Erythroidine Alkaloids: A Novel Class of Phytoestrogens

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Abstract

Erythrina poeppigiana is a medicinal plant which is widely used in Asia, Latin America, and Africa in traditional remedies for gynecological complications and maladies. In continuation of studies for the discovery of novel phytoestrogens, four erythroidine alkaloids, namely α-erythroidine, β-erythroidine, and their oxo-derivatives 8-oxo-α-erythroidine and 8-oxo-β-erythroidine, were isolated and structurally characterized from the methanolic extract of the stem bark of *E. poeppigiana*. Due to the high amounts of erythroidines in the extract and considering the widespread utilization of *Erythrina* preparations in traditional medicine, the exploration of their estrogenic properties was performed. The estrogenicity of the isolated erythroidines was assayed in various estrogen receptor-(ER)-dependent test systems, including receptor binding affinity, cell culture based ER-dependent reporter gene assays, and gene expression studies in cultured cells using reverse transcription polymerase chain reaction techniques. α-Erythroidine and β-erythroidine showed binding affinity values for ERα of 0.015 ± 0.010% and 0.005 ± 0.010%, respectively, whereas only β-erythroidine bound to ERβ (0.006 ± 0.010%). In reporter gene assays, both erythroidines exhibited a significant dose-dependent estrogenic stimulation of ER-dependent reporter gene activity in osteosarcoma cells detectable already at 10 nM. Results were confirmed in the MVLN cells, a bioluminescent variant of MCF-7 breast cancer cells. Further, α-erythroidine and β-erythroidine both induced the enhanced expression of the specific ERα-dependent genes trefoil factor-1 and serum/glucocorticoid regulated kinase 3 in MCF-7 cells, confirming estrogenicity. Additionally, using molecular docking simulations, a potential mode of binding on ERα, is proposed, supporting the experimental evidences. This is the first time that an estrogenic profile is reported for erythroidine alkaloids, potentially a new class of phytoestrogens.

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Medicinal plants used in traditional medicine can be considered as an almost permanent source of medication of significant importance. A high percentage of individuals, particularly in third world countries, depend on medicinal plant-based preparations for their primary health care. In particular, about 80% of the population in developing countries still resorts to medicinal plants for various reasons. Most important, they are affordable and, according to their long-term use in humans, they are usually considered to be safe, although their consumption may pose risks undetected so far [1,2]. Unfortunately, very often the knowledge base for traditional use is empirical. More importantly, it is usually not known whether the plants or plant-derived products contain bioactive compounds, which may account for the traditional use.

Among these traditional medicinal plants, *Erythrina poeppigiana*, from the *Erythrina* species (Fabaceae), is of particular interest since it is widely used for the treatment of various disorders and complications including hormone-related conditions [3,4]. It is distributed and utilized in traditional remedies in South America, Africa, and Asia. Irrespective of the geographical site of use, this plant has been reported as being used in the folk empirical system of medicine as an abortive agent and for the treatment of amenorrhea, microbial infections, jaundice, agitation, and insom-

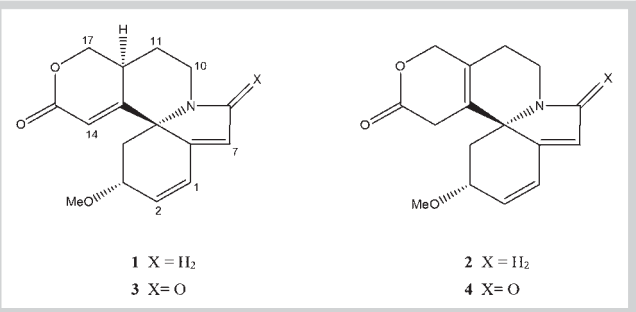

Fig. 1 Chemical structures of isolated compounds **1** (*α*-erythroidine), **2** (*β*-erythrodine), **3** (8-oxo-*α*-erythrodine), and **4** (8-oxo-*β*-erythrodine) on MVLN cells (breast cancer cell line derivative).

nia [5]. *E. poeppigiana* is particularly rich in secondary metabolites with estrogen-like activities commonly referred to as phytoestrogens [6]. In a recent study, the dichloromethane extract of *E. poeppigiana* was found to be exceptionally rich in isoflavones, among them eight specifically prenylated genistein derivatives of which seven exhibited significant estrogenic activities *in vitro* [6, 7].

Chemically speaking, the large majority of the newly discovered phytoestrogens belongs either to flavonoids (flavones, flavanones, chalchones), isoflavonoids (isoflavones, coumestanes, arylbenzofurans), or lignans, with isoflavones being the best characterized class [8]. Reports on plant secondary metabolites for which the estrogenic activity originates from an alkaloid structure are rather scarce. A study published in 1994 by Ng et al. reported for the first time estrogenic activity of an alkaloid. Yuehchukene, a *bis*-indole alkaloid isolated from *Murraya paniculata* (Rutaceae), exhibited mixed estrogenic and antiestrogenic properties *in vivo* and *in vitro* [9]. In 2001, Nazrullaev et al. reported the estrogenic activity of several quinoline alkaloids [10]. Recently, Allred et al. described the estrogenic activity of trigonelline, an alkaloid from coffee beans structurally related to vitamin B3 [11]. A possible reason for alkaloids being neglected towards the exploration of their estrogenicity could be that they are generally considered toxic. However, the striking structural variation of a vast number of natural alkaloids is the basis of a wide spectrum of quite diverse activities. Moreover, several pharmacological studies have shown that several alkaloids belonging to different chemical subgroups exhibit only a low toxicity [12, 13].

