dark-colored solution, MeOH was added in the mixture and it was evaporated to dryness. Then the crude mixture was submitted to column chromatography for the isolation of the produced compounds.

Modification of the reaction and application on I3A (1) and indole (8)

The modified protocol for the investigation of the above reaction was based on a method described by Santoro *et al.*[11] 0.4 mmol of I3A (**1**) or indole (**8**) were added to a mixture of 2.1 mL H_2O_2 6% and 0.7 mL acetonitrile containing a catalytic amount of $(PhSe)_2$ (0.04 mmol). The reaction remained under mechanical stirring for 72h at 23°C. The precipitate was filtered, washed with water, then solubilized with THF, collected and evaporated to dryness. The isolation of the synthesized compounds **2** and **3** was performed with preparative TLC and their structure elucidation with NMR spectroscopy.

Optimization of the reaction conditions

For the optimization of the reaction, numerous trials were performed by modifying and investigating parameters such as the oxidative means, the catalyst or the quantity of the reagents. These efforts were carried out in parallel, under the same environmental conditions and by modifying only one parameter at the time. In all cases the starting material was I3A (**1**) and the mixture was stirred for 72h at 23°C. All the attempts are summarized in Table **1,** leading to some interesting remarks and conclusions for this synthesis.

For further examination of the optimal conditions, the environmental conditions were then modified, namely the temperature and the reaction time. The starting material was once again I3A **(1)** and the reagents the same as the ones referred to entry 1 of Table **1**. These experiments are listed in Table **2**.

Investigation of the reaction mechanism

To explore the mechanism of the reaction and its' potentials, we then applied the reaction on compounds that bear longer chains in the position 3 of the indolic scaffold (e.g. tryptophan and tryptamine). Moreover, aiming to examine the necessity and the nature of the nitrogen atom in position 1 of the aromatic core, we used benzo*[b]*thiophene and *N*-Boc-indolo-3-carbaldehyde as staring materials. In all cases the aforementioned developed protocol was applied.

General protocol for the synthesis of compounds 2-2h and 3-3h

A quantity (0.4 mmol) of the appropriately substituted indolic compound was added to a mixture of 2.1 mL H_2O_2 6% and 0.7 mL acetonitrile containing a catalytic amount of $(PhSe)_2$ (0.04 mmol). The reaction remained under mechanical stirring for

72h at 23°C. The produced precipitate was filtered, washed with water, then solubilized with THF, collected and evaporated to dryness.

The isolation of the synthesized compounds was performed with preparative TLC and their structure elucidation with NMR Spectroscopy. The synthesized compounds and the reaction yields are presented in Tables **3** and **4**.

Synthesis of indirubin (2) and tryptanthrin (3):

The general method was followed using as starting material either I3A (**1**) or indole (**8**). In both cases the produced alkaloids and their yields were equivalent.

*Indirubin (**2**)*

Purple solid; Yield: 5%; R_f=0.50 (cyclohexane:EtOAc – 1:1 + 1.5% acetic acid). Spectral data are in accordance to Adachi *et al.*[12] and are given in Supporting Information.

*Tryptanthrin (**3**)*

Yellow solid; Yield: 12%; R_f=0.63 (cyclohexane:EtOAc – 1:1 + 1.5% acetic acid). Spectral data are in accordance to Jao *et al.*[13] and are given in Supporting Information.

Synthesis of 5,5'-difluoroindirubin (2a) and 2,8-difluorotryptanthrin (3a):

The general method was followed using as starting material the 5-fluoroindole.

*5,5'-difluoroindirubin (**2a**)*

Purple solid; Yield: 5%; R_f=0.18 (cyclohexane:EtOAc – 7:3 + 1.5% acetic acid. Spectral data are in accordance to Wang *et al.*[14] and are given in Supporting Information.

*2,8-difluorotryptanthrin (**3a**)*

Yellow solid; Yield: 16%; R_f=0.53 (cyclohexane:EtOAc – 7:3 + 1.5% acetic acid). Spectral data are in accordance to Wang *et al.*[15] and are given in Supporting Information.

Synthesis of 5,5'-dichloroindirubin (2b) and 2,8-dichlorotryptanthrin (3b):

The general method was followed using as starting material the 5-chloroindole.

*5,5'-dichloroindirubin (**2b**)*

Purple solid; Yield: 5%; R_f=0.21 (cyclohexane:EtOAc – 7:3 + 1.5% acetic acid). Spectral data are in accordance to Riepl et Urmann[16] and are given in Supporting Information.

*2,8-dichlorotryptanthrin **(3b)***

Yellow solid; Yield: 10%; R_f=0.63 (cyclohexane:EtOAc – 7:3 + 1.5% acetic acid). Spectral data are in accordance to Wang *et al.*[15] and are given in Supporting Information.

Synthesis of 5,5'-dibromoindirubin (2c) and 2,8-dibromotryptanthrin (3c):

The general method was followed using as starting material the 5-bromoindole.

*5,5'-dibromoindirubin **(2c)***

Purple solid; Yield: 6%; R_f=0.44 (cyclohexane:EtOAc – 7:3 + 1.5% acetic acid). Spectral data are in accordance to Beauchard *et al.*[17] and are given in Supporting Information.

*2,8-dibromotryptanthrin (**3c**)*

Yellow solid; Yield: 12%; R_f=0.64 (cyclohexane:EtOAc – 7:3 + 1.5% acetic acid). Spectral data are in accordance to Wang *et al.*[15] and are given in Supporting Information.

Synthesis of 5,5'-dimethylindirubin (2d) and 2,8-dimethylotryptanthrin (3d):

The general method was followed using as starting material the 5-methylindole.

*5,5'-dimethylindirubin **(2d)***

Purple solid; Yield: 6%; R_f=0.23 (cyclohexane:EtOAc – 7:3 + 1.5% acetic acid Spectral data are in accordance to Wang *et al.*[14] and are given in Supporting Information.

*2,8-dimethylotryptanthrin (**3d**)*

Yellow solid; Yield: 15%; R_f=0.46 (cyclohexane:EtOAc – 7:3 + 1.5% acetic acid). Spectral data are in accordance to Wang *et al.*[15] and are given in Supporting Information.

Synthesis of 5,5'-dicarbomethylindirubin (2e) and 2,8-dicarboxymethylotryptanthrin (3e):

The general method was followed using as starting material the 5-carboxymethylindole.

*5,5'-dicarboxymethylindirubin **(2e)***

Red solid; Yield: 17%; R_f=0.27 (cyclohexane:EtOAc – 1:1 + 1.5% acetic acid). ^{1}H-NMR ($(CD_3)_2SO$, 600MHz): δ 11.44 (brs, -NH); 11.34 (brs, -NH); 9.45 (d, H-4, *J*=1.7Hz); 8.18 (brs, H-4'); 8.16 (dd, H-6', *J*=8.5Hz, 1.6Hz); 7.93 (dd, H-6, *J*=8.2Hz, 1.7Hz); 7.54 (d, H-7', *J*=8.5Hz); 7.02 (d, H-7, *J*=8.2Hz); 3.88 (s, 3H, $-CH_3$); 3.86 (s, 3H, $-CH_3$).

