JOURNAL OF NATURAL PRODUCTS

Note

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Amorfrutins Are Natural PPARγ Agonists with Potent Anti-inflammatory Properties

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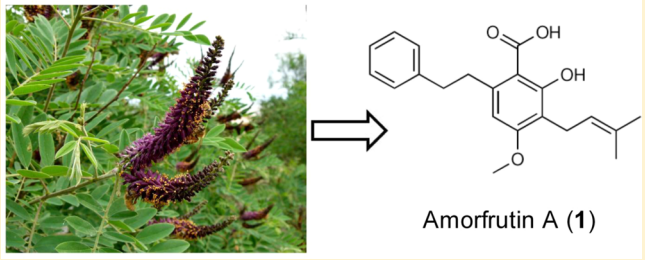
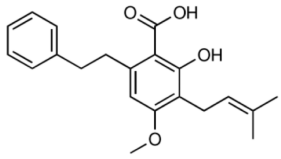
Supporting Information

ABSTRACT: Amorfrutins are isoprenoid-substituted benzoic acid derivatives, which were found in *Amorpha fruticosa* L. (bastard indigo) and in *Glycyrrhiza foetida* Desf. (licorice). Recently, amorfrutins were shown to be selective activators of the nuclear receptor PPARγ. Here, we investigated the effects and PPARγ-based mechanisms of reducing inflammation in colon cells by treatment with amorfrutins. In TNF-α-stimulated colon cells amorfrutin A (**1**) reduced significantly the expression and secretion of several inflammation mediators, in part due to interaction with PPARγ. These results support the hypothesis that amorfrutins may have the potential to treat inflammation disorders such as chronic inflammatory bowel diseases.

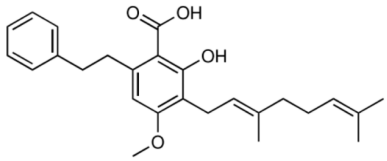


Amorfrutins are natural products composing a family of isoprenoid-substituted benzoic acid derivatives, which were originally identified from fruits of *Amorpha fruticosa* (bastard indigo)[1] and in edible roots of *Glycyrrhiza foetida* (licorice).[2] Recently, we showed that amorfrutins have potent antidiabetic *in vivo* activity that results from partial activation of the nuclear receptor PPARγ. Using reporter gene assays, EC_{50} values of 458 nM were reported for amorfrutin A (**1**) and 73 nM for amorfrutin B (**2**).[2−4] Furthermore, X-ray analyses indicated interaction of amorfrutins with the helix H3 and the β-sheet of PPARγ.[2,3] Amorfrutins improved in particular insulin resistance through activating PPARγ in white adipose tissue. In contrast to full agonists of PPARγ such as rosiglitazone, amorfrutins act as selective PPARγ modulators (SPPARγMs) in fat cells, activating only a subset of metabolic genes that are under the control of PPARγ.[2,4] This partial activation mechanism may have contributed to reduce undesirable side effects that are commonly observed with synthetic PPARγ ligands such as the thiazolidinediones.[5]

In addition to its role in metabolic regulation, PPARγ can in principle also inhibit inflammatory gene expression through several different mechanisms, including for example direct interference with the transcription factor NFκB,[6] competition for limited amounts of shared coactivators with NFκB,[7] nuclear export of p65,[8] the repression of inflammatory genes via a SUMOylation-dependent pathway,[9] and the capability of PPARγ to act as an E3 ubiquitin ligase.[10] Notably, several clinical studies have been initiated to explore the anti-inflammatory effects of PPARγ ligands, for example for treating inflammatory bowel diseases such as ulcerative colitis (https://clinicaltrials.gov/).



Amorfrutin A (**1**)



Amorfrutin B (**2**)

Recently, phenolic constituents derived from *Amorpha fruticosa*, such as **1**, were shown to inhibit in micromolar concentration ranges the NFκB pathway and consequently expression of genes involved in inflammation.[11,12] However, these studies could not directly link these interesting observations to a distinct target protein. In this Note, we show that anti-inflammatory effects of amorfrutins may in part be derived from interaction with PPARγ.