Along these lines and in a continuing effort to characterize the chemical and biological properties of medicinal plants traditionally used for the treatment of gynecological problems, we investigated the methanolic extract of *E. poeppigiana*. The alkaloid-rich fraction was targeted and isolated, and the corresponding constituents were purified and structurally elucidated using chromatographic and spectroscopic methods, respectively. Moreover, various estrogen receptor (ER)-dependent test systems, including receptor binding and reporter gene assays, in two independent cellular systems, namely MVLN breast cancer cells (a bioluminescent variant of MCF-7 breast cancer cells) [14] and U2OS human osteosarcoma cells [15], were used. These cell lines were utilized for determination of potential estrogen-like activities of the isolated alkaloids. We finally evaluated the impact of the most potent constituents of the methanolic extract of *E. poeppigiana* on the expression of the endogenous estrogen regulated genes SGK3 (serum/glucocorticoid regulated kinase 3) and trefoil factor-1 (TFF1) in MCF-7 cells. In addition, to further substantiate observed estrogenic activities of *α*- and *β*-erythroidine *in silico,* docking calculations were performed. The interactions of isolated erythroidines to the ligand binding domain (LBD) of the estrogen receptor alpha (ER*α*) have been studied considering ER plasticity as expressed by different conformational preferences derived by crystallography.

Results

The relative binding affinities (RBA) to ER*α* and ER*β* for **1**–**4** (**Fig. 1**) were determined using purified recombinant proteins and a fluorescence polarization approach (**Table 1** and **Fig. 1S**, Supporting Information). Compounds **1** and **2** displayed RBA values for ER*α* of 0.015 ± 0.010 and 0.005 ± 0.010, respectively. For **3** and **4** at the concentrations tested, no dose-dependent competition curve could be obtained. Therefore, no RBA could be determined, implying a very low or no affinity for ER*α*. The same holds for binding to ER*β*, except for **2**, for which a relatively low RBA of 0.006 ± 0.010 to ER*β* could be determined. IC_{50} values of estradiol, **1**, and **2** for ER*α* were 8.31×10^{-9} M, 5.73×10^{-5} M, and 1.11×10^{-4} M, respectively, whereas the IC_{50} of **2** to ER*β* was 9.61×10^{-5} M as compared to 5.47×10^{-9} M for estradiol.

To verify whether this binding affinity to the ER translates into receptor transactivation, we performed reporter gene assays. As toxicity of alkaloids is a concern, we additionally performed the MTT cytotoxicity assay in U2OS osteosarcoma cells. This test showed that both erythroidines are void of cytotoxicity in doses up to 10 µM (**Fig. 13S**, Supporting Information).

Estrogenic activity of the alkaloids was first assayed in a derivative of ER-positive MCF-7 breast cancer cells (MVLN). In accordance with the aim of our study, we assessed the estrogenic activity of the four isolated alkaloids as well as a positive (E2 10 nM) and a negative (DMSO 0.1%) control in MVLN cells, and verified observations for the most potent molecules in U2OS human osteosarcoma cells. Compound **1** induced significant and dose-dependent luciferase reporter gene activity in MVLN cells, starting from 10 nM, while compound **2** induced significant estrogenic

Table 1 Ligand binding data.

	RBA (%)		IC_{50} (µM)	
	Er*α*	ER*β*	Er*α*	ER*β*
Estradiol	100	100	0.0083	0.0055
α-Erythroidin	0.015 ± 0.010	n. m.	57.3	n. m.
β-Erythroidin	0.005 ± 0.010	0.006 ± 0.010	111	96.1
8-Oxo-*α*-erythroidin	n. m.*	n. m.	n. m.	n. m.
8-Oxo-*β*-erythroidin	n. m.	n. m.	n. m.	n. m.