*2,8-dicarboxymethylotryptanthrin **(3e)***

Yellow solid; Yield: 27%; R_f=0.52 (cyclohexane:EtOAc – 1:1 + 1.5% acetic acid). ^{1}H-NMR ($CDCl_3$, 600MHz): δ 9.11 (d, H-1, *J*=1.8Hz); 8.73 (d, H-10, *J*=8.4Hz); 8.59 (d, H-7, *J*=1.4Hz); 8.51 (dd, H-3, *J*=8.4Hz, 1.8Hz); 8.49 (dd, H-9, *J*=8.4Hz, 1.4Hz); 8.10 (d, H-4, *J*=8.4Hz); 4.02 (s, 3H, -CH_3); 3.99 (s, 3H, -CH_3)

Synthesis of 6,6'-dibromoindirubin (2f) and 3,9-dibromotryptanthrin (3f):

The general method was followed using as starting material the 6-bromoindole-3-carbaldehyde.

*6,6'-dibromoindirubin (**2f**)*

Red solid; Yield: 5%; R_f=0.28 (cyclohexane:EtOAc – 7:3 + 1.5% acetic acid). Spectral data are in accordance to Clark et Cooksey[18] and are given in Supporting Information.

*3,9-dibromotryptanthrin (**3f**)*

Yellow solid; Yield: 13%; R_f=0.65 (cyclohexane:EtOAc – 7:3 + 1.5% acetic acid). ^{1}H-NMR ($CDCl_3$, 600MHz): δ 8.84 (d, H-10, *J*=1.6Hz); 8.28 (d, H-1, *J*=8.5Hz); 8.19 (d, H-4, *J*=1.8Hz); 7.80 (dd, H-2, *J*=8.5Hz, 1.8Hz); 7.70 (d, H-7, *J*=8.1Hz); 7.61 (dd, H-8, *J*=8.1Hz, 1.6Hz)

Synthesis of 6,6'-dicarbomethylindirubin (2g) and 3,9-dicarboxymethylotryptanthrin (3g):

The general method was followed using as starting material the 6-carboxymethylindole.

*6,6'-dicarboxymethylindirubin (**2g**)*

Purple solid; Yield: 14%; R_f=0.40 (cyclohexane:EtOAc – 1:1 + 1.5% acetic acid). ^{1}H-NMR ($(CD_3)_2SO$, 600MHz): δ 11.45 (brs, -NH); 11.08 (brs, -NH); 8.82 (d, H-4, *J*=8.2Hz); 8.06 (brs, H-7'); 7.79 (d, H-4', *J*=8.0Hz); 7.67 (d, H-5', *J*=8.0Hz); 7.60 (d, H-5, *J*=8.2Hz); 7.43 (brs, H-7); 3.90 (s, 3H, -CH_3); 3.86 (s, 3H, -CH_3)

*3,9-dicarboxymethylotryptanthrin **(3g)***

Yellow solid; Yield: 17%; R_f=0.59 (cyclohexane:EtOAc – 1:1 + 1.5% acetic acid). Spectral data are in accordance to Wang *et al.*[15] and are given in Supporting Information.

Synthesis of 6'-bromoindirubin (2h) and 3-bromotryptanthrin (3h):

The general method was followed using as starting material equivalent quantities of I3A (**1**) and 6-bromoindole-3-carbaldehyde (**8**). The quantities of all the reagents were doubled.

*6'-bromoindirubin (**2h**)*

Purple solid; Yield: 5%; R_f=0.38 (cyclohexane:EtOAc – 7:3). Spectral data are in accordance to Clark et Cooksey[18] and are given in Supporting Information.

*3-bromotryptanthrin (**3h**)*

Yellow solid; Yield: 12%; R_f=0.53 (cyclohexane:EtOAc – 7:3). Spectral data are in accordance to Li *et al.*[19] and are given in Supporting Information.

Calculation of the reaction yields with NMR Spectroscopy

The calculation of the reaction yields was performed using quantitative ^{1}H-NMR spectroscopy in a similar way as previously described for metabolite quantitation in complex natural mixtures.[20] The spectra were acquired in solution of the reaction mixtures after the work up described above at specific concentration in $(CD_3)_2SO$, where a known quantity of syringaldehyde was added as Internal Standard. Using the peak of the aldehyde group of syringaldehyde at 9.79 ppm for comparison, the ratio of the reactants/products was measured in the reaction mixture.

Protocol for the evaluation of AhR activity

The synthesized alkaloids were evaluated for their ability to activate AhR-dependent gene expression in two different recombinant cell lines (human hepatoma (HG2L7.5c1) and mouse hepatoma (H1L7.5c3),) containing the identical stably transfected AhR-responsive luciferase reporter gene plasmid, pGudLuc7.5.[21, 22] The results (EC_{50} (M)) calculated from concentration-response experiments were compared to those of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the prototypical and highly potent AhR agonist. The AhR activity protocol was derived from He *et al.*[23] and was as follows:

Human and mouse cell lines were grown in 100-mm tissue culture plates (Corning Glass Works; Corning, NY) using sterile techniques, maintained in culture medium (alpha-minimum essential media (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and G418 (1 mg/mL)) and incubated at >80% humidity and 37°C.

For AhR-dependent gene expression analysis, plates of human or mouse cells (approximately 80–100% confluent) were trypsinized and resuspended in 20 mL culture media. An aliquot (100 μL) of the indicated cell suspension was added into sterile 96-well tissue culture plates (Corning) and plates were incubated for 24 h prior to chemical exposure, allowing 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 culture media containing DMSO (10 μL/mL) or the indicated concentration of TCDD or test compounds in DMSO for 6h or 24h at 37°C. For AhR antagonist studies, cells were incubated with TCDD or 3-bromotryptanthrin in the absence or presence of the indicated concentration of the AhR antagonist CH223191 (Chembridge Corporation; San Diego, CA) or StemRegenin1 (SR1) (Cayman Chemicals; Ann Arbor, MI)) for 6h at 37°C.

After incubation cells were washed twice with PBS, 100 μL of 1X Lysis buffer (Promega; Madison, WI) was added to each well and the plate shaken at room temperature until cells were lysed (approximately 20 min). Luciferase activity was measured using an automated microplate luminometer (Anthos Lucy2, Austria) in enhanced flash mode with the automatic injection of 50 μL of Promega stabilized luciferase reagent.

RESULTS

Initial methodology of the reaction on I3A (1)

The performance of the initial reaction led to the formation and isolation of indirubin (**2**) after treatment of I3A (**1**) with H_2O_2 under Baeyer-Villiger oxidation conditions (Figure 1). However, several practical difficulties were observed in the above reaction such as the need for renewal of the reagents in the course of the reaction or the way of evaluating its success. Therefore, we investigated the possible ameliorations that could increase the reaction yield.

Modification of the reaction and application on I3A (1) and indole (8)

A reaction described by Santoro *et al*.[11] was selected as alternative synthetic approach. It seemed preferable mainly due to its reagents, which were coinciding with the ones used for the transformation of I3A (**1**) to indirubin (**2**). According to this protocol, H_2O_2 has to be at a concentration of 6%, the solvent H_2O:ACN in a ratio 3:1 and the optimal conditions are achieved when the selenium derivative is added in a catalytic amount (10%) and the reaction mixture is stirred for 72h at 23°C. This synthetic path is being characterized as an "eco-friendly olefin dihydroxylation", probably due to the eco-friendly reagents used, and is proposed for the formation of *vic* diols (*cis*- or *trans*-) from the corresponding alkenes.