On the basis of the established assumption that PPARγ is a prime target protein of amorfrutins and that PPARγ (isoform 1) is known to be abundant in the intestine,[13−18] we aimed to mechanistically analyze the so far unexplored role of this interaction for anti-inflammatory effects in a disease-relevant cellular context. Therefore, we evaluated the anti-inflammatory

Received: October 6, 2014
Published: May 4, 2015

DOI: 10.1021/np500747y
J. Nat. Prod. 2015, 78, 1160−1164

potential of **1** and **2** in TNF-α-stimulated human HT-29 colon cells, a well-established model for inflammation processes in the gut.[19]

To evaluate the cell-specific cytotoxicity of amorfrutins in HT-29 colon cells and to optimize treatment concentrations for subsequent experiments, we initially treated these cells with different concentrations of **1** or **2** for 54 h. As shown in Supplementary Figure 1, using a widely applied antibody for PPARγ, we detected expression of this intestinal protein in our cellular model, consistent with previous results showing in particular expression of the PPARγ1 isoform.[13] The determined IC_{50} (inhibitory concentration 50%) value for **1** was 50.8 μM. Up to a concentration of 10 μM, no cytotoxic effects or impact on cell viability could be observed in HT-29 colon cells (Figure 1, Supplementary Figure 2). The IC_{50} for **2** was 30.5 μM,

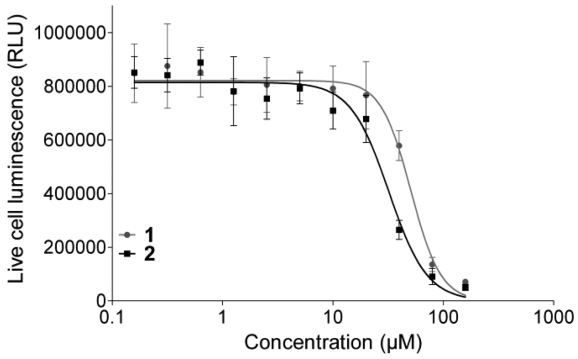


Figure 1. Concentration-dependent cytotoxic effects of amorfrutins. For the determination of cytotoxicity, cells were treated with different concentrations of **1** or **2** for 54 h. Cell viability was determined by the luminometric CytoToxGlo assay. Data are expressed as mean ± SD (n = 4).

indicating a slightly higher cytotoxic potential. Compound **2** did not induce cytotoxic effects or affect cell viability up to a concentration of 5 μM (Figure 1, Supplementary Figure 3). On the basis of these results, treatment concentrations of 10 μM for compound **1** and 1 μM for compound **2** were chosen for subsequent experiments.

To study the effects of **1** and **2** on the expression of different inflammation marker genes, cells were incubated with **1** or **2** for 48 h and subsequently stimulated with TNF-α for 6 h. Treatment with **1** resulted in a downregulation of the expression of several marker genes compared to untreated TNF-α-stimulated cells (Figure 2A). Genes COX-2, GRO-α, IL-8, and MIP-3α were significantly downregulated due to treatment with **1**. Weaker effects were observed for downregulation of expression of GRO-γ, IL-1β, and MIP-2 (Figure 2A). Treatment with **2** showed similar effects. Expression of genes COX-2, IL-8, and MIP-3α was significantly reduced, whereas GRO-α, GRO-γ, IL-1β, MIP-2, and TNF-α showed only by trend decreased gene expressions.

In general, more significant cellular effects were observed by treatment with **1** than with **2**. Therefore, and due to the fact that **1** seems to be more abundant in plants than **2**,[4] we focused on this compound in further experiments. As **1** binds and activates PPARγ in the nanomolar range and thereby potentially inhibits NFκB target genes involved in inflammation, we hypothesized that this natural product exerts its anti-inflammatory potential at least in part in a PPARγ-dependent