* n. m. = not measurable

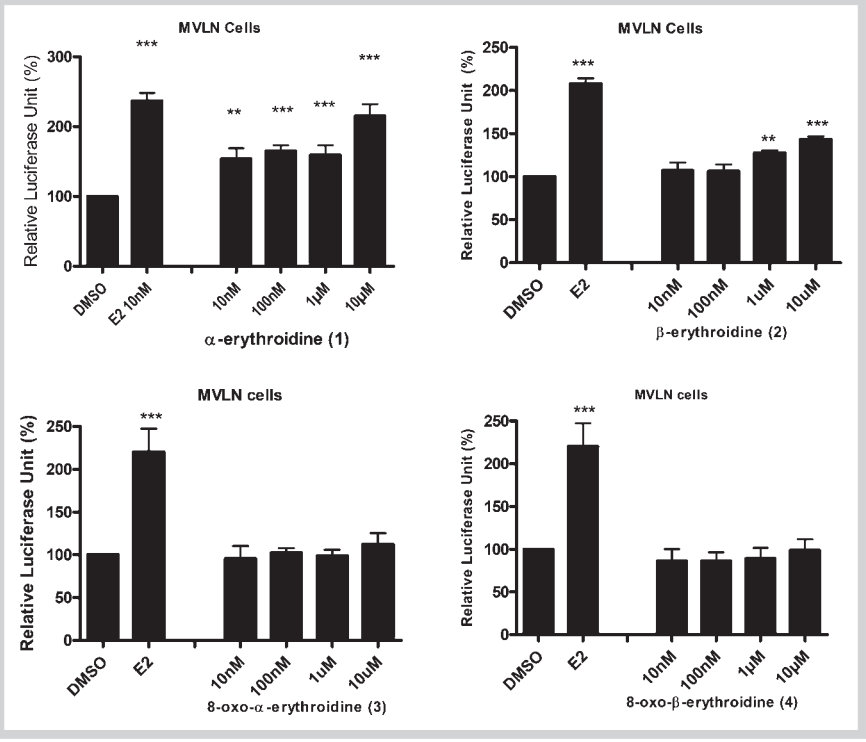

Fig. 2 Estrogenic activity of erythroidines **1** and **2** activated ERα-dependent reporter gene expression in a statistically significant manner, reaching a maximum response at 10 µM, while **3** and **4** had no effects. Significance was calculated against DMSO (set to 100 %): ** $p < 0.01$, *** $p < 0.001$. Data represent the mean ± SD of at least three independent triplicate experiments. MVLN cells: breast cancer cell line derivative.

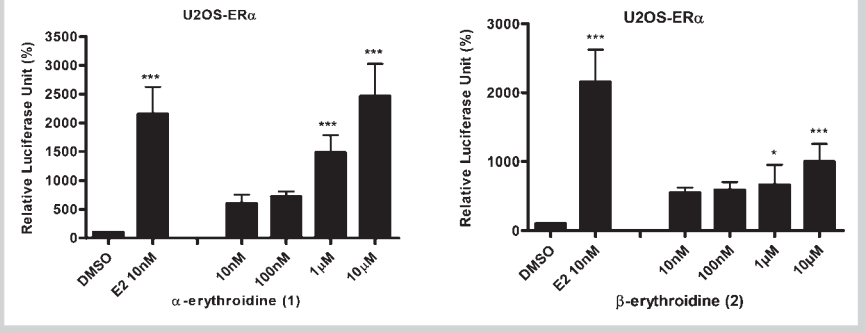

Fig. 3 ERα-dependent luciferase reporter gene activation by α-erythroidine (**1**) and β-erythroidine (**2**) in U2OS cells. Compound **1** appears to be more potent than **2** in U2OS-ERα cell systems. Significance was calculated against DMSO (set to 100 %): ** $p < 0.01$, *** $p < 0.001$. Data represent the mean ± SD of at least three independent triplicate experiments. U2OS-ERα: human osteosarcoma cell line expressing estrogen receptor α.

activity starting from 1 µM (**Fig. 2**). On the other hand, compounds **3** and **4**, which are the corresponding 8-oxo derivatives of **1** and **2**, failed to induce any significant estrogenic activity in MVLN cells (**Fig. 2**). From these results, we made two decisions for additional experiments. First, only **1** and **2** exhibited sufficient activity to be studied in depth. Second, we decided to continue to investigate interactions of erythroidines with ERα only to address the issue of structure-function relationships.

As a consequence of this decision, compounds **1** and **2** have been investigated for their estrogenic potential in a cell line of a different organ, namely, bone-derived U2OS human osteosarcoma cells expressing ERα (U2OS-ERα). Compound **1** induced significant and dose-dependent reporter gene activity in U2OS cells starting from 10 nM (**Fig. 3**), while **2** also induced a significant and dose-dependent activation of luciferase reporter gene activity in U2OS-ERα cells starting from 10 nM (**Fig. 3**). As a general observation, the maximum induction in response to compound **2** is by far less pronounced than that of compound **1**.

In order to verify whether the activation of the reporter gene activity by **1** and **2** is mediated through the ER, U2OS-ERα cells were coincubated with the higher effective dose of **1** and **2**, and the pure antiestrogen Fulvestrant. Fulvestrant completely inhibited the induction of the reporter gene transcription by these alkaloids, indicating that their effect is ER-dependent (**Fig. 4**).

Aiming to further characterize the estrogenicity of these alkaloids, we investigated whether the erythroidines are also capable of inducing endogenous target genes in MCF-7 breast cancer cells. The impact of **1** and **2** on the expression of the estrogen-regulated genes TFF1 (**Fig. 5A** and **B**) and serum and glucocorticoid-inducible kinase 3 (SGK3; **Fig. 5C** and **D**) was evaluated in MCF-7 breast cancer cells. The expression of both genes was upregulated in response to 10^{-8} M of estradiol and to 10^{-5} M concentrations of both test substances (**Fig. 5**). This effect was completely inhibited by coincubation with the antiestrogen fulvestrant (data not shown).

According to the chemical structure of erythroidines, their affinity for the ER was not expected. Most molecules showing strong estrogenic activity usually possess two OH groups in a distance of 10–12 Å. However compounds, both natural and semisynthetic, possessing only one OH group has showed a significant affinity to the ER [16]. Likewise, compounds **1** and **2**, although they lack OH groups, exhibited low but surprising binding affinities. This

