Inhibitors

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# Phloroglucinol Derivatives Guttiferone G, Aristoforin, and Hyperforin: Inhibitors of Human Sirtuins SIRT1 and SIRT2**

*Claudia Gey, Sergiy Kyrylenko, Lothar Hennig, Lien-Hoa D. Nguyen, Anita Büttner, Hung D. Pham, and Athanassios Giannis**

*Dedicated to Professor Peter Welzel on the occasion of his 70th birthday*

Sirtuins are class III histone deacetylases and are homologous to silent information regulator 2 (Sir2) of yeast.[1] Deacetylation by sirtuins was shown to be $NAD^+$ dependent, with the acetyl group being transferred to the ADP-ribose portion of $NAD^+$ to yield 2′-*O*-acetyl-ADP-ribose (*O*AADPR) and free nicotinamide.[2] Seven enzymes of this class have been identified in humans so far (SIRT1–7), but the function of most of them remains elusive.[3] However, SIRT1 and SIRT2 have been intensively investigated.[4,5]

SIRT1 was shown to inactivate tumor suppressor protein p53 by deacetylating Lys382.[4] In this way, SIRT1 prevents cells from apoptosis induced by DNA damage and stress. Other transcription factors deacetylated by SIRT1 are the Forkhead transcription factors FOXO1, FOXO3, and FOXO4 as well as Ku70, NF$\kappa$-B, and MyoD.[5] Furthermore, SIRT1 regulates HIV replication by deacetylation of the viral transcription factor Tat.[6] SIRT2 together with HDAC6 were shown to deacetylate $\alpha$-tubulin,[7] thus rendering them control elements in the formation of microtubules. In accordance with that finding, SIRT2 was shown to control mitosis within the cell cycle.[8]

After the identification of the yeast Sir2 protein as a regulator of gene expression, sirtuins have now been proposed to influence a variety of cellular processes, among them energy metabolism, cell-cycle progression, muscle differentiation, fat mobilization, and aging.[5] Therefore, the use of small molecules as regulators of sirtuin activity is of high pharmacological interest. However, only a few inhibitors of sirtuins have been reported so far. With the exception of nicotinamide, which is released during the reaction with the deacetylase and which serves as an internal inhibitor by blocking $NAD^+$ hydrolysis,[9] most of the inhibitors are synthetically derived and were identified in library screening studies.

Sirtuin inhibitors can roughly be grouped into $NAD^+$ derivatives (nicotinamide, carba-$NAD^+$, NADH), coumarin derivatives (dihydrocoumarin, A3, splitomicin, HR73), and 2-hydroxynaphthaldehyde derivatives (2-OH-naphthaldehyde, sirtinol, *para*-sirtinol, M15, cambinol).[6,10] Other inhibitors are CD04097 (which consists of three highly substituted benzene rings), the tricyclic derivative JFD00244, and indole derivative EX527.[11] The most potent inhibitor among them is EX527, which inhibits SIRT1 at a nanomolar concentration and SIRT2 at a low micromolar concentration;[11b] sirtinol was shown to inhibit only SIRT2 ($IC_{50} = 38\ \mu M$).[12a] Recently, several adenosine mimetics were identified as inhibitors of sirtuins.[12b]

Herein we present a new class of compounds originating from natural sources that inhibit both SIRT1 and SIRT2 at a low micromolar concentration: guttiferone G, which was extracted from *Garcinia cochinchinensis*, a tree growing in Vietnam; hyperforin, a well known pharmacological agent initially extracted from *Hypericum perforatum* (St. John's wort), and its synthetic derivative aristoforin. Furthermore, we propose that the pharmacological effects of the phloroglucinol derivative hyperforin and of the guttiferones are associated with sirtuin activity.

