Mariannaeapyrone – a New Inhibitor of Thromboxane A_2 Induced Platelet Aggregation

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Mariannaeapyrone ((*E*)-2-(1,3,5,7-tetramethyl-5-nonenyl)-3,5-dimethyl-6-hydroxy-4*H*-pyran-4-one) is a new fungal metabolite isolated from fermentations of the common mycophilic deuteromycete *Mariannaea elegans*. The chemical structure of the 4-pyrone was determined by spectroscopic techniques. Mariannaeapyrone is a selective inhibitor of the thromboxane A2 induced aggregation of human platelets, whereas only weak cytotoxic and antimicrobial effects could be observed.

Introduction

Platelet activation and aggregation are essential events in thrombus formation. For this reason, platelets do not only play an important role in haemostasis, but they are also involved in the pathophysiology of cardiovascular disorders, which may result in myocardial or ischemic cerebral infarction.

One important inducer of platelet aggregation is the prostanoid thromboxane A_2 (TXA_2), additionally inducing constriction of vascular and bronchiolar smooth muscle by receptor activation and thus being involved in the pathomechanism of a series of diseases. Many antagonists of the thromboxane A_2 receptor (TP receptor) have been described during the last years and some of them are under further investigation. But very often strong side effects have been detected and clinical trials on these compounds have produced very disappointing results. For this reason only a few antagonists of the TP receptor are available for use in man and only one of them, the quinone derivative **2**, is marketed so far (Bronica® by Takeda Company, Japan). There is therefore a need for novel and more potent antagonists, which are structurally different from the known entities, as they may provide more information about the TP receptor and its various functions as well as facilitate the development of novel potentially useful drugs. In the following we describe the fermentation, isolation, structure elucidation and the biological properties of mariannaeapyrone (**1**), a new fungal metabolite inhibiting the thromboxane A2 induced platelet aggregation.

Material and Methods

General

UV and IR spectra were measured with a Perkin-Elmer Lambda 16 UV/VIS spectrometer and a Bruker IFS 48, respectively. For analytical HPLC a Hewlett Packard 1100 series instrument and for preparative HPLC a Jasco PU-980 instrument were used. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in CD_3OD, and the solvent signals (3.31 and 49.15 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for ^1JCH=145 Hz and ^nJCH=10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). Mass spectra were recorded with a Jeol

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SX102 spectrometer and a HP 1100 LC-MSD, Hewlett Packard, while the optical rotation were measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Producing organism

Mariannaea elegans is a very common mycophilic deuteromycete. The strain UR 742 was obtained from Dr. H. Besl, University of Regensburg, and is deposited in the culture collection of the LB Biotechnology, University of Kaiserslautern.

Fermentation and isolation

For maintenance on agar slants and submerged cultivation, *Mariannaea elegans* was grown in YMG medium composed of : yeast extract 0.4%, malt extract 1%, glucose 1%, pH 5.5 and agar 1.5% for solid media. Fermentations were carried out in a Biolafitte C6 fermenter containing 20 l of YMG medium with aeration (2 l air/min) and agitation (120 rpm) at room temperature. 250 ml of a well grown culture in YMG medium were used as inoculum. During fermentation 100 ml samples were taken every day. The culture fluid was separated by filtration from the mycelia and then extracted with 100 ml ethylacetate. The evaporated extract was dissolved in methanol and tested in the platelet aggregation assay. After seven days of fermentation the fermenters were harvested and the culture broth was separated from the mycelia. Mariannaeapyrone (**1**) was removed from the culture fluid (17 l) that was extracted with 10 liters of ethylacetate. 1016 mg crude extract were purified by silica gel chromatography (0.063–0.2 mesh, Merck 60; column: 55 × 95 mm), using 200 ml cyclohexane: ethylacetate 70 : 30 v/v as eluent, to afford 186 mg of an intermediate product. Finally, 2.4 mg of the pure compound **1** were obtained by preparative HPLC, elution with water : methanol 13 : 87 v/v (Rt 85 min).