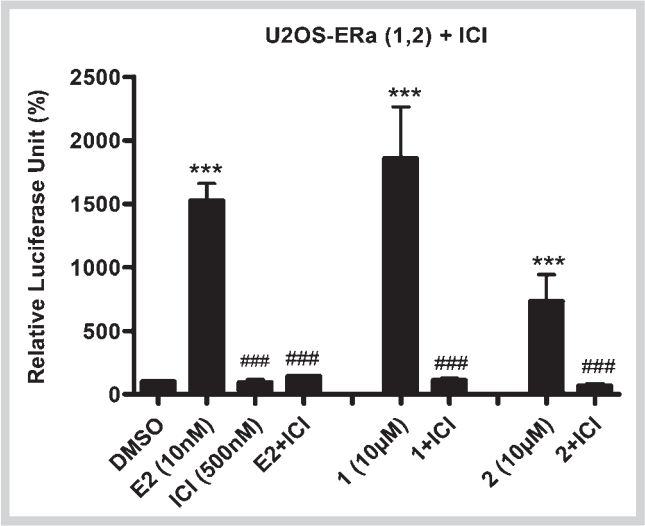

Fig. 4 Inhibition of the ER-dependent reporter gene activity of E2, **1**, and **2** by Fulvestrant. Inhibition of estrogenic responses by Fulvestrant is indicative for the mediation of the effect by the ER. Significance comparing results with 0.1% DMSO (= 100%): *** $p < 0.001$; significance comparing combined effects against individual treatment: ### $p < 0.001$. Data represent the mean ± SD of at least three independent, triplicate experiments.

affinity was proven to be translated into a significant activity regarding ER-dependent reporter gene expression in cells of different organs and regarding regulation of endogenous gene expression. So we considered it necessary to perform molecular docking studies *in silico* to further understand the interaction of erythroidines with the ER LBD on a molecular level. Our hypothesis was that the nitrogen atom existing in **1** and **2** could trigger binding to the ER. This nitrogen can be protonated and form a hydrogen bond with known important hydrophilic amino acids such as His 524, Asp353, and Arg394 inside the binding pocket of the receptor [16]. One the other hand, the nitrogen in oxo-erythoidine derivatives cannot be protonated and, thus, the binding ability is prohibited in compounds **3** and **4**.

Specifically, in order to investigate the binding of compounds **1** and **2** to the ER, all possible conformations and stereochemistry patterns considering the nitrogen inversion were investigated [17, 18]. The theoretical pKa value of each nitrogen atom was calculated as 9.26 and 9.12, respectively, using ChemAxon software (www.chemaxon.com) suggesting that at pH 7, the nitrogen atom is protonated and each compound can exist in an *R/S* nitrogen configuration. Additionally, calculated logD values on pH 7 were found to be − 1.43 and − 1.86, respectively, showing very low lipophilicity. After 1000 runs of a conformational search using the Monte Carlo/Low Mode (MC/LMOD) algorithm, the global minimum of each compound was depicted (**Fig. 14S**, Supporting Information). These structures reveal that in the *S* nitrogen configuration, the NH group is better exposed to the solvent or to the interaction with the receptor pocket, while in the *R* configuration, NH is sheltered by the core of the molecule.

Furthermore, docking calculations were performed considering the lowest energy structure obtained for the interaction of each compound with the ERα LBD. Starting from the crystal structure of LBD-ERα in a complex with diethylstilbestrol (DES) (PDB entry 3ERD) or ortho-trifluoromethylphenylvinyl estradiol (EZT) (PDB entry 2P15), the crystallographic ligand was replaced manually by compounds **1** and **2**. Both ligands fit within the ligand binding cavity of the 3ERD LBD structure of ERα, exhibiting only weak Van Der Waals (VdW) interactions. In the case of the 2P15 ERα LBD structure, the binding pocket is larger, so both ligands were suitably fitted. In both cases, the *S* enantiomer (regarding the N+ configuration) forms a hydrogen bond with His 524 since the NH is better exposed to the receptor as described above (**Fig. 6** and **7**).

Discussion

In third world countries, people depend on traditional medicine for their primary health care, which comprises medicinal plants as a fundamental element. Efficacy is mostly evidenced by the traditional use without knowing the molecular basis of the activity. Likewise, (toxic) side effects presumably often remain unnoticed. With our studies on estrogenic activities of compounds

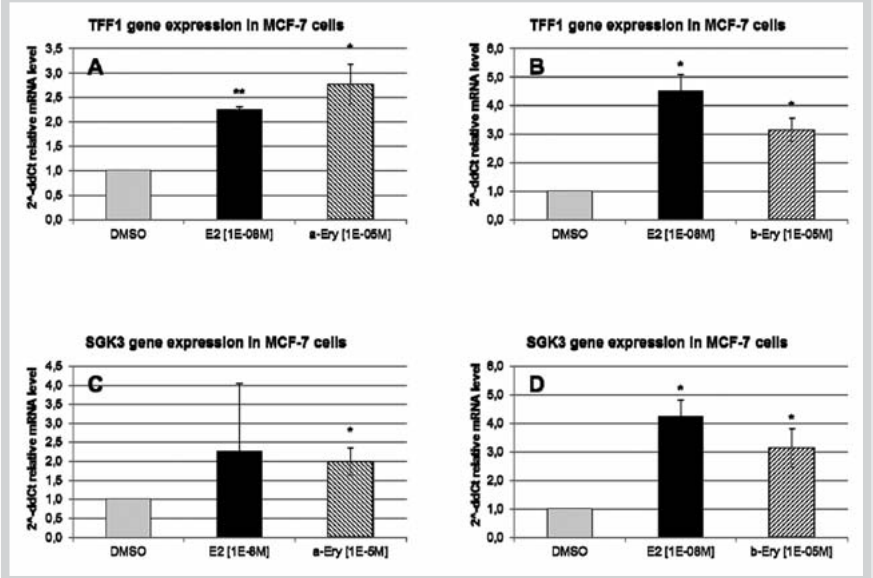

Fig. 5 Regulation of the TFF1 gene (pS2; **A**, **B**) and serum and glucocorticoid-inducible kinase 3 (**C**, **D**) gene expression by α- and β-erythroidine (**A**, **C**) and (**B**, **D**) in MCF-7 cells. Significance was calculated against DMSO (set to 1): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data represent the mean ± SD of at least three independent experiments. MCF-7 cells: breast cancer cell line.