Fractionation of the petroleum ether extract of the bark of *Garcinia cochinchinensis* afforded compound **1**. The molecular formula was established as $C_{43}H_{58}O_6$ from high-resolution mass spectrometry ($[M+H]^+$: $m/z$ 671.4318). Detailed examination by 1D and 2D NMR methods and comparison with the literature data resulted in the identification of **1** as guttiferone G (Scheme 1). The $^1H$ and $^{13}C$ NMR data (see the Supporting Information) are in good agreement with the literature data of guttiferone G.[13]

The compound isolated from *Garcinia cochinchinensis*, however, showed positive specific rotation ($[\alpha]_D = +27.1\ deg\,cm^3\,g^{-1}\,dm^{-1}$, $c = 1.7\ g\,cm^{-3}$, $CHCl_3$), while the specific rotation of guttiferone G isolated from *Garcinia macrophylla* was negative ($[\alpha]_D = -25\ deg\,cm^3\,g^{-1}\,dm^{-1}$, $c = 0.04\ g\,cm^{-3}$, $CHCl_3$).[13a] Thus it is presumed that our isolated guttiferone G is the (+) enantiomer (Scheme 1).

[*] C. Gey, Dr. L. Hennig, A. Büttner, Prof. Dr. A. Giannis
Institut für Organische Chemie
Universität Leipzig
Johannisallee 29, 04103 Leipzig (Germany)
Fax: (+49) 341-973-6599
E-mail: giannis@uni-leipzig.de
Dr. S. Kyrylenko
Department of Biochemistry
University of Kuopio
Yliopiostonranta 1E, 70210 Kuopio (Finland)
E-mail: kyrylenk@messi.uku.fi
Dr. L.-H. Nguyen, Prof. Dr. H. D. Pham
Department of Organic Chemistry
University of Natural Sciences
Ho Chi Minh City, 227 Nguyen Van Cu, District 5, HCMC (Vietnam)
E-mail: lienhoa@saigonnet.vn

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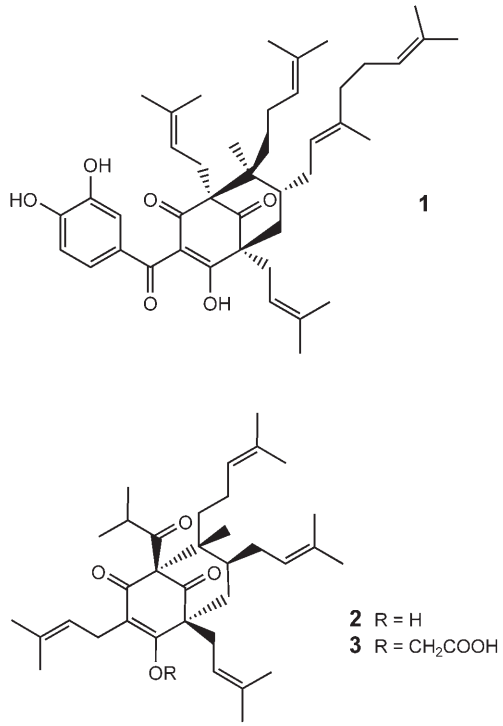


***Scheme 1.*** (+)-Guttiferone G (**1**), hyperforin (**2**), and aristoforin (**3**). The absolute stereochemistry of compound **1** is tentative.

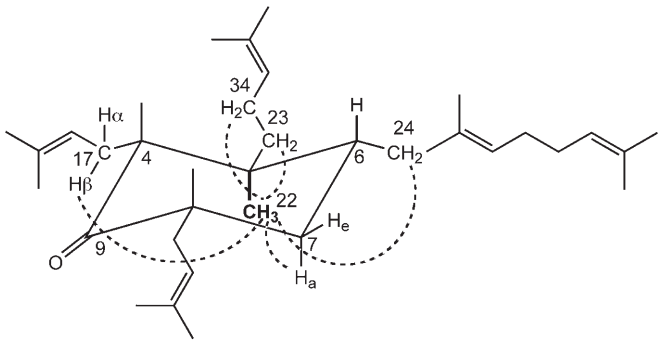


***Figure 1.*** Selected NOESY correlations of **1**; a = axial, e = equatorial.

1D NOESY measurements at 700 MHz (irradiation at the resonance frequency of $CH_3$-22, H-6, and H-7a) and the coupling constant of 13.0 Hz between H-6 and H-7a revealed that *ent*-guttiferone G has a chair conformation with all the isoprene units in equatorial positions. Figure 1 shows the NOESY correlations of guttiferone G obtained with irradiation at the resonance frequency of $CH_3$-22.