When the above conditions were applied on I3A (**1**), they resulted once again in the production of indirubin (**2**) along with a mixture of several co-products where a

fluorescent yellow compound was predominant. The yellow compound was isolated and identified as indolo[2,1-*b*]quinazolin-6,12-dione or tryptanthrin (**3**) after comparison of its spectroscopic data with literature[13]. Tryptanthrin (**3**) is an already known AhR inducer which possesses also significant anti-inflammatory, antimicrobial and cytostatic activity.[24-30] It has been isolated from many plants as well as the yeast *Candida* (*Yarrowia*) *lipolytica*[31-33] and has also been detected and isolated in *Malassezia furfur* yeasts.[3, 4] Additionally, the spectroscopic analysis of the mixture showed the formation of indolo[3,2-*b*]carbazole (**4**), another known *Malassezia* metabolite, in traces (Figure 2).

Those results prompted us to investigate further the optimal conditions for the reaction as well as its mechanism. During this effort, the reaction was also applied on simple indole (**8**), another abundant metabolite of *Malassezia* spp. [5] and interestingly the formation of the metabolites **2** and **3** was also achieved, while indolo[3,2-*b*]carbazole (**4**) was not detected.

Optimization of the reaction conditions

The successful application of the oxidation on indole (**8**) and I3A (**1**) paved the road for a further investigation of the optimal reaction conditions as well as for the role of each reagent. For this reason, several trials were performed and the attempts are summarized in Table **1**. Further details on the optimization of the reaction conditions are presented as supporting information.

Synthesis of tryptanthrin and indirubin derivatives

After the described synthesis was successfully applied on indole (**8**) and I3A (**1**) and the optimal reaction conditions were clarified, the next step was the application on substituted indoles and indole-3-carbaldehydes. A wide variety of substituted indole compounds bearing halogens, alkyl-, hydroxyl-, alkoxy-, carboxymethyl- and amino-groups in positions 4, 5, 6 or 7 of the indole ring was chosen to be studied.

The most active among them seemed to be the compounds with substituents in positions 5 or 6 of the core and, especially, the ones that bore halogens, alkyl- and carboxymethyl- groups. This observation let us proceed in the synthesis of all the corresponding bisubstituted indirubins and tryptanthrins in yields that varied from 5 to 17% for indirubins (**2-2g**) and from 10 to 27% for tryptanthrins (**3-3g**), depending on the substitution. The highest yields for both molecules were achieved when 5- and 6-

carboxymethylindoles were used as reactants. On the contrary, the application of this protocol on indoles bearing easily oxidizable groups (e.g. amino-, cyano-, hydroxyl- or methoxy- groups), led to the formation of dark colored, non-soluble precipitates. Furthermore, the reaction didn't evolve as predicted when applied on either azaindoles or compounds with substituents in positions 4 or 7 of the indolic core.

Yet, another reaction performed on the mixture of I3A (**1**) and 6-bromo-I3A resulted in the simultaneous synthesis of all 8 bis-, mono- and unsubstituted analogues of the two alkaloids. Amongst the monosubstituted compounds, the formation of 6'-bromoindirubin and 3-bromotryptanthrin was favored by the reaction conditions, probably due to differences in the kinetics of the reactions.

Evaluation of AhR Agonist Activity

The AhR agonist activity of 15 out of the 18 synthesized compounds was evaluated in recombinant HG2L7.5c1 human hepatoma cells containing a stably transfected AhR-responsive luciferase reporter gene.[22] Indirubins were tested only in the human cell line due to previous evidence for the high selectivity of indirubins for the human AhR as compared to the mouse AhR.[3, 34, 35] In contrast, tryptanthrin analogues were tested in both stably transfected human (HG2L7.5c1) and mouse (H1L7.5c3)hepatoma cell lines, not only to determine their relative potencies and efficacies, but also to evaluate whether these analogues also exhibited a greater selectivity for human AhR compared to mouse AhR. The 6 hour concentration-response results of tryptanthrins obtained using the human HG2L7.5c1 cells are presented in Figure 3. The EC_{50} (M) results from concentration response curve for the indirubins and tryptanthrins were calculated and compared to the EC_{50} (M) obtained from TCDD concentration response analysis (Tables **5** and **6**).

While most of the synthesized indirubins showed very strong activity (two of them (**2b**, **2f**) had potencies comparable to or greater than TCDD), insertion of substituents reduced activity in comparison to the highly potent unsubstituted indirubin (Table 5). Concerning the tryptanthrin analogues, it is clear that this skeleton can afford extremely active derivatives with very simple modifications.

Among the tryptanthrin derivatives (Table 6), 3,9-dibromotryptanthrin (**3f**) interestingly showed a 350-fold increased potency compared to tryptanthrin (**3**) in the human HG2L7.5c1 cells and was comparable to the potency of TCDD when evaluated at 6h (EC_{50} $5x10^{-10}$M. In contrast, the monosubstituted analogue **3h** (3-

bromotryptanthrin) was 3,000-times more potent than tryptanthrin and 10 times more potent than TCDD in the human cells. Given the previously documented high degree of selectivity of indirubin for human AhR, as compared to mouse AhR,[3,34,35] and similarities in several structural aspects of indirubin and tryptanthrin, the relative potencies of tryptanthrin analogues in human and mouse cells were compared. These analyses revealed that while most tryptanthrin analogues were about 10-fold less potent in the mouse cell line, as compared to that of the human cell line, all were significantly less potent than TCDD in the mouse cells (Table 6, Figure 4). Interesting, 3,9-dibromotryptanthrin and 3-bromotryptanthrin were 4,000- and 10,000-fold less potent in the mouse cells, respectively, than in the human cells, where these compounds were equipotent to or more potent than TCDD (Table 6, Figure 4). These results clearly demonstrate that 3,9-dibromotryptanthrin and 3-bromotryptanthrin are highly potent and efficacious human selective AhR agonists.

To confirm that the induction of reporter gene expression in human cells was mediated by the AhR, the effect of AhR antagonists (CH223191 and SR1[36,37] on the induction of reporter gene expression by 3-bromotryptanthrin was examined in the human HG2L7.5c1 cells (Figure 4). These results clearly demonstrate that AhR antagonists inhibit both TCDD- and 3-bromotryptanthrin-dependent reporter gene induction in the human cell line, consistent with these responses being mediated by the AhR

The gene induction experiments were carried out at 6h of incubation due to the metabolic lability of the tryptanthrins.

At 24h of incubation, the EC_{50} for gene induction in human cells by TCDD remained as low as $3x10^{-10}$ M but the potency of tryptanthrins was significantly reduced (EC_{50}s of 10^{-6} to 10^{-7}) and this results from differences in the metabolism of these compounds. While TCDD cannot be metabolized to any significant degree and therefore its relative potency is retained at 24h, tryptanthrins are metabolized by the CYP enzymes induced in these cells, resulting in induction of AhR-dependent gene expression that is transient, with a maximum peak between 6h and 12h.

Discussion

Here we describe the one-step transformation of the aldehyde **1** and/or indole (**8**) to the alkaloids indirubin (**2**) and tryptanthrin (**3**), under Baeyer-Villiger oxidation conditions.