Mariannaeapyrone (**1**) was obtained as a yellowish oil, $[\alpha]_D$ −50° (c 0.2 MeOH). UV (MeOH) λ nm (ε): 283 (6,100). IR (KBr): 3425, 2960, 2925, 1650, 1565, 1505, 1455, 1380, 1235 and 1090 cm^{-1}. EI-MS, *m/z*: 320.2359 (M^+, 25%, $C_{20}H_{32}O_3$ requires 320.2351), 291 (18%), 263 (7%), 223 (7%), 181 (70%), 167 (21%), 153 (100%), 139 (48%). LC-APCI-MS (acetonitrile/water, positive ions), *m/z*: 321 (100%). ^1H NMR (CD_3OD, 500 MHz), δ, mult., *J* (Hz): 4.85, m, 11-H; 3.07, m, 6-H; 2.24, m, 12-H; 1.95, s, 16-H_3; 1.92, dd, 7.5 and 13.5, 9-Ha; 1.86, s, 15-H_3; 1.83, ddd, 4.0, 10.2 and 13.5, 7-Ha; 1.77, dd, 5.5 and 13.5, 9-Hb; 1.49, s, 19-H_3; 1.45, m, 8-H; 1.32, m, 13-Ha; 1.20, m, 13-Hb; 1.17, d, 6.8, 17-H_3; 1.15, ddd, 4.8, 9.2 and 13.5, 7-Hb; 0.89, d, 6.7, 20-H_3; 0.85, t, 7.4, 14-H_3; 0.83, d, 7, 18-H_3. ^{13}C NMR (CD_3OD, 125 MHz), δ: 176.8 C-3; 169.9 C-1; 161.6 C-5; 134.3 C-11; 133.7 C-10; 112.4 C-4; 97.2 C-2; 49.6 C-9; 43.1 C-7; 35.4 C-12; 33.7 C-6; 31.8 C-13; 30.1 C-8; 21.5 C-20; 20.1 C-18; 19.9 C-17; 16.3 C-19; 12.6 C-14; 10.5 C-16; 9.2 C-15.

Biological assays

In the platelet aggregation assay (Fabian *et al.*, 1999) the aggregation of concentrated human platelets (1×10^9 cells/ml) was induced with the following agonists ADP (16 μM), collagen (0.4 mg/ml), U46619 (3 μM) and TPA (12-o-tetradecanoylphorbol 13-acetate) (0.3 μg/ml). Cytotoxic effects were determined using the following monolayer cell lines: HeLa S3 (epitheloid carcinoma, cervix, human; ATCC CCL 2.2) and COS-7 (SV-40 transformed kidney fibroblasts, monkey; ATCC CRL-1651) grown in DMEM medium and the suspension cell lines: COLO-320 (colon adenocarcinoma, human; DSMZ ACC 144), HL 60 (promyelocytic leukemia, human; ATCC CCL 240) and L 1210 (lymphocytic leukemia, mouse; ATCC CCL 219) all grown in RPMI medium. Cytotoxicity was measured in microtiter plates with 1×10^5 cells/ml. After 48 hours incubation with the tested compound the cells were examined microscopically. The effect on the growth of monolayer cell lines was measured with Giemsa stain and the viability of suspension cell lines was measured by the XTT test as described in the product information (Roche Diagnostics, Mannheim). The tested cell lines were cultivated as described elsewhere (Zapf *et al.*, 1995 and Fabian *et al.*, 1998). The antimicrobial activity (Anke *et al.*, 1989) was carried out as described previously.