Guttiferones are an interesting class of natural products found in plants of the Guttiferae family that exhibit a variety of biological activities. For example, guttiferones A–F were shown to reduce the cytopathic effects in HIV-infected cells.[14] Guttiferone E inhibits the depolymerization of the microtubules into tubulin,[15] and guttiferone I was reported to be a ligand of liver X receptors.[13a] Since SIRT1 is a regulator of HIV transcription[6] and SIRT2 a tubulin deacetylase,[7a] we assayed the isolated guttiferone G for inhibitory activity against sirtuin. Indeed, guttiferone G strongly inhibited recombinant human SIRT1 and SIRT2 at low micromolar concentrations, with $IC_{50}$ values of 9 and 22 μM, respectively (Table 1).

Guided by this discovery, we investigated the inhibitory properties of the structurally related natural compounds hyperforin (**2**) and its synthetic derivative aristoforin (**3**).[16] We were pleased to see that both compounds were also potent inhibitors of SIRT1 and SIRT2 (Table 1), and it is presumed that the phloroglucinol scaffold is the common principle of action. Furthermore, the inhibitory activity of the phloroglucinols on SIRT1 was double that of SIRT2. Interestingly, $Zn^{2+}$-dependent histone deacetylases (class I and II) were not inhibited by compounds **1**–**3** at concentrations of up to 300 μM.

To examine the cytotoxic effects of the phloroglucinol derivatives on cells, HUVE cells were incubated with compounds **1**–**3** and then assayed for cell viability by using the WST-1 assay (Roche). Cell proliferation was monitored by incorporation of 5-bromo-2′-deoxyuridine (BrdU), with quantification by an enzyme-linked immunosorbent assay (ELISA). In this way, **1** and **3** were found to reduce the metabolic activity of the cells to a lesser extent than **2** ($IC_{50}$ values are given in Table 1). However, both compounds **1** and **3** were stronger inhibitors of cell proliferation (Figure 2), and thus act more specifically.

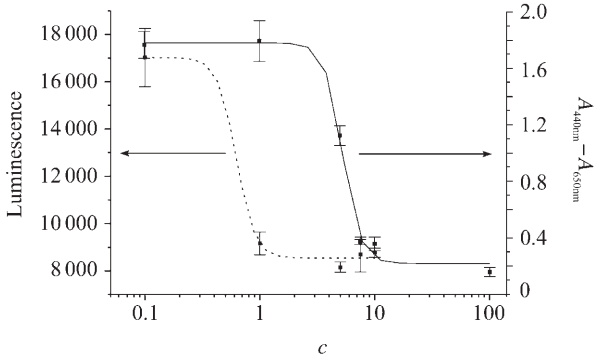


***Figure 2.*** Determination of antiproliferative (-----) and cytotoxic effects (——) of **1** on HUVE cells (see also the Experimental Section); the concentration *c* is in μm. Three determinations made at seven different inhibitor concentrations.