As already analyzed, the established reaction protocol is the result of an amelioration process, with one of the most important and consistent characteristics to be the catalyst used, diphenyl diselenide, the reaction of which with H_2O_2 results in the *in situ* formation of the corresponding seleninic acid (aka *Syper method* of activation).[38-40] The necessity for further investigation and optimization of the procedure led us to perform an extensive bibliographic search on oxidation of indoles and on reactions using either H_2O_2 as an oxidative means or organic derivatives of selenium as a catalyst, identified a number of different relevant publications.

We chose to follow the procedure as it is described by Santoro *et al*.[11] as it combined the use of H_2O_2 and diphenyl diselenide with the organoselenic derivatives to be a category of catalysts preferred in several organic reactions due to their easy synthesis and their stability under various conditions. Throughout this protocol, we achieved the one-step transformation of the aldehyde **1** to the alkaloids **2** and **3** in good yields and the compound **4** in traces. This can be considered as a biomimetic reaction, showing for the first time the possible common biosynthetic origin of the three metabolites (Figure **2**), assumption that is verified by the synthesis of the same two out of three alkaloids when indole (**8**), also a metabolite found in abundance in *Malassezia* cultures, was used as starting material. The one-pot conversion of both I3A (**1**) and indole (**8**) to indirubin (**2**) and tryptanthrin (**3**) simultaneously *via* the same oxidative reaction has never been reported.

The up-to-date data on the oxidation of indoles refer to studies conducted in many different ways, using a variety of reagents (usually expensive and difficult to handle) and leading mainly to the formation of the corresponding 2-indolinones or oxindoles.[41,42,45,47] In some cases also isatin[50] or indigo were formed. [48,49] Additional research on the attempted syntheses of the two alkaloids revealed that both are formed by the coupling of an isatin (**5**) molecule with either 3-acetoxyindole for indirubin (**2**) [53]or isatoic anhydride for tryptanthrin (**3**).[31,54,55] Many other alternative paths have been used for the synthesis of **3**, including even electrochemical methods and condensation of isatin (**5**).[31, 56-60] A review on the synthetic and medicinal advances for tryptanthrin has been recently published, summarizing most of the available methods for tryptanthrin synthesis.[61] Among the most recent publications, some newer interesting methods for the synthesis of thryptanthrin (**3**) were described that are very similar to the aforementioned biomimetic reaction. In two of them, I3A (**1**) was submitted to oxidative dimerization using either Oxone in a mixture of water and ACN [62] or urea

peroxide in toluene.[63] Especially the last one is very efficient for tryptanthrin but not for indirubin and probably this is related with the use of toluene and heat that make the reaction conditions much more different than those used in our case which are closer to those of a living organism (aqueous medium and ambient temperature). Three more publications have used indole (**8**) as starting material which is dimerized either with copper iodide as a catalyst under a continuous flux of oxygen[15] or *via* irradiation by LED light combined with the use of selected catalysts and simultaneous exposure to oxygen.[19,64]

However, the developed synthetic protocol followed in our case seems to have certain advantages. This synthetic path is being described as an "eco-friendly olefin dihydroxylation" leading to the formation of *vic* diols from the corresponding alkenes.[11] The eco-friendly character of the reaction is of great importance and reassured by the solvents (water and ACN) and the reagents (H_2O_2 and $(PhSe)_2$) used, that are not harmful for the environment, especially after the treatment of the residue. Other advantages are the simple overall procedure, the low-cost, easily accessible reagents and the possibility of application on a significant number of substrates, comprising both indoles and indole-3-carbaldehydes. All these features, along with the simultaneous formation of two different categories of alkaloids, establish the reaction as an attractive alternative for their synthesis. To the very best of our knowledge, the successful application of the same dimerization reaction on both indole (**8**) and I3A (**1**) hasn't been reported before whereas the parallel synthesis of both indirubin (**2**) and tryptanthrin (**3**) has been mentioned only once again,[62] where indirubin (**2**) is referred to as one of the byproducts of the reaction.

The mechanism that mediates the oxidative transformation of I3A (**1**) and indole (**8**) is difficult to be confirmed with certainty. A primary concept can be based on the mechanism described by Santoro, involving the formation of an intermediary epoxide which then opens to the *cis*- or *trans*- diol[11] and the catalyst is reborn through this cycle.

In accordance to the above assumption, we came up with a hypothesis involving the synthesis of 3-indolyl formate (**5**), which would be originally expected under Bayer-Villiger conditions, and the subsequent formation of an intermediary epoxide on the double bond of the positions 2 and 3. Hydrolysis of the intermediate results in isatin (**6**), creating thus an equilibrium between the indole derivatives **5** and **6,** the coupling of which gives indirubin (**3**). This mechanism is depicted in Figure 5 and is close to a

well-known methodology of indirubin (**3**) synthesis[53] which involves a coupling reaction between 3-acetoxyindole and isatin (**6**).

A quite similar mechanism is proposed for the synthesis of tryptanthrin (**3**) which is thought to be produced by the coupling reaction between anthranilic acid (**7**) and isatin (**6**). As mentioned, isatin (**6**) is formed in the reaction medium, whereas anthranilic acid (**7**) can result by the hyperoxidation of either I3A (**1**) or isatin (**6**) and subsequent *in situ* formation of the isatoic anhydride which will be hydrolyzed to yield the acid **7**, a hypothesis supported by previous bibliographic data.[60] This synthetic path is shown in Figure 6 and the concept behind this proposed mechanism is similar to the ones suggested by the most relevant publications.[15, 62, 63,]

It's worth mentioning that, like in the case of indirubin (**2**), the reaction between anthranilic acid (**7**) and isatin (**6**) is a common synthetic method for the production of tryptanthrin (**3**),[65,66] with distinct reference to the biosynthesis of the alkaloid.[31] However, when an ACN solution of the proposed intermediates isatin (**6**) and anthranilic acid (**7**) was stirred for 72h at 23°C none of the desirable alkaloids were formed, suggesting the necessity of an oxidative means in the reaction medium, an assumption that is also in accordance with bibliographic data.[60, 65, 66]

The aforementioned synthetic schemes represent largely the assumed mechanisms for the transformation of indole (**8**) to the two alkaloids when the exact same reaction conditions. The hypothesis is slightly altered, involving the condensation of two molecules of the produced isatin (**6**). This is supported also by recent data that describe the synthesis of indirubins by isatin coupling in reductive conditions.[14] The proposed synthetic scheme for this reaction is in Figure 7.

As far as the formation of indolo[3,2-*b*]carbazole (**4**) is concerned, a different mechanism is proposed, involving the direct coupling of two I3A (**1**) units (Figure 8) and is based on relevant bibliographic data.[9, 67-70] As it has already been mentioned, compound **4** is formed only in the reaction of I3A (**1**). This observation can be easily explained as indolo[3,2-*b*]carbazole (**4**) is a molecule with 18 carbon atoms whilst the dimerization of indole (**8**), can offer only up to 16 carbon atoms in the reaction. On the other hand, in the reaction of I3A (**1**) the carbon atoms participating are sufficient enough for the formation indolo[3,2-*b*]carbazole (**4**).