Results and Discussion

Fermentation, isolation and structural elucidation

The fermenters were harvested when the activity of the crude extract in the platelet aggregation

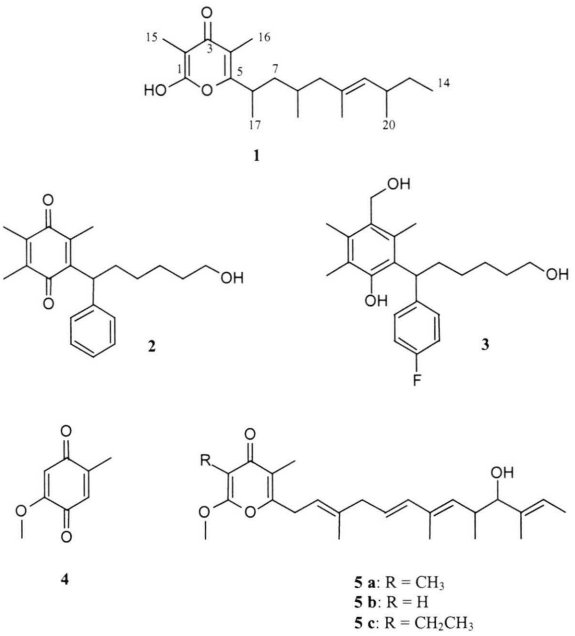

Fig. 1. Structure of mariannaeapyrone (**1**) and other TXA_2 receptor antagonists.

assay reached a maximum, approximately after seven days. The extraction of the culture broth and the isolation of mariannaeapyrone (**1**) are described in the experimental section. The mass spectra of **1** suggested that its elemental composition is $C_{20}H_{32}O_3$, and this was confirmed by the NMR data. The structure of **1** could be determined from the 2D COSY, NOESY, HMQC and HMBC NMR data, of which pertinent HMBC correlations are shown in Figure 2. The HMBC correlations

Fig. 2. Pertinent HMBC correlations observed with mariannaeapyrone (**1**).

from the protons of the 7 methyl groups to the neighbouring carbons establish the carbon skeleton of **1**, which is in agreement with the correlations between the protons in the open chain observed in the COSY spectrum. C-3 is obviously a keto functionality, and the extreme chemical shifts of the carbons in the remaining two carbon-carbon double bonds support the suggestion that they are both conjugated with an EWG and have oxygen attached to the β-carbon. With three carbon-carbon double bonds and one keto function 4 of the 5 unsaturations of mariannaeapyrone (**1**) are accounted for, and the remaining must be a ring. This can only be formed between C-1 and C-5, in the form of a 4-pyrone.

Biological properties

Mariannaeapyrone (**1**) did not inhibit the aggregation of human platelets stimulated by ADP, collagen and the protein kinase C activator TPA up to 312 μM (100 μg/ml) and interfered only weakly with the thrombin induced aggregation starting from 156 μM (50 μg/ml). **1** is a potent inhibitor of the aggregation caused by the thromboxane A2 analogue U46619 with an IC_{50} value of 15.6 μM (5 μg/ml). In Figure 3, a dose-response curve for the inhibition of the U46619 induced aggregation by mariannaeapyrone (**1**) is shown. The inhibitory effect of **1** is strongly dependent on the concentration of the inducer U46619 (Fig. 4), suggesting a competitive inhibition of the thromboxane A2 receptor mediated platelet aggregation by **1**.

Mariannaeapyrone showed moderate cytotoxic effects against different cell lines. The IC_{50} values varied between 125 and 250 μM (40 and 80 μg/ml) and are shown in Table I. In the agar diffusion assay a visible inhibition of *Bacillus brevis*, *Bacillus subtilis* and *Micrococcus luteus* started at concentrations 156 μM (50 μg/ml) whereas no antimicro-

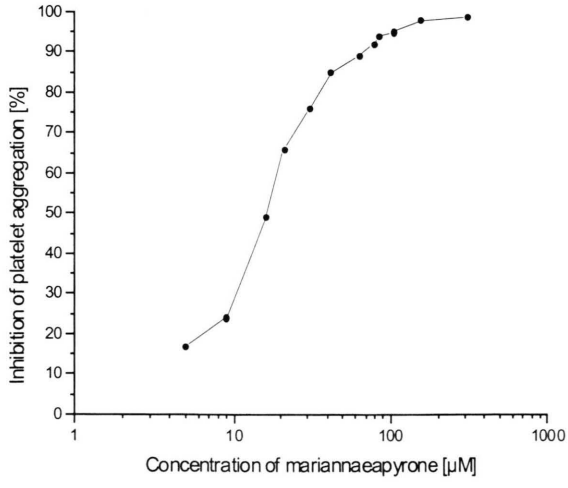

Fig. 3. Dose response curve of mariannaeapyrone inhibiting platelet aggregation.