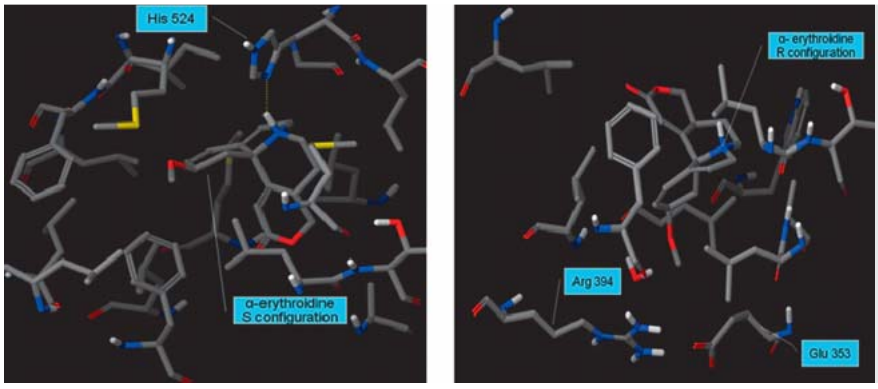

Fig. 6 Minimum energy structure of ERα-LBD (2P15) in complex with α-erythroidine (*S* configuration) (**A**) and α-erythroidine (*R* configuration) (**B**). (Color figure available online only.)

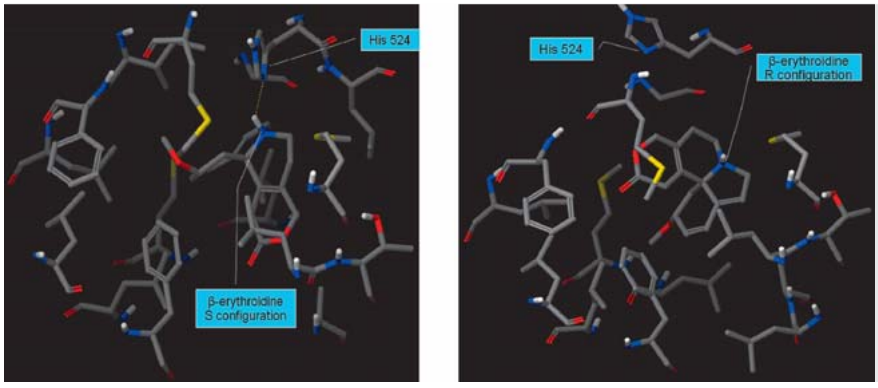

Fig. 7 Minimum energy structure of ERα-LBD (2P15) in a complex with β-erythroidine (*S* configuration) (**A**) and β-erythroidine (*R* configuration) (**B**). (Color figure available online only.)

from *E. poeppigiana*, we aimed to contribute to the evaluation of the efficacy and safety of this medicinal plant.

Generally, plants belonging to *Erythrina* species are very rich in various subgroups of flavonoids. The estrogenic activity of *E. poeppigiana* could initially be linked to derivatives of the soy isoflavone genistein, which were isolated from its dichlomethane extract [6, 7]. Nevertheless, information about the possible estrogenicity of more polar compounds of the plant, including the estrogenicity of erythroidines, was missing. Erythroidine alkaloids represent major constituents of the methanol extract in *Erythrina* species. Thus, the aim of the present study was to phytochemically investigate the polar constituents of the stem bark of *E. poeppigiana* and to evaluate their estrogenic properties using *in vitro* and *in silico* models.

Along these lines, we first characterized the relative binding affinities of the isolated erythroidines **1, 2, 3** and **4** to the ERs in comparison to 17β-estradiol. Overall, we showed that two (**1, 2**) of the four compounds bound to ERα and, additionally. one of them (**2**) to ERβ. With RBA values ≤ 0.1 %, α- and β-erythroidines have to be characterized as weak binders of the ERs; however, with this relative binding affinity, they are still in the range of the binding affinities of the red clover isoflavones biochanin A and formononetin or the endocrine disruptor bisphenol A [19]. In contrast, both oxo-derivatives, 8-oxo-α-erythroidine (**3**) and 8-oxo-β-erythroidine (**4**), showed no affinity for the ERs.

Considering that the binding affinity to a receptor does not always correlate with receptor transactivation [20], the estrogenic activity of these four alkaloids was additionally assessed on cell culture models of human origin. Prior to hormonal stimulation experiments, we performed cytotoxicity assays. The MTT test did not reveal cytotoxic properties of erythroidines (**Fig. 13S**, Supporting Information).

The initial reporter gene assays were performed in MVLN cells, ERα-expressing MCF-7 breast cancer cell line-derived reporter cells [14]. The results exactly matched the ligand binding data, meaning measurable activity of **1** and **2** and neglectable activity of **3** and **4**. This observation could also be correlated with the docking results. The oxo-erythroidine derivatives **3** and **4** have lost their ability for nitrogen protonation, so they lose their only group to form a hydrogen bond with His 524 as shown for compounds **1** and **2**. Since His 524 is not so important for ER recognition compared to Arg394 and Asp353 [16], binding only to His 524 could explain the low binding affinity for compounds **1** and **2**.