Summing up the details of the reaction mechanism, we need to state the importance of the order of addition of the reagents as, according to the general reaction mechanism proposed [11], the catalyst has first to be activated meaning that the reaction

of the oxidative means with the catalyst has to take place prior to its reaction with the starting material. For this reason, the addition of H_2O_2 in the solution of the catalyst before the reactant is mandatory.

Finally, examining the products resulting from the reaction on the mixture of I3A (**1**) and 6-bromo-I3A (**8**) we observed the favored synthesis of certain compounds; combined with the mechanisms proposed, they can lead to some remarks for the kinetics of the transformations. Given the bibliographic data[31, 71] the 6'-substitution on indirubin scaffold derives from the substitution of acetoxyindole and, respectively, the 3-subsitution on tryptanthrin from the anthranilic acid (**7**). Our observation can suggest that the reaction for the formation of 6-bromoisatin (**10**) progresses more slowly than the corresponding acetoxyindole (**9**) and anthranilic acid (**11**), favoring thus the one for the non-acylated compound (Figure 9). According to this hypothesis, the synthesis of 6'-bromoindirubin (**2h**) and 3-bromotryptanthrin (**3h**) seems to be favored compared to the 6- and 9- substituted compounds.

The final step of this work comprised the evaluation of the AhR agonist activity of the purified indirubin and tryptanthrin analogues and revealed some interesting results for these classes of alkaloids. Although most of the synthesized indirubin analogues were relatively potent AhR agonists, all of the resulting compounds were less potent than that of unsubstituted indirubin. Additionally, it was clear that even simple modification of the tryptanthrin skeleton could produce analogues that are extremely potent and efficacious AhR agonists, particularly in the human cell line.

The tryptanthrin structure activity relationship bioassay results in human cells revealed that insertion of bromine at positions 3 and 9 (**3f**) not only increased the relative potency (EC_{50}) of unsubstituted tryptanthrin (**3**) from 170 nM to 490 pM, but induced maximal AhR-dependent reporter gene activity (Figure 3). These observations are significant in that the relative potency and efficacy of the 3,9-dibromotryptanthrin as an AhR agonist in human cells is comparable to that of TCDD, the prototypical and one of the most potent known AhR agonists. Even more significant is that a tryptanthrin analogue with only a single bromine at position 3 (3-bromotryptanthrin (**3h**)) had a relative potency in human cells that was even 10-fold higher (57 pM), not only suggesting that the additional bromine at position 9 in compound 3f actually reduced the overall relative AhR affinity/potency compared to that of compound 3h, but producing a compound that was 10-fold more potent than TCDD. 3-Bromotryptanthrin (**3h**) was also found to be a highly efficacious AhR agonist, stimulating AhR-dependent

reporter gene expression to a level comparable to that produced by a maximally inducing concentration of TCDD (Figure 3). Previous mutagenesis and molecular docking analysis studies have clearly demonstrated that some specific interactions of a ligand with residues within the AhR ligand binding site are critical and can drive ligand-selective interactions,[72-75] such that a simple modification like the insertion of a carbomethoxy group in the same scaffold positions (**3g)** instead of bromine can lead to a completely inactive derivative. While induction of reporter gene expression in the human cells by a given compound strongly suggests that it is an AhR agonist and activates reporter gene expression via the AhR, the role of the AhR in the ability of the most potent tryptanthrin analogue (3-bromotryptanthrin (**3h**)) was confirmed by demonstrating the ability of two AhR antagonists (CH223191 and SR1) to inhibit the induction response (Figure 4). Together, these analyses have identified two new and novel tryptanthrin analogues (3-bromotryptanthrin and 3,9-dibromotryptanthrin) as highly potent and efficacious agonists of the human AhR and AhR signaling pathway.

Studies from several laboratories have demonstrated that indirubin is a highly potent and efficacious human-selective AhR agonist that induces AhR-dependent gene expression in human cells with an EC_{50} that is ~10-fold greater than that of TCDD.[3, 34, 35, 76] In contrast, in mice or mouse cells, indirubin is ~100-less less potent than TCDD, but still highly efficacious. [34, 35, 76] Site-directed mutagenesis and functional analysis studies have identified specific amino acid residues within the mouse and human AhR ligand binding pockets that contributes to this differential specificity and responsiveness.[35] The similarities in structual aspects of indirubins and tryptanthrins suggest that similar species differences in tryptanthrin ligand specificity may also exist. Comparison of the relative potencies of the indirubins (**3-3h**) to stimulate AhR-dependent reporter gene analysis in both human and mouse hepatoma cell lines containing the identical reporter gene plasmid (Table 6) revealed that while the potency of tryptanthrin (**3**) was comparable between human and mouse cell lines, all tryptanthrin analogues were less potent in the mouse cell lines. The divergent species-specificity was particularly evident for the tryptanthin analogues that were most potent human AhR agonists (i.e., 3-bromotryptanthrin (**3h**) and 3,9-dibromotryptanthrin (**3f**)). While 3-bromotryptanthrin (**3h**) was 10-times more potent than TCDD in human cells, it was 10,000-times less potent than TCDD in the mouse cells; the difference in relative AhR agonist potency between the human and mouse cells was >17,000-fold (Table 6). Similarly, although 3,9-dibromotryptanthrin (**3h**) was equipotent to that of TCDD in

human cells, it was 4,000-times less potent than TCDD in the mouse cells; the difference in relative AhR agonist potency between the human and mouse cells was ~800-fold (Table 6). These results confirm that these two tryptanthrin analogues, like indirubin, are human-selective AhR agonists. However, the AhR antagonist results with 3-bromotryptanthrin suggest that it interacts with residues within the AhR ligand binding pocket in a manner that is distinctly different than that of indirubin. Previous studies have demonstrated that while the AhR antagonist CH223191 could effectively inhibit the ability of TCDD to bind to and activate the AhR, this antagonist had little effect on AhR activation by indirubin,[37] suggesting that TCDD and indirubin differentially interact with residues within the AhR ligand binding domain. The ability of CH223191 to inhibit AhR-dependent reporter gene activity by both TCDD and 3-bromotryptanthrin (Figure 5) suggests that binding of 3-bromotryptanthrin to the AhR is more similar to that of TCDD than indirubin, which is not inhibited by CH223191. However, since 3-bromotryptanthrin is a more potent AhR activator of the human AhR than TCDD and a much less potent activator of the mouse AhR than TCDD, 3-bromotryptanthrin must interact with the AhR ligand bidning pocket in a manner similar to, but distinctly different from that of TCDD; the differences in potency of TCDD between the human and mouse cell lines was only ~6-fold (Table 6). These induction and inhibition responses suggest that 3-bromotryptanthrin (and perhaps other tryptanthrins) may represent a novel group of AhR agonists that interact with residues within the AhR ligand binding pocket in a manner distinctly different from TCDD, indirubin and other ligands characterized to date.[34, 35, 73-78]. However, further detailed QSAR and docking analysis of these analogues into both human and mouse AhR ligand binding sites generated by molecular modeling are needed to understand the molecular mechanisms responsible for the high affinity/potency of selected tryptanthrins and the dramatic species differences in ligand specificity.