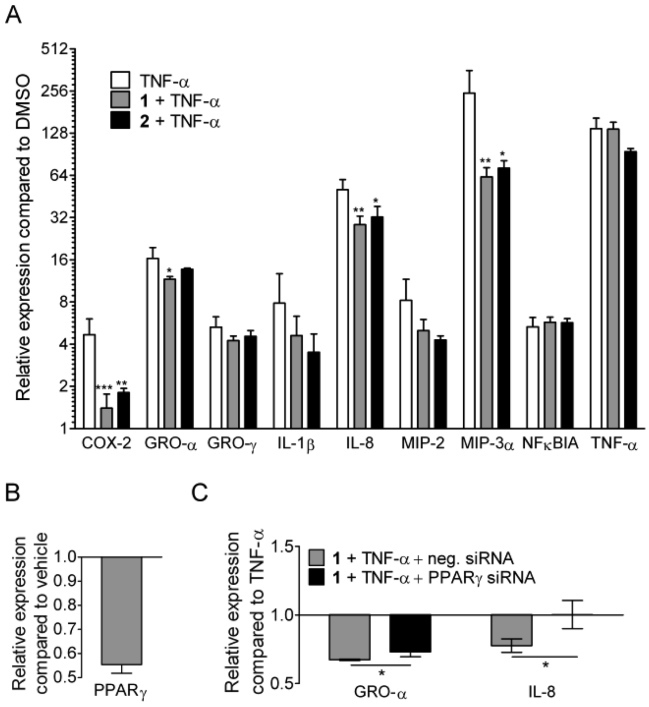


Figure 2. PPARγ-binding amorfrutins lower the expression of various inflammatory genes. (A) Cells were treated with 0.1% DMSO, 10 μM **1**, or 1 μM **2** for 48 h and subsequently treated with 1 ng/mL TNF-α for 6 h. Real-time qPCR was applied to detect gene expression of various inflammatory marker genes. Data are shown relative to DMSO-treated cells. Data are expressed as mean ± SD (n = 3). *$p \leq$ 0.05, **$p \leq$ 0.01, ***$p \leq$ 0.001 vs TNFα. (B) siRNA-mediated knockdown of PPARγ in HT-29 cells. Cells were transfected with PPARγ Silencer Select validated siRNA or Silencer Select Negative Control #1 siRNA (vehicle control) using HT-29 transfection reagent (knockdown efficiency: 45%). Knockdown efficiency was determined via real-time qPCR. The significant knockdown efficiency was further validated by detection of PPARγ protein by Western blotting (Supplementary Figure 5). (C) Gene expression analysis of pro-inflammatory genes in HT-29 cells after siRNA-mediated PPARγ knockdown via real-time qPCR. Data are shown relative to TNF-α-stimulated cells. Data are expressed as mean ± SD (n = 3). *$p \leq$ 0.05 vs TNF-α.

manner.[16,17] Hence, knockdown of PPARγ should abolish some effects of ligands such as **1** on gene expression.

Interestingly, we found that PPARγ knockdown strongly affected the decrease of IL-8 and GRO-α gene expression induced by compound **1**, indicating PPARγ-based ligand-dependent activation of anti-inflammatory effects (Figure 2B,C). Decrease of GRO-α gene expression after treatment with PPARγ–ligand **1** was significantly reduced after PPARγ knockdown, whereas decreases of IL-8 gene expression were even almost completely abolished (Figure 2B,C).

For the other two tested inflammation marker genes (COX-2 and MIP-3 α), we did not observe significant PPARγ-dependent gene expression changes after treatment with compound **1** (Supplementary Figure 4). This result could have potentially been observed due to significant but only slight technical PPARγ-knockdown efficiency on the protein level (Supplementary Figure 5), compensatory effects in the cells after knockdown of PPARγ, or other unidentified targets of **1**, which require further study. Since natural products such as amorfrutins tend to promiscuously bind to proteins, for example other related receptors of the PPAR family such as

DOI: 10.1021/np500747y
J. Nat. Prod. 2015, 78, 1160−1164

PPARα or PPARβ/δ or proteins of the NFκB pathways, these factors may play an additional role in amorfrutin-dependent cellular phenotypes. But notably, in contrast to the strong PPARγ-agonist rosiglitazone, amorfrutins showed in general a more pronounced anti-inflammatory gene expression profile in HT-29 cells, which might be a result of reported differential PPARγ activation of these compounds (Supplementary Figure 6).[2,3]

On the basis of the gene expression analyses of PPARγ target genes, we next analyzed the cellular effects of treatment with **1** on secretion of pro-inflammatory molecules such as chemokines. Since gene expression of MIP-3α, IL-8, and GRO-α was downregulated after treatment with **1**, we analyzed by ELISA the effects of **1** on protein levels of these chemokines in cell culture supernatants. In accordance with the results obtained from gene expression analysis, secretion of MIP-3α (Figure 3A), IL-8 (Figure 3B), and GRO-α (Figure 3C) was significantly reduced after treatment with **1**.