In order to further investigate the estrogenic activity of the two active alkaloids **1** and **2**, they were assayed in the U2OS-ERα variant of the bone-derived human U2OS osteosarcoma cell line [15]. The rationale for this procedure stems from the observation that compounds which are structurally different from estradiol, but still exhibit estrogen-like properties, may have organ-specific functional qualities, like, for example, the synthetic estrogen receptor modulator Tamoxifen. As shown by the results in **Fig. 3**, α-erythroidine (**1**) and β-erythroidine (**2**) significantly induced reporter gene activity in U2OS-ERα cells, with a more pronounced magnitude of stimulation for **1**. The stimulation pattern of the reporter genes in **Fig. 2** and **3** did not result in a steadily increasing dose-response pattern. So far, we are missing a mechanistic clue, but nonlinear dose-response patterns have been extensively discussed for endocrine disrupting chemicals (for review see [21]). The pure antiestrogen Fulvestrant® completely inhibited the estrogenic activity of these alkaloids (**Fig. 4**), implying that the observed effect was primarily mediated through the ER. In essence, α- and β-erythroidines are capable of inducing ER-mediated reporter gene activity in cells originating from mam-

mary glands and bones, thereby not exhibiting organ selective properties.

Reporter gene constructs usually contain minimal or reduced promotor element arrangements with a low number of DNA base pairs comprising the respective estrogen response element and, therefore, do not entirely mimic complex promotors of responsive genes, which often comprise several thousand base pairs. For weak estrogenic compounds, it is therefore important to test whether they are capable of triggering the ER-dependent regulation of expression of endogenous genes. We therefore investigated the impact of **1** and **2** on the regulation of expression of specific estrogen-regulated genes in MCF-7 cells, namely TFF1 and SGK3, by semiquantitative real-time PCR. TFF1, formerly known as pS2, was the gene from which an estrogen response element as a molecular switch for the ER was initially described [22,23]. It also represents a gene which, in the presence of estradiol, is significantly upregulated both in human MCF-7 breast cancer cells and in breast cancer biopsies of estrogen-dependent breast cancers, and, therefore, qualifies as an E2 responsive marker gene [22–24]. In our study, E2, as well as both erythroidines, induced a significant upregulation of TFF1 (**Fig. 5A** and **B**), thus confirming their estrogenic activity, which appears to be ER-dependent because it was completely inhibited by Fulvestrant®. It has been shown that E2 dose-dependently induces the expression of the SGK3 gene [25], which promotes ER-positive mammary adenocarcinoma cell survival. In the current study, E2, **1**, and **2** induced an upregulation of SGK3 (**Fig. 5C** and **D**), thus confirming the estrogenic properties of these alkaloids even on a complex promotor in a natural genomic organization. Regarding the regulation of expression of these primary estrogen response genes, α-erythroidine seemed to be slightly more potent than β-erythroidine.

In order to decipher the molecular mechanism by which these alkaloids bind the ER, molecular docking simulations were performed starting from two distinct crystal structures of ERα. In both structures, ERα adopts the so-called "agonist conformation" regarding the orientation of the C-terminal H12 helix. The first structure is the one derived in the complex with DES, a classical estradiol agonist, usually adopted for this type of docking calculation. In these conditions, ERα did not show any remarkable interaction with both erythroidine molecules. However, the crystal structure of ERα in the complex with 17β-estradiol (EZT) had proven that the LBD cavity can display a remarkable structure plasticity, notably on loop Asp411-Val418, in order to accommodate the bulky analog of EZT. Considering this specific conformational snapshot of the ERα structure, α/β-erythroidines (**1** and **2**) could adequately fit and, more importantly, form a hydrogen bond between His 524 and the nitrogen atom (in the *S* configuration). However, one has to consider that ERα-LBD is lipophilic, while α/β-erythroidines are protonated at a physiological pH exhibiting negative logD values. This definitely would influence the entropic term (ΔS) of the system's free energy of binding, lowering the overall binding affinity. Overall molecular docking calculations support binding of α/β-erythroidines for ERα. As shown at **Fig. 6** and **7**, the *S* nitrogen configuration of both **1** and **2** forms a hydrogen bond with His 524 inside the receptor pocket. The resulting interactions provide evidence for the *in vitro* binding affinity and activity of these alkaloids toward the ERα.

Overall, based on ER-dependent test systems including competitive binding analyses, reporter gene assays, and gene expression as well as *in silico* studies, we provide various pieces of evidence for the estrogenicity of α- and β-erythroidine, a prominent class of *Erythrina* species alkaloids contained in the methanol extract of the stem bark of *E. poeppigiana*. Thus, we suggest that α- and β-erythroidine alkaloids contribute to the estrogenic activity of the medicinal plant *E. poeppigiana*. Whether it contributes to the estrogenic properties recently shown in an experimental rat model [26] remains open. From our *in vitro* study, it is not possible to assess the overall contribution of the erythroidines to the total estrogenic activity of the plant or how it is relative to the less polar isoflavones [6,7,27] or arylbenzofurans [28]. Overall, our results from four different *in vitro* test systems justify studies in animal models to better define efficacy and safety profiles of the test compounds.

In conclusion, our study presented here is important in the field of molecular endocrinology as well as in the area of alkaloid biochemistry, disclosing the existence of potentially estrogenic agents with this particular chemistry.