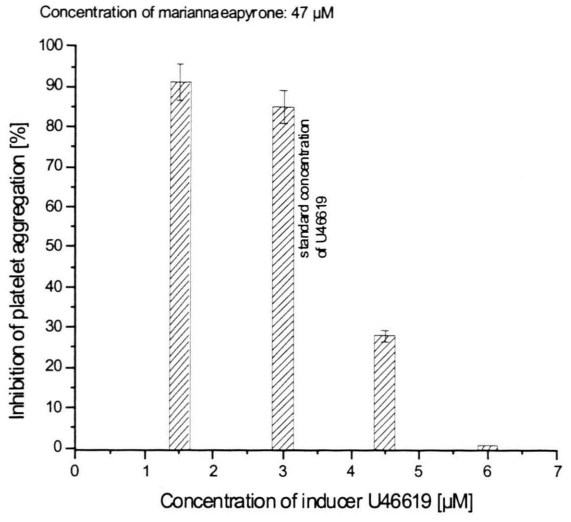

Fig. 4. The concentration of U46619 affects the potency of mariannaeapyrone in inhibiting platelet aggregation.

Table I. Cytotoxic activities of mariannaeapyrone.

Cells	IC_{50} [µM]
HeLa S3	188
COS-7	219
Colon	250
L1210	125[a]
HL 60	156[a]

[a]: Lysis of cells observed after 48 h.

bial activity was detected against *Enterobacter dissolvens* and the fungi *Nematospora coryli*, *Paecilomyces variotii*, *Penicillium notatum* and *Mucor miehei*.

In conclusion, mariannaeapyrone (**1**) interferes with platelet aggregation by binding to the thromboxane A2 receptor of human platelets. Interestingly, **1** belongs to the small group of antagonists of the TP receptor lacking a carboxyl group. As reported by Yamamoto *et al.* (1993), the terminal carboxyl group of **2** and **3** (Fig. 1) plays an important role in receptor binding by interacting with Arg 295 of the receptor. Lacan *et al.* (1999), investigating 2,3-dihydrothiazole derivatives, found that the affinity of the inhibitors for the TP receptor was strongly dependent on the position of the carboxyl group in the side chain. Analyzing the receptor-ligand interactions in the model of Yamamoto *et al.* (1993) established that a polar group of the ligand (e.g. a hydroxyl group) interacting with Ser 201, as well as a hydrophobic moiety are required for TP receptor antagonists of type **2** and **3**. Wouters *et al.* (1999) showed that the benzoquinone (e.g. of **2**) and benzene (e.g. of **2** and **3**) rings, which are part of many inhibitors, can be replaced by other groups provided that they fit the two hydrophobic pockets identified in the receptor. On the other hand, compounds that do not fit completely to the postulated binding sites but still inhibiting the TP receptor have been described (Lauer *et al.*, 1991), and an exampel is 2-methoxy-5-methyl-1,4-benzoquinone (**4**). In the case of mariannaeapyrone (**1**), the 4-pyrone moiety might fit into the site where the benzoquinone moiety of **2** or the phenolic moiety of **3** bind. The lipophilic side chain, however, is expected to bind to another part of the receptor. In 1986, the actinopyrones (**5**) isolated from a strain of *Streptomyces pactum* possessing vasodilatoric properties in dogs were reported (Yano *et al.*, 1986), but unfortunately no data of possible antiplatelet activity have been published. For this reason, it remains unclear if the mechanism of the vasodilatoric effects reported for the actinopyrones (**5**) are due to an interference with the TXA_2 receptor mediated signal transduction.

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