Consistent with downregulation of gene expression of COX-2 (Figure 2A), protein levels of COX-2 were reduced after treatment with **1**, as determined by Western blotting (Figure 3D,E). On the basis of the PPARγ-knockdown analyses (Supplementary Figure 4) we concluded that this effect was potentially independent from direct activation of PPARγ and rather a result of indirect effects derived from modulating PPARγ, or due to reported interference of compound **1** with NFκB pathway proteins, or due to other effects.[11,12]

In particular the inducible form COX-2 is transcribed and synthesized during inflammation and responsible for the production of prostaglandins involved in inflammatory processes.[20] Cyclooxygenases are the main targets for non-steroidal anti-inflammatory drugs (NSAIDs) to decrease prostaglandin production through the inhibition of COX activity or expression.[21] Nevertheless, NSAIDs targeting both isoforms are likely to evoke side effects, which are thought to occur due to the inhibition of the constitutively expressed COX-1, whereas the anti-inflammatory potential of NSAIDs is thought to be mediated by the inhibition of COX-2.[21] Interestingly, **1** strongly inhibited the expression of COX-2 on the gene and the protein level, suggesting further potential of amorfrutins to develop natural product inhibitors of the COX-2 pathway. Since COX-2 was not directly affected by PPARγ knockdown, the exact mechanism of action including target proteins involved requires further study.

In this Note we focused on the anti-inflammatory effects of the natural PPARγ ligands amorfrutin A (**1**) and amorfrutin B (**2**) in inflamed colon cells. Our data suggest that specific interaction of amorfrutins with PPARγ led in part to inhibited gene expression of inflammatory mediators that could otherwise activate other (immune) cells to amplify inflammation processes.[22] Due to the complex and still poorly understood interference of PPARγ with inflammatory processes including transcription factors such as NFκB,[5] further basic research is needed to enable better study of ligand-based effects. Furthermore, natural products such as amorfrutins can induce nonspecific off-target effects, which are difficult to pinpoint to distinct interactions. Interestingly, the here observed anti-inflammatory effects of amorfrutins seem to be mild compared to strong synthetic molecules such as cortisone that show severe side effects during long-term treatment.

Dietary amorfrutins may provide perspectives for complementary application, in particular for treating and preventing chronic inflammatory (bowel) disorders. Future *in vivo* studies seem promising to further explore the potential of these relatively unknown natural products. Future comparative analyses of amorfrutins with structurally related PPARγ ligands such as 5-amino salicylates and other anti-inflammatory molecules that target alternative pathways shall allow for fine-tuning anti-inflammatory treatments. Amorfrutins or edible plant extracts thereof may become useful alternatives for managing chronic inflammatory bowel diseases with fewer side effects.