Materials and Methods

General experimental procedures

Analytical TLC was performed on Merck precoated silica gel 60 F_{254} plates. Spots were visualized by fluorescence extinction using UV light and vanillin-sulfuric acid reagent. Column chromatography was carried out using Si gel 0.04–0.06 mm (Merk). Amberlite XAD-4 resin (Rhom and Hass) was used for the preparation of the enriched fraction of the MeOH extract. A Thermo Finnigan HPLC system connected to a spectral system UV2000 PDA detector was employed for the profiling of the extracts, and ChromQuest 2.1 software was used for the operation of the system and data management. High-speed countercurrent chromatography was performed using fast centrifugal partition chromatography equipment (FCPC®, Kromaton) equipped with a 1000-mL rotor and a preparative pump (LabAlliance®). 1 and 2D NMR spectra (COSY, COSYLR, HSQC-DEPT, HMBC) were recorded in deuterated chloroform ($CDCl_3$ – Merck) on a Bruker Avance III spectrometer (Bruker Biospin GmbH) operating at 600.11 MHz for ^1H and at 150.11 MHz for ^{13}C, with a 5-mm inverse detection probe. The residual ^1H (7.26 ppm) and ^{13}C (77.0 ppm) signals of $CDCl_3$ were used as an internal standard. 1 and 2D NMR experiments were performed with standard pulse programs, at room temperature

Plant material

The stem bark of *E. poeppigiana* was collected in November 2006 in Sancta Cruz (Bolivia), and identified by Ing. Mario Saldias Paz. A specimen was deposited in the Museo de Historia Natural, Facultad de Ciencias Agricolas de Santa Cruz-Bolivia under the voucher number USZ: 71775.

Extraction, isolation, and identification

Air-dried and pulverized bark of the plant (1.66 kg) was extracted at room temperature, successively with CH_2Cl_2 (3 × 2 L), MeOH (3 × 2 L), and H_2O (3 × 2 L), for 48 h per extraction. The MeOH extract was concentrated to give a residue (90.5 g) from which 50 g was subjected to amberlite resin XAD-4HP (Rhom and Hass) dissolved in hot water and remained for two days with controlled and smoothed shaking. After filtration, the resin was washed with MeOH (2 L) and the eluent was concentrated to give 14.5 g of enriched extract. From this enriched extract, 10 g were subjected to FCPC-based separation using a biphasic system consisting of EtOAc/isopropanol/H_2O (ethyl acetate/isopropanol/water)

in the proportion of 3/2/5. The rotor speed was set at 1000 cycles/min and the flow rate at 15 ml/min. From this analysis, 119 fractions of 50 ml each were collected. Fractions 63–67 were put together to provide 4.1 g, which were further subjected to column liquid chromatography (4.5 cm Ø) on Si gel (0.015–0.04 mm) leading to the isolation of **1** (40.0 mg) and **2** (60.0 mg). Fraction 19 of the last separation was subjected to preparative TLC using a dichloromethane/methanol, 95/5, solvent system, leading to the purification of compound **4** (5.5 mg), while fractions 17–18 (131.2 mg) were concentrated together and subjected to column liquid chromatography (2.1 cm Ø) on Si gel (0.015–0.04 mm), leading to **3** (7.0 mg). The structural elucidation of the purified alkaloids was carried out using 1 and 2D NMR and comparison with literature data [17,29,30]. The isolated compounds (**Fig. 1**) were identified as *α*-erythroidine (**1**), *β*-erythroidine (**2**), and their oxo-derivatives, 8-oxo-*α*-erythroidine (**3**) and 8-oxo-*β*-erythroidine (**4**).

Receptor binding assay

The fluorescent derivative of E2, recombinant human ER*α*, and recombinant human ER*β* were purchased from Invitrogen®, while EZT (purity > 98%) was obtained from Sigma-Aldrich. The receptor binding affinities, RBA of **1**–**4**, were assessed using a fluorescence polarization approach as previously described [31,32]. In brief, the concentration for EZT and **1**–**4** that inhibited the binding of fluorescent estrogen ES2 (1 nM) (Invitrogen®) to isolated recombinant human ER*α* or ER*β* (Invitrogen) by 50% (IC_{50}) was determined and used to derive the receptor binding affinity values: [RBA = (IC_{50} 17*β*-estradiol/IC_{50} compound) × 100].

Cells and plasmids

U2OS cells were obtained from ATCC/Promochem. U2OS cells stably transfected with ER*α* (U2OS-ER*α* cells) and the $(ERE)_2$-tk-Luc reporter plasmid were kindly provided by Dr. Luisella Toschi (Schering AG). MVLN cells, a derivative of an ER-positive MCF-7 cell line stably transfected with the vitellogenin-A2-promoter/luciferase reporter construct, were from Dr. Michel Pons (INSERM U439). ER*α*-positive MCF-7 cells were from the German national center for biomedical material and resources (DSMZ).

3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assay

MTT was from AppliChem. The MTT test, as such, was performed as described in the literature [33], with 7500 U2OS cells per well of a 96-well plate.

Reporter gene assays

U2OS cells were routinely cultured in phenol red-free DMEM-F12 medium containing 10% fetal calf serum (FCS) and 0.5 mg/ml gentamycin (Calbiochem/VWR). Experiments were performed in phenol red-free DMEM-F12 medium containing 5% dextran-coated charcoal (DCC) stripped FCS, and 0.5 mg/ml gentamycin. For transfection, U2OS-ER*α* cells were plated in a 24-well plate (30 000 cells/well) and transfected with 100 ng of the $(ERE)_2$-tk-Luc reporter plasmid using the liposomal protocol (DOTAP; Roth) and DOTAP : DNA in a ratio of 3 : 1 [15].