In contrast to guttiferone G, for which very limited knowledge about its biological properties exists, a plethora of pharmacological actions have been described for hyper-

***Table 1:*** $IC_{50}$ values (μM) of phloroglucinols **1**–**3** for the inhibition of SIRT1 and SIRT2, cell viability, and proliferation of HUVE cells.

| Compound | SIRT1 | SIRT2 | Cytotoxicity | Proliferation |
|---|---|---|---|---|
| guttiferone G (**1**) | 9 ± 0.2 | 22 ± 0.5 | 5.3 ± 0.2 | 0.6 ± 0.1 |
| hyperforin (**2**) | 15 ± 0.5 | 28 ± 0.2 | 1.3 ± 0.2 | 1.15 ± 0.1 |
| aristoforin (**3**) | 7 ± 0.2 | 21 ± 1 | 4.9 ± 0.1 | 0.59 ± 0.09 |

  

forin.[17] In addition to its well-known antidepressive effects, it also promotes apoptosis of various cancer cells from solid tumors and haematological malignancies, it is antiangiogenic and antimetastatic, and also displays antibacterial effects. Furthermore, hyperforin induces the expression of the CYP3A4 isoform of cytochrome P450 by binding to the pregnane X receptor. Interestingly, the antidepressant effects of hyperforin were recently shown to be associated with an activation of cation channels of neurons. This activation led to an increase in the intracellular sodium concentration followed by a reduced sodium-gradient-driven uptake of neurotransmitters from the synaptic cleft.[18] In this context it is worth noting that the co-product of sirtuin-catalyzed deacetylation, *O*-AADPR, was reported to regulate TRPM2, a nonselective cation channel mainly expressed in the brain.[19]

On the basis of our observations it is tempting to speculate that several of the above mentioned effects of hyperforin (and guttiferone G) can be attributed at least in part to its inhibitory properties against SIRT1 and SIRT2. We assume phloroglucinols such as **1** and **2** to be modulators of protein–protein interactions based on bromodomains (protein domains able to recognize acetylated lysine residues on the N-terminal tails of histones).

In summary, we have shown that the natural products (+)-guttiferone G and hyperforin as well as the synthetic hyperforin derivative aristoforin are inhibitors of human SIRT1 and SIRT2, with SIRT1 being more greatly affected than SIRT2. Moreover, we have shown that (+)-guttiferone G and aristoforin are less toxic than hyperforin, but are stronger inhibitors of cell proliferation. These natural compounds may represent valuable tools in the area of epigenetics and could also be used to shed more light on the biological role of sirtuins in processes such as cancer, ageing, neurodegenerative diseases, adipositas, and diabetes. It will also be interesting to investigate the ability of other members of the phloroglucinol class[20] of natural products to modulate the activity of histone-modifying enzymes.[21,22]

## Experimental Section

Isolation and structure elucidation of guttiferone G:

The bark of *Garcinia cochinchinensis* was collected in south Vietnam. The air-dried and powdered bark (2.1 kg) was extracted by soxhlet with hot petroleum ether for 32 h. The solvent was then evaporated under reduced pressure to give the petroleum ether extract (78 g). The extract was fractionated by column chromatography on silica gel, using EtOAc/petroleum ether (step gradient) to give 7 fractions. Fractions which failed to give clear spots when analyzed by thin-layer chromatography or contained fats were not further studied. Fraction 5 (14 g) was subjected to further column chromatography (silica gel, acetone/petroleum ether gradient) to yield 7 fractions. Fraction 2 was then further purified (silica gel, acetone/petroleum ether gradient) to give 9 fractions of which fraction 3 (1.1 g) was used to furnish guttiferone G. The major fraction of fraction 3 was separated by a combination of flash column chromatography (silica gel, acetone/petroleum and EtOAc/petroleum ether gradients) followed by gel-permeation chromatography (Sephadex LH-20, $CHCl_3$/MeOH 1:1) and column chromatography over DIOL silica (EtOAc/petroleum ether gradient) to afford 80 mg of guttiferone G.