Conclusion

The total of the collected data is of significant chemical and toxicological value. The synthesis of all three alkaloids -indirubin (**2**), tryptanthrin (**3**) and indolo[3,2-*b*]carbazole (**4**)- through the same oxidative mechanism from I3A (**1**), the main product of *L*-tryptophan metabolism in *Malassezia* yeasts, seems to be used also by the fungus for the production of these secondary metabolites which are implicated in the *Malassezia*-related skin diseases. Taking into consideration that indirubin (**2**) and

tryptanthrin (**3**) are often together isolated from several plant species, like *Isatis* spp. and *Polygonum tinctorium*,[78,79] the existence of a common biosynthetic path for the two metabolites is a reasonable hypothesis. The inhibition of the oxidative transformation of I3A to indirubin and tryptanthrin by *Malassezia* could be a potential future target for the inhibition of *Malassezia* toxicity to skin.

Supporting Information

Analytical data for the synthesized compounds and results of the optimization trials and reaction mechanism investigation

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Abbreviations

AhR: Aryl-hydrocarbon Receptor
I3A: Indole-3-carbaldehyde
H_2O_2: Hydrogen Peroxide
ICZ: Indolo[3,2-*b*]carbazole
6-FICZ: 6-Formylindolo[3,2-*b*]carbazole
NMR: Nuclear Magnetic Resonance
TLC: Thin Layer Chromatography
DCM: Dichrolomethane
$(PhSe)_2$: Diphenyl diselenide
THF: Tetrahydrofuran
R_f: Retardation factor
EtOAc: Ethyl Acetate
$(CD_3)_2SO$: Deuterated Dimethyl Sulfoxide
$CDCl_3$: Deuterated Chloroform
EC_{50}: Half maximal effective concentration
TCDD: 2,3,7,8-Tetrachlorodibenzodioxin
α-MEM: alpha-minimum essential media
FBS: Fetal bovine serum
PBS: phosphate-buffered saline
DMSO: Dimethyl Sulfoxide

ACN: Acetonitrile

CYP: Cytochrome P450

Table 1: Trials of the biomimetic reaction with modification of the reagents' quantities

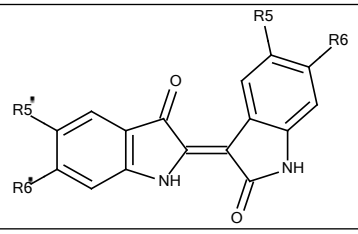
Entry	Oxidative Means	Catalyst	Quantity of catalyst	Solvent	Results
1	H_2O_2 6%	$(PhSe)_2$	10%	ACN	Indirubin&Tryptanthrin
2	H_2O_2 6%	$(PhSe)_2$	10%	-	Hyperoxidation
3	H_2O_2 6%	-	-	ACN	n.d.
4	H_2O_2 6%	$(PhSe)_2$	20%	ACN	Indirubin&Tryptanthrin
5	H_2O_2 6%	$(PhSe)_2$	50%	ACN	Indirubin&Tryptanthrin
6	$H_2O_2$12%	$(PhSe)_2$	10%	ACN	Indirubin&Tryptanthrin
7	$H_2O_2$12%	$(PhSe)_2$	10%	-	Hyperoxidation
8	$H_2O_2$12%	-	-	ACN	n.d.
9	$H_2O_2$12%	$(PhSe)_2$	20%	ACN	Indirubin&Tryptanthrin
10	$H_2O_2$12%	$(PhSe)_2$	50%	ACN	Indirubin&Tryptanthrin

n.d.: not detected

Table 2: Trials of the biomimetic reaction with modification of the conditions

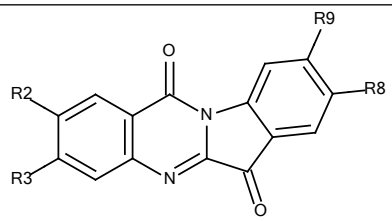
Entry	Temperature	Reaction Time	Result
11	0-23°C	72h	Indirubin&Tryptanthrin
12	~10°C	72h	Lower yield
13	40°C	72h	Lower yield
14	23°C	120h	Indirubin&Tryptanthrin

Table 3: Synthetic Analogues of Indirubin **(2)**



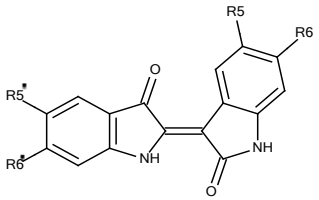
	Substitution	Yield
2	R_5=R_6=$R_{5'}$=$R_{6'}$=H	5%
2a	R_5=$R_{5'}$=F, R_6=$R_{6'}$=H	5%
2b	R_5=$R_{5'}$=Cl, R_6=$R_{6'}$=H	5%
2c	R_5=$R_{5'}$=Br, R_6=$R_{6'}$=H	6%
2d	R_5=$R_{5'}$=CH_3, R_6=$R_{6'}$=H	6%
2e	R_5=$R_{5'}$=$COOCH_3$, R_6=$R_{6'}$=H	17%
2f	R_5=$R_{5'}$=H, R_6=$R_{6'}$=Br	5%
2g	R_5=$R_{5'}$=H, R_6=$R_{6'}$=$COOCH_3$	14%
2h	R_5=$R_{5'}$= R_6=H, $R_{6'}$=Br	5%

Table 4: Synthetic Analogues of Tryptanthrin **(3)**



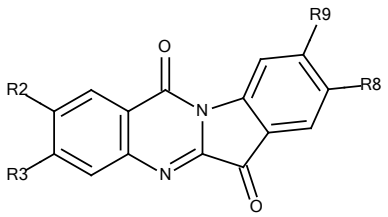
	Substitution	Yield
3	R_2=R_3=R_8=R_9=H	12%
3a	R_2=R_8=F, R_3=R_9=H	16%
3b	R_2=R_8=Cl, R_3=R_9=H	10%
3c	R_2=R_8=Br, R_3=R_9=H	12%
3d	R_2=R_8=CH_3, R_3=R_9=H	15%
3e	R_2=R_8=$COOCH_3$, R_3=R_9=H	27%
3f	R_2=R_8=H, R_3=R_9=Br	13%
3g	R_2=R_8=H, R_3=R_9=$COOCH_3$	18%
3h	R_2=R_8= R_9=H, R_3=Br	12%

Table 5: EC_{50}values for the induction of AhR-dependent gene expression in human hepatoma (HG2L7.5c1) cells by Indirubins



	EC_{50}, 6h Human
2	**$2.8x10^{-11}$**
2a	$1.0x10^{-9}$
2b	$3.0x10^{-10}$
2e	$8.2x10^{-8}$
2f	$8.6x10^{-10}$
2g	$1.0x10^{-6}$
TCDD	$5.7x10^{-10}$

Table 6: EC_{50}values for the induction of AhR-dependent gene expression in human (HG2L7.5c1) and mouse (H1L7.5c3) hepatoma cell lines by tryptanthrins.