MVLN cells as well as MCF-7 cells were cultured in DMEM-F12 medium as previously described [14], while experiments were performed using DMEM-F12 supplemented with 1% DCC in 24-well plates (80 000 cells/well).

Treatment with the test substances and luciferase assay

All tested substances were serially diluted in DMSO (final DMSO concentration in test well plate of 0.1% v/v). All assays were performed dose-dependently, thereby specifically adapting the test substance concentration to the respective assay in order to obtain a reliable dose-response curve. Ten nM E2 was used as a positive control, while 0.1% DMSO was used as a negative control. To investigate whether the estrogenic activity of the isolated neutral alkaloids is mediated by ER activation, cells were incubated with the effective doses of the substances in the absence or presence of the pure antiestrogen Fulvestrant, also referred to as Faslodex and ICI 182 780 (purity > 99%; Tocris) at the test concentration of 500 nM [34]. In all of the reporter gene experiments, cells were exposed to the test substances 24 h prior to the measurement of luciferase activity. All experiments were done in triplicate and were independently repeated three times.

RNA isolation, cDNA synthesis, and mRNA quantification using realtime PCR

After a 24-h treatment period, the total cytoplasmic ribonucleic acid (RNA) was extracted from adherent MCF-7 cells using Trizol® reagent (PeqLab) according to the manufacturer's protocol. RNA samples were qualitatively examined on a 1% agarose-formaldehyde gel. DNA contamination was enzymatically eliminated by digestion (deoxyribonuclease I, Ambion). Absence of genomic DNA was checked by PCR. M–MLV reverse transcriptase (Promega) and oligo (dT) 12–18 primers were used for the first-strand cDNA synthesis. Quantitative real-time PCR using Platinum® Taq DNA polymerase (Life Technologies) and a thermal cycler with an iQ real-time detection system (BioRad) was performed for mRNA quantification. SybrGreen I (Sigma-Aldrich) was used as a detection probe. The reactions were run three times in triplicate. After vortexing, 50 µl aliquots of the total mix were pipetted to each well of the 96-well PCR plate (BioRad). PCR reactions consisted of a first denaturing cycle at 95 °C for 3 min, followed by 50 cycles of 10 s at 95 °C, 15 s at 60 °C, and 30 s at 72 °C. Fluorescence was quantified at the end of the 60 °C annealing step and product identity was confirmed by a melting curve analysis (60–95 °C). Primer sequences are summarized in **Table 1S**, Supporting Information. For all genes measured, we obtained three different biological replicates (mRNA preparations) from three independent cell culture experiments resulting in at least three independently synthesized cDNAs from independent cell culture experiments. Each cDNA was subjected to qPCR analysis in triplicate. The relative mRNA amounts of target genes TFF1/pS2 and SGK3 were calculated after normalization to an endogenous reference gene (ribosomal protein 18, RPS18). Results are expressed as the relative amount of mRNA of the gene of interest compared to the mRNA levels of the housekeeping gene hRPS18, using the $2^{-\Delta\Delta CT}$ formula [35].

Molecular simulation

All calculations were run using Macromodel 9.0 (Schrödinger, Inc.). Compounds' **1** and **2** virtual structures (both *R*/*S* enantiomers regarding the NH group) were generated using Maestro 9.3.5 (Schrödinger, Inc.). The full search in the conformational space for each molecule was achieved using the OPLS2005 force field with an MC/LMOD search algorithm. One thousand starting conformers were produced and minimized using the TNCG algorithm (rmsG < 0.01 kJ/mol Å). No solvent model was used.

The ligand binding domains of ERα in the agonist conformation (PDB entries 3ERD and 2P15) were chosen as starting structures for docking calculations. On each complex, the crystallographic ligand was replaced by compound **1** or **2** (*R*/*S* enantiomers). Docking calculations were performed using a 1000 step search of the mixed MC/LMOD search algorithm as implemented in Macromodel with a ratio of 0.5 and an OPLS2005* force field. A distance-dependent dielectric "constant" of 4 r was used. All residues within 6.0 Å from the ligand were allowed to move freely, while the remaining residues were treated as "frozen atoms". After each successful run, the complex was minimized using the TNCG algorithm (rmsG < 0.01 kJ/mol Å). Unique conformations were stored only if they were within the lowest 50 kJ/mol.

Data presentation and statistical analysis

All data from the receptor binding affinity are expressed as mean ± standard deviation of at least three independent experiments. Data from luciferase reporter gene assays were obtained from three independent cell culture experiments, within which treatments were performed in triplicate. Data from real-time PCR experiments were obtained from three different cell culture experiments, RNA extractions, and cDNA syntheses. Statistical analysis included one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test in order to determine significant differences. Results are defined as significant at $^{*}\ p \le 0.05$, $^{**}\ p \le 0.01$, and $^{***}\ p \le 0.001$.

Supporting information

Competition curves of ligand binding assays and 1 and 2D NMR data of isolated compounds, an HPLC-DAD chromatogram of the methanol extract, data of the MTT test, and global minimum structures of α- and *β*-erythroidine using molecular docking simulations are available as Supporting Information.

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Conflict of Interest

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The authors declare no conflict of interest.

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