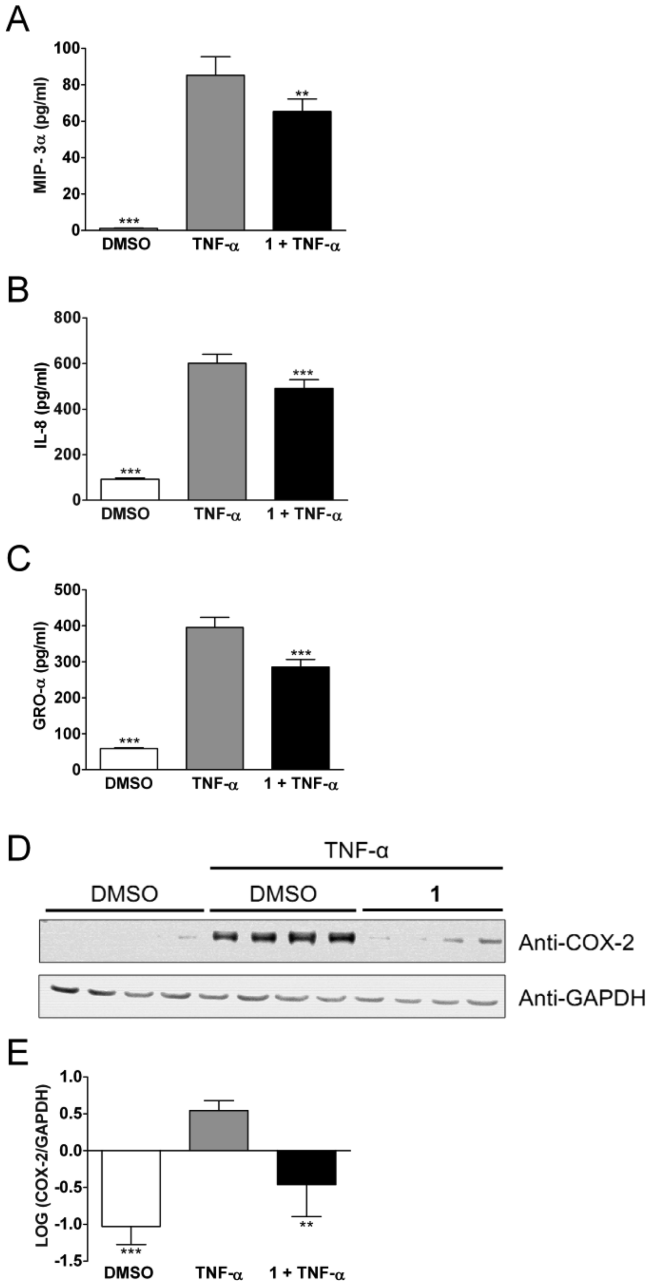


Figure 3. Amorfrutin A (**1**) decreases chemokine secretion and COX-2 protein levels. Cells were incubated with 0.1% DMSO (vehicle control) or 10 μM **1** for 48 h and subsequently treated with 1 ng/mL TNF-α for 6 h. MIP-3α/CCL20 (A), IL-8 (B), and GRO-α (C) amounts were determined via ELISA. COX-2 protein levels were determined via Western blotting (D, E). Data are expressed as mean ± SD. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ vs TNF-α.

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EXPERIMENTAL SECTION

General Experimental Procedures. Amorfrutin A (**1**) and amorfrutin B (**2**) were originally isolated from *A. fruticosa* L. (Fabaceae) (bastard indigo) and in *G. foetida* Desf. (Fabaceae) (licorice) and can be obtained from Analyticon Discovery (Potsdam, Germany). Detailed information on the plant material, extraction and isolation, and analytical procedures and data can be gained from this company. However, to reduce any potential experimental variation and compound background, in this mechanistic study we used the same synthetic amorfrutins **1** and **2**, which have recently been synthesized and for which detailed analytical data have already been published elsewhere.[2−4] For reported multigram synthesis, triflate served as precursor for the generation of **1** and **2** by using 2,4,6-trihydroxybenzoic acid as starting material. Sonogashira coupling was performed to introduce aromatic chains. Prenylation was achieved by modified phenoxide ortho-alkylation as described.[2] An overall yield of 35% could be achieved with this synthesis route. ^{1}H and ^{13}C NMR spectra were obtained on 400, 500, and 600 MHz Varian NMR spectrometers using $CDCl_3$ and acetone-d_6 (Cambridge Isotope Laboratories) as solvents. The purity of the synthetic materials was assessed by proton NMR spectroscopy and HPLC. For **1** and **2**, protons and carbons were assigned based on (^{1}H,^{1}H)-dqfCOSY, (^{1}H,^{1}H)-NOESY, (^{1}H,^{13}C)-HSQC, and (^{1}H,^{13}C)-HMBC spectra. Purities of **1** and **2** were reported to be greater than 99%.[2−4]