	EC_{50}, 6h Human Cells	EC_{50}, 6h Mouse Cells
3	**$1.7x10^{-7}$**	**$8.1x10^{-7}$**
3a	$5.4x10^{-8}$	$2.0x10^{-6}$
3b	$8.4x10^{-8}$	$9.1x10^{-7}$
3c	$5.6x10^{-7}$	$3.6x10^{-6}$
3d	$6.8x10^{-8}$	$7.2x10^{-7}$
3e	$4.5x10^{-7}$	$3.7x10^{-6}$
3f	**$4.9x10^{-10}$**	**$3.9x10^{-7}$**
3g	Inactive	Inactive
3h	**$5.7x10^{-11}$**	$>1.0x10^{-6}$

TCDD	**5.7×10^{-10}**	**1.0×10^{-10}**

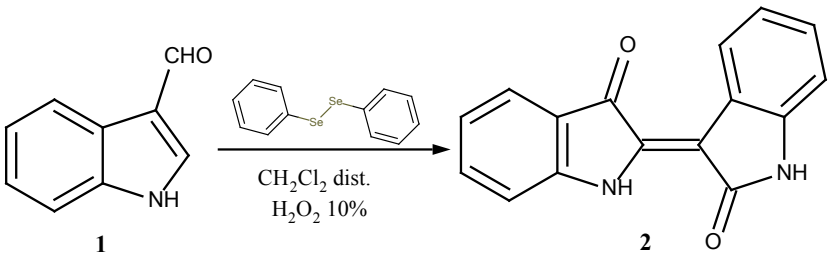


Figure 1: Initial attempt for the formation of indirubin (**2**)

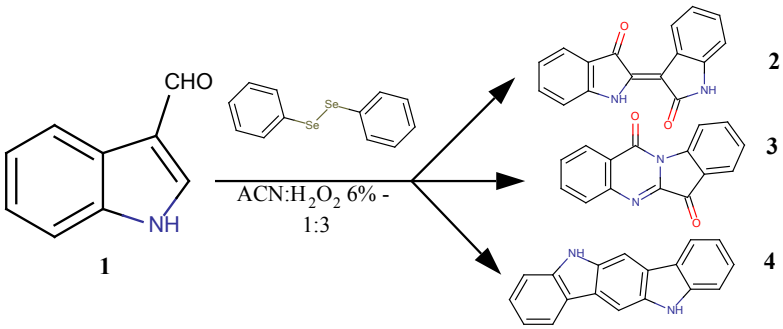


Figure 2: General scheme of the biomimetic reaction

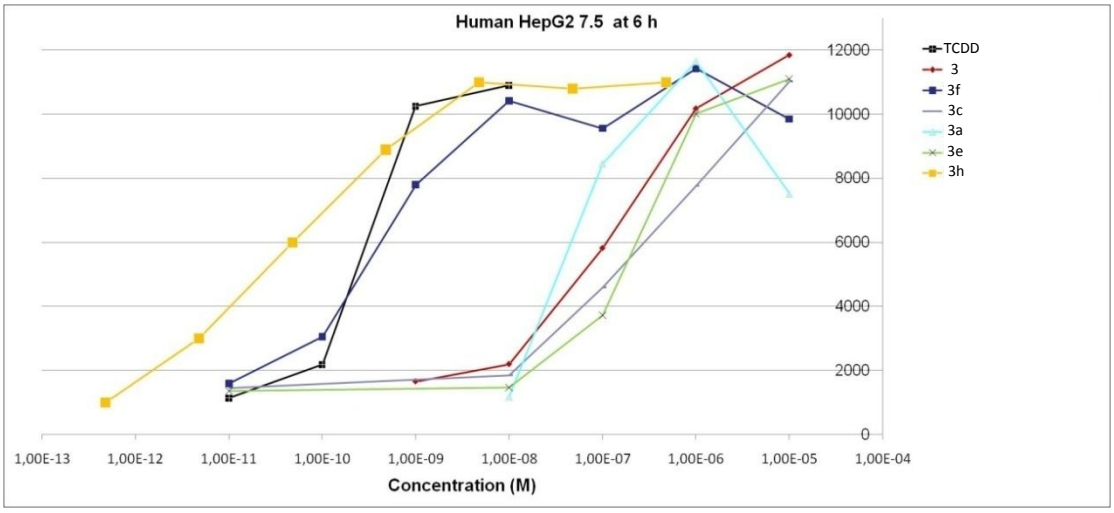


Figure 3: Concentration-response analysis of the induction of AhR-dependent luciferase reporter gene expression in human hepatoma (HG2L7.5c1) cells by TCDD, tryptanthrin and tryptanthrin analogues. See Table 4 for specific chemical substitutions.

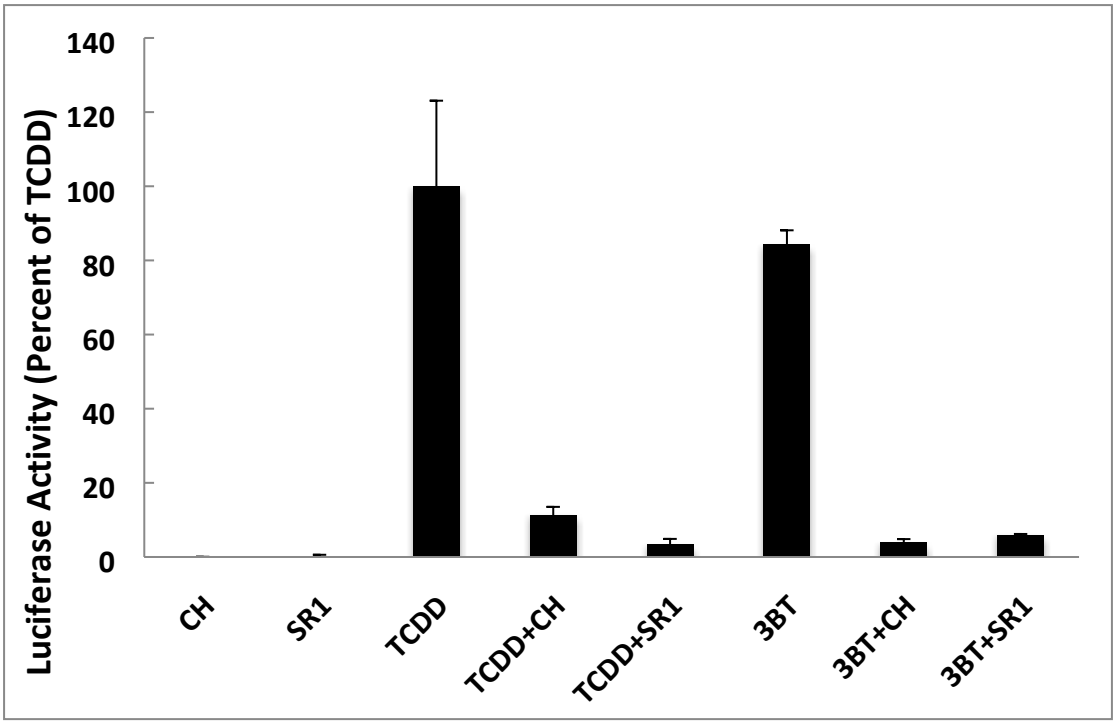


Figure 4: The AhR antagonists CH223191 (CH) and StemRegenin1 (SR1) inhibit TCDD- and 3BT-dependent induction of luciferase reporter gene expression in recombinant human hepatoma (HG2L7.5c1) cells.