Assignment of the NMR spectra was performed using standard NMR pulse sequences and 1D and 2D NMR methods ($^1H$, $^{13}C$, APT, H,H-COSY, HMQC, HMBC, NOESY). The spectra were recorded on a Varian Mercury 400 MHz, a Bruker DRX-600, and a Bruker Avance-700 spectrometer in [$D_4$]methanol and [$D_5$]pyridine. Specific rotation was determined with a Schmidt and Haensch Polartronic D polarimeter by applying the natural product at a concentration of 1.7 g/100 mL in $CHCl_3$ and a cell of 5-cm length. The molecular mass was determined by high-resolution mass spectrometry by using a Bruker 7 T APEX II FT-ICR mass spectrometer.

Isolation of hyperforin and synthesis of aristoforin:

Hyperforin was isolated directly from St. John's wort according to the method of Adam et al.[23] and derivatized as described.[16] Briefly, hyperforin was first alkylated with ethyl bromoacetate to give the C- and the O-alkylated derivatives. The latter was then saponified with aqueous NaOH solution to afford aristoforin. Hyperforin and aristoforin were stored at −80 °C, with hyperforin in an argon atmosphere and with exclusion of light.

Enzyme expression and purification:

Human deacetylases SIRT1 and SIRT2 were overexpressed as glutathione S-transferase (GST) fusion proteins. Vectors were cloned as described in Ref. [11a] The *E. coli* strain DH5α was used for protein expression, which was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after the culture had reached an $OD_{600}$ value ($OD_{600}$ = optical density at 600 nm) of 0.6. The temperature was then lowered to 25 °C. After 5 h incubation in LB medium which contained ampicillin, cells were harvested by centrifugation and solubilized either by French pressing or by lysozyme/sonification treatment. The soluble recombinant proteins were purified by affinity chromatography on GSTrap HP columns (Amersham Biosciences) by elution with 10 mM glutathione. GST-fusion proteins showed $NAD^+$-dependent deacetylase activity, which could be inhibited with nicotinamide. Furthermore, the identity of the purified proteins was verified by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli.[24]

Inhibition assay:

A radioactivity-based deacetylation assay using the [$^3H$]-labeled tubulin peptide MPSDKTIGG as a substrate was utilized to determine sirtuin inhibition.[11a] Briefly, the peptide was chemically acetylated with [$^3H$]acetic acid and BOP reagent (BOP = benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate) according to the histone deacetylase kit (Upstate Technology). The deacetylase reaction was performed in 100 μL HDAC buffer (15 mM Tris/HCl, pH = 7.9, 0.25 mM EDTA, 10 mM NaCl, 10% glycerol (v/v), 10 mM mercaptoethanol; Upstate Technology), with 40000 cpm peptide substrate and 500 μM $NAD^+$ as cosubstrate. The reaction was started by adding 1 μg of recombinant GST-SIRT1 and GST-SIRT2, respectively, and incubated at 37 °C overnight. The released [$^3H$]acetyl product was extracted with ethyl acetate and quantified with a liquid scintillation counter. Measurements were performed in triplicate, and the $IC_{50}$ values were determined by sigmoidal fitting of at least seven measurement points (seven different inhibitor concentrations) using Origin graphic program (version 6.0).

Cell viability and proliferation assays:

Cell proliferation reagent WST-1 (Roche) was used to determine the viability of the cells in response to incubation with the natural products. 5000–10000 human umbilical vein endothelial (HUVE) cells (PromoCell) were cultured in 96-well microplates for 24 h in a final volume of 100 μL culture medium per well. After washing the cells and replacing the medium (final volume: 95 μL), 5 μL of the natural products were added at different concentrations, so that the DMSO content did not exceed 0.5% (v/v). After 24 h of incubation, WST-1 was added and the absorbance was measured according to the test protocol.

Cell proliferation was investigated by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU, Roche Elisa assay) into

  

HUVE cells. To obtain proliferating cells, 3000–4000 HUVE cells were seeded into each well of a 96-well microplate 24 h prior to incubation with the natural products, which were then applied as described for the cell-viability assay. Cells were incubated with the natural products for 72 h and the proliferation status was determined according to the test instructions using an Orion Microplate Luminometer (Bertholt) to measure chemiluminescence.

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