Cell Culture. HT-29 cells were purchased from the Leibniz-Institut DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and cultured in Dulbecco's modified Eagle's medium: Ham's Nutrient Mixture F-12, 1:1 (DMEM-F12, ATCC) medium supplemented with 5% fetal bovine serum (FBS, Biochrom), and 1% penicillin/streptomycin (Biochrom, Berlin, Germany). Cells were incubated at 37 °C, 5% CO_2, and 95% humidity. Before reaching complete confluency, cells were subcultured. One day prior to treatment, cells were seeded into cell culture plates (Nunc, ThermoScientific, Bonn, Germany) or flasks (TPP, Trasadingen, Switzerland). Cells were treated with 10 μM **1** or 1 μM **2** and incubated for 48 h. Afterward, cells were treated with 1 ng/mL TNF-α (AbD Serotec, München, Germany) for 6 h and then harvested for gene expression analysis, ELISA, or Western blotting. Samples were run either as biological triplicates or as biological quadruplicates. DMSO (AppliChem, Darmstadt, Germany) concentration was 0.1% in all experiments and all treatments. Cells treated with 0.1% DMSO only served as a control.

Investigation of Cytotoxic Effects. One day prior to treatment, cells were seeded into 12-well plates (270 000 cells/well, Thermo Scientific) and incubated overnight at 37 °C. Cells were then treated with 1 μM, 10 μM, 20 μM, 50 μM, or 100 μM **1** or **2**. DMSO concentration was 0.1% in all experiments and all treatments. Cells treated with 0.1% DMSO only served as a control. After 24 h of incubation, the treated cells were observed under the microscope and checked for changes in morphology and appearance in contrast to the DMSO control. All samples were run as biological quadruplicates.

CytoTox-Glo Cytotoxicity Assay. Cells were seeded into 384-well plates 1 day prior to treatment. Cells were then treated with different concentrations (0, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10, 20, 40, 80, 160 μM) of **1** and **2** for 54 h. Subsequently, cytotoxicity was determined using the CytoTox-Glo cytotoxicity assay (Promega, Fitchburg, WI, USA). The assay was carried out according to the manufacturer's manual, whereby the viable cell luminescence was determined. All samples were run as biological quadruplicates.

Gene Expression Analysis. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's manual. Prior to the purification procedure, medium was discarded and cells were washed twice with PBS (Sigma-Aldrich, Steinheim, Germany) and lysed in RLT buffer (Qiagen) with β-mercaptoethanol (10 μL/mL, Sigma-Aldrich). To digest genomic DNA, an optional on-column DNase digestion was performed, using the RNase-free DNase set (Qiagen). RNA was eluted in RNase-free water. The final RNA concentration was measured using the Nanodrop 2000 (Thermo Scientific). RNA was then stored at −80 °C until use. For qPCR, RNA was reverse transcribed into cDNA with the high capacity cDNA reverse transcription kit (Applied Biosystems, Darmstadt, Germany) with random primers. The following reverse transcription program was performed using a thermocycler (MJ Research, Ramsey, MN, USA): 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, afterward cooling to 4 °C. After the reverse transcription, the cDNA was diluted to a concentration of 20 ng/μL with nuclease-free water (Ambion) and stored at −20 °C. Quantitative real-time PCR was performed on the ABI Prism 7900HT sequence detection system using the Power SYBR Green PCR Master Mix (all Applied Biosystems). After an initial denaturation at 95 °C for 10 min, the cDNA was amplified by 40 cycles of PCR (95 °C, 15 s; 60 °C, 60 s). The relative gene expression levels were normalized using GAPDH and quantified by the $2^{-\Delta\Delta Ct}$ method.[23] Primer sequences are summarized in Supplementary Table 1. Analysis of significance was performed using one-way ANOVA followed by Dunnett's multiple comparisons test.

PPARγ Knockdown. siRNA-mediated PPARγ knockdown was performed to investigate the specificity of compound-dependent gene expression effects. The knockdown experiments were carried out in 24-well plates using the HT-29 transfection kit (Altogen, Austin, TX, USA) according to the manufacturer's manual. Cells were transfected with 30 nM of PPARγ Silencer Select validated siRNA (ID s10888, Ambion, Applied Biosystems) or Silencer Select Negative Control #1 siRNA (Ambion, Applied Biosystems). All samples were run as biological triplicates.