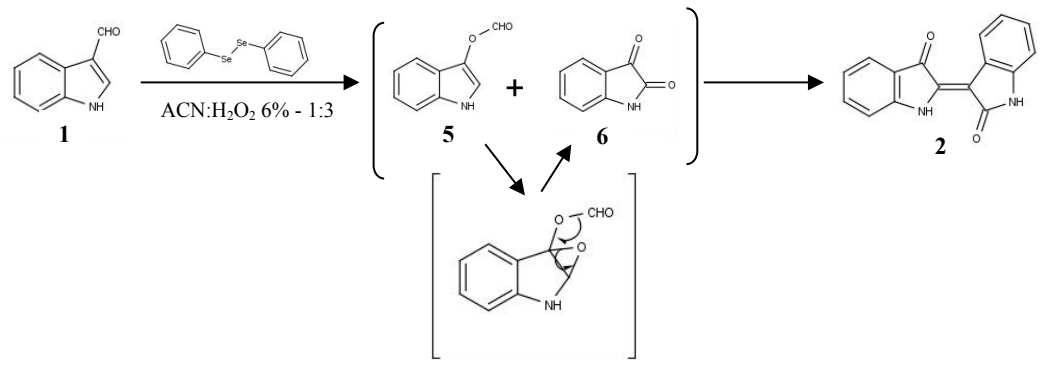


Figure 5: Proposed mechanism for the formation of Indirubin (**2**)

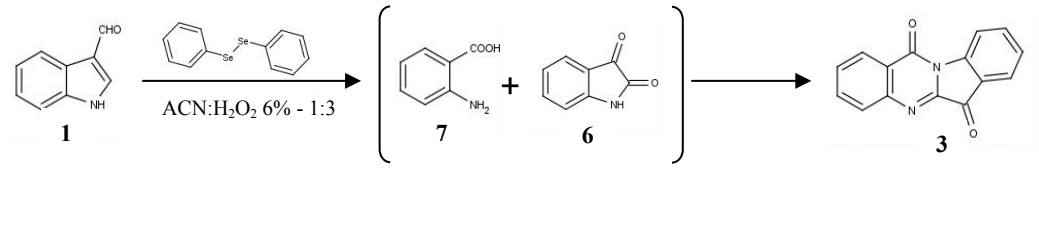


Figure 6: Proposed mechanism for the formation of Tryptanthrin (**3**)

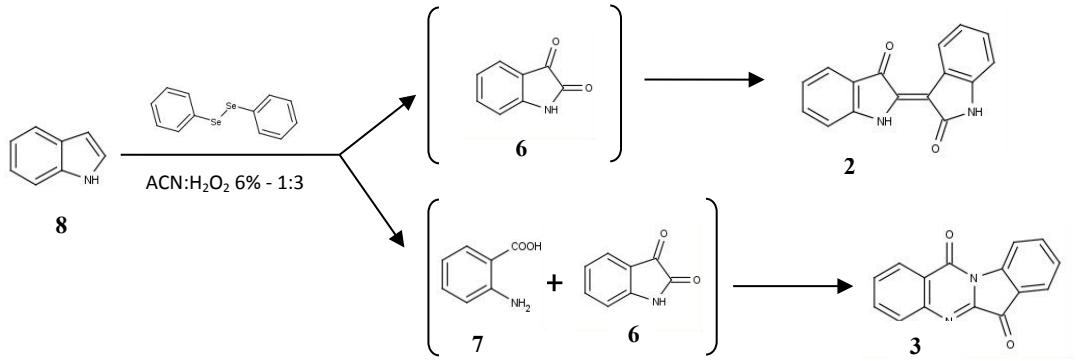


Figure 7: Proposed mechanism for the transformation of indole (**8**)

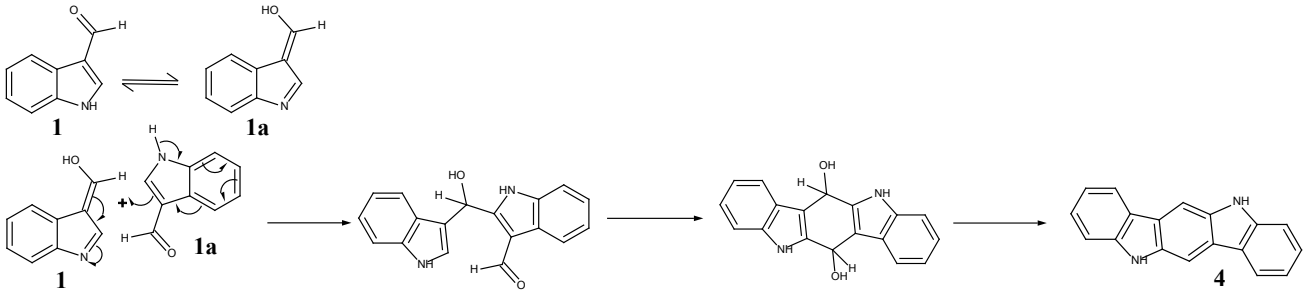


Figure 8: Proposed mechanism for the formation of Indolo[3,2-*b*]carbazole (**4**)

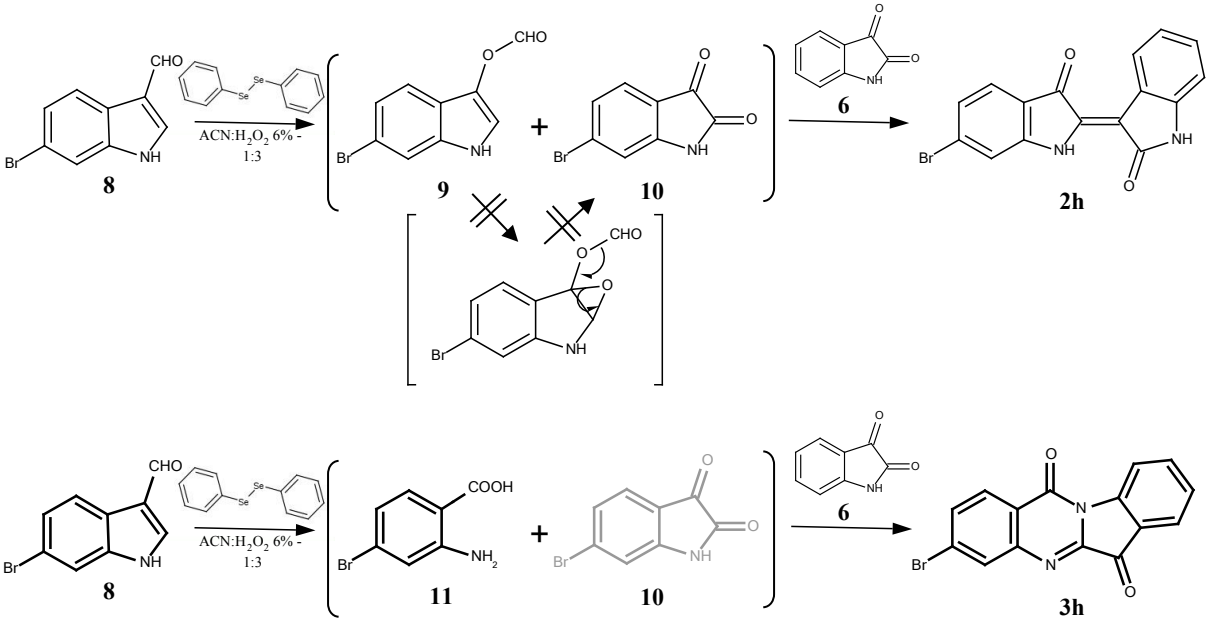


Figure 9: Proposed mechanism for the formation of the monosubstituted derivatives **2h** and **3h**

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