Immunoblotting. For protein harvest, cells were washed once with cold PBS (Sigma-Aldrich), then scraped with 5 mL of cold PBS with 1× phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 1× protease inhibitor cocktail (Roche, Basil, Switzerland) and transferred into 15 mL tubes. Samples were centrifuged for 5 min at 1000*g* and 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 mL of cold PBS + 1× phosphatase inhibitor cocktail + 1× protease inhibitor cocktail and transferred into 1.5 mL tubes. Samples were again centrifuged for 5 min at 1000*g* and 4 °C. The supernatant was discarded, and pellets were resuspended in 100 μL of lysis buffer (50 μM Tris-HCL; pH 8.0, 10 mM EDTA, 1× phosphatase inhibitor cocktail, 1× protease inhibitor cocktail, 1% SDS), vortexed, and then incubated for 10 min on ice. Afterward, the suspension was sonicated to lyse the cells. The samples were centrifuged for 10 min at 14000g at 4 °C and stored at −80 °C. For determination of protein concentration, samples were diluted 20-fold in water. Analyses were carried out in 96-well plates. A 10 μL amount of diluted sample and 150 μL of Pierce solution (Thermo Scientific) with 1 g/20 mL IDCR (Thermo Scientific) were mixed and incubated for 1 min with continuous shaking (600 rpm); afterward, the plate was incubated without shaking at RT. After 3−7 min, absorbance was measured at 660 nm using a plate reader (POLARstar Omega, BMG Labtech, Ortenberg, Germany). BSA (Sigma Life Sciences) standards were as follows: 0, 0.05, 0.09, 0.19, 0.38, 0.76, 1.53 mg/mL.

Protein samples were denatured and separated using a Novex NuPAGE 4−12% Bis-tris-gel (Invitrogen, Carlsbad, CA, USA) and blotted onto nitrocellulose membranes (Hybond ECL nitrocellulose membrane 0.45 μM, GE Healthcare/Amersham Biosciences, Freiburg, Germany). Membranes were blocked in a solution containing 5% milk in 1× TBS + 0.1% Tween for 1 h at RT on a shaker. After blocking, membranes were incubated with primary antibodies against PPARγ (E-8, Santa Cruz, CA, USA, sc-7273), GAPDH (Santa Cruz, sc-48167), or COX-2 (Abcam, Cambridge, UK, ab52237) in incubation solution containing 5% milk in 1× TBS + 0.1% Tween overnight at 4 °C on a shaker and subsequently incubated with anti-goat IgG-HRP (Santa Cruz, sc-2020) or anti-rabbit IgG-HRP (Santa Cruz, sc-2004), respectively, prior to detection with Western Lightning ECL solution (PerkinElmer, Waltham, MA, USA). Quantitative analysis of the protein bands was performed using the GelAnalyzer2010 software. Analysis of significance was performed using one-way ANOVA followed by Dunnett's multiple comparisons test.

ELISA. For the measurement of chemokines in cell culture supernatants, 1 mL of supernatant was taken and transferred into 1.5 mL Eppendorf tubes. Supernatants were centrifuged at 4 °C for 15

DOI: 10.1021/np500747y
J. Nat. Prod. 2015, 78, 1160−1164

min at 1000*g*. The supernatant was then distributed into stripes and immediately stored on dry ice and later at −80 °C. For the detection of MIP-3α/CCL20 in cell culture supernatants, the Human CCL20/MIP-3α ActivELISA kit [Imgenex (now Novus Biologicals, Ltd., Cambridge, UK)] was used. GRO-α was detected using the USCN Life Science (Wuhan, China) GRO-α ELISA (SEA041 Hu), and the detection of IL-8 was performed using the Biolegend (San Diego, CA, USA) IL-8 ELISA. The assays were performed according to the manufacturer's manual. The color development was detected using a plate reader (POLARstar Omega, BMG Labtech). Analysis of significance was performed using one-way ANOVA followed by Dunnett's multiple comparisons test.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/np500747y.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the German Ministry for Education and Research (BMBF, grant numbers 0315082, 01EA1303 to S.S.) and TRIAD Foundation (to F.C.S.) for financial support. We would like to thank C. Weidner for experimental help and fruitful discussions and A. Prasad and K. Kawamoto for providing chemically synthesized amorfrutins that were used in this and in recent studies. This work is part of a university thesis of L.F.

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DOI: 10.1021/np500747y
J. Nat. Prod. 2015, 78, 1